Involvement of myosin V and associated proteins in protein trafficking and neuronal morphogenesis

by

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ABSTRACT

Proper neuronal development and function requires precise sorting and delivery of various elements from the soma to the synapse. Important mediators of intracellular transport events are the actin-based class V myosin motors, which are involved in organelle transport in various cell types. Two myosin V family members, myosin Va and Vb, are present in the brain, however, the identity of cargoes transported by these motors is unknown. The objective of this thesis was to conduct molecular and cell biological studies to identify and characterize novel myosin V cargoes in neurons.

The first approach I used was to characterize the distribution of candidate protein cargoes after blocking the function of endogenous myosin Va and Vb with dominant-negative (DN) versions. I found that in developing neurons, expression of DN myosin Vb, but not DN myosin Va, resulted in the accumulation in the soma of the AMPA-type glutamate receptor subunit, GluR1, and a reduction of its surface expression. I also found that myosin Vb-mediated trafficking of GluR1 required an interaction with the GTPase Rab11. These results reveal a novel mechanism for the transport of a specific glutamate receptor subunit mediated by myosin Vb and Rab11.

As an alternative approach to identify myosin Va binding partners in the brain, we conducted a yeast-two hybrid screen of a rat brain cDNA library using the cargo binding domain of myosin Va. Among the proteins identified in our screen, I selected a protein of unknown function previously identified as Rab-lysosomal-interacting protein like 2 (RILPL2) and further assessed its function. I found that RILPL2 expression in non-neuronal cells resulted in morphological changes and activation of the Rho GTPase Rac1. In developing neurons, gain or loss of RILPL2 function altered the density of dendritic spine protrusions and increased phosphorylation of the Rac1 effector Pak. These findings uncover a novel role for the myosin Va-interacting protein, RILPL2, in regulating dendritic spine development, possibly through Rac signaling.

Taken together, the work presented in this thesis provides novel insights into the function of class V myosins in neurons, and into the critical machinery involved in trafficking of AMPARs and dendritic spine morphogenesis.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole propionate</td>
</tr>
<tr>
<td>Bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CA</td>
<td>cornu ammonis</td>
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<tr>
<td>CaM</td>
<td>calmodulin</td>
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<td>CaMKII</td>
<td>calcium/calmodulin-dependent kinase II</td>
</tr>
<tr>
<td>CBD</td>
<td>cargo binding domain</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CP</td>
<td>cortical plate</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DIV</td>
<td>days in vitro</td>
</tr>
<tr>
<td>DLC</td>
<td>dynein light chain</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant-negative</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dop</td>
<td>dilute-opisthotonus</td>
</tr>
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<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>GCL</td>
<td>ganglion cell layer</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GKAP</td>
<td>guanylate kinase domain-associated protein</td>
</tr>
<tr>
<td>GS</td>
<td>Griscelli syndrome</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HC</td>
<td>Heavy chain</td>
</tr>
<tr>
<td>IC</td>
<td>interhippocampal commissural fibers</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filament</td>
</tr>
<tr>
<td>in vitro</td>
<td>experiments performed in cultured cells</td>
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<tr>
<td>in vivo</td>
<td>experiments performed in/from rat tissue</td>
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<td>IP3R</td>
<td>inositol 1,4,5-triphosphate receptors</td>
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<td>IZ</td>
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<td>KIF</td>
<td>kinesin superfamily</td>
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<td>LC</td>
<td>Light chain</td>
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<td>MAP</td>
<td>Microtubule-associated protein</td>
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<tr>
<td>MDCK</td>
<td>Martin-Darby canine kidney</td>
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<td>mEPSC</td>
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<td>mIPSC</td>
<td>miniature inhibitory postsynaptic current</td>
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<td>microtubule-organizing centre</td>
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<td>mRNP</td>
<td>messenger ribonucleoprotein</td>
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<tr>
<td>MyoV</td>
<td>myosin V</td>
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<tr>
<td>MT</td>
<td>microtubule</td>
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<tr>
<td>Myrip</td>
<td>Myosin- and Rab-interacting protein</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>Nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>Oligo</td>
<td>oligonucleotide</td>
</tr>
<tr>
<td>PC</td>
<td>Purkinje cells</td>
</tr>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDZ,</td>
<td>PSD-95, Discs large, Zona occludens 1</td>
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<td>PMSF</td>
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<td>PTV</td>
<td>Piccolo-bassoon Transport Vesicles</td>
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<tr>
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<td>synaptic vesicle precursor</td>
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<td>VZ</td>
<td>ventricular zone</td>
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<td>WM</td>
<td>white matter</td>
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ACKNOWLEDGEMENTS

There are many people I would like to thank for their support during my graduate studies.

First, I wish to thank and remember my late supervisor Alaa El-Husseini, who died in a tragic accident on December 23, 2007. I am deeply grateful to Alaa for taking me under his wings. As my mentor and friend, he always encouraged me to be an independent and critical thinker, and provided me with numerous opportunities to grow as a scientist. I will always remember his Great Spirit and contagious laugh.

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Especially, I wish to thank Pamela Arstikaitis, Kun Huang, Joshua Levinson and Andy Shi, my hard working peers accompanying my late nights in the lab, for inspiring me and making me laugh day after day.

Special thanks to Kim Gerrow, Rochelle Hines, and Josh who have shown incredible reserve of patience in answering my questions and providing constructive feedback on my work. Many thanks also to our outstanding lab manager, Rujun Kang, who generously shared her expertise and always answered my technical questions with kindness.

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I am especially indebted to Deepak Srivastava and Peter Penzes, for their continuous support and critical feedback for the advancement of the RILPL2 project.

Finally, endless thanks and love to my family and friends, who have provided encouragements and emotional support in this adventure of graduate studies in Vancouver. Special thanks to Jack Chevalier, my life partner, for his everlasting support and patience.
CO-AUTHORSHIP STATEMENT

CHAPTER 2

I designed, performed and analyzed all the immunocytochemical experiments in cell lines and cultured neurons. I also designed and performed the myosin Vb and GluR1 co-immunoprecipitations experiments. I performed the bioinformatics and data analysis for all experiments, generated figures, and wrote the manuscript. Rujun Kang and Alex Trinh generated and purified the GST-MyoVb CC fusion protein used as an antigen to raise the polyclonal myosin Vb antibody. Rujun and Alex also purified the myosin Vb antibody, which I characterized when I joined the lab. Alaa and Alex generated and characterized the GFP-MyoVb CT and GFP-MyoVa CT cDNA used in this study. Jim Goldenring provided the GFP-Myosin Vb full-length construct. I generated the myosin Vb mutant forms lacking the Rab 11 binding region. Tak Pak Wong and Lidong Liu performed and analyzed the electrophysiological experiments presented in Figure 2.11. Rochelle Hines provided the myosin Vb immunohistochemistry data presented in Figure 2.1. Rujun Kang and Xiao-Yan Jiang performed the rat brain subcellular fractionation and provide me with samples used to prepare figure 2.5C. Dustin Hines provided technical support for extraction of brains from P16-P19 rats. Esther Yu and Yu-Ping Li prepared the dissociated primary neuronal cultures used in this study.

CHAPTER 3

The identification of RILP-like protein 2 (RILPL2) as a myosin Va-interacting protein was performed in Alaa El-Husseini’s lab. This was a mutli-step process involving the help of several people. The initial yeast two-hybrid screen using myosin Va as bait was performed by Alaa El-Husseini. RILPL2-positive cDNA was then isolated from yeast by Alex Trinh. Full-length RILPL2 mouse cDNA was generated by Vijay Viswanathan. To confirm the interaction of RILPL2 full-length with myosin Va, I repeated the yeast-two hybrid assay with these proteins. I designed and performed the co immunoprecipitations, pull-down assays and RT-PCR reactions. I also designed and characterized RILPL2 small interfering RNAs. For RNA extraction from brain and other tissues, I received technical assistance from Pascale Fretier, Rujun Kang and Esther Yu. Robyn Lett performed the in situ hybridization in brain tissue, and prepared the data presented in Figure 2. Deepak Srivastava performed the Rac activation biochemical assays in
COS-7 cells, presented in Figure 5. Pamela Arstikaitis performed neuronal transfections and image acquisition for the experiments presented in Figure 4. She also provided expertise for the quantification of dendritic filopodia and spines, which I performed. Except for the collaborations with Deepak and Pam reported above, I performed all the data collection and analysis of the experiments. Finally, I wrote the manuscript, and Deepak Srivastava, Peter Penzes, Pamela Arstikaitis, Joshua Levinson, and Timothy O’Connor provided intellectual support and constructive feedback.
CHAPTER 1

1.1 Introduction

1.2 The challenge of neuronal transport

All brain functions, ranging from the ability to learn and remember, to those involved in our emotions, consciousness and all human behaviors, are possible because of the amazingly complex connectivity of neurons in the brain. Moreover, our ability to process information and learn from experience is due to continual modifications in neuronal communication. Neurons communicate with one another at specialized sites of contact called synapses. In humans, the majority of synapses form during early prenatal and postnatal development until about 1 year after birth. By the end of this critical period, the 100 billion neurons in the brain have each formed thousands of synapses.

Development, maintenance and plasticity of synapses rely on precise sorting and trafficking of a wide range of molecules, including neurotransmitter receptors, ion channels, cell adhesion, signaling and scaffolding proteins, from their site of synthesis to the synaptic compartments. Given their elaborate shape and complex architecture, regulation in time and space of the mechanisms that deliver synaptic materials to the developing synapse and subsequently sustain the synapse represents a challenge of the utmost importance for neurons. Indeed, mutations in motor proteins and other defects in intracellular transport are increasingly linked to neurological and neurodegenerative diseases (De Vos et al., 2008; Hirokawa and Takemura, 2003; Hirokawa and Takemura, 2004; Langford and Molyneaux, 1998; Morfini et al., 2002; Saxena and Caroni, 2007; Seabra et al., 2002; Stokin and Goldstein, 2006).

Despite the major advances in identifying factors that regulate trafficking and clustering of proteins at the synaptic site, the factors that mediate transport of synaptic components from their site of synthesis to the synapse remain enigmatic. Potential candidates involved in this process are molecular motors that carry cargoes along microtubules (MTs) or actin filaments (Figure 1.1). It is widely accepted that long-range transport of membranous organelles and vesicles along dendrites and axons is mediated by MT-based motors of the kinesin and dynein families (Goldstein et al., 2008; Goldstein and Yang, 2000; Hirokawa and Noda, 2008; Hirokawa and Takemura, 2005; Nakata and Hirokawa, 2007; Vallee et al., 1989). In sub-compartments lacking
MTs, such as the presynaptic terminals and dendritic spines, local transport events are likely to be mediated by actin-based motors of the myosin family. In particular, the class V of unconventional myosins has previously been implicated in the transport of organelles and vesicles in different cell types, including neurons (Bridgman, 2004; Desnos et al., 2007a). However, the function of myosin V family members, particularly their potential involvement in trafficking of synaptic proteins remains poorly characterized.

In the first part of this chapter, I will discuss evidence for the involvement of MT- and actin-based motor proteins in the transport of various cargoes in axonal and dendritic processes. I will also provide an overview of the factors that regulate maturation and morphogenesis of dendritic spines, as well as factors involved in local trafficking of postsynaptic elements within dendritic spines during synapse development and plasticity. I will then introduce the class V myosins and highlight their properties and known functions that are consistent with a role in cargo transport in neurons.
1.3 Cytoskeletal tracks and motors for intraneuronal transport

1.3.1 Microtubule-based transport

Microtubules (MTs) constitute the major cytoskeletal element supporting fast and long-range transport along neuronal processes. MTs are polymers of $\alpha$- and $\beta$-tubulin that are nucleated at the microtubule-organizing center (MTOC) located in the cell body, before being delivered throughout the length of the neuron's processes (Baas and Qiang, 2005). Tubulin heterodimers polymerize in an end-to-end fashion, with the $\alpha$ subunit of one tubulin dimer contacting the $\beta$ subunit of the next to form protofilaments with alternating $\alpha$ and $\beta$ subunits. The protofilaments then assemble to form a hollow cylinder that is typically comprised of 13 protofilaments. Since the protofilaments bundle parallel to each other, every MT will display a plus-end, with only $\beta$ subunits and a minus-end with only $\alpha$ subunits exposed. This specific orientation of the protofilaments makes the MT a polarized structure that allows for directional transport by motor proteins.

During neuronal development, MTs are segregated into axonal and dendritic MTs, which differ in their distribution and orientation, as well as their interaction with different MT-associated proteins (MAPs) (Mandell and Banker, 1995; Sato-Harada et al., 1996). In the axon, MTs are unipolar, with the plus-ends pointing away from the soma (Baas et al., 1988). This uniform
orientation allow the formation of an overlapping set of tracks that span the length of the axon that is favorable for long-range transport. In dendrites, MTs have mixed polarity in the proximal region, and are unipolar, with the plus-end pointing away from the cell body in distal regions (Baas et al., 1988). The differences in distribution, orientation and interaction with MAPs between axonal and dendritic MTs dictate the direction, and possibly influence the speed, of MTs-based transport by motor proteins of the kinesin and dynein families.

1.3.1.1 Kinesins

Members of the kinesin superfamily (KIFs) typically mediate anterograde transport toward MTs plus-end (Goldstein and Yang, 2000; Hirokawa and Takemura, 2005; Miki et al., 2005; Nakata and Hirokawa, 2007). There are 45 KIF genes identified in mice and humans, and they are classified in 14 large kinesin families (Kinesin 1 to 14). KIFs generally exist as tetramers formed by two heavy chains (HCs) and two light chains (LCs). Each HC comprise a conserved globular motor domain that binds to MTs and hydrolyzes ATP, followed by a neck region, a stalk region and a tail region that encompasses the cargo binding domain (CBD). Several KIF members are critical for proper delivery within axons and dendrites of a wide variety of cargoes involved in neuronal morphogenesis, function and survival. These include plasma membrane components, synaptic vesicles, ion channels, adhesion molecules, mitochondria, postsynaptic scaffolding proteins, neurotransmitter receptors, ion channels and specific mRNAs (Figure 1.2) (Goldstein et al., 2008; Hirokawa and Takemura, 2005; Nakata and Hirokawa, 2007).
Figure 1.2. KIFs and cargoes for axonal and dendritic transport.

(A) A typical neuron, extending several dendrites (left) and a single thin axon (right) from the cell body. MTs form special bundles at the initial segment, which might serve as the cue for axonal transport. Tubulovesicular organelles are transported anterogradely along MTs by KIFs. Rough endoplasmic reticula are abundant in most parts of the cell body, except for the axon hillock. Dendrites contain some rough endoplasmic reticula. Membranous organelles and RNA-containing granules are transported along MTs by KIFs. (B) KIF5 transports vesicles containing APP (amyloid precursor protein) and APOER2 (apolipoprotein E receptor 2) by interacting with KLC (Inomata et al., 2003; Kamal et al., 2000; Matsuda et al., 2003; Nakata and Hirokawa, 2003; Verhey et al., 2001). Mitochondria are transported by KIF5 and KIF1B (Nangaku et al., 1994; Tanaka et al., 1998). KIF3 transports vesicles associated with fodrin (Takeda et al., 2000). KIF1A and KIF1B\(\alpha\) both transport synaptic vesicle precursors (Okada et al., 1995; Yonekawa et al., 1998; Zhao et al., 2001). JIPs, scaffolding proteins of the c-Jun amino (N)-terminal kinase (JNK) signalling pathway; KAP3, kinesin superfamily-associated protein 3. (C) In dendrites, KIF5 transports vesicles containing AMPA-type glutamate receptors through an interaction between KIF5 and GRIP1 (glutamate receptor-interacting protein 1) (Setou et al., 2002). RNA-containing granules are also transported by interacting directly with KIF5 (Kanai et al., 2004). KIF17 transports vesicles containing NMDA-type glutamate receptors by interacting through the LIN complex, a tripartite protein complex containing mammalian homologues of the Caenorhabditis elegans scaffolding proteins mLin-2, mLin-7 and mLin-10 (Setou et al., 2000). Reprinted from (Hirokawa and Takemura, 2005), with permission.
1.3.1.2 Dyneins

The second category of MT-based motor proteins is formed by dyneins, which generally mediate retrograde cargo transport toward MTs minus-ends (Goldstein and Yang, 2000; Levy and Holzbaur, 2006; Vallee et al., 2004). There are approximately 15 forms of dynein found in vertebrates, most being “axonemal,” referring to their role in ciliary and flagellar movement. Only two forms are “cytoplasmic,” and are referred to as cytoplasmic dynein 1 and 2 (or 1b). All dyneins are multi-subunit complexes composed of two large HC and a variable number of accessory subunits, termed intermediate, light intermediate and light chains (ICs, LICs, and LCs). The HC contains the motor domain that mediates MT attachment and ATP binding and hydrolysis. The motor domain is followed by a stalk region and an N-terminal CBD. Each HC associates with a certain number of IC, LIC and LC, which in turn bind to dynactin subunits, such as p150Glued and p50dynamitin, that can mediate cargo attachment (Hirokawa et al., 1998; Vallee et al., 2004). The differential assembly of dynein HC and accessory subunits allows the formation of various dynein complexes with common features, but which discriminate between cargoes and are specialized for individual functions. Cytoplasmic dynein 1, the most studied cytoplasmic dynein, is widely expressed in neurons and is implicated in axonal and dendritic transport of membrane-bound organelles and proteins, including mitochondria, signaling endosomes, Golgi outposts and ion channels (Goldstein and Yang, 2000; Ibanez, 2007; Susalka et al., 2000; Vallee et al., 2004). Figure 1.3 summarizes data from recent studies that have established a role for dynein in regulating the pattern of dendritic branching through transport of endosomes and other organelles into dendrites (Tear, 2008; Zheng et al., 2008).
Figure 1.3. Dynein-mediated transport of organelles and endosomes is required for elaboration of the dendritic tree.

(A) *Drosophila melanogaster* dendritic arborization (ddaC) neurons are shown to the left. In the wild-type, the dendritic tree of the neuron is extensive, with increased branching in distal regions. A mutation that disrupts the function of dynein light intermediate chain (dLIC) causes a marked change in the morphology of the ddaC dendritic tree. In dlic mutants, the ddaC dendritic tree is much more compact, with branching now occurring in proximal regions of the arbor.

(B) Schematic illustration of a proximal dendrite, where MTs have mixed polarities. Dynein is required to transport endosomes and other organelles toward the minus-ends of dendritic MTs. This cargo is unloaded to allow extension and branching of the dendrite. Rab5 endosomes seem to have an active role in promoting dendrite branching. Once the dynein molecules have reached the distal ends of the MTs, they may be returned to the soma by kinesin. Reprinted from (Tear, 2008), with permission.

### 1.3.2 Actin-based transport

In addition to MTs, another independent system for intracellular transport is provided by the actin cytoskeleton. Actin exists in two states, as monomeric globular (G)-actin and as an asymmetric two-stranded helical filament (F-actin) composed of G-actin. The assembly and disassembly of F-actin can be rapid owing to the weak non-covalent interaction of G-actin. At steady state, F-actin preferentially polymerizes at one end of the filament (the barbed end), while G-actin monomers are lost at the opposite end (the pointed end). The difference in
polymerization rates between the two ends results in a net turnover of the filaments. The precise orientation of the polarized actin filaments, concentrated adjacent to the plasma membrane along dendrites and axons, is not well defined; however, it is believed that actin filaments of both polarities co-exist side by side. F-actin is also enriched at the pre- and post-synaptic compartments (Figure 1.6). In dendritic spines, there is emerging evidence for the presence of two coexisting populations of F-actin: a highly dynamic pool that is a substrate for structural plasticity, and a stable longitudinal F-actin pool that is consistent with a role as a polarized track supporting directional transport along the core of spines (Cingolani and Goda, 2008; Honkura et al., 2008).

At least two mechanisms of actin-based intracellular movement have been identified in multiple cell types: one is dependent upon the Arp2/3 complex for actin nucleation and polymerization, and the other is myosin dependant (Fehrenbacher et al., 2003). Although both mechanisms are likely to contribute to transport in neuron, here we focus on myosins that can actively carry cargoes along F-actin. Similar to KIFs, all myosin HCs share three functional domains: a motor, a neck and a tail domain (Reck-Peterson et al., 2000; Sellers, 2000). The core motor region (head) contains the catalytic domain responsible for ATP binding and hydrolysis, and an actin binding interface. This is followed by a “converter” region which links the core motor domain to the neck region (or lever arm). The neck region is composed of a long helix of variable length depending on the number of IQ motifs (from none to six) which bind to either calmodulin or other LCs. Following the motor and neck domains is the tail domain, which is highly variable in sequence, length, composition and organization. This domain is thought to determine cellular localization and function of the various myosins, as well as their oligomeric structure. Myosins with tail regions containing predicted α-helical coiled-coil domains are believed to be dimeric with two motor domains, whereas those without a coiled-coil region are believed to be monomeric with a single motor domain.

Phylogenetic analysis based on a comparison of myosin motor domain sequences has divided the myosin superfamily into 18 distinct classes, designated (I to XVIII) (Berg et al., 2001; Foth et al., 2006; Hodge and Cope, 2000; Sellers, 2000). Myosin classes are further divided into “conventional” filament-forming myosins, such as myosin II that is abundant in muscle fibers, and “unconventional” myosins, including myosin V, that do not form filaments and instead function as monomers or dimers (Cheney and Mooseker, 1992). Classes of myosins identified in
neurons, likely to expand, include myosin I, II, V, VI, IX and X (Figure 1.4). These myosins are involved in several processes, including neuronal migration, process outgrowth, growth cone motility, neuronal morphogenesis and axonal transport (Bridgman, 2004; Bridgman and Elkin, 2000; Brown and Bridgman, 2004). Some myosins, such as nonmuscle myosin II and I, can exert tensions between actin filaments or actin filaments and membrane domains, whereas others, such as myosin V and VI, are well-suited for transport since they are processive and can perform many consecutive steps along F-actin without dissociating. In neurons, classes I, V, and VI myosins have been implicated in the transport of organelles and vesicles in the axonal compartments (Figure 1.4), and accumulating evidence suggests they are involved in dendritic transport events as well (Bridgman, 2004; Bridgman and Elkin, 2000; Brown and Bridgman, 2004).
Figure 1.4. Myosin-mediated transport in axonal compartments.

Diagram showing the distribution of the different myosin classes present in the axonal compartments. (A) In growth cones myosin I is associated with the cortical actin meshwork adjacent to the cell membrane. In proximal portions of neurites, myosin I is found to associate with tubulovesicular organelles. (B) Nonmuscle myosin II generates tension in growth cones through the formation of bipolar filaments that pull against oppositely oriented actin filaments (Matsumura et al., 1985). (C-D) Myosin V and VI are processive and are implicated in the transport of membranous organelles along the axon and at terminals. (E) Myosin IX is unique because the tail domain contains a region that stimulates the GTPase-activity of Rho, which regulates neuronal morphology. Synaptic vesicles are depicted as yellow; other membranous vesicles are shown in magenta and blue. Actin filaments are depicted as fine short lines, or intermediate length lines, while microtubules are depicted as long curvilinear lines. Reprinted from (Bridgman and Elkin, 2000), with permission.

1.4 Vesicular/cytoskeletal trafficking during synaptogenesis

1.4.1 Development of excitatory glutamatergic synapses

In the mammalian central nervous system (CNS), excitatory synapses are highly asymmetric junctions composed of a presynaptic terminal and a postsynaptic density (PSD). The axonal presynaptic terminal is filled with synaptic vesicles (SVs) containing neurotransmitters, the chemical basis of neuron to neuron communication. These vesicles are docked on the presynaptic membrane, attached by a complex cytomatrix of proteins, and primed for release
with the proper signal. In response to depolarization, fusion of these vesicles with the plasma membrane results in the release of glutamate into the synaptic cleft, a ~20-25 nm space between the pre- and the postsynaptic cells (Schikorski and Stevens, 1997). At the dendrite, or postsynaptic site, neurotransmitter receptors and a wide array of transmembrane, cytoskeletal and signaling proteins are clustered at the PSD, an electron dense meshwork of proteins in the dendrites (Kennedy, 2000). The PSD can be contained within small actin-rich protrusions that emerge from the dendritic shaft: the dendritic spines. The opening of the two major classes of glutamate receptors, the N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (NMDARs and AMPARs) (Hollmann and Heinemann, 1994) leads to an influx of ions, local depolarization and activation of voltage-gated ion channels as well as various signaling cascades. This series of events is called synaptic transmission and forms the basis of most information transfer within the CNS.

Since glutamatergic synapses are responsible for most of the fast excitatory transmission in the brain, much effort has been made in understanding the molecular and cellular mechanisms that govern their development and plasticity. New discoveries in the past decade have identified several key molecules, including cell adhesion, signaling and scaffolding molecules, which control one or more steps of excitatory synapse formation (Dityatev and El-Husseini, 2006; Gerrow and El-Husseini, 2006; McAllister, 2007; Waites et al., 2005; Yamagata et al., 2003). From contact initiation, recruitment of presynaptic and postsynaptic proteins to stabilization/maturation and elimination (Figure 1.5), synapse formation requires tight spatial and temporal regulation in the delivery of these various key elements.

How do neurons achieve the exquisite task of precise sorting and delivery? Although this fundamental question remains largely unsolved, a growing body of evidence suggests that vesicular/cytoskeletal trafficking by motor proteins is critical for proper synapse development, maintenance and plasticity.
1.4.2 Recruitment of pre- and postsynaptic proteins

For a synapse to form, contact must first be made between a presynaptic axon and a postsynaptic dendrite. While dendrites develop in an elaborate dendritic arbor, the axon will travel over long distance, guided by several cues, before it reaches a precise target field and recognizes a specific cell type. Both axons and dendrites display growth cones with dynamic filopodia extending and
retracting in search of the proper target (Figure 1.5). Regardless of whether dendrites or axons are responsible for the initiation of contact, target recognition is thought to be specific, since proper connectivity is essential to the function of a neuronal network. Compelling evidence suggests the specificity of initial contacts is, at least in part, mediated by cell adhesion molecules (Gerrow and El-Husseini, 2006; Shen, 2004). A small subset of these contacts will then be stabilized and undergo the second step of synapse formation which is the coordinated recruitment of pre- and postsynaptic molecules at the newly formed contact site (Figure 1.6).

In the presynaptic cell, molecular machinery necessary for neurotransmitter release is delivered to the axonal compartment. In parallel, a specific set of molecules, including glutamate receptors and scaffold molecules, are targeted to the postsynaptic site and gather in a structure known as the PSD, a multi-protein complex responsible for anchoring neurotransmitter receptors near sites of neurotransmitter release. Trans-synaptic cell adhesion systems, such as the neuroligins/neurexins and SynCams, are thought to avoid mismatching by the recruitment of the proper neurotransmitters at the presynaptic site with the cognate receptors at the postsynaptic site (Dean and Dresbach, 2005; Lise and El-Husseini, 2006; Washbourne et al., 2004a). Scaffolding proteins, including members of the PSD-95 family, Shank, and homer, are responsible for clustering receptors, ion channels, and associated signaling proteins at the synapse (Figure 1.6) (Kim and Sheng, 2004). Overall, a functional synapse can form within minutes after initial contact (McAllister, 2007; Rao et al., 1998; Shapira et al., 2003). This speed is thought to be achieved because the transport machinery has already brought the key components of the synapse into the growing neurites.
Figure 1.6. Protein complexes at excitatory synapses.

(A) An image of a neuron stained with the presynaptic marker synaptophysin (green) to identify synaptic contacts. This panel illustrates steps involved in the assembly of proteins at contact sites. Synapse formation is generally thought to involve three basic steps which include production of proteins in the cell soma (A-1), transport of these proteins to early sites of contact between axon and dendrites (A-2), and assembly of protein complexes at synapses (A-3). (B, C) The dense clustering of proteins seen at the PSD of excitatory synapses is highlighted in the electron micrograph shown in B. A schematic diagram is enlarged in C, illustrating the role of scaffolding molecules such as PSD-95, in the assembly of large protein complexes. PSD-95 forms the core of the protein network, which is associated with the membrane through palmitoylation, and anchored within the postsynaptic compartment by several proteins that associates with actin. Coupling of PSD-95 to adhesion molecules such as neuroligins allows for trans-synaptic signaling. Reprinted from (Hines and Husseini, 2006), with kind permission of Springer Science and Business Media.
In general, pre- and postsynaptic proteins are generally present in neurons before initial contacts form (Fletcher et al., 1991; Fletcher et al., 1994; Rao et al., 1998). Recently, many of these synaptic proteins have been localized to small, heterogeneous clusters of proteins, called transport packets (Craig et al., 1993; Gerrow et al., 2006; Washbourne et al., 2002; Washbourne et al., 2004b). Time-lapse imaging studies in cultured neurons have revealed that these mobile transport packets are recruited to sites of axo-dendritic contact with a rapid time course but variable hierarchy, depending of the nature of the target and possibly the age of the neurons (McAllister, 2007; Rao et al., 1998; Shapira et al., 2003). Although the precise mechanisms regulating the delivery to synaptic sites is poorly understood, the emerging evidence linking particular motor proteins to these organelles suggests a role for active cytoskeletal transport in the recruitment of pre- and post-synaptic proteins important for synaptogenesis.

1.4.2.1 Axonal transport of presynaptic proteins

In axons of young neurons, at least two types of presynaptic precursors have been identified: Piccolo-bassoon Transport Vesicles or PTVs, named after the active zone proteins piccolo and bassoon (Zhai et al., 2001), and Synaptic Vesicle Precursors (SVPs) (Ahmari et al., 2000; Sabo et al., 2006). The PTVs, are dense-core 80 nm vesicles with a coat of electron dense material that contains active zone proteins. In contrast, the SVPs are positive for markers of synaptic vesicles (Jin and Garner, 2008). Though PTVs and SVPs cluster together while moving and are recruited together to new synaptic sites, they may in fact use distinct kinesins (Goldstein et al., 2008).

The Kinesin-1 motor KIF5b and the adaptor protein syntabulin have been implicated in the transport of PTVs in hippocampal neurons. Knockdown of syntabulin or interference with its binding to KIF5B impaired the transport of several PTV components without disrupting the dynamics of SVPs (Cai et al., 2007; Su et al., 2004). For SVPs three anterograde MT-based motors have been identified in mammals: conventional kinesin-1 (KIF5) (Leopold et al., 1992; Sato-Yoshitake et al., 1992), and kinesin-3 members KIF1A (Okada et al., 1995; Yonekawa et al., 1998) and KIF1Bα (Nakamura et al., 2002; Zhao et al., 2001) (Figure 1.2). When mutations disrupt these motors, the vast majority of SVPs are trapped in the cell body and do not enter the axon leaving little doubt that these motors are the primary motor for axonal transport of SVPs (Goldstein et al., 2008). Why several KIFs are implicated in transport of SVPs is not clear.
Possibilities are that different motors may cooperate to make long range movement within the axon more efficient, or that the use of one motor or the other may differ according to the cell type and developmental stage.

Interestingly, myosin Va was identified as one of the many components of purified synaptic vesicles (Takamori et al., 2006) and shown to be present on vesicles moving along axonal microtubules (Ohyama et al., 2001; Prekeris and Terrian, 1997). Myosin Va associates with the SNARE protein synaptobrevin/VAMP2 and synaptophysin (Ohyama et al., 2001; Prekeris and Terrian, 1997), and myosin-V-associated vesicles label with the synaptic vesicle marker protein, SV2 (synaptic-vesicle protein 2) (Evans et al., 1998; Ohyama et al., 2001; Prekeris and Terrian, 1997). The presence of myosin Va on synaptic vesicles, as well as on a population of vesicles larger than synaptic vesicles (Cremona and De Camilli, 1997; Evans et al., 1998; Miller and Sheetz, 2000), suggest that myosin V may contribute to the delivery of SVPs and PTVs to the presynaptic terminal during synapse development.

1.4.2.2 Dendritic transport of postsynaptic proteins

At the postsynaptic site, one of the most critical events during synaptogenesis is the recruitment of ionotropic glutamate receptors. Different processes have been implicated in the synaptic recruitment of glutamate receptors, including insertion at extrasynaptic sites and traveling along the dendrite via lateral diffusion in the plasma membrane until reaching the synapse, local protein synthesis in dendrites and direct insertion into the synapse, as well as intracellular trafficking by cytoskeleton-associated motors (Chen et al., 2007; Shepherd and Huganir, 2007; Triller and Choquet, 2005; Wenthold et al., 2003). Although the overall delivery is likely the result of these combined processes, here we review the evidence for the involvement of motor proteins in directional transport of glutamate receptors and associated proteins into dendrites.

Similar to what is observed in axons, packets of receptors and scaffolding proteins traveling along dendrites are observed early in development (Gerrow et al., 2006; Washbourne et al., 2002). Live-imaging experiments of fluorescently-tagged NMDARs have shown that NMDARs are moved as discrete transport packets within dendrites (Washbourne et al., 2002; Washbourne et al., 2004b). Retrospective immunostaining indicates that NMDA transport packets carry the scaffold protein SAP-102 and the exocyst protein Sec8 (Sans et al., 2003; Washbourne et al.,
2004b) and that a subset of NMDA transport packets also carries AMPARs in young neurons (Washbourne et al., 2002). Experiments with purified vesicles containing NMDAR subunit 2B (NR2B) show that packets are transported along microtubules by the anterograde motor KIF17. This selective transport is accomplished by direct interaction of the KIF17 tail domain with a PDZ domain of mLin-10 (Mint1/X11), which is a constituent of a large protein complex including mLin-2 (CASK), mLin-7 (MALSVelis), and the NR2B subunit (Figure 1.14A) (Setou et al., 2000). Notably, the rate of transport of KIF17 is an order of magnitude faster than that for NMDAR transport packets in young cortical neurons (Guillaud et al., 2003; Washbourne et al., 2002), indicating that additional and/or alternative motor proteins must be involved in the rapid, bidirectional transport of NMDARs. Kinesin motors have also been implicated in the anterograde recruitment of AMPAR. For instance, the GluR2/3-binding protein GRIP1/ABP directly interacts with KIF5 (Figure 1.14B). A complex of GluR2, GRIP1 and KIF5 can be co-immunoprecipitated from brain lysates and expression of dominant-negative versions of kinesin decreases synaptic abundance of AMPAR (Setou et al., 2002). Another kinesin motor, KIF1A also associates with GluR2/GRIP1 through interaction with liprin-α (Wyszynski et al., 2002). KIF1A and AMPARs can be immunoprecipitated with liprin-α in brain lysates (Shin et al., 2003), and expression of mutant forms of liprin-α that cannot bind to GRIP1 blocks synaptic targeting of AMPARs (Wyszynski et al., 2002). Taken together, these results indicate MT-based motors are implicated in transport of postsynaptic proteins before and during synaptogenesis. Moreover, it provides evidence that PSD scaffolding proteins can act as adaptors for anterograde transport of membrane-associated AMPARs and NMDARs by motor proteins.

In addition to neurotransmitter receptors packets, other mobile transport packets containing the scaffold proteins PSD-95, GKAP, and Shank have been observed within dendrites in young neurons and shown to accumulate at synapses during their formation (Gerrow et al., 2006). What motor proteins are involved in transport of these scaffolds remain to be elucidated. PSD-95 is known to interact with the kinesin motor KIF1B (Mok et al., 2002), however, transport velocities of mobile PSD-95/GKAP/Shank preassembled complexes were slower than reported values for microtubule-based vesicular transport (Gerrow et al., 2006). Treatment with cytochalasin B and nocodazole to disrupt actin-and microtubule-based transport suggested that that the mobility of preformed scaffold complexes involves an actin-based motor. Interestingly, myosin Va was shown to co-immunoprecipitate with a protein complex containing DLC, GKAP and PSD-95
from brain lysates (Naisbitt et al., 2000), suggesting a role for myosin Va in dendritic transport of postsynaptic proteins.

### 1.4.3 Synapse maturation and dendritic spine morphogenesis

Once a synapse is established, new components must continue to arrive to replace proteins and organelles targeted for degradation. Whether or not the arrival and removal of components are balanced may determine whether a synapse is strengthened, weakened, or in a steady state. Maturation of pre- and post-synaptic sites occurs in a coordinated fashion as both the number of synaptic vesicles in the presynaptic compartment and the size and protein content of the PSD increase. For immature glutamatergic synapses, maturation is observed as the dramatic transformation of the immature postsynaptic compartment into a mature spine, a protrusion with a bulbous head (Figure 1.7). Within spines, actin has the dual roles of being the major scaffold for transport, as well as being the substrate responsible for structural changes at the synapse. Actin polymerization and depolymerization are dynamic processes that require tight regulation. Regulators of the actin cytoskeleton include the Rho family of GTPases, as well as actin-binding proteins such as Arp2/3 and cortactin (Ethell and Pasquale, 2005).

In particular, Rho GTPases are emerging as critical regulators of dendritic spine morphogenesis and structural plasticity during synapse formation and maturation (Ethell and Pasquale, 2005; Koh, 2006; Luo, 2000; Luo, 2002). Rho GTPases are signaling G proteins that form a subfamily in the larger Ras superfamily. The best studied Rho family members are Cdc42 (cell division cycle 42), Rac1 (Ras-related C3 botulinum toxin substrate 1), and RhoA (Ras homologous member A). These small GTPases act as molecular switches, alternating between an active GTP-bound form to an inactive GDP-bound form. This cycling is modulated by guanine-nucleotide exchange factors (GEFs), which promote the formation of active GTP-bound GTPase and GTPase-activating proteins (GAPs) which promote GTP hydrolysis and increase levels of inactive, GDP-bound GTPase (Van Aelst and Cline, 2004). A great deal of progress has been made in identifying GEFs and GAPs as upstream regulators of specific Rho GTPases that control processes such as spinogenesis, neurite elongation and branching (Koh, 2006). Several downstream signaling pathways linking Rho GTPases to the actin cytoskeleton have also been characterized (Figure 1.8). The emerging picture is that Rho GTPases act as integrators between...
extracellular cues conveyed by cell surface receptors, ions channels or cell adhesion molecules, and actin-binding proteins that regulate actin polymerization and depolymerization to promote several aspect of neuronal morphogenesis, including spine formation, maturation and plasticity (de Curtis, 2008; Ethell and Pasquale, 2005; Koh, 2006; Luo, 2000; Luo, 2002; Penzes et al., 2008; Van Aelst and Cline, 2004)

Figure 1.7. Dendritic spines are small protrusions along dendrites that contain postsynaptic densities.

(A) Example of a cortical neuron expressing green fluorescent protein (GFP). The main dendrite is branched and has dendritic spines along its length. Dashed box indicates area magnified in the inset. The axon of the neuron is much thinner than the dendrite and has no spines. Scale bar, 8 μm. (B) Dendrite of a cortical neuron expressing GFP-fused β-actin. Note the enrichment of β-actin in the dendritic spines. Scale bar, 5 μm. (C) Schematic of a mature dendritic spine making contact with an axon. (D) During brain development, dendritic spines mature from filopodia into shorter, mushroom-like structures. In both developing and mature brains, spines form and grow, and can undergo either maintenance or elimination, processes that contribute to overall spine density of a neuron. In mature brains, spines change morphology concurrent with synaptic plasticity. Reprinted from (Penzes et al., 2008), with permission.
Besides controlling the states of actin polymerization and depolymerization, Rho GTPases regulate the structure and function of spines through an actin-based motor of the myosin family, the nonmuscle myosin II (Figure 1.8). Myosin II is a dimeric, non-processive myosin that forms bipolar filaments which can cross-link and contract F-actin (Figure 1.4). In a recent study, postsynaptic myosin IIB was shown to be essential for normal spine morphology and dynamics as well as synaptic function (Ryu et al., 2006). At this point, it remains unclear whether other myosins, such as myosin V and VI which can processively move cargo along actin, are also involved in spine morphogenesis through actin. The ability of myosin Va to regulate actin organization in vitro and in vivo (Cheney et al., 1993; Eppinga et al., 2008; Tauhata et al., 2001) suggests myosin Va is involved in the regulation of the actin cytoskeleton. However, no significant abnormalities were found in the ultrastructural cytoskeletal arrangement in spines of cerebellar cortex neurons (Petralia et al., 2001) or spine morphology in developing mouse hippocampal neurons (Yoshimura et al., 2006) lacking a functional myosin Va. Neurons from myosin VI-deficient mice exhibit a decreased number of synapses and dendritic spines (Osterweil et al., 2005). Taken together, these observations suggest that the function of myosin motors and the regulation of synapse morphogenesis may be mechanistically linked.
1.4.4 Local trafficking and plasticity at dendritic spines

The enlargement and increased content of dendritic spines during maturation creates a novel micro-environment that is physically and chemically isolated from dendrites. Synaptic components that are delivered to this distinct compartment will be subjected to a new set of local trafficking rules that will dictate whether the components are inserted of removed from the synaptic plasma membrane, and whether they are recycled or degraded. The exclusively actin-based cytoskeleton of spines points to the potential involvement of actin-based myosin motors for directed transport of synaptic elements.
Rapid excitatory synaptic transmission at glutamatergic synapses is mediated by AMPA-type glutamate receptors and considerable effort has been made to uncover the mechanisms that control their trafficking to and from the postsynaptic membrane (Derkach et al., 2007; Elias and Nicoll, 2007; Esteban, 2008; Newpher and Ehlers, 2008; Shepherd and Huganir, 2007). AMPARs are composed of four types of subunits, designated as GluR1-4, which combine in the ER to form tetramers. Most AMPARs are heterotetrameric, consisting of symmetric “dimer of dimers” of GluR2 and either GluR1, GluR3 or GluR4. AMPAR are highly mobile at the postsynaptic membrane (Bredt and Nicoll, 2003; Cognet et al., 2006). They can diffuse laterally into and out of the PSD (Ashby et al., 2006; Borgdorff and Choquet, 2002; Groc et al., 2004; Tardin et al., 2003), as well as undergo dynamic intracellular trafficking through endocytosis and recycling (Ehlers, 2000; Luscher et al., 1999; Park et al., 2004). Two pathways referred to as the “constitutive” and the “regulated” pathways have been described to explain AMPARs exo/endocytic trafficking at the synapse. A number of studies have established that subunit-specific rules orchestrate this differential trafficking (Passafaro et al., 2001; Shi et al., 2001). The current model is that GluR2-3 receptors constitutively cycle in and out the synapse independently of synaptic activity (constitutive pathway), preserving the total number of synaptic AMPAR, whereas GluR1-2 (and GluR4) receptors are added into the synapse in an activity-dependant manner during synaptic plasticity (regulated pathway). The existence of constitutive and activity-dependant synaptic delivery of AMPARs leaves open the possibility that these trafficking events may have different requirements for active transport.

Activity-dependent regulation of AMPARs mobility and synaptic abundance may mediate diverse forms of synaptic plasticity, including long-term potentiation (LTP) and depression (LTD) (Figure 1.9) (Bredt and Nicoll, 2003; Malenka, 2003; Turrigiano and Nelson, 2004). One intensely studied form of synaptic plasticity is LTP at CA1 synapses in the hippocampus. LTP-inducing stimuli activate synaptic NMDARs, resulting in the entry of Ca^{2+} into the spine and activation of signaling pathways that ultimately lead to increased synaptic AMPARs (Derkach et al., 2007; Kennedy and Ehlers, 2006; Newpher and Ehlers, 2008; Shepherd and Huganir, 2007). In most but not all cases (Cingolani and Goda, 2008), increased synaptic AMPAR is coupled with rapid alteration of dendritic spine morphology (Alvarez and Sabatini, 2007; Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Carlisle and Kennedy, 2005; Ethell and Pasquale, 2005; Schubert and Dotti, 2007; Tada and Sheng, 2006). Recently, work from Ehlers’ group has established a link between recycling endosomes and increased AMPAR insertion and
spine size during LTP (Cooney et al., 2002; Ehlers, 2000; Park et al., 2004; Park et al., 2006). They demonstrated that recycling endosomes (REs) located within or at the base of spines contain a pool of AMPARs to support local receptor cycling. During LTP, REs are rapidly mobilized into spines where their contents are delivered to the plasma membrane (Figure 1.9). Disrupting RE trafficking blocks not only activity-dependent AMPAR insertion but also spine growth and new spine formation (Park et al., 2004; Park et al., 2006), indicating that dendritic REs are the local reservoir of cargo and membrane needed for plasticity-induced spine modification.

![Figure 1.9. Integrating models for receptor trafficking and diffusion during synaptic plasticity.](image)

Induction of LTP by Ca\(^{2+}\) influx through NMDARs leads to activation (lightning bolt) of PKA and CaMKII, which in turn promotes the mobilization of recycling endosomes (RE) into spines, exocytosis from recycling endosomes, and appearance of AMPARs at the spine membrane. The number of available slots in the PSD increases through unknown mechanisms, which can be filled by increased levels of extrasynaptic AMPARs. Receptor diffusion inside synapses decreases due to stronger scaffold interactions and/or receptor confinement. The endocytic zone (EZ) may also contribute to LTP by maintaining local recycling of AMPAR and preventing their escape from the spine membrane. On the other hand, induction of LTD leads to activation of protein phosphatases (lightning bolt), including PP2B and PP1, triggering clathrin-, dynamin-, and Rab5-dependent endocytosis of AMPA receptors, likely at the spine EZ. Receptor downregulation occurs by trafficking through early (EE) and late endosomes (LE). Loss of synaptic slot positions through unknown mechanisms reduces AMPA receptor capacity and increases the diffusion of synaptic AMPARs. P-GluR1: phosphorylated GluR1. Reproduced from (Newpher and Ehlers, 2008), with permission.
Candidate actin-based motors to mediate delivery and local trafficking in dendritic spines include class V and VI myosins. Myosin VI was shown to localize to endocytic vesicles and play a role in clathrin-mediated endocytosis of AMPA receptors (Osterweil et al., 2005). Myosin Va was implicated in translocation of an mRNA/protein complex into hippocampal neuron dendritic spines following mGluR1 activation (Yoshimura et al., 2006), and in spine localization of smooth ER at cerebellar Purkinje cell synapses (Miyata et al., 2000). Moreover, myosin Va was shown to associate with the postsynaptic density protein complex containing the scaffolding proteins GKAP and PSD-95, suggesting it may have a role in transport or anchoring of a part of the complex (Naisbitt et al., 2000). Myosin Vb is another class V myosin present in the brain (Zhao et al., 1996) and was reported to associate with Rab11-positive REs in non-neuronal cells (Lapierre et al., 2001). Thus, myosin V family members are ideal candidates to mediate local delivery of organelles and synaptic proteins to dendritic spines.

1.5 Class V myosins are candidates for neuronal trafficking

Class V myosins are among the most thoroughly studied forms of unconventional myosins and considerable evidence supports a role in organelle and vesicle transport in various cell types, including neurons (Bridgman, 2004; Desnos et al., 2007a). This evidence arises from both the known structural and functional properties of myosin V as well as from direct cellular studies in non-neuronal and neuronal cells.

1.5.1 Myosin V family members

Myosin V is expressed in most if not all eukaryotic cells (Reck-Peterson et al., 2000; Titus, 1997). In yeast there are two myosin V subclasses, Myo2p and Myo4p (Brown, 1997). Myosin V is also found in squid (Cohen, 2001), Drosophila melanogaster (Bonafe and Sellers, 1998; MacIver et al., 1998) and Caenorhabditis elegans (Baker and Titus, 1997) and in plants (Kinkema and Schiefelbein, 1994; Kinkema et al., 1994). In vertebrates, myosin V exists in three distinct isoforms (Va, Vb, and Vc). Myosin Va, the first mammalian member of the myosin-V family to be identified (Mercer et al., 1991), is the gene product of the classical coat-color locus dilute in mouse (Green, 1989; Silvers, 1979). The second member of the myosin-V family, myosin Vb (originally referred to as myr6), was identified by degenerate polymerase chain
reaction (PCR) from rat brain stem cDNA (Zhao et al., 1996). The third member, myosin Vc, was recently identified through extensive analysis of the human EST database (Rodriguez and Cheney, 2002). All three myosin V family members share similar structural features and major functional domains. Myosin V consists of two HCs that dimerize to form a two-headed molecule (Cheney et al., 1993; Sellers and Veigel, 2006; Vale, 2003). Each HC can be divided into three major domains: a motor head, a neck, and a tail domain (Figure 1.10) (Provance and Mercer, 1999; Rodriguez and Cheney, 2002; Trybus, 2008). Myosin Va and Vb share ~78% overall identity, whereas myosin Vc shares ~50% overall identity with myosin Va and Vb (Figure 1.10) (Rodriguez and Cheney, 2002; Zhao et al., 1996). The most divergent region is the C-terminal tail containing the cargo binding domain (CBD), which is consistent with the different cargoes carried by myosin V family members.

1.5.1.1 Transcriptional regulation and tissue expression

Myosin Va is alternatively spliced in a region located in the medial tail region (Figure 1.10) (Lambert et al., 1998; Seperack et al., 1995). Splicing occurs in different tissues and four isoforms, which have different combinations of tissue-specific spliced exons, have been identified to date (Huang et al., 1998b; Mercer et al., 1991; Seperack et al., 1995). The myosin Va isoform containing exon B is exclusively expressed in brain. The other isoforms, which do not contain exon B and are not expressed in brain, are expressed in skin and other tissues at different levels. In skin, alternative splicing takes place between exons A and G and generates three isoforms with different combinations of exons D and F (Huang et al., 1998b; Mercer et al., 1991; Seperack et al., 1995). Similar to myosin Va, myosin Vb contains exons A, B, C, D and E, while no exons F has yet been identified. Notably, while myosin Va lacks exon D in the brain, two isoforms of myosin Vb, with and without exon D, have been recently identified (Roland et al., 2009). Conversely, only the exon-D lacking isoform of myosin Vb is found in tissues such as kidney, pancreas, and testis (Roland et al., 2009; Zhao et al., 1996).

Interestingly, exon F is necessary for the selective binding of myosin Va to melanosomes (Au and Huang, 2002), while exon B is responsible for binding to DLC (Hodi et al., 2006; Wagner et al., 2006). The alternatively spliced exon D in myosin Va and Vb is required for binding to Rab10 (Roland et al., 2009). Thus, alternative splicing is a mechanism that may control the
selectivity of cargo binding to myosin Va and Vb. Myosin Vc is mainly enriched in epithelial tissue and expression in brain appears to be limited to the cerebellum (Rodriguez and Cheney, 2002). In contrast to myosin Va and Vb, no evidence for tissue-specific alternative splicing has been reported yet for myosin Vc.

Figure 1.10. Predicted structure of class V myosins in vertebrates.

(A) Linear diagram representing the functional features of class V myosins. All three myosins share an N-terminal head domain that contains the actin binding domain (BD; blue) and the catalytic ATP binding domain (BD) (black). This is followed by a neck region containing six IQ motifs (yellow) that associate with calmodulins (CaMs), or related light chains (Benashski et al., 1997; Cheney et al., 1993; Espindola et al., 2000). Following is a proximal tail region encompassing a PEST site (green, absent in myosin Vc), a medial tail region that is subjected to alternative splicing (in the case of myosin Va and Vb), and a globular tail region that is responsible for cargo binding. The position of the AF6/canoe homology region (orange) and the conserved phosphorylation site for CaMKII are shown within the C-terminal globular tail. (B) Predicted structure shared by class V myosins. The coiled-coil regions are responsible for dimerization.
1.5.1.2 Subcellular localization

Myosin Va is highly enriched in the CNS (Espindola et al., 1992; Mercer et al., 1991; Tilelli et al., 2003). Myosin Va is detected in several cell types, including neurons, astrocytes, microglia, and oligodendrocytes (Bridgman, 1999; Esprefico et al., 1992; Rodriguez and Cheney, 2002; Sloane and Vartanian, 2007; Stachelek et al., 2000; Tilelli et al., 2003). Biochemical fractionation of brain lysates revealed myosin Va is enriched in the PSD fraction (Naisbitt et al., 2000; Walikonis et al., 2000). Immunocytochemical localization of myosin Va in cultured hippocampal neurons shows that it partially colocalizes with PSD-95 at synapses and is also diffusely localized in cell bodies, dendrites, and axons (Walikonis et al., 2000). Other immunolocalization studies indicated the presence of myosin Va at the MTOC located in the cell body of various cell types in culture, including primary neurons (Esprefico et al., 1998; Wu et al., 1998c). The presence of myosin Va at the postsynapse suggests it may participate in local transport events within the dendritic spine. The presence of myosin Va in microtubule-rich regions in the soma suggests myosin Va may facilitate the transfer of organelles and vesicles from their site of formation to the microtubule fast transport tracks. Myosin Vb and Vc are also detected in the brain (Rodriguez and Cheney, 2002; Zhao et al., 1996), however their cellular distribution and subcellular localization remain poorly characterized.

1.5.1.3 Developmental regulation

Myosin Va is clearly abundant in adult brain, however, whether its expression is regulated during development is not clear. One study showed that myosin Va protein is detectable at low level at early stages of embryonic development (E10-E13), and levels are seen to gradually increase until they are maximal at E-19 (Bridgman and Elkin, 2000; Espindola et al., 1992). In a another study, myosin Va protein levels were monitored during postnatal development and shown to slightly decrease between (P1-P19) and remain constant during adulthood (Sloane and Vartanian, 2007). The presence of myosin Va in postnatal development and adult brain is consistent with a role in trafficking events during synapse formation, maturation and plasticity. Whether, myosin Vb and Vc are subjected to developmental regulation in the brain is not known.
1.5.2 Structural and functional features of myosin V

In vitro studies of myosin V mechanoenzymatic and conformational properties have provided insightful data that allows researchers to predict myosin V molecular behavior in vivo (Sellers and Veigel, 2006; Taylor, 2007; Trybus, 2008; Vale, 2003)

1.5.2.1 Myosin V is a processive motor

A salient feature of the two-headed myosin V molecule is processivity, meaning that it can “walk” along an actin filament for many steps before detaching. A series of elegant experiments using single molecule-imaging, X-ray crystallography and electron microscopy have provided evidence that myosin Va moves along F-actin tracks in a ‘hand-over-hand’ fashion where the two heads alternate between leads and trails, with steps of approximately 36 nm which corresponds to the pseudo-repeat of the helical actin filament (Figure 1.11) (Mehta et al., 1999; Rief et al., 2000; Sakamoto et al., 2000; Veigel et al., 2002; Walker et al., 2000; Yildiz et al., 2003). Myosin Vb is also a processive motor (Watanabe et al., 2006), while myosin Vc appears to be non-processive (Watanabe et al., 2008). The ability of myosin Va and Vb to perform several consecutive steps along a filament before dissociating is consistent with a role in intracellular cargo transport. Given that a single processive run by myosin Va is at least 40 steps (Mehta et al., 1999; Rief et al., 2000; Sakamoto et al., 2000), which is approximately the average spine length (~1.5 μm), myosin Va (and likely Vb) is well-suited for delivery of organelles from the dendritic shaft into the spine head and for short-range transport in other actin-rich regions inside the neuron.
Figure 1.11. A general model for myosin Va processive movement.

Starting in state 1, both heads are strongly bound to actin with ADP at the active site. The trailing head is in a post-power stroke conformation, and the leading head is in a strained, pre-power stroke state. The trailing head releases ADP at ~12 s⁻¹ (k₁), the rate limiting step of the ATPase cycle (state 2). ATP binds to the trailing head and dissociates it from actin, allowing the attached head to complete its power stroke. At the same time, the detached head is thrust forward, hydrolyzes ATP, and becomes the new leading head in a pre-power stroke conformation (state 3). The detached head undergoes a diffusive search for the next actin binding site, releases Pi, and undergoes a transition to the strong binding state. State 1’ shows that the leading head may pass through an isomerized ADP* state before returning to state 1 (Purcell et al., 2005). The darker gray actin monomers are located ~36 nm apart to illustrate the size of each step. Reprinted from (Trybus, 2008), with permission.

The motor activity of myosin V has been extensively studied in vitro (Sellers and Veigel, 2006; Vale, 2003). In the sliding filament motility assay, purified myosin Va translocated F-actin with an average velocity of 0.3-0.4 µm/s (Cheney et al., 1993). In the same assay, the reported actin sliding velocity of myosin Vb is 0.2 µm/s (Watanabe et al., 2006). Myosin Va’s ability to transport cargo was also shown in vitro using beads of 800 and 300 nm in diameter or purified organelles, which were transported at rates of 0.45µm/s and 0.6µm/s, respectively (Wolenski et al., 1993). Furthermore, purified organelles can also move along actin tracks in a myosin Va-dependent manner and at velocities similar to purified myosin Va (0.3-0.4 µm/s) (Chabrillat et al., 2005; Evans et al., 1998). Thus, myosin V-mediated movement along F-actin is generally slower than for kinesins and dyneins that mediate long-range transport along MTs at velocities varying between 0.2-1.5 µm/s and 0.3-0.7 µm/s, respectively (Hirokawa and Takemura, 2005; Mallik et al., 2005).

The first evidence of myosin Va-dependent transport in living cells came from the visualization of melanosomes, the pigmented organelles of melanocytes. Timelapse imaging showed that the motion of melanosomes was only partially inhibited by the microtubule-depolymerizing drug nocodazole in wild-type melanocytes, but was almost completely abolished in melanocytes from
myosin Va-null mice. (Wu et al., 1998a). Similar observations were subsequently made for myosin Va–associated movement of organelles monitored in living neurons using microinjected fluorescently labeled antibodies to myosin Va (Bridgman, 1999). Although indirect, these initial studies suggest that myosin Va is processive and contributes to movement of cargoes in living cells.

1.5.2.2 Regulation of myosin Va conformation and motor activity

The finding that myosin V is a processive motor that contributes to organelle movement raised the following question: how are myosin Va motor activity and attachment to cargo regulated? Some clues came from hydrodynamic and electron microscopy analysis showing that myosin Va undergoes a change in conformation as it goes from an active “open” extended state to a compact “inactive” one (Krementsov et al., 2004; Li et al., 2004; Wang et al., 2004a). In the inactive state myosin Va adopts a compact conformation in which the cargo-binding globular tail domain folds back on the motor head domain, rendering myosin Va incompetent for transport (Figure 1.12) (Liu et al., 2006; Thirumurugan et al., 2006). A series of in vitro studies with purified myosin V revealed that Ca\(^{2+}\) controls conformational switches of full-length myosin Va and regulates its actin-activated MgATPase activity (Krementsov et al., 2004; Li et al., 2004; Wang et al., 2004a). In the absence or at nanomolar Ca\(^{2+}\) concentrations, actin-activated MgATPase activity is low and the molecule adopts the inactive compact fold. Micromolar Ca\(^{2+}\) concentrations favor the extended conformation and activate the MgATPase activity. However, reduced myosin Va motility was reported at high Ca\(^{2+}\) levels (10mM) (Cheney et al., 1993). Therefore, it appears that moderate increase in Ca\(^{2+}\) may activate the catalytic cycle of myosin Va, whereas stronger increase in Ca\(^{2+}\) concentration may signal the end of a processive run. Although Ca\(^{2+}\) levels alter the actin-activated MgATPase activity and motility in vitro, whether Ca\(^{2+}\) is the only physiological mechanism that regulates myosin V activity in vivo is debatable. For instance, it was suggested that the physiological signal that activates myosin Va mechanoenzymatic activity is the attachment to cargo, which facilitates the transition to the open and active conformation by disrupting the interaction between the motor domain and the CBD (Sellers et al., 2008). In agreement with this model, the MgATPase activity of myosin Va increases on binding of melanophilin to the CBD (Li et al., 2005). In summary, myosin Va motor activity appears to be tightly regulated by factors that control its conformational state, such as Ca\(^{2+}\) and cargo binding.
Figure 1.12. Diagram of myosin Va in the active and inactive conformation.

(a) Schematic view of active myosin Va with a vesicle bound to the cargo binding domain (CBD). Actin is light blue. Each myosin Va heavy chain has a motor domain (MD; red) and a lever arm (cyan) that has six calmodulin light chains (green) bound. An elongated rod domain has three predicted coiled-coil segments; the first (cyan) is broken at the PEST site, which begins at residue V1105, the second (blue) after residue A1234, the third (blue) extends to the CBD. The dynein light chain, labeled DLC, binds within the second uncoiled region. (b) Myosin Va in the inactive conformation folds up using interactions between the CBD and the motor domain. Binding to actin is limited to one head per actin filament. The structure has an asymmetric placement of the first coiled-coil segment relative to the CBDs. This might allow for further folding of the rod along the paired CBDs as suggested by (Li et al., 2006). If the inactive conformation binds actin strongly, it will stay bound while actin monomer addition to the (+) end displaces the actin filament toward the (−) end. (c) Electron micrograph of folded myosin-V bound to actin showing single headed actin binding. Same coloring scheme as in (b). Reprinted from (Taylor, 2007), with permission.
1.5.3 Myosin V-dependant organelle transport: insights from non-neuronal cells

Accumulating evidence from non-neuronal cells points to a general role of myosin V family members in transport of membranous organelles. In yeast, Myo2p is involved in the transport of vacuole-derived vesicles to the bud site (Catlett et al., 2000; Catlett and Weisman, 1998; Govindan et al., 1995; Johnston et al., 1991). In mammalian cells, myosin Va is detected on the membrane of several organelles, including melanosomes in melanocytes, secretory granules (SGs) in adrenal chromaffin cells, and insulin secreting cells (Ivarsson et al., 2005; Rose et al., 2003; Rudolf et al., 2003; Varadi et al., 2005) and phagosomes in macrophages (Al-Haddad et al., 2001; Swanson et al., 1999). The current model for myosin Va function in organelle transport has been established mainly through studies of melanosome transport in murine melanocytes and Xenopus melanophores (Gross et al., 2002; Kural et al., 2007; Rogers and Gelfand, 1998; Tuma and Gelfand, 1999; Wu et al., 1998a), although a similar model has been proposed to describe myosin Va involvement in SGs transport in secretory cells (Desnos et al., 2007a; Desnos et al., 2007b). In this model, referred to as the “dual filament model”, melanosomes are carried rapidly and bidirectionally by MT-based motors before being captured, translocated, and actively dispersed in the actin-rich periphery by myosin V, and transferred to the adjacent keratinocyte (Rogers and Gelfand, 1998; Wu et al., 1998a). In the absence of a functional myosin Va, melanosomes quickly shuttle back and forth and cannot be tethered to the peripheral actin cytoskeleton meshwork, leading to an abnormal accumulation at the perinuclear region near the MOTC or at the plus ends of MTs, depending on the balance between plus-end- and minus-end-directed MT-based motors (Wu et al., 1998a). In agreement with a transitional role for myosin V between different cytoskeletal elements, the cargo-binding domain of myosin V directly binds kinesin (Brown et al., 2001; Huang et al., 1999) in yeast and mice (Beningo et al., 2000; Brown et al., 2001; Huang et al., 1999). Moreover, myosin V is able to bridge across microtubule and actin cytoskeletons by virtue of separate interactions with actin (through its N-terminal motor domain) and with tubulin (through its C-terminal cargo-binding domain) (Cao et al., 2004)).

Taken together, these studies suggest a dual role for myosin Va in melanocytes: it captures and translocates cargoes from MTs to F-actin in peripheral dendrites, and mediates local transport and dispersion in the actin-periphery. The lack of translocation of IP3-sensitive Ca^{2+} stores and mRNA/protein complexes in the actin-rich dendritic spines observed in myosin Va-deficient neurons (Dekker-Ohno et al., 1996; Miyata et al., 2000; Petralia et al., 2001; Yoshimura et al.,
2006) indicates the proposed model of myosin Va function in non-neuronal cells can be generalized to neurons.

Most of what is known of myosin Vb function comes from studies using a dominant-negative (DN) strategy, consisting of the expression of a truncated form of myosin Vb lacking the N-terminal motor domain but containing the C-terminal CBD. This “headless” form of myosin Vb cannot move along actin and accumulates in a perinuclear compartment and has a DN effect on cargo transport by dimerizing and competing with endogenous wild-type myosin Vb. Using this technique, Lapierre and colleagues initially revealed that myosin Vb was associated with the plasma membrane recycling systems by showing that expression of myosin Vb DN disrupts trafficking of Rab11a-associated REs (Lapierre et al., 2001). Since this initial finding, the list of cargoes whose recycling is dependant on myosin Vb has been rapidly growing (Table 1.1). Overall, the involvement of myosin Vb in recycling/trafficking through Rab11 of various integral membrane proteins in various cell types points to a similar role for myosin Vb in neurons.
<table>
<thead>
<tr>
<th>Motor</th>
<th>Cargo</th>
<th>Adaptor</th>
<th>Cell type</th>
<th>Reference</th>
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<tr>
<td><strong>Myosin Va</strong></td>
<td>Skin melanosomes</td>
<td>Rab27a/melanophilin</td>
<td>melanocytes</td>
<td>(Fukuda et al., 2002; Strom et al., 2002; Wu et al., 2002)</td>
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<td></td>
<td>Secretory granules</td>
<td>Rab27a/Myrip</td>
<td>PC12 cells</td>
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<td></td>
<td>Polyribosomes mRNA/protein complexes</td>
<td>Pura/mStauufen/Fragile X TLS/Nd1-L RNA</td>
<td>Hippocampal neurons</td>
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<td></td>
<td>“Neuronal vesicles”</td>
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<td>SCG neurons</td>
<td>(Bridgman, 1999; Lalli et al., 2003)</td>
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<td></td>
<td>IP3-sensitive Ca^2+ stores in dendritic spines</td>
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<td>Purkinje neurons</td>
<td>(Dekker-Ohno et al., 1996; Miyata et al., 2000; Petralia et al., 2001; Tabb et al., 1998; Takagishi et al., 1996)</td>
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<tr>
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<td>Rab11a/Rab FIP2</td>
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<td>(Hales et al., 2002; Lapierre et al., 2001; Provance et al., 2008; Provance et al., 2004)</td>
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<td>(Rodriguez and Cheney, 2002)</td>
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</table>
1.5.4 Regulation of myosin V interaction with cargo

The interaction of myosin V with its cargo needs to be regulated in time and space. We have discussed earlier that Ca\(^{2+}\) levels regulate myosin V conformation and motor activity. In the absence of Ca\(^{2+}\), myosin V is folded and the globular tail is unavailable for cargo binding. In the presence of micromolar concentration of Ca\(^{2+}\), the myosin V molecule adopts an extended conformation that allows for cargo binding (Figure 1.12). In addition to the conformational changes, other mechanisms likely participate in the recruitment and association of myosin V with its cargo. These include Rab GTPases, adaptor/scaffolding protein complexes, phosphorylation, and proteolytic cleavage.

1.5.4.1 Rab GTPases

While the tail domain of myosin V appears to mediate the binding to cargo, sequences known to directly interact with phospholipids are not present in the tail domain of myosin V family members (Li et al., 1994). This suggests that myosin V’s interaction with its cargo requires the presence of an intermediary protein (or protein complex) on the cytoplasmic face of the cargo. One likely family of candidate adaptor proteins are the Rab GTPases, which have been shown to associate with membranous organelles via a lipid anchor and be implicated in every steps of vesicular transport (Pereira-Leal and Seabra, 2001; Pfeffer, 2001; Seabra et al., 2002). With more than 60 independent genes encoding Rab proteins in mammalian genomes, Rab GTPases represent the largest family within the Ras superfamily and may provide the diversity required to precisely connect a specific motor protein with the desired organelle. Moreover, Rab GTPases function as molecular switches that cycle between a GTP-bound active form and a GDP-bound inactive form and may provide the means to regulate in time and space the association and dissociation of motor proteins with the appropriate organelle.

Evidence from yeast secretory vesicles and mitochondria, as well as from mammalian melanosomes and endosomes, suggest that Rab GTPases are crucial components of the myosin organelle receptor machinery (Figure 1.13) (Seabra and Coudrier, 2004). In melanocytes, Rab27a recruits myosin Va via melanophilin/ Slac2a (synaptotagmin-like protein homologue lacking C2 domain A), a rabphilin-like effector protein (Fukuda et al., 2002; Hume et al., 2002;
In insulin secreting cells, Rab27a recruits myosin Va SGs membrane via Myrip (myosin- and Rab-interacting protein)/Slac2c, a protein with strong similarity to melanophilin (Desnos et al., 2003; Fukuda et al., 2002; Waselle et al., 2003). Notably, Rab27a and melanophilin/Slac2a are barely detectable in neurons (Desnos et al., 2007a), and the exon F responsible for binding to melanophilin is absent from the neuronal form of myosin Va (Au and Huang, 2002), suggesting other unknown adaptor proteins are mediating myosin Va association with cargo in neurons.

Figure 1.13. Molecular mechanism of Rab-dependant, myosin-driven organelle motility.
(A) The *Saccharomyces cerevisiae* Myo2p is another class V myosin involved in organelle motility. Two Rab proteins, Sec4p and Ypt11, have been implicated in the docking of Myo2p onto secretory vesicles and mitochondria, respectively. Genetic evidence suggests that Sec4p interacts with the tail domain of Myo2p, but a direct interaction has not been demonstrated (Schott et al., 1999). Furthermore, mutations in Sec2p, GEF and thus an activator of Sec4p, uncouple Myo2p from secretory vesicles (Schott et al., 1999). These observations support a mode of action of a Rab GTPase and a myosin motor in yeast, whereby a class V myosin is recruited to the surface of an organelle upon activation of a Rab protein (Sec4p).
(B) Rab27a localizes to melanosomes irrespective of the presence of melanophilin or myosin Va, suggesting that it represents the organelle-recognition element in the system (Hume et al., 2002; Provance et al., 2002; Wu et al., 2002). In wild-type melanocytes, Rab27a associates with mature melanosomes, and upon activation via GTP loading, Rab27a-GTP recruits melanophilin. Melanophilin stabilized at the melanosome surface then binds the tail of the melanocyte-specific spliced form of myosin Va. The motor domain of this myosin then allows association of melanosomes with the actin network and leads to their accumulation in dendrite extensions.
(C) In mammals, another member of the class V myosin, myosin Vb, has been shown to regulate a membrane traffic step (plasma membrane recycling) through its association with a distinct Rab GTPase (Rab11a) (Lapierrr et al., 2001). It is not yet clear whether the interaction is direct or whether it requires the Rab11a and myosin Vb-binding protein, Rab11 family interacting protein-2 (Rab11-FIP2) (Hales et al., 2002). The interaction of one Rab and one myosin via a linker protein is reminiscent of the Rab27a/melanophilin/myosin Va interaction. Adapted from (Seabra and Coudrier, 2004), with permission.

Myosin Vb is recruited to REs through association with Rab11a and Rab11-FIP2 (Rab11 family interacting protein-2) (Hales et al., 2002; Lapierrr and Goldenring, 2005; Lapierrr et al., 2001). In addition to Rab11a, myosin Vb was shown to associate with Rab8a (Roland et al., 2007), suggesting that myosin Vb might regulate the trafficking of distinct populations of endosomes.
In contrast to Rab27a, both Rab11a and Rab8a are present in neurons (Horton and Ehlers, 2004), and therefore are prime candidates for recruiting myosin Vb to cargo in neurons.

1.5.4.2 Adaptor/scaffolding protein complex

In addition to the Rab GTPases that can reversibly mediate association between a motor protein and the membrane of a specific organelle, there is evidence that scaffolding protein complexes can also allow for specific association of motors proteins with transmembrane proteins present at the surface of organelles or vesicles (Kim and Sheng, 2004; Kneussel, 2005). As mentioned earlier, several scaffolding protein complexes have been identified for kinesin motors. For example, NMDAR are coupled to the kinesin motor KIF17 through direct binding of the KIF17 tail domain with the PDZ domain of mLin-10 (Mint1/X11), which is a constituent of a large protein complex including mLin-2 (CASK), mLin-7 (MALS/Velis), and the NR2B subunit (Figure 1.14A) (Setou et al., 2000). Conversely, AMPAR are coupled to KIF5 through direct interaction with the GluR2/3-binding protein GRIP1/ABP (Figure 1.14B) (Setou et al., 2002).

Interestingly, myosin Va associates with the synaptic scaffolding proteins GKAP and PSD-95 through interaction with dynein light chain (DLC; also referred as PIN or Dlc8) (Espindola et al., 2000; Naisbitt et al., 2000), to synaptic scaffolding protein GKAP (Naisbitt et al., 2000). PSD-95 is a master scaffold protein that clusters AMPARs at excitatory synapses via its interaction with stargazin family members (Chen et al., 2000; El-Husseini et al., 2000; Kim et al., 1997; Kim and Sheng, 2004; Schnell et al., 2002; Tomita et al., 2005). DLC also associates with gephyrin (Fuhrmann et al., 2002), a scaffolding protein involved in clustering of glycine and GABA receptors at inhibitory synapses. Although the functional relevance of these interactions remains to be elucidated, this data points to the possibility that synaptic scaffold proteins form complexes that couple myosin V to transmembrane proteins to be delivered to the synaptic membrane.
Figure 1.14. Scaffolding protein complexes for kinesin-mediated transport of NMDARs and AMPARs.

Kinesin-mediated transport of NMDA receptors (A) and AMPA receptors (B). Schematic representation of microtubule-dependent transport complexes, which consist of secretory vesicles and associated proteins. These contain receptor (shown in red) and adaptor proteins, which link receptors to kinesin motors (in blue) through PDZ-domain-mediated interactions. Note that proteins depicted in yellow represent polypeptides with dual functions at both the intracellular transport pathway and the postsynaptic membrane specialization. Reproduced from (Kneussel, 2005), with permission.

1.5.4.3 Phosphorylation

Compelling evidence suggests that phosphorylation of motor proteins is a critical mechanism that regulates interaction with cargo (Hirokawa, 1993; Hirokawa and Noda, 2008; Karcher et al., 2002). For example, phosphorylation of the dynein intermediate chain (DIC) controls dynein interaction with dynactin subunit pl50Glued (Figure 1.15A) (Dell et al., 2000). Another example is phosphorylation of KIF17 tail by the serine/threonine kinase Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), which regulates the interaction between KIF17 and the scaffold.
protein mLIN-10 (Guillaud et al., 2008). In *Xenopus* melanophores, CaMKII-dependent phosphorylation results in release of myosin Va from melanosomes (Figure 1.15B) (Karcher et al., 2001).

![Diagram of motor proteins membrane association by phosphorylation.](image)

**Figure 1.15. Regulation of motor proteins membrane association by phosphorylation.**

**(A)** Phosphorylation of the dynein intermediate chain causes release of dynein from the organelle owing to loss of p150Glued interaction and therefore dynactin binding.

**(B)** Regulation of myosin V organelle binding by phosphorylation of the globular tail domain. Phosphorylation in a conserved CaMKII site of the globular tail of myosin V releases the motor from melanosomes and downregulates its activity in mitosis. From (Karcher et al., 2002), with permission.

In neurons, both myosin Va and CaMKII are major components of the PSD (Walikonis et al., 2000). Myosin Va was shown to both interact directly and be phosphorylated by CaMKII in the brain (Costa et al., 1999). Thus, it is tempting to speculate that activation of CaMKII by Ca$^{2+}$ influx through NMDA receptors may modulate release of cargo from myosin V in dendritic spines. However, a recent study reported that co-immunoprecipitation of myosin Va and its mRNPs cargo from brain lysates is reduced in the presence of CaMKII inhibitors (Yoshimura et al., 2006). Thus, myosin Va phosphorylation by CaMKII may facilitate interaction with cargo. Although it is not clear whether phosphorylation mediates release or attachment to cargo, these studies indicate phosphorylation is a potential mechanism to reversibly regulate myosin V association with cargo in neuronal cells.
1.5.4.4 Proteolytic cleavage

In addition to protein adaptor complexes and phosphorylation, myosin V binding to cargo can be regulated by proteolytic cleavage and degradation of its receptor on the vesicle. For instance, the vacuole-specific Myo2p receptor, Vac17p, has a polypeptide sequence enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST) that targets the protein for rapid degradation (Rechsteiner and Rogers, 1996). Proteolytic cleavage of Vac17p regulates vacuole transport and localization in yeast (Tang et al., 2003). In melanocytes, protein degradation of melanophilin/Slac2a, which also possesses PEST-like sequences, controls the proper distribution of melanosomes (Fukuda and Itoh, 2004). These studies indicate proteolytic cleavage and degradation of the myosin V receptor complex can control its association with cargo.

The presence of a PEST sequence in both myosin Va and myosin Vb is indicative that the motor itself could also be subjected to proteolytic cleavage (Figure 1.10 and Figure 5.1). In vitro cleavage experiments of purified myosin Va by the Ca\(^{2+}\)-dependent protease calpain results in a \(~80\) kDa tail fragment similar to the motorless construct used as a dominant-negative in several studies (Nascimento et al., 1996). Calpain-dependant proteolysis of myosin Va also occurs in purified synaptosomes upon depolarization and in the presence of Ca\(^{2+}\) (Casaletti et al., 2003), as well as in cultured cerebellar granule neurons in the presence of excitotoxic concentrations of glutamate, kainate or NMDA (Alavez et al., 2004). Similar to myosin Va, enhanced neuronal depolarization with KCl or enhanced neuronal excitability by blocking GABAergic inhibitory transmission with bicuculline results in the production of a \(~75-80\) kDa myosin Vb tail fragment (see Appendix A). Moreover, increased synaptic activity also triggers changes in the distribution of endogenous myosin Vb in neurons. Remarkably, treatment with bicuculline resulted in loss of myosin Vb puncta in the dendritic shafts and spines (see Appendix A). Taken together, these observations point to an activity-regulated mechanism that could be used by neurons to regulate cleavage and/or degradation of members of the myosin V family members and thus control the amount of cargo transported to the synapse.

1.5.5 Defects associated with myosin V lack of function

Mutations and genetic defects of myosin V have been reported and studied in both mice and humans, and lead to neurological defects and premature death. Although the underlying causes
of the neurological abnormalities are still debated, results from these studies have implicated myosin V in a variety of processes in the brain including organelle transport into dendritic spines, synaptic plasticity, and myelination (Desnos et al., 2007a; Perlson and Holzbaur, 2008; Takagishi and Murata, 2006).

1.5.5.1 Dilute animals

Genetic studies reveal that dilute mice display chromosomal alteration in the MYO5A gene due to the ectopic insertion of a murine leukemia provirus, Emv-3 (Seperack et al., 1995). Mutations in a number of alleles of MYO5A have been molecularly characterized to regions in both the head and tail domains (Huang et al., 1998a; Huang et al., 1998b). All mutations at the MYO5A locus cause a dilution in coat color due to defects in melanosome trafficking. Less severe alleles, such as dilute-viral, exhibit only pigment dilution since the mutation primarily affects the synthesis of the melanocyte-specific alternatively spliced form (containing exon F) of myosin Va (Seperack et al., 1995). The most severe allele, referred as dilute-lethal, is associated with a loss of function of brain myosin Va and the mice display severe neurological abnormalities characterized by ataxia, opisthotonus, seizures and death at about 3 weeks of age (Mercer et al., 1991; Searle, 1952). Spontaneous mutations of MYO5A gene have also been reported in the dilute-opisthotonus (dop) rat (Futaki et al., 2000). The dop homozygotes show diluted coat color and ataxic behavior around 12 days after birth. They develop convulsive limb movement, opisthotonus, body weight loss, and weakness approximately 3 weeks after birth, and die, presumably owing to inability to feed (Dekker-Ohno et al., 1996).

The underlying causes of the neurological disorders observed in MyoVa deficient animals are unclear. An attractive explanation is the observed depletion of Ca\(^{2+}\) stores in dendritic spines of Purkinje cells (PC) in the cerebellum of dilute-lethal animals. Ultrastructural studies in both the dilute-lethal mouse (Takagishi et al., 1996) and dop rat (Dekker-Ohno et al., 1996) indicate that spines of PC lack smooth SER and inositol 1,4,5-triphosphate receptors (IP3R) (Petralia et al., 2001; Takagishi et al., 1996). Although synaptic transmission at the PC synapses remains largely normal, LTD of parallel fiber synapse is abolished (Miyata et al., 2000), due to a decrease in IP3-mediated Ca\(^{2+}\) release from the SER in the PC spines of the dop cerebella. (Takagishi and Murata, 2006). Consistent with the argument that loss of IP3R could be, at least in part, responsible for the observed neurological defect, inactivation of the IP3R type I gene results in a
phenotype similar to that of *dilute-lethal* animals, with ataxia and seizures (Matsumoto et al., 1996). Recently, a role for myosin Va in the formation of proper synaptic circuitry in the cerebella was reported. Climbing fiber (CF) projections to Purkinje cells are reduced in the *dilute-lethal* mice and *dop* rats, suggesting that myosin Va is essential for terminal CF extension and for the establishment of CF synapses within the proper dendritic territories of PCs (Takagishi et al., 2007). Taken together, these studies outline a critical role for myosin Va for proper plasticity and connectivity in the cerebellum.

Beside the circuitry abnormalities recently reported in the cerebellum, no gross alteration of brain architecture was found in *dilute-lethal* mice or *dop* rats that lack functional myosin Va (Dekker-Ohno et al., 1996; Takagishi et al., 1996). Myosin Va-null cortical neurons have normal neuronal growth cone size and filopodia morphology, suggesting myosin Va is not necessary for normal outgrowth, morphology, or cytoskeletal organization of this type of neuron (Evans et al., 1997). Although myosin Va-null dorsal root ganglia (DRG) neurons exhibit impaired anterograde synaptic vesicle movement (Bridgman, 1999), electrophysiological analyses of synaptic transmission and synaptic vesicle cycling at hippocampal CA1 synapses from *dilute-lethal* mice (Schnell and Nicoll, 2001) and in cultured superior cervical ganglion (SCG) neurons from *dop* rats (Takagishi et al., 2005) revealed no significant defect in neurotransmission and plasticity. Ultrastructural immunogold study of the cerebellar cortex revealed that spines from *dilute-lethal* mice retain a normal distribution of glutamate receptors (δ1/2, GluR2/3 and mGluR1α), at least one associated MAGUK (membrane-associated guanylate kinase) protein, Homer (which interacts with mGluR1α and IP3Rs), the actin cytoskeleton, the reticulum-associated protein BiP, and the motor-associated protein, DLC. (Petralia et al., 2001). Thus, while myosin Va may maintain the SER and IP3R in the spine for proper calcium regulation, other mechanisms are likely to be involved in the delivery of glutamate receptors and associated proteins to synapses. Other possible mechanisms include diffusion along the extrasynaptic membrane and compensation by other myosin motors traveling along the spine's actin cytoskeleton. Overall, these data suggest that myosin Va is not essential for the neuronal morphology, synaptic release machinery, postsynaptic receptor composition, or plasticity in the type of neurons tested. However, these data do not exclude significant roles for myosin Va in other cell types nor potential compensation by other myosin V isoforms.
The work presented above investigated the effects of myosin Va loss-of-function in neuronal cells. Notably, myosin Va is also expressed in glial cells in the CNS (Sloane and Vartanian, 2007). Based on previous observations, oligodendrocytes from myosin Va-null mice exhibit a significant reduction in 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) activity involved in myelinogenesis (Noguchi et al., 1983) and dilute-lethal brains show a prominent delay in CNS myelination (Kelton and Rauch, 1962; Winterbourn et al., 1971). Sloane and colleagues (2007) proposed that myosin Va could function as a molecular motor that transports material essential for glial cell growth and myelin formation. Indeed, they found severely impaired myelination in the brains and spinal cords of myosin Va-null mice. Moreover, oligodendrocytes had significantly smaller lamellas, shorter processes, decreased process and branching, as well as impaired localization of the vesicle-associated protein VAMP2. Together, these findings established a role for myosin Va in oligodendrocyte function as it relates to myelination. Defects in myelination associated with the lack of myosin Va may account, at least in part, to the neurological deficits observed in rodents and humans that lacks myosin Va.

1.5.5.2 Flailer mice

Normal expression of myosin Va is also compromised in another mouse line, referred to as flailer mouse (Jones et al., 2000). These mice express a novel gene that combines the promoter and first two exons of guanine nucleotide binding protein beta 5 (Gnb5), with the C-terminal exons of the closely linked MYO5 gene. This combination results in the flailer protein, which is expressed predominantly in brain and contains the N-terminal Gnb5 fused in-frame with the C-terminal CBD of myosin Va. Biochemical and genetic studies indicate that the flailer protein competes with wild-type myosin Va in vivo. Similar to the dilute-lethal mice, flailer mice display neurological abnormalities by 14 days of age, including convulsions, opisthotonus and ataxia. However, in contrast with dilute-lethal mice that die before weaning, the neurological symptoms in flailer mice improve with age and their fertility and lifespan is normal (Jones et al., 2000). Immunocytochemical analysis of flailer visual cortical neurons in culture and superior colliculus (SC) neurons in vivo, show pronounced PSD-95 immunoreactivity in dendritic shafts but sparse PSD-95 puncta at spines, suggesting that delivery of PSD-95 to spines is dependent on myosin Va (Van Zundert, 2005). Moreover, electrophysiological recordings of synaptic currents in flailer SC neurons using whole-cell patch clamping revealed that flailer mice display AMPAergic mEPSCs with significantly larger amplitudes and frequency than wild-type animals.
(Van Zundert, 2005). Although further analysis is required to clarify the discrepancies between results obtained from *dilute-lethal* and *flailer* mice, the fact that both mice display a neurological phenotype indicate that myosin Va function is critical for proper neuronal function.

### 1.5.5.3 Griscelli syndrome in humans

In humans, mutations of MYO5A are associated with Griscelli syndrome type I, a rare autosomal recessive disorder characterized by a pigmentary dilution of the skin and the hair, and severe neurological deficits, including hypotonia, quadraparesis, marked motor developmental delay, mental retardation, seizures, and ataxia (Ivanovich et al., 2001; Pastural et al., 1997; Pastural et al., 2000; Sanal et al., 2002). This phenotype is remarkably consistent with the *dilute* phenotype seen in myosin Va-null mice and rats. Notably, these neurological defects are not observed in Griscelli syndrome types II and III, which are due to mutations in RAB27A and MLPH genes encoding for Rab27a and melanophilin/Slac2a, respectively (Anikster et al., 2002; Bahadoran et al., 2003b; Menasche et al., 2002; Menasche et al., 2000).

In addition to Griscelli disease, two other rare hypopigmentation syndromes in humans are characterized by symptoms similar to those of myosin Va mutant mice. Cross-syndrome patients are described as having a metallic sheen to their hair and are unresponsive, with hypotonic characteristics (Cross et al., 1967), and Elejalde syndrome patients have silver hair and exhibit neurological defects including hypotonia, seizures, ataxia (Bahadoran et al., 2003a; Bahadoran et al., 2003c; Duran-McKinster et al., 1999; Elejalde et al., 1979; Ivanovich et al., 2001; Sanal et al., 2000). A few reports have suggested that the neurologic deficits seen in GS1 patient may be a result impaired myelination (Anikster et al., 2002; Kelton and Rauch, 1962; Noguchi et al., 1983; Pastural et al., 1997; Sloane and Vartanian, 2007; Winterbourn et al., 1971). Consistent with this is the necessity of having a functional myosin Va for normal oligodendrocyte morphology and function in myelination (Sloane and Vartanian, 2007). Further investigation is required to clarify how myosin Va loss-of-function in neuronal cells may underlie the neurological defects associated with GS1, Cross and Elejalde syndromes in humans.
1.5.5.4 Microvillus inclusion disease in humans

Nonsense and missense mutations in MYO5B have been recently associated to an extremely rare intestinal disorder referred to as microvillus inclusion disease (MVID) (Muller et al., 2008b). This disorder is also referred to as congenital familial protracted diarrhea, congenital microvillus atrophy, Davidson's disease, or familial enteropathy, microvillus. MVID is characterized by lack of microvilli on the surface of enterocytes, a class of intestinal cells that line the lumen of the intestine, and the occurrence of intracellular vacuolar structures containing microvilli. As a consequence, affected newborns are not able to absorb nutrients. Mislocalization of transferrin receptor in MVID enterocytes suggests that MYO5B deficiency causes defective trafficking of apical and basolateral proteins in MVID (Muller et al., 2008a). This study reveals the importance of myosin Vb for normal intestinal function. Presently, myosin Vb has not been linked to any neuronal disease in humans.

1.6 Thesis hypothesis and objectives

Proper sorting and delivery of newly synthesized neurotransmitter receptors and associated proteins from the soma to the synapse is essential for neuronal development, activity and plasticity. Cargo transport is mediated by molecular motor proteins that travel along cytoskeletal tracks. Microtubule-based motors are implicated in long-range transport within dendrites and axons, while the contribution of actin-based myosin motors to neuronal trafficking remains poorly understood.

Potential candidates for actin-based transport in neuronal cells are class V myosins. Myosin Va, the prototype member of the family, is widely expressed in the brain and has previously been implicated in transport of vesicles and organelles in neuronal cells (Bridgman, 2004; Desnos et al., 2007a). Loss of function of myosin Va in rodents and humans results in grave neurological defects and premature death, suggesting a critical role for myosin Va-mediated trafficking in normal neuronal function. A second myosin V family member, myosin Vb, which has also been implicated in trafficking events in several cell types, is also expressed in the brain but its role in neurons is unknown.
The presence of both myosin Va and Vb in the brain raises several questions: what neuronal proteins are trafficked by myosin Va and myosin Vb? Do myosin Va and Vb share similar mechanisms/adaptor proteins for delivering cargo to specific subcellular locations? Is there functional redundancy between these closely related family members? The primary objective of this thesis is to provide a better understanding of the function of myosin Va and Vb in neuronal processes such as trafficking and neuronal morphogenesis, through the identification and characterization of cargoes and binding partners in neurons. The specific goals of this thesis were to:

1- Identify novel myosin Va and Vb cargoes in neuronal cells using a DN approach

A popular approach to study the function of myosin V family members consists of the expression of truncated forms lacking the N-terminal motor domain but containing the C-terminal cargo binding domain, which results in competitive inhibition of the wild-type myosin. This DN strategy, coupled with immunocytochemistry to visualize the localization of endogenous proteins or organelles inside the cell, has been used to demonstrate the involvement of myosin Va-mediated melanosome transport in melanocytes and myosin Vb-mediated recycling of receptors in non-neuronal cells. Several questions will be addressed: Will synaptic proteins show altered distribution upon expression of myosin Va or myosin Vb DN constructs in neurons? Will expression of myosin Va or myosin Vb DN constructs have similar or differential effects on trafficking of a given cargo? What adaptor proteins couple myosin Va or Vb to a given cargo?

2- Identify and characterize the function of novel myosin V-interacting proteins in neuronal cells

As a complementary strategy to identify novel myosin V binding partners in the brain, we performed a yeast two-hybrid screen of a brain cDNA library with the tail domain of myosin Va. From this screen, I selected for further characterization a protein identified as RILP-like protein 2 (RILPL2) that is related to Rab-lysosomal interacting protein (RILP) (Wang et al., 2004b). In contrast to the well-documented role of RILP in regulating late endosomal and lysosomal compartment morphology (Cantalupo et al., 2001; Progida et al., 2007; Wang et al., 2004b), the cellular function of RILPL2 remains unknown. Questions I addressed include: What is RILPL2 distribution in the brain and subcellular localization in neuronal cells? What is RILPL2 function
in neuronal cells? Does RILPL2 interact with myosin Va in the brain? Is RILPL2 function dependant on myosin Va-mediated trafficking?
1.7 References


2.1 Involvement of myosin Vb in glutamate receptor trafficking

2.2 Introduction

Proper sorting and transport of excitatory neurotransmitter receptors and associated proteins is essential for neuronal activity and plasticity. Recent studies have identified several proteins that regulate clustering of neurotransmitter receptors at the synapse (Kim and Sheng, 2004). However, it remains unknown what proteins mediate sorting and delivery of receptors from the soma to postsynaptic sites. Molecular motors that regulate cargo trafficking on both actin filaments and microtubules have been implicated in initial transport and delivery to specific subcellular sites (Bridgman, 2004; Hirokawa and Takemura, 2005). In particular, class V of unconventional myosins are actin based motors thought to regulate trafficking of organelles and associated proteins in neuronal cells (Bridgman, 2004; Langford, 2002).

Three known members of the myosin V family have been detected in brain extracts. The most studied member, myosin Va, is widely expressed in the brain (Tilelli et al., 2003). Dilute mice, which possess mutation in the myosin Va gene, suffer from impaired melanosome transport, severe seizures, and die within 2-3 weeks after birth (Mercer et al., 1991). These observations suggest that alteration in the transport of important yet unknown cargos contributed to the observed defects in neuronal function. In neurons, myosin Va is enriched at the postsynaptic density (PSD) of excitatory synapses (Walikonis et al., 2000) and associates with the scaffolding guanylate kinase domain-associated protein (GKAP) through interaction with dynein light chain (DLC) (Naisbitt et al., 2000). The association of GKAP with the postsynaptic density protein-95 (PSD-95), a protein involved in glutamate receptor clustering, may functionally couples these proteins to myosin Va (Kim et al., 1997; Kim and Sheng, 2004).

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Myosin Vb and myosin Vc are two additional members of the myosin V family that are also expressed in the brain, however their exact localization in neurons remains unclear (Rodriguez and Cheney, 2002; Zhao et al., 1996). All of these motors share ~ 42% identity and contain a conserved N-terminal motor domain, followed by a coiled-coil region and a globular C-terminal tail. The globular tail of class V myosins contains the cargo binding domain (Bridgman, 2004; Karcher et al., 2002). Expression of truncated forms of various members of the myosin V family lacking the N-terminal motor domain but containing the globular tail domain leads to a dominant-negative (DN) phenotype in different cultured cell systems (Fan et al., 2004; Hales et al., 2002; Lapierre et al., 2001; Rodriguez and Cheney, 2002; Volpicelli et al., 2002; Wu et al., 1998c). This DN approach revealed an important role of myosin Va in the control of melanosome transport in melanocytes (Wu et al., 1998b). A similar strategy has been used to delineate the involvement of myosin Vb in recycling of several receptors in non-neuronal cells. These include M4 subtype of muscarinic acetylcholine receptors in PC12 cells (Volpicelli et al., 2002), transferrin and polymeric IgA receptors in HeLa and MDCK cells (Hales et al., 2002; Lapierre et al., 2001; Rodriguez and Cheney, 2002), and the chemokine receptor CXCR-2 in immune cells (Fan et al., 2004).

Members of the Rab family of small GTPases have recently emerged as potential mediators of vesicle transport by members of the myosin V family (Hammer and Wu, 2002; Langford, 2002; Seabra and Coudrier, 2004). In melanocytes, myosin Va associates with melanosomes through interaction with a receptor complex containing Rab27a and melanophilin/Slac2a (Bahadoran et al., 2001; Fukuda et al., 2002; Provance et al., 2002; Wu et al., 2001; Wu et al., 2002). Association of myosin Vb with Rab 11a has also been reported to regulate plasma membrane recycling (Lapierre et al., 2001). In contrast, association of myosin Vc with Rab8-positive endosomes has been involved in transferrin trafficking in HeLa cells (Rodriguez and Cheney, 2002). These observations indicate that coupling of individual members of the myosin V family to a particular subset of endosomal proteins may control the specificity of cargo transported by these motors.

Although these studies revealed some of the mechanisms underlying myosin V-mediated trafficking, the identity of cargo transported by these proteins in neurons remains unknown. Also unclear is whether various members of the myosin V family share similar mechanisms for delivering cargo to specific subcellular locations. In this study, we show that myosin Vb is
widely distributed in the brain and that it is expressed in several neuronal populations. In brain tissue, myosin Vb associates with the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-type glutamate receptor subunit GluR1. Expression of the tail region of myosin Vb reduced surface expression and clustering of GluR1. These observations correlated with a decrease in the frequency of excitatory currents. Using myosin V mutants lacking regions required for coupling to Rab11, we further report that the effects of myosin Vb on GluR1 require coupling to Rab11. Taken together, these results uncover a novel role for myosin Vb in trafficking of a specific subunit of the AMPA–type excitatory neurotransmitter receptors in neurons.

2.3 Material and methods

2.3.1 Antibody generation and immunohistochemistry

Myosin Vb expression in tissue sections was detected using affinity-purified rabbit polyclonal antibodies, raised against a GST fusion protein of the coiled-coil region (amino acids 895-1221) of rat Myosin Vb. Adult female Wistar rats were perfused with 4% paraformaldehyde (PFA), pH 7.4. Brain sections (15 µm) were incubated for 1 hour with 2 µg/ml anti-myosin Vb antibodies and the ABC technique (Elite ABC kit; Vector Laboratories) was used for detection.

2.3.2 Cloning and mutagenesis

The cDNA encoding rat brain myosin Va was a gift from Dr. P. Bridgman (Washington University, St. Louis). The truncated form of myosin Va (MyoVa CT) was generated by subcloning GFP in frame with amino acids 1005-1830 of myosin Va in pCDNA3.1 (Gibco-Invitrogen). The generation of full length rat myosin Vb construct fused to GFP was described earlier (Lapierre et al., 2001). The truncated form of myosin Vb (MyoVb CT) was generated by subcloning GFP in frame with amino acids 1221-1846 of myosin Vb into pCR3.1 vector (Gibco-Invitrogen). The truncated form of myosin Vb lacking the Rab11 interacting region was generated by deleting the last 49 amino acids at the C-terminus of the GFP-MyoVb CT (MyoVb CT ΔRab11; 1221-1797). Full-length myosin Vb lacking the Rab11 interacting region was generated by deleting 15 amino acids corresponding to 1797-1811 (MyoVb FL ΔRab11; 1-1796; 1812-1846) using QuickChange site-directed mutagenesis kit (Stratagene). The generation of hemagglutinin (HA)-tagged wild type GluR1 and flag-tagged full length BERP was previously
described (El-Husseini and Vincent, 1999; Man et al., 2000). The generated constructs were verified by sequencing.

### 2.3.3 Cell culture and transfections

Dissociated primary neuronal cultures were prepared from hippocampi of E18/E19 rats. Briefly, hippocampi were dissociated by papain enzymatic digestion. Cells were plated on poly-D-lysine (Sigma)-treated coverslips. Cultures were maintained in neurobasal media (Gibco-Invitrogen), supplemented with B27, penicillin, streptomycin, and L-Glutamine as described elsewhere (Brewer et al., 1993). COS-7 or HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) or Minimum Essential medium (MEM) (Gibco-Invitrogen) containing 10% fetal bovine serum, penicillin and streptomycin. PC12 cells were cultured in RPMI 1640 medium (Gibco-Invitrogen) containing 2 mM L-glutamine, penicillin and streptomycin. Transfections were performed using Lipofectamine 2000 (Gibco-Invitrogen) according to manufacturer’s protocol or calcium phosphate method (Clontech) as previously described (Jiang et al., 2004). Neurons were transfected at least three days prior to fixation and immunostaining. For COS-7, PC12 and HEK cells, immunostaining or biochemistry were performed 24 to 48 hours post-transfection.

### 2.3.4 Immunocytochemistry

Neurons on coverslips were fixed at room temperature for 10 min in 2% PFA or in -20°C methanol when staining for synaptic proteins. Cells were washed three times (1 minute each wash) with phosphate-buffered saline (PBS) containing 0.3% Triton-X-100 before and after each antibody incubation. The following primary antibodies were used (source and dilution as indicated): GluR1 (rabbit: 1:1000; Upstate Biotechnology), GluR2/3 (rabbit: 1:1000; Chemicon Int.), GABA (A) γ2 (rabbit: 1:2000; Alomone); Rab11 (rabbit: 1:500; Zymed Laboratories Inc.; mouse: 1:200; BD Transduction Lab), calnexin (rabbit: 1:200; Sigma); Rab5 (mouse: 1:200; BD Transduction Lab); NR1 (mouse: 1:1000; Synaptic Systems), GluR2 (mouse: 1:500; MAB397; Chemicon Int.), PSD-95 (mouse: 1:200; Affinity Bioreagents), synaptophysin (mouse: 1:500; Pharmingen), MAP-2 (mouse: 1:500; BD Pharmingen), GM130 (mouse, 1:200; Transduction Laboratories), HA (mouse: 1:1000; Upstate Biotechnology); acetylcholine receptor α4 (guinea
pig: 1:1000; Chemicon Int.), and GFP (guinea pig: 1:1000; custom made by Affinity Bioreagent). All antibody incubations were performed in blocking solution containing 2% normal goat serum for 1 hr at room temperature or overnight at 4°C. Cells were then incubated 1 hour at RT in blocking solution containing the appropriate Cy3 or Alexa conjugated secondary antibody (1:200; Jackson ImmunoResearch; 1:1000; Molecular Probes). Coverslips were then mounted on slides (Frost Plus; Fisher) with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). GluR1 surface labeling experiments were carried out using antibodies directed against an extracellular region of GluR1 under non-permeabilized conditions (rabbit: 1:15; Oncogene Research) as previously described (Swayze et al., 2004).

2.3.5 Imaging and analysis

Images of neurons and cell lines used in this study were taken using a 63X objective affixed to a Zeiss Axiovert M200 motorized microscope and AxioVision software. For analysis of total and surface cluster density (number), the images were analyzed in Northern Eclipse (Empix Imaging, Mississauga, Canada) by using custom written software routines as described elsewhere (Prange et al., 2004). Briefly, dendrites of cells of interest were manually outlined. Puncta were defined as sites of intensities at least 1.5 times the dendritic background. The average puncta number per dendrite length in transfected cells was compared to either GFP transfected or untransfected cells present in the same field. For changes in perinuclear accumulation in neuronal and non-neuronal cells, a minimum of 30 transfected cells per group were analyzed from at least 3 independent experiments. Image J 1.33u software (Wayne Rasband, NIH, USA) was used to represent graphically the fluorescence intensity patterns per µm. Statistical analysis were performed using Student-t-test.

2.3.6 Immunoprecipitation, western blotting and subcellular fractionation

For immunoprecipitation, whole brains from adult or postnatal day 16-19 Wistar rats were quickly removed. Brain tissue was homogenized in TEEN buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA, 150 mM NaCl) supplemented with 10 mM ATP and 10 mM MgCl2, 2.5 mM Na3Va4, 1mM phenylmethylsulfonyl fluoride (Sigma), and 1 protease inhibitor cocktail tablet/10ml (Roche). For myosin Vb immunoprecipitation, cells were lysed in TEEN by the addition of 0.1% SDS, 0.8% Triton X-100. For GluR1 immunoprecipitation, cells were lysed in
TEEN containing 0.5% deoxycholate and 1% NP-40. After rotation for 1 hr at 4°C, insoluble material was removed by centrifugation at 50 000 rpm for 30 min at 4°C. Samples were then incubated for 1 hr at 4°C with 5µg anti-myosin Vb or anti-GluR1 polyclonal antibodies, raised against GST fusion proteins of coiled-coil region of myosin Vb and carboxy tail of GluR1, respectively. After addition of 40 µl protein A sepharose 4 Fast Flow beads (Amersham), samples were incubated at 4°C for 1 hr or overnight. Immunoprecipitates were washed 3 times with TEEN buffer containing 1% Triton X-100. Samples were boiled in SDS-PAGE sample buffer with 10% β-mercaptoethanol for 3 min, and analyzed by SDS-PAGE. Western blots signals were detected with an Odyssey machine (Li-Cor) as previously described (Swayze et al., 2004) or ECL (Amersham Pharmacia Biotech). Co-immunoprecipitations from HEK-293 cells were performed in lysis buffer containing 1% Triton X-100 and samples were processed as described above. For subcellular fractionation, cerebral cortices from 7 adult rats were homogenized and fractionated as previously described (Fleisig et al., 2004). 10µg of each fraction was loaded on gel (P1, debris and nuclei; S1, postnuclear supernatant; P2, crude synaptosomal fraction; S2, small compartments; P3, microsomal pellet; S3, soluble protein fraction; LP1, synaptosomal membrane-enriched fraction; LS1, supernatant; LP2, synaptic vesicle-enriched fraction; LS2, presynaptic cytosol).

2.3.7 Electrophysiology

Recording of miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) were performed 3 days post-transfection. Hippocampal neurons on coverslips were transferred to a recording chamber continuously perfused with extracellular solution [pH 7.4; 320–330 milliosmolar (mosM)] containing 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 1.3 mM CaCl₂, 25 mM HEPES, 33 mM glucose, and 0.0005 mM tetrodotoxin (Alomone, Jerusalem). Transfected cells with GFP signal were identified under a fluorescent upright microscope. Intracellular solution (pH 7.2; 300–310 mOsm) was composed of 115 mM Cs gluconate, 17.5 mM CsCl, 10 mM HEPES, 2 mM MgCl₂, 10 mM EGTA, 4 mM ATP, 0.4 mM GTP, and 0.1% Lucifer yellow (Sigma-Aldrich). A MultiClamp 700A amplifier (Axon Instruments, Foster City, CA) was used for recording. Access resistance was monitored, and recordings where series resistance varied by >10% were rejected. No electronic compensation for series resistance was used. Whole-cell patch-clamp recordings were performed in voltage-clamp mode while maintaining the membrane potential either at the reversal potential for GABA (A)
receptor-mediated miniature PSCs (−60 mV) to isolate mEPSCs or at the reversal potential for ionotropic glutamate receptor-mediated miniature PSCs (+10 mV) to isolate mIPSCs. Recorded mEPSCs and mIPSCs were antagonized completely by the ionotropic glutamate receptor antagonist cyano-7-nitroquinoxaline-2, 3-dione (Sigma-Aldrich) and the GABA (A) receptor antagonist bicuculline (Sigma-Aldrich), respectively (*data not shown*). Recordings were low-pass-filtered at 2 kHz, sampled at 10 kHz, and stored in a computer by using CLAMPEX 8.0 (Axon Instruments).

2.4 Results

2.4.1 Myosin Vb localization in the brain

Previous *in situ* hybridization studies revealed that myosin Vb is present in the brain, however its exact subcellular distribution remained unknown (Zhao et al., 1996). To determine whether myosin Vb is expressed in neurons, we raised specific antibodies against the coiled-coil domain of myosin Vb, the region least conserved among members of the myosin V family (Bridgman, 2004). Western blot analysis confirmed that the generated antibodies specifically recognize the coiled-coil region of myosin Vb but not myosin Va (Figure 2.1A). A major band of ~ 200 kDa was detected in homogenates obtained from various brain regions of juvenile P17 (postnatal day 17) and adult rats (Figure 2.1B). Myosin Vb was mainly detected in neurons in the cortex, hippocampus, septum, striatum, midbrain and in specific neuronal subpopulations in the brain stem and cerebellum (Figure 2.1C, and *data not shown*). Intense myosin Vb immunoreactivity was also found in the posterior dentate gyrus of the hippocampus and pyramidal neurons in somatosensory cortex (Figure 2.1D). In both adult neurons in brain slices and hippocampal neurons in culture, myosin Vb showed a prominent punctate staining in the perinuclear region and dendrites (Figure 2.1D, Figure 2.2, and Figure 2.3). Myosin Vb staining was also more intense in GAD-65-positive interneurons relative to other neurons present in the hippocampus and cortex (*data not shown*).
Figure 2.1. Distribution of myosin Vb in the brain.

(A) Lysates from COS-7 cells expressing GFP fusion proteins of constructs containing the coiled-coil region of myosin Vb (MyoVb CC) or myosin Va (MyoVa CT) were analyzed by western blotting using antibodies against GFP (top panel) and antibodies raised against the coiled-coil region of myosin Vb (MyoVb) (middle panel). Myosin Vb antibodies specifically recognized MyoVb CC but not MyoVa CT and the signal was blocked using the fusion protein (immunogen; 5X) used for antibody preparation (bottom panel). (B) Western blot analysis of extracts obtained from various brain areas probed with myosin Vb (top panels) and actin (lower panels) antibodies. A major band migrating at ~200 kDa was detected in multiple brain regions in both young postnatal day 17 (P17) (left panel) and adult rats (right panel). (C) Immunohistochemical detection of myosin Vb protein in various regions of adult rat brain, including hippocampus, cortex and striatum. Immunostaining for myosin Vb (left panel) was blocked by myosin Vb immunogen (right panel). (D) Immunohistochemical analysis shows that myosin Vb is enriched in neurons in the posterior dentate gyrus (upper panels) and somatosensory cortex (lower panels). Enlarged boxed areas in D are shown to the right. Scale bars, 2 cm (C); 100 μm (D, left panels); 20 μm (D, right panels).
Figure 2.2. Distribution of endogenous myosin Vb in cultured hippocampal neurons varies with maturation stage.

Hippocampal neurons were double labeled using myosin Vb and the synaptic marker PSD-95 antibodies. In neurons at DIV 7 (left panels) and DIV 14 (middle panels), myosin Vb was mainly absent from sites positive for PSD-95 (arrowheads). In mature neurons at DIV 28 (right panels), myosin Vb was detected at sites positive for PSD-95 (arrowheads). Scale bar, 1 μm.

To further characterize the subcellular distribution of myosin Vb in neurons, we compared the localization of myosin Vb to the synaptic markers PSD-95 and synaptophysin at days in vitro (DIV) 7 and DIV 14, as well as in mature (DIV 21-28) neurons. In DIV 7-14 neurons, myosin Vb was present in the soma and dendrites but was mainly lacking from synaptic sites (Figure 2.2 and Figure 2.3). In DIV 21-28, myosin Vb was mainly observed in the soma. However, at this stage myosin Vb was also detected in dendritic spines, and at sites positive for synaptophysin and PSD-95 (Figure 2.2 and Figure 2.3). The enrichment of myosin Vb in the perinuclear region in both developing and adult neurons indicates that myosin Vb may participate in initial cargo transport from the soma to neuronal processes (Figure 2.3). Moreover, the modest localization of myosin Vb in dendritic spines in mature neurons suggests that myosin Vb may also regulate some aspects of local cargo delivery and/or recycling at the synapse.
Figure 2.3. Localization of myosin Vb in cultured neurons varies with maturation stage.

(A-B) Localization of myosin Vb in cultured hippocampal neurons: (A) in DIV 7 neurons myosin Vb shows a somato-dendritic distribution with punctate staining in the dendritic shaft and enrichment in the perinuclear region (white arrowheads). (B) In DIV 28 neurons, myosin Vb is enriched in the perinuclear region (white arrowheads), and is found in dendritic spines (black arrowheads). (C) Neurons were labeled with antibodies against myosin Vb and the presynaptic marker synaptophysin (Syn). In DIV 7 (left panels) and DIV 14 neurons (middle panels), myosin Vb was mainly absent from syn-positive sites (arrowheads). In DIV 28 neurons (right panels), myosin Vb was detected at sites positive for synaptophysin (arrowheads). (D) Distribution of GluR1 also varies with maturation stage. Double labeling of hippocampal neurons with GluR1 and Syn shows that GluR1 is weakly clustered and mainly lacking form synaptophysin-positive puncta at DIV 7 (left panels; arrowheads). At DIV 14, GluR1 is present in clusters that colocalize with synaptophysin (right panels; arrowheads). Scale bars, 10 µm (A and B); and 1 µm (C and D).
2.4.2 Expression of a mutant form of myosin Vb alters the distribution of specific glutamate receptor subunits

It is well established that the tail domains of myosin Va-Vc are critical for cargo transport (Bridgman, 2004; Karcher et al., 2002). Truncated forms expressing the tail domain of either myosin Va or myosin Vb accumulate in a perinuclear vesicular compartment that co-localizes with recycling endosomal markers and alter cargo transport in non-neuronal cells (Hales et al., 2002; Volpicelli et al., 2002; Wu et al., 1998b). To assess their role in neuronal protein trafficking, we transfected DIV 9 hippocampal neurons with GFP fusion constructs containing the tail domain of either myosin Va (MyoVa CT) (Bridgman, 1999) or myosin Vb (MyoVb CT) (Zhao et al., 1996). Three to four days post-transfection, neurons were fixed and the distribution of several neuronal proteins was assessed using immunocytochemistry. Remarkably, neurons expressing MyoVb CT but not MyoVa CT showed a striking perinuclear accumulation of the AMPA-type glutamate receptor subunit GluR1 (Figure 2.4, A-C). In contrast, no significant change in the accumulation of several other neurotransmitter receptors was observed, including the N-methyl-D-aspartic acid (NMDA)-type glutamate receptor subunits NR1 and NR2B, γ-amino butyric acid (GABA)(A) γ2, and the nicotinic acetylcholine receptor α4 (Figure 2.4, D-G, and J). Moreover, MyoVb CT did not alter the distribution of microtubule-associated protein MAP-2 or synaptophysin, suggesting a specific involvement of myosin Vb in GluR1 trafficking from a site emanating from the perinuclear region (Figure 2.4, H-J).
Figure 2.4. Altered distribution of the AMPA receptor subunit GluR1 in neurons expressing a mutant form of myosin Vb.

(A-I) Cultured neurons (DIV 9) were transfected with GFP mutant forms of myosin Vb (MyoVb CT) and myosin Va (MyoVa CT) fused to GFP. At DIV 12, neurons were stained for GluR1, NR1, α4 nicotinic acetylcholine receptor (α4 nAChR) and synaptophysin (Syn). Left panels show representative images of untransfected cells stained for endogenous GluR1 (A), NR1 (D), α4 nAChR (F), and Syn (H). MyoVb CT (B), but not MyoVa CT (C), enhanced GluR1 accumulation in a perinuclear region (arrowheads). No significant perinuclear accumulation of NR1 (E), α4 nAChR (G) and Syn (I) was observed (arrowheads). (J) Summary of changes in perinuclear accumulation of GluR1 and several other neuronal proteins examined (accumulation: +++, high; +/-, low; -, absent). Scale bar, 10 µm.

Consistent with previous studies, the truncated form of myosin Vb did not induce a gross rearrangement of internal organelles such as the Golgi and endoplasmic reticulum (ER) (Figure 2.5) (Lapierre et al., 2001; Rodriguez and Cheney, 2002). Moreover, the perinuclear compartment containing GluR1 and myosin Vb mutant did not colocalize with either the ER marker calnexin or with the Golgi marker GM130, indicating that the accumulation of GluR1 with MyoVb CT is not due to aberrant trapping of GluR1 in these compartments.
Figure 2.5. Overexpression of mutant myosin Vb does not alter localization of Golgi and ER resident proteins.

(A,D) Localization of endogenous Golgi resident protein GM130 and the endoplasmic reticulum (ER) marker calnexin in DIV 12 neurons. (B) Relative distribution of endogenous GluR1 and GM130. (C, E) neurons (DIV 9) were transfected with the tail region of myosin Vb (MyoVb CT) fused to GFP and stained at DIV 12 for GluR1 and GM130, or calnexin. MyoVb CT expression resulted in enhanced GluR1 accumulation in a perinuclear compartment that did not colocalize with GM130 (C) (arrowheads). Also, MyoVb CT did not alter the distribution of GM130 (C) or calnexin (E), suggesting an intact Golgi apparatus and ER compartment exist in these cells. At least 20 cells per group were analyzed. Scale bar, 5 µm.

Altered GluR1 localization suggests that myosin Vb exists in a protein complex containing GluR1. To assess this, myosin Vb was immunoprecipitated from brain tissue lysates and possible binding partners were probed for using western blotting. This analysis revealed that GluR1 co-immunoprecipitates with myosin Vb, however neither NR1 nor the presynaptic protein synaptotagmin I, were detected (Figure 2.6A). Conversely, myosin Vb was also detected in GluR1 immunoprecipitates (Figure 2.6B). To further characterize the relation between myosin Vb and GluR1 in developing hippocampal neurons, we next assessed GluR1 localization in DIV 7 and DIV 14 neurons at synaptic and non-synaptic sites (Figure 2.3D). GluR1 was weakly clustered at DIV 7 neurons. Moreover, synaptophysin-positive puncta were mainly lacking.
GluR1. This pattern resembles the distribution of myosin Vb in these young neurons. In DIV 14 neurons, GluR1 showed an accumulation at synaptic sites, which was more prominent than myosin Vb at the same age. However, at this stage significant amounts of both proteins were still detectable in the soma and the dendrites at non-synaptic sites. Subcellular fractionation analysis of extracts obtained from adult brain tissue revealed the presence of myosin Vb in crude synaptosomal membranes (P2) and synaptosomal membrane-enriched fraction (LP1), which are enriched in GluR1 (Figure 2.6C). This is consistent with the detection of both myosin Vb and GluR1 in the dendritic spines of mature (DIV 28) neurons. However, myosin Vb was not restricted to these fractions, and was also enriched in the soluble synaptosomal extracts (LS1 and LS2). This wide subcellular distribution resembles the one previously reported for myosin Va (Lee et al., 2001). These results suggest that in the adult brain, myosin Vb exists in a complex containing GluR1 in both synaptic and non-synaptic sites.

Surprisingly, expression of MyoVb CT had no effects on GluR1 localization in several heterologous expression systems tested, including COS-7 cells, HEK-293 cells and the neuronal-like cell line PC12 (Figure 2.7, and data not shown). The inability of MyoVb CT to disrupt GluR1 localization in these cell types suggests a lack of direct association.

**Figure 2.6. Myosin Vb exist in a complex containing GluR1.**

(A, B) Immunoprecipitation of myosin Vb and GluR1 from brain tissue extract using myosin Vb or GluR1 specific antibodies. (A) GluR1, but not NR1 or synaptotagmin I (Syt I) co-immunoprecipitated with myosin Vb (n=5 independent experiments). (B) Myosin Vb and GluR2, but not NR1, co-immunoprecipitated with GluR1 (n=3 independent experiments). (C) Cortices from adult rats were homogenized, fractionated by differential centrifugation, and analyzed by sequential immunoblotting for the indicated proteins. Myosin Vb is detected in crude synaptosomal membranes (P2) and synaptosomal membrane-enriched fraction (LP1), which are enriched in GluR1. Myosin Vb is also enriched in the soluble synaptosomal extracts (LS1 and LS2).
Figure 2.7. The effects of mutant myosin Vb on GluR1 trafficking is neuron-specific.

(A-H) COS-7 or PC12 cells were transfected with the tail region of myosin Vb (MyoVb CT) fused to GFP with or without GluR1. Cells were fixed 48 hours later and stained with Rab11 or GluR1 antibodies. A, C, E, and G show representative images of endogenous Rab11 (Rab endo) or transfected GluR1 distribution in COS-7 and PC12 cells in the absence of MyoVb CT. B and D, in both COS-7 cells (B) and PC12 cells (D), MyoVb CT expression induced Rab11 redistribution in a perinuclear compartment (arrowheads). (F–H) MyoVb CT did not trigger perinuclear accumulation of GluR1 in either COS-7 (F) or PC12 cells (H) (arrowheads). Scale bars: 5 µm.
To further assess this possibility, HEK-293 were co-transfected with either C-terminal tail or full-length myosin Vb fused to GFP and HA-tagged GluR1 or FLAG-tagged BERP, a known binding partner of myosin Vb. Consistent with previous studies, myosin Vb constructs co-immunoprecipitated BERP (Figure 2.8A) (El-Husseini and Vincent, 1999). However, GluR1 was absent from myosin Vb immunoprecipitates (Figure 2.8B). These results indicate that myosin Vb association with GluR1 may require an adaptor molecule exclusively expressed in neurons. Alternatively, differences in the vesicular machinery involved in sorting of GluR1 in heterologous cells and neurons may have contributed to the differential effects of mutant myosin Vb on GluR1.

Next, we analyzed whether enhanced perinuclear accumulation of GluR1 in neurons expressing mutant myosin Vb results in aberrant GluR1 trafficking and/or sorting to the plasma membrane. For this analysis, DIV 9 neurons were transfected with GFP or truncated forms of either myosin Va or Vb. Four days later, neurons were incubated with antibodies that recognize the extracellular domain of GluR1, fixed and incubated with a fluorescently conjugated secondary antibody under non-permeabilized conditions. Quantitative analysis of the number of GluR1

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**Figure 2.8. Myosin Vb does not directly associate with GluR1.**

HEK-293 cells were transfected with either the full-length (GFP MyoVb FL) or the C-terminal tail domain of myosin Vb (GFP MyoVb CT) fused to GFP, and either with HA-tagged GluR1 or Flag-tagged BERP. 24 hours later, cells were harvested and GFP antibodies were used to immunoprecipitates GFP-fused myosin Vb constructs. Left panels in A and B show the expression of the different constructs in starting lysates. Right panels in A and B show the co-immunoprecipitates using GFP, HA, or FLAG antibodies. Both GFP MyoVb CT (A) and GFP MyoVb FL (B) co-immunoprecipitated BERP, but not GluR1 (n=2 independent experiments).
Figure 2.9. Expression of a truncated form of myosin Vb reduces GluR1 surface expression.

DIV 9 neurons were transfected with either GFP, the C-terminal tail domain of myosin Vb (MyoVb CT) or myosin Va (MyoVa CT) fused to GFP. Four days later, cells were incubated with antibodies raised against the extracellular portion of GluR1, fixed and stained with fluorescently conjugated secondary antibodies without permeabilization. (A-C) Reduced amounts of surface GluR1 puncta in neurons expressing MyoVb CT but not in GFP transfected or untransfected cells. The boxed areas are shown enlarged below. (D) Summary of changes in GluR1 surface expression in cells transfected with GFP (n=10), MyoVb CT (n=10), or MyoVa CT (n=11). The dashed lines indicate 100% (untransfected control) levels. **p<0.01. Scale bars, 10 μm (full view images) and 1 μm (enlarged panels).
puncta per dendrite length in transfected cells compared to untransfected controls revealed reduced amounts of GluR1 (0.48 ± 0.05-fold) on the surface of neurons expressing MyoVb CT. In contrast, no change in GluR1 surface localization was observed in cells expressing either MyoVa CT (1.0 ± 0.2-fold) or GFP alone (1.0 ± 0.1-fold) (Figure 2.9). These results show that expression of the mutant form of myosin Vb in neurons hinders the delivery of GluR1 to the cell surface.

To further characterize the role of myosin Vb in AMPA receptor trafficking, we also analyzed whether altered surface expression of GluR1 results in reduced clustering of total GluR1 at the synapse. For this analysis, DIV 9 neurons were transfected with either full-length myosin Vb or the truncated forms of myosin Va and Vb. Cells were fixed 4 days later, permeabilized and stained for GluR1 and the synaptic marker synaptophysin (Syn). Quantitative analysis showed that expression of MyoVb CT but not full-length myosin Vb results in a 35% ± 6% decrease in the total number of GluR1 clusters (Figure 2.10, A-D). The reduction of GluR1 clustering at sites positive for synaptophysin indicates a reduction in GluR1 accumulation at synapses (Figure 2.10E). Expression of MyoVb CT, however, did not alter clustering of the NMDA receptor subunit NR1 (1.1 ± 0.2-fold) (Figure 2.10, F-G). Moreover, the effects on GluR1 clustering were specific to MyoVb CT since the expression of MyoVa CT had no significant effect on the clustering of either GluR1 or NR1 (0.92 ± 0.07-fold and 0.95 ± 0.07-fold, respectively) (Figure 2.10, D and G).
Figure 2.10. Reduced total GluR1 clustering in neurons expressing a mutant form of myosin Vb.

Cultured neurons (DIV 9) were transfected with either the full-length (MyoVb FL) or the C-terminal tail domain of myosin Vb (MyoVb CT) or myosin Va (MyoVa CT) fused to GFP. At DIV12-13, neurons were stained for GluR1, NR1 or synaptophysin (Syn). (A-C) Compared to untransfected cells (A), expression of MyoVb CT (C) but not MyoVb FL (B) drastically reduced GluR1 clustering. (D) Summary of changes in GluR1 puncta density with different constructs (MyoVb FL, n=14; MyoVb CT, n=24; MyoVa CT, n=12). (E) MyoVb CT expression decreased the number of Syn-positive GluR1 puncta (arrowheads). (F-G) NR1 clustering was unaffected by MyoVb CT or MyoVa CT expression (n=17 and 10, respectively). ***p<0.001. Scale bars, 10 µm (A-C) and 1 µm (E, F).
To examine the functional correlates of the immunocytochemical changes observed upon expression of mutant myosin Vb, an electrophysiological approach was taken. DIV 9 neurons were transfected with either MyoVb CT or GFP. After 3 days of expression, changes in the frequency of tetrodotoxin-insensitive miniature excitatory (mEPSCs) and inhibitory (mIPSC) postsynaptic currents were compared using whole cell voltage clamp recordings. Consistent with the reduced clustering of GluR1, expression of MyoVb CT significantly reduced mEPSC frequency (0.52 ± 0.09-fold), without affecting the mIPSC (0.8 ± 0.2-fold) (Figure 2.11). The reduced frequency of excitatory but not inhibitory currents may reflect an increase in the number of silent excitatory synapses due to the preferential loss of AMPA receptors at existing synapses.

Figure 2.11. Reduced GluR1 clustering and basal excitatory synaptic transmission in neurons expressing a mutant form of myosin Vb.

Spontaneous mEPSCs and mIPSCs were recorded in voltage clamp mode at holding potential of −60mV and +10mV, respectively. Representative traces of mEPSCs (A, upper panels) and mIPSCs (B, upper panels) recorded from neurons transfected with GFP (control) or MyoVb CT. Frequency of mEPSC frequency, but not mIPSC, was decreased in hippocampal cells expressing MyoVb CT. **p<0.01.
2.4.3 Myosin Vb modulates trafficking of a specific subunit of AMPARs

Previous studies showed that specific interacting proteins differentially regulate trafficking of AMPA receptor subunits GluR1 and GluR2 (Bredt and Nicoll, 2003; Kim and Sheng, 2004; Lee et al., 2004; Passafaro et al., 2001; Shi et al., 2001). To explore whether myosin Vb regulates the localization of various AMPA receptor subunits, we stained transfected hippocampal neurons with antibodies against GluR1 and GluR2/3. Remarkably, MyoVb CT did not induce GluR2/3 redistribution to the perinuclear region, which contains MyoVb CT and GluR1 (Figure 2.12, A-D). The pattern of fluorescence intensity of GluR1 in the perinuclear region highly correlated with MyoVb CT (Figure 2.12B, right panel). In contrast, there was no correlation between MyoVb CT signal and GluR2/3 fluorescence intensity (Figure 2.12D, right panel). Consistent with the lack of change in GluR2 distribution in the soma, no significant change in the total number of GluR2 puncta (1.1 ± 0.1-fold) was observed in neurons expressing MyoVb CT (Figure 2.12, E and F). Moreover, GluR2 was not detected in myosin Vb immunoprecipitates obtained from both juvenile (P17) and adult rat brain extracts (Figure 2.12, G and H). These results are surprising since the majority of GluR1 are thought to exist as heteromers. This suggests that myosin Vb may regulate the transport of a specific pool of GluR1 containing homomeric AMPA receptors.
Figure 2.12. Differential effects of mutant myosin Vb on the clustering of specific AMPAR subunits. (A-F) Cultured neurons (DIV 9) were transfected with MyoVb CT and stained 3-4 days later for GluR1, GluR2/3 and GluR2 subunits of AMPARs. (A, C) Representative images of untransfected cells stained for endogenous GluR1 or GluR2/3. MyoVb CT expression triggered GluR1 (B), but not GluR2/3 (D), redistribution into a perinuclear compartment (arrowheads). (B, D) Right panels show relative fluorescent intensities of GFP signal and GluR1 staining calculated by drawing a line crossing the perinuclear region where MyoVb CT accumulates. (E) Quantitative analysis of GluR1 and GluR2 puncta density upon expression of different constructs (GluR1: MyoVb CT, n=24; GluR2: MyoVb CT, n=18; GFP, n=9). The dashed lines indicate 100% (untransfected control) levels. (G, H) Co-immunoprecipitation of GluR1 and myosin Vb from brain tissue extracts using myosin Vb specific antibodies. GluR1, but not GluR2 or synaptotagmin (Syt I), co-immunoprecipitates with myosin Vb from P17 (G) or adult rat brain lysates (H). These results are representative of at least 5 independent experiments. ***p<0.001. Scale bars, 10 µm (A-D) and 1 µm (E).
2.4.4 Myosin Vb-mediated effects on GluR1 involves coupling to Rab11

Recent studies in non-neuronal cells showed that myosin Vb associates with the recycling endosome protein Rab11, and that this protein is involved myosin Vb-mediated cargo transport (Hales et al., 2001; Hales et al., 2002; Lapierre et al., 2001; Volpicelli et al., 2002; Wakabayashi et al., 2005b). In neurons, endogenous myosin Vb and Rab11 show partially overlapping colocalization in puncta present in the soma and dendrites (Figure 2.13).

Thus, a plausible mechanism for vesicular transport of GluR1 in neurons may involve coupling of myosin Vb to Rab11. Consistent with previous studies, expression of the tail region of myosin Vb resulted in enhanced perinuclear accumulation of endogenous Rab11 in COS-7 cells, PC12 cells and in cultured neurons (Figure 2.7 and Figure 2.15, A and B). In contrast, no change in the distribution of early endosome associated proteins, including Rab5, and the early-endosome-associated protein 1 (EEA1) was observed upon MyoVb CT expression (Figure 2.15, C and D, and data not shown).
Figure 2.13. Colocalization of endogenous myosin Vb and Rab11 in hippocampal neurons.

Staining of myosin Vb and Rab11 shows a partial overlap in the perinuclear region and in dendrites. Enlarged boxed area is shown below. This analysis reveals two populations of myosin Vb- and Rab11-positive structures. White arrows point to examples of Rab11-positive structures surrounded by myosin Vb-positive puncta. Black arrows point to examples of overlapping Rab11- and myosin Vb positive puncta. Scale bars, 10 μm (full view image) and 1 μm (enlarged panels).

To further address the involvement of Rab11 in myosin Vb-mediated cargo transport, we tested whether coupling of myosin Vb to Rab11 is required for GluR1 trafficking. For this analysis we first generated a MyoVb CT mutant (MyoVb CT ΔRab11) consisting of the C-terminal globular tail but lacking amino acids 1797-1846, which contains the region required for interaction with Rab11 (Lapierre et al., 2001). This construct is expected to neither associate with actin nor with Rab11. In transfected neurons, MyoVb CT ΔRab11 was detected in the soma and dendrites and
was not restricted to the perinuclear region. Overexpression of this mutant form did not alter the localization of endogenous Rab11 when expressed in COS-7 cells or in neurons (Figure 2.14), nor did it result in any significant change in GluR1 clustering (0.90 ± 0.08-fold) (Figure 2.15, F and I).

We also examined whether expression of a full-length version of myosin Vb lacking the region required for interaction with Rab11 (amino acids 1797-1811; MyoVb FL ΔRab11) (Lapierre et al., 2001), interferes with GluR1 trafficking (Lapierre et al., 2001). Since this mutant contains the actin binding motor domain, it is expected to associate and move along actin filaments, however because it lacks the Rab11 binding site, such a mutant may interfere with cargo transport that requires coupling of Rab11 to myosin Vb. Unlike truncated form of myosin Vb, this mutant was detected in both the soma and dendrites of transfected neurons and its localization was not restricted to the perinuclear region. Neurons transfected with MyoVb FL ΔRab11 exhibited a decrease in both total GluR1 clustering (0.68 ± 0.05 fold) and surface expression (0.73 ± 0.05 fold) (Figure 2.15, H and I, Figure 2.16, C and D). These results support the notion that coupling of myosin Vb to Rab11 is involved in GluR1 trafficking.

Finally, to directly assess the role of Rab11 in this process, we expressed a DN form of Rab11 (Rab11-S25N) in DIV 8-9 neurons and analyzed its effect on GluR1 and myosin Vb localization. Overexpression of Rab11-S25N in hippocampal neurons moderately enhanced perinuclear accumulation of GluR1 and myosin Vb (Figure 2.16).
Figure 2.14. Effects of myosin Vb mutant lacking Rab11 binding region on endogenous Rab11 distribution.

COS-7 cells or neurons were transfected with the tail region of myosin Vb that lacks the Rab11 binding region (MyoVb CT ΔRab11) fused to GFP. Cells were fixed 24 to 72 hrs later, and stained for endogenous Rab11. (A, C) Representative images of endogenous Rab11 (Rab endo) distribution in untransfected COS-7 and neuronal cells. Expression of MyoVb CT ΔRab11 failed to redistribute endogenous Rab11 in COS-7 cells (B) (arrowheads) or in neurons (D). Scale bars: 5 µm.
Figure 2.15. Myosin Vb mediated effects on GluR1 involves coupling to Rab11.

Cultured neurons (DIV 9) were transfected with the tail region of myosin Vb (MyoVb CT), MyoVb CT lacking the Rab11 binding region (amino acids 1221-1797; MyoVb CT ΔRab11), myosin Vb full-length (MyoVb FL) or myosin Vb full-length lacking the Rab11 binding region (amino acids 1796-1812; MyoVb FL ΔRab11), and stained with antibodies against Rab11, Rab5 or GluR1. (A, C) Representative images of untransfected cells stained for endogenous Rab11 (Rab11 endo) or Rab5 (Rab5 endo). MyoVb CT induced redistribution of Rab11 (B) but not Rab5 (D) in a perinuclear region (arrowheads). Expression of MyoVb CT (E) but not MyoVb CT ΔRab11 (F) altered GluR1 localization. Expression of MyoVb FL (G) did not alter GluR1 localization, while expression of MyoVb FL ΔRab11 (H) decreased GluR1 clustering. (I) Graph shows quantitative analysis of GluR1 puncta density in cells expressing MyoVb FL (n=14), MyoVb FL ΔRab11 (n=22), MyoVb CT (n=24), MyoVb CT ΔRab11 (n=23). The dashed lines indicate 100% (untransfected controls). **p<0.01; ***p<0.001. Scale bars, 5 µm.
Figure 2.16. Expression of myosin Vb and Rab11 mutants reduces surface expression of GluR1.

Cultured neurons (DIV 9) were transfected with full-length myosin Vb (MyoVb FL), myosin Vb lacking the Rab11 binding region (MyoVb FL ΔRab11), or a DN form of Rab11 (Rab11-S25N) fused to GFP. At DIV13, cells were incubated with antibodies raised against the extracellular portion of GluR1, fixed and stained with fluorescently conjugated secondary antibodies without permeabilization. (A-C) Reduced amounts of surface GluR1 puncta in neurons expressing MyoVb FL ΔRab11 but not MyoVb FL, when compared to untransfected cells. (D) Expression of Rab11-S25N also reduces surface expression of GluR1. Graph shows summary of changes in GluR1 surface expression in cells transfected with MyoVb FL (n=18), MyoVb FL ΔRab11 (n=22), or Rab11-S25N (n=20). The dashed lines indicate 100% (untransfected controls) levels. **, p<0.01; ***p<0.001. Scale bar, 5 μm.
Figure 2.17. Expression of a mutant form of Rab11 alters myosin Vb and GluR1 distribution in neurons.

Cultured neurons (DIV 9) were transfected with a DN form of Rab11 (Rab11-S25N) or MyoVb CT. At DIV 13, neurons were stained for myosin Vb (MyoVb endo) and GluR1. (A, B) Representative images of untransfected cells stained for endogenous GluR1 (A) and myosin Vb (B). Expression of Rab11-S25N results in redistribution of myosin Vb (C) and GluR1 in the perinuclear region (D) (arrowheads). However the effect of Rab11-S25N on GluR1 in the perinuclear region was less dramatic when compared to MyoVb CT. (E) (arrowheads). Scale bar: 5 µm.
Further analysis revealed that expression of Rab11-S25N results in a significant decrease in surface expression of GluR1 (0.71 ± 0.08 fold) (Figure 2.16D). This suggests that blocking Rab11 function interferes with recycling of GluR1 in an endocytic compartment in the soma/dendrites and hampers its reinsertion at the plasma membrane. Consistent with this, recent findings showed that Rab11 controls recycling of AMPARs (Park et al., 2004). Taken together, the findings presented here provide further evidence that both myosin Vb and Rab11 cooperate in the regulation of GluR1 trafficking.

2.5 Discussion

In this study, we have investigated the involvement of myosin V family members in the trafficking of a subset of neuronal proteins. We elucidate a selective role for myosin Vb in the trafficking of GluR1, an AMPA-type glutamate receptor subunit important for synaptic plasticity (Bredt and Nicoll, 2003; Malenka, 2003). Moreover, we show that in neurons, this effect requires association with the vesicular recycling protein Rab11. Since synaptic efficacy is modulated by AMPAR trafficking, our findings provide a new mechanism for the modulation of synaptic transmission mediated by a member of the myosin V family.

Our analysis shows that myosin Vb is present in several neuronal populations in the brain. In neurons, the enrichment of endogenous myosin Vb in the perinuclear region suggests that myosin Vb is involved in regulating constitutive transport of cargo from a vesicular compartment in the soma. Notably, myosin Vb staining was absent from synaptic sites in young hippocampal neurons but was present in spines in mature neurons, suggesting that myosin Vb may also mediate cargo transport at synaptic sites at later stages of neuronal development. The presence of myosin Vb at dendritic spines of adult neurons, sites where myosin Va is also enriched (Takagishi et al., 1996; Walikonis et al., 2000), suggests that both motor proteins may control some aspects of glutamate receptor trafficking at the synapse.

Previous studies implicated myosin Va in glutamate receptor trafficking. Myosin Va associates with a complex containing DLC and the scaffolding protein GKAP, which associates with PSD-95 (Kim et al., 1997; Naisbitt et al., 2000). In turn, coupling of PSD-95 and AMPAR through members of the stargazin family of transmembrane proteins suggests a role for a protein complex
containing myosin Va/DLC/GKAP/PSD-95 in regulating glutamate receptor trafficking (Chen et al., 2000; Kim and Sheng, 2004). However, in this study we show that expression of a mutant form of myosin Va does not alter GluR1 trafficking. A lack of change in glutamate receptor clustering and localization has been also reported in myosin Va mutant mice (Petralia et al., 2001; Schnell and Nicoll, 2001). Taken together, these findings indicate that myosin Va may not be essential for regulating glutamate receptor trafficking. The differential effects of myosin V subtypes on GluR1 trafficking is reminiscent of the selective disruption of transferrin receptor recycling in HeLa cells by a mutant form of myosin Vb but not myosin Va (Lapierre et al., 2001). It is possible that coupling to specific endosomal proteins, possibly various members of the Rab family, regulates the type of cargo transported by these motors.

AMPAR subunits are thought to be synthesized and assembled in the ER and directed to the Golgi for subsequent post-translational modifications (Vandenberghe and Bredt, 2004). A surprising finding from our analysis is the effect of mutant myosin Vb on the clustering of the AMPAR subunit GluR1, but not GluR2. Also, GluR2 was absent from myosin Vb immunoprecipitates. This suggests that myosin Vb preferentially regulates a specific vesicular pool containing GluR1 but not GluR2. Biochemical analysis of AMPAR complexes from CA1/CA2 hippocampal pyramidal neurons revealed that about 8% of the total AMPAR complexes contains homomeric GluR1 (Wenthold et al., 1996). Moreover, recent evidence suggests the existence of a pool of homomeric GluR1 in cultured hippocampal neurons (Thiagarajan et al., 2005). However, considering that only a small amount of GluR1 is expected to be homomeric in pyramidal hippocampal neurons, it was surprising to see a noticeable change in the localization of AMPARs. Taking into consideration that changes in AMPAR localization were analyzed 3-4 days after expression of mutant forms of myosin Vb, this may have accounted for the observed accumulation of GluR1 in the perinuclear region and its reduced surface expression. The partial reduction rather than total loss of GluR1 from the cell surface is consistent with this notion. Another possibility is that myosin Vb was selectively immunoprecipitated from a neuronal population containing homomeric GluR1. Indeed, the cortex has a large population of interneurons expressing homomeric GluR1. Although it is unlikely that we have selectively immunoprecipitated myosin Vb from a specific neuronal population mainly containing homomeric GluR1, this possibility cannot be excluded.
Trafficking of AMPARs is regulated through interaction of different subunits with a variety of cytoplasmic proteins and members of the transmembrane AMPAR regulatory protein (TARP) family (Bredt and Nicoll, 2003; Fukata et al., 2005; Kim and Sheng, 2004; Malenka, 2003). Thus, it is possible that association with some of these molecules may explain the selective effects of myosin Vb on specific subunits of AMPARs; however, further studies are needed to explore this possibility. Our observations imply the existence of an alternative mechanism to transport GluR2-containing AMPARs. In dendrites, a complex formed of the GluR2-interacting protein GRIP-1 and KIF5, a member of the kinesin superfamily, has been implicated in the transport of AMPARs along microtubules (Setou et al., 2002). KIF17 is another member of the kinesin family that has been shown to regulate transport of a protein complex containing the NR2B subunit of NMDA receptor (Guillaud et al., 2003; Setou et al., 2000). From these findings, it is evident that multiple mechanisms control the trafficking of various subunits of glutamate receptors from the soma to the synapse. In the future, it will be important to determine whether actin- and microtubule-dependent AMPAR transport involves cargo exchange between myosin Vb and kinesin motors. Myosin VI is another unconventional myosin that is enriched at the PSD and has been implicated in AMPAR trafficking (Osterweil et al., 2005). Specifically, myosin VI has been shown to control insulin-induced AMPAR endocytosis. Further studies are required to assess whether myosin VI and V family members act in concert to regulate insertion/removal of AMPARs.

Several lines of evidence presented in this study suggest that coupling of myosin Vb to Rab11 is involved in GluR1 transport. First, expression of a truncated form of myosin Vb lacking the N-terminal motor domain but containing the globular tail domain, interferes with both Rab11 and GluR1 trafficking. However, further deletion of the C-terminal region that encompasses the Rab11 binding site eliminates the DN effects of this mutant on both Rab11 and GluR1. Second, results obtained from expression of full-length myosin Vb lacking amino acids required for binding to Rab11 further support this notion. This mutant contains the actin binding motor domain, and thus it is expected to associate and move on actin filaments. Still, the failure of this mutant to associate with Rab11 may interfere with myosin Vb-associated cargo transport. Indeed, expression of this mutant resulted in a significant decrease in GluR1 clustering and surface expression. Third, expression of a DN form of Rab11 (Rab11-S25N) redistributes GluR1 and myosin Vb in the soma and reduces surface expression of GluR1.
However, it remains unclear why myosin Vb induced changes in GluR1 localization were only restricted to neuronal cells. This result was unexpected, since Rab11 localization was altered in both neuronal and non-neuronal cells. It is possible that GluR1 is differentially sorted to various populations of transport vesicles, and that coupling to Rab11-positive endosomes only occurs in neurons. Nevertheless, we were unable to detect Rab11 in immunoprecipitates of both GluR1 and myosin Vb obtained from brain extracts (not shown). Taken together, these results suggest that interaction of myosin Vb with GluR1 is mediated by a neuron-specific adaptor protein rather than through direct association with Rab11. The presence of GluR1 in myosin Vb immunoprecipitates derived from brain lysates, but not from heterologous expression systems, lends further support to this concept. In neurons, putative adaptor proteins may include PDZ-containing proteins, which have been implicated in sorting of glutamate receptors (Braithwaite et al., 2000; Kim and Sheng, 2004; Sans et al., 2003; Setou et al., 2002; Wenthold et al., 2003). Coupling of myosin Vb to a neuronal protein complex containing the endosome-associated protein hrs, actinin-4 and BERP may have also contributed to the specific modulation of GluR1 trafficking observed in neurons (Yan et al., 2005). Despite the lack of a clear mechanism on how GluR1 is coupled to myosin Vb and Rab11, we believe that the substantial evidence presented here demonstrates that the link between myosin Vb and Rab11 is critical for GluR1 trafficking.

In summary, our data reveal a selective action of myosin Vb on the trafficking of specific neuronal proteins. However, it should be noted that only a subset of proteins have been examined in this study and thus, one cannot exclude the possibility that myosin Vb regulates trafficking of many other neuronal proteins. Further work will be required to define other cargoes transported by this motor in neurons. Nevertheless, the robust effects observed on GluR1 localization indicate that this motor is critical for AMPAR trafficking.
2.6 References


CHAPTER 3

3.1 Novel myosin Va interacting protein, RILPL2, activates Rac1 and controls cell shape and neuronal morphogenesis

3.2 Introduction

Throughout development, cells differentiate and undergo cytoskeletal and membrane rearrangements to establish a distinct morphology, specific for their physiological function. One of the most complex morphologies is exhibited by neurons, which typically extends one long and thin axon housing the molecular machinery for releasing signals for neuronal transmission, as well as numerous, relatively shorter dendrites containing neurotransmitter receptors for receiving signals. These dendrites are thicker in diameter and are the processes from where dendritic filopodia and spines emerge. Filopodia, thought to be immature spines, may serve an exploratory role to identify presynaptic partners. Dendritic spines are actin-rich protrusions containing the postsynaptic density (PSD), where many important synaptic proteins are found (Tada and Sheng, 2006). These structures have been implicated in the structure, function and plasticity of glutamatergic synapses (Harris and Kater, 1994; Matus, 2000; Yuste and Bonhoeffer, 2004). The establishment and maintenance of neuronal morphology is intimately linked to the actin cytoskeleton, which has the unique capacity to function as a stable structural component or as a dynamic filament. However, despite intense research, the mechanisms that control actin dynamic in neurons have yet to be fully understood.

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Members of the Rho family of small GTPases, including Rac1, Cdc42 and RhoA, are important regulators of the actin cytoskeleton (Hall, 1998; Hall and Nobes, 2000). In neurons, Rho GTPases play a pivotal role in various developmental and maintenance processes, including cell migration, establishment of cell polarity, axon growth and guidance, dendritic growth and maintenance, as well as in the formation and plasticity of dendritic spines and synapses (Arimura and Kaibuchi, 2007; Linseman and Loucks, 2008; Luo, 2002; Newey et al., 2005; Tada and Sheng, 2006). A growing body of evidence indicates that the small GTPase Rac is particularly critical for dendritic spine formation, maintenance and structural plasticity (de Curtis, 2008; Penzes et al., 2008). For instance, overexpression of Rac1 in young dissociated hippocampal neurons is sufficient to induce the formation of spines (Wiens et al., 2005), whereas expression of a dominant-negative (DN) Rac in rat hippocampal slice culture results in a progressive elimination of dendritic spines (Nakayama et al., 2000). The activity of small GTPases such as Rac is directly modulated by guanine-nucleotide exchange factors (GEFs), that promote the formation of active GTP-bound Rac and GTPase-activating proteins (GAPs) that promote the hydrolysis of GTP and increase levels of inactive, GDP-bound Rac (Van Aelst and Cline, 2004). Following its activation, GTP-bound Rac activates downstream targets that are involved in spine morphogenesis, such as the p-21 activated kinase (Pak), which regulates dendritic spine density and length by regulating effectors of actin polymerization (Bagrodia and Cerione, 1999; Hayashi et al., 2007; Hayashi et al., 2004).

Besides its critical role in dendritic spine morphogenesis and structural plasticity (Carlisle and Kennedy, 2005; Cingolani and Goda, 2008; Luo, 2002; Matus, 2000), the actin cytoskeleton also acts as a track for transport mediated by actin-based motor protein of the myosin family. Recent studies have implicated myosin V isoforms in trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-type glutamate receptors and in the delivery of mRNA/protein complexes within dendrites and dendritic spines in hippocampal neurons (Correia et al., 2008; Lise et al., 2006; Wang et al., 2008; Yoshimura et al., 2006). These studies suggest a critical role for myosin V in trafficking events required for proper neuronal function, however, the identity of neuronal cargoes transported by myosin V family members remains largely unknown. Also unclear is whether myosin V, in addition to its role in local transport events within the dendritic spine compartment, plays a role in the regulation of the actin cytoskeleton during neuronal development and maturation.
In this study, we identify RILP-like protein 2 (RILPL2) as a novel myosin Va-interacting protein. RILPL2 is related to the Rab-lysosomal interacting protein (RILP) (Wang et al., 2004b), a protein that regulates morphology of the lysosomal and late endosomal compartments via interactions with the small GTPase Rab7, and the microtubule-based motor dynein (Cantalupo et al., 2001; Jordens et al., 2001; Progida et al., 2007). In contrast to RILP, RILPL2 lacks the Rab7-interacting region, and ectopic expression of RILPL2 failed to alter lysosomal compartment morphology (Wang et al., 2004b). To date, the cellular function of RILPL2 remained unknown.

In this study, we show that RILPL2 is expressed in the developing and adult brain. Ectopic expression of RILPL2 in non-neuronal cells resulted in a change in cell shape, as well as activation of the Rho GTPase Rac. Both morphological changes and Rac activation induced by RILPL2 were blocked by the expression of a DN form of myosin Va. In cultured dissociated hippocampal neurons, RILPL2 expression increased the density of spine-like protrusions, as well as phosphorylation of the Rac effector Pak. In contrast, short interfering RNA (shRNA)-mediated knockdown of RILPL2 blocked dendritic spine development, an effect that was rescued by expression of a shRNA-resistant form of RILPL2. While early expression of RILPL2 in young neurons specifically reduced axonal growth, the presence of RILPL2 appeared not to be critical for neuritogenesis at this stage. These findings uncover a novel role for RILPL2 in the regulation of spine morphogenesis during development by controlling the actin cytoskeleton by acting, at least in part, through Rac-Pak signaling pathway.

### 3.3 Material and methods

#### 3.3.1 cDNA Cloning

The generation of a GFP-tagged version of rat brain myosin Va (MyoVa CT; aa 1005-1830) and myosin Vb (MyoVb CT; aa 1221-1846) has been described elsewhere (Lise et al., 2006). Truncated versions of myosin Va corresponding to the medial (MyoVa MT; aa 1152-1395) and globular (MyoVa GT; aa 1396-1830) tails were obtained by PCR with specific primers and subcloned into pEGFPC1 (Clonetech). Full-length mouse RILPL2 cDNA (aa 1-197) was obtained from whole brain tissue by RT-PCR using specific primers and subcloned into a N-HA-GW1 vector (Sala et al., 2001) containing a CMV promoter for expression in mammalian cells. Truncated forms of RILPL2 (RILPL2 ΔCT; aa 1-113 and RILPL2 ΔNT; aa 114-197) were
obtained by PCR amplification from full-length cDNA and subcloned into N-HA-GW1. For pull down assays, RILPL2 was amplified by PCR with specific primers and subcloned in frame with glutathione-S-transferase (GST) into pGEX4T-1 (GE Healthcare). For RNA interference experiments, short hairpin RNA (shRNA) vectors were constructed using pSUPER (OligoEngine) or a modified version of pSUPER, pSUPERneo+GFP (OligoEngine), in which a short interfering RNA and GFP are dually expressed under the H1 and the PGK promoter, respectively. To create RILPL2 shRNA 493 and 496, two complementary 60 bp oligonucleotides containing the sense and antisense sequences for AGAGAGAAAGACGCTATGG (19 bp, corresponding to nucleotides 768-786 of rat RILPL2) and for GAGAAAGACGCTATGGTTA (corresponding to nucleotides 771-789 of rat RILPL2) were annealed and ligated to pSUPER vector in accordance with OligoEngine’s instructions. For the rescue experiments, we used a vector expressing the mouse cDNA version of RILPL2 (HA-RILPL2-res). All DNA constructs were confirmed by DNA sequencing.

3.3.2 Yeast two-hybrid analysis

Brain myosin Va C-terminal tail (aa 1005-1830) was subcloned into pGBKT7 (GAL4 DNA binding domain (DB) vector) and used to screen an adult rat brain cDNA library subcloned into pGADT7 (GAL4 activation domain (AD) vector) (MATCHMAKER system; Clonetech, Palo Alto, CA). Three independent clones corresponding to RILPL2 (aa 7-197) were obtained from our screen. Full-length rat RILPL2 cDNA (aa 1-197) was isolated from whole brain tissue by RT-PCR using specific primers and subcloned into pGBKT7. For assays of specificity and binding domains, desired cDNA fragments were amplified by PCR with specific primers and subcloned into pGBKT7 and pGADT7. These fusion proteins were tested for interaction using HIS3 and β-gal as reporter genes and induction levels were semi-quantified as previously described (Kim et al., 1995)

3.3.3 RT-PCR analysis

RNA from embryonic day 18 (E18), adult rat tissues and cultured cortical neurons was isolated using the RNeasy Mini Kit (Quiagen) following the manufacturers protocol. We used 1 µg of
RNA for each reverse transcription reaction with oligo (dt) primers (Invitrogen). The RT reaction was followed by PCR amplification using specific primers for rat RILPL2 (forward 5’-ATGGAGGAGCCCCCAGTACGG -3’ and reverse 5’-CGGTCAGGGAAGCACACCTAG-3’) and β-actin (forward 5’-AGCCATGTACGTAGCCATCC-3’ and reverse 5’-TTCACCACCACAGCTGAGAG-3’) as a control.

3.3.4 In situ hybridization

*In situ* hybridization was conducted as previously described (de Lecea et al., 1997), with some modifications. E18 rat brains were perfused with 4% PFA, and cryoprotected in 30% sucrose in 4% PFA. Brains were then embedded in grade IV-V bovine albumin (Fisher Scientific), cut into blocks and further cryoprotected in the sucrose/PFA solution prior to sectioning on a Leica tabletop cryostat microtome. Rat full length RILPL2 cDNA was subcloned into pSPT-19 between EcoRI and XmaI. Probes were synthesized with the Dig-RNA labeling kit (Roche). Hybridization with 1µg/ml digoxigenin-labeled RILPL2 probes proceeded at 65°C in a solution of 50% deionized formamide, 10% dextran sulphate, 5x Denhardt’s solution, 0.62M NaCl, 10mM EDTA, 20mM PIPES-Na, 0.2% SDS, 250µg/ml heat-denatured salmon sperm DNA and 250µg/ml heat-denatured yeast tRNA.

3.3.5 Cell culture and transfections

Dissociated primary neuronal cultures were prepared from hippocampi or cortices of E18/E19 rats. Briefly, the brain regions were dissociated by papain enzymatic digestion. Cells were cultured on poly-D-lysine (Sigma)-treated coverslips at a density of 125,000-150,000 cells per coverslip (24 well plates). Cultures were maintained in neurobasal media (Gibco-Invitrogen), supplemented with B27, penicillin, streptomycin, and L-glutamine as described elsewhere (Brewer et al., 1993). COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-Invitrogen) containing 10% fetal bovine serum, penicillin and streptomycin. Transfections were performed using Lipofectamine 2000 (Gibco-Invitrogen) according to manufacturer’s protocol. Neurons were transfected at least 3 days prior to fixation and immunostaining. Experiments using COS-7 cells were performed 24 to 72 hours post-transfection.
For experiments in young neurons, freshly dissociated cells (DIV0) were electroporated using the AMAXA system following manufacturer’s protocol. Neurons were then plated at a density of 300,000 cells per coverslips and grown for 3 days prior to fixation and immunostaining.

### 3.3.6 Immunofluorescence

Cells were fixed at room temperature with 4% paraformaldehyde/4% sucrose for 10 minutes, then washed 3 times with phosphate-buffered saline (PBS) containing 0.3% Triton-X-100 before and after each antibody incubation. The following primary antibodies were used (source and dilution as indicated): GFP (chicken: 1:1000; AbCam; rabbit 1:1000; Synaptic systems), HA (mouse: 1:1000; Upstate Biotechnology; rat: 1:500, Roche); phospho-PAK1 (Thr423)/PAK2 (Thr402) (rabbit; 1:1000; Cell Signaling); Tau (mouse; 1:1000; Millipore, Clone PC1C6) and MAP2 (mouse; 1:500; Millipore; Clone AP20; chicken; 1:10000; AbCam). All antibody incubations were performed in blocking solution containing 2% normal goat serum for 1 hr at room temperature or overnight at 4°C. Cells were incubated 1 hour at RT in blocking solution containing the appropriate Alexa conjugated secondary antibody (1:1000; Invitrogen Molecular Probes). Coverslips were then mounted on slides (Frost Plus; Fisher) with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL).

### 3.3.7 Imaging and analysis

Images were taken using a 63X or 20X objective affixed to a Zeiss Axiovert M200 motorized microscope using AxioVision software. For analysis of cell morphology in COS-7 cells, an experimenter blinded to treatment conditions used the 10X objective to randomly select 10-15 fields for analysis. Morphological changes were quantified in terms of the proportion of transfected cells showing at least one cellular extension which had a length at least twice the diameter of the nucleus. Analysis was performed on 2-3 coverslips from 2-3 independent experiments.

For spine-like protrusion analysis, images were exported as 16 bit images and analyzed using Northern Eclipse software (Empix Imaging, Mississauga, Canada). Briefly, an experimenter blinded to the treatment conditions used the RFP or GFP fluorescent signal to detect transfected
cells and manually outline spines present on all dendritic processes in the field of view. Any protrusions with a clear head greater than 0.35μM (as measure with the curve measurement tool at the widest part and parallel to dendrite) were classified as spine-like structures, while any thin protrusions lacking a head and with a length between 2 and 10μM were considered as filopodia. The data presented represent the average number of spine-like protrusions per dendritic length (100 μm) from three coverslips and two to three independent experiments. Statistical analyses were performed using unpaired Student-t-test to compare two groups. For three and more groups, ANOVA with Tukey B Post-Hoc was performed using XLSTAT.

For quantitative analysis of changes in Pak phosphorylation, cultures that were directly compared were stained simultaneously and imaged with the same acquisition parameters. For each condition, seven to nine neurons from two separate experiments were analyzed using Northern Eclipse software (Empix Imaging, Mississauga, Canada). An experimenter blinded to the treatment conditions used the GFP or HA fluorescent signal to manually outline approximately 50-100 μm of primary dendrites (including dendritic protrusions). In parallel, regions corresponding to areas without cells were outlined to create a background mask. For summary average gray intensities (total immunofluorescence intensity /pixel area), average gray values of background areas were subtracted to average gray values of the outlined dendritic segments. Statistical analyses were performed using unpaired Student-t-test.

For quantitative analysis of morphology in young neurons, images were captured with the 20X objective based on immunoreactivity against GFP. Dendrites and axons were identified based on standard morphological criteria and based on immunoreactivity against the axonal marker Tau or the dendritic marker MAP-2. Neurons presenting no classifiable processes were excluded from analyses. The total length of the axon and dendrites, as well as the number of primary and secondary branches were determined manually using Neuron J 1.4.0 (Meijering et al., 2004), a plug-in software for Image J (NIH). Analysis was performed by an experimenter blinded to the identity of the transfected constructs.
3.3.8 Immunoprecipitation, pull down assays and immunoblotting

For co-immunoprecipitation experiments, transfected COS-7 cells were quickly harvested and lysed in TEEN buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA, 150 mM NaCl) containing 1% triton X-100, 1mM PMSF (Sigma), and a protease inhibitor cocktail tablet (Roche). After rotation for 1 hr at 4°C, insoluble material was removed by centrifugation at 13,200 rpm for 15 min at 4°C. Samples were then incubated for 1 hr at 4°C with 3µg anti-GFP polyclonal antibody (rabbit; custom made by Affinity BioReagents). After addition of 40 µl protein A sepharose 4 Fast Flow beads (GE Healthcare), samples were incubated on a rotator at 4°C for 1 hr or overnight. Immunoprecipitates were washed 3 times with TEEN buffer containing 0.1% Triton X-100. Samples were heated at 90-100°C in SDS-PAGE sample buffer containing 5 mM DTT for 5 min, and analyzed by SDS-PAGE. Western blots signals were detected with an Odyssey system (Li-Cor) or ECL reagents (GE Healthcare).

For pull down assays, whole brains from adult Wistar rats were quickly removed. Brain tissue was homogenized in TEEN buffer supplemented with 10 mM ATP and 10 mM MgCl₂, 1 mM PMSF (Sigma), 1 mM benzamidine (Sigma) and a protease inhibitor cocktail tablet (Roche). Cells were lysed by the addition of 1% Triton X-100 followed by rotation for 1 hr at 4°C. Insoluble material was removed by centrifugation at 50,000 rpm for 25 min at 4°C. Samples were then incubated on a rotator at 4°C overnight with sepharose 4B beads (GE Healthcare) coupled to 30 µg purified GST-RILPL2 (aa 1-197) or GST. Beads were washed 3 times with TEEN buffer containing 0.5% Triton X-100. Sample buffer containing 5 mM DTT was added to the beads and samples were heated at 90-100°C for 5 min before analysis by SDS-PAGE. Membranes were blotted with anti-myosin Va (Esprefico et al., 1998), anti-Rac1 (mouse; Cell Biolabs) and anti-GST (rabbit; custom made by Affinity Bioreagent).

3.3.9 Rac GTPase activation assay and quantification

COS-7 cells were grown in 6 well dishes until 60% confluent. 1 to 3 µg of DNA was transfected using Lipofectamine 2000 reagent (Gibco-Invitrogen) according to the manufacturer’s protocol. Two days after transfection, cells were lysed by the addition of ice cold 400 µl MBL with protease inhibitors provided with the Rac assay kit (Millipore). Samples were briefly sonicated,
spun down at 14,000 RPM, and the supernatant retained. For input/loading control, 40 μl was removed from the samples, while the rest of the sample was used to perform Rac activation assay as previously described (Xie et al., 2007). Quantification was performed by densitometry as previously described (Srivastava et al., 2005; Xie et al., 2007). Intensities were averaged and a one way ANOVA with Tukey B Post-Hoc was performed using SPSS. Experiments were repeated 3-4 times.

3.4 Results

3.4.1 Identification of RILPL2 as a novel myosin Va-interacting protein

To identify novel myosin Va binding partners in the brain, we performed a yeast two-hybrid screen of a rat brain cDNA library with the myosin Va C-terminal tail (CT) as bait. This region encompasses the myosin Va proximal, medial and globular tail regions, the latter being the cargo binding domain (Figure 3.1A). One of the proteins detected with our screen was a small protein of 197 residues previously identified as RILP-like protein 2 (RILPL2) (Wang et al., 2004b). RILPL2 is related to RILP (Rab-lysosomal-interacting protein) and RILPL1, based on the presence of 2 small regions of significant amino acid similarities, referred to as RILP homology region -1 and -2 (RH1 and RH2), respectively (Figure 3.1B; (Wang et al., 2004b)). Overall, RILPL2 shares ~22% and ~32% amino acid identity with RILP and RILPL1, respectively (Wang et al., 2004b). Similar to RILP and RILPL1, RILPL2 is predicted to adopt 2 α-helical coiled-coil secondary structures, between amino acids 62 and 96, and 121 and 154 (Figure 3.1B) (Lupas et al., 1991). Database searches failed to identify any other conserved structural or functional domains in RILPL2.

As our original myosin Va yeast two-hybrid bait contained a large portion of myosin Va CT tail, we sought to determine more precisely the regions of interaction between myosin Va and RILPL2. To do this, we generated myosin Va medial tail (MyoVa MT) and globular tail (MyoVa GT) constructs, as well as RILPL2 N- or C-terminally truncated forms (Figure 3.1B). Yeast two-hybrid analysis revealed that the N-terminal portion of RILPL2, encompassing amino acid residues 1 to 113, was sufficient for association with the myosin Va globular tail region (Figure 3.1C). We further confirmed myosin Va and RILPL2 interaction by co-immunoprecipitation from COS-7 cells co-expressing epitope-tagged versions of full-length RILPL2 and various
myosin Va CT constructs (HA-RILPL2 FL and GFP-MyoVa CT constructs, respectively; Figure 3.2A). Notably, we found that RILPL2 associated with the CT region of myosin Va but not myosin Vb, a closely related family member also expressed in the brain (Figure 3.2A) (Lise et al., 2006; Zhao et al., 1996)). These results reveal a novel and specific interaction of RILPL2 with one member of class V myosins.

Next, we assessed whether RILPL2 associates with endogenous myosin Va present in the brain. The lack of a commercially available RILPL2 antibody and our unsuccessful attempts to
generate specific RILPL2 antiserum prevented us from detecting endogenous RILPL2 protein and performing RILPL2 co-immunoprecipitation assays from brain lysates. As an alternative, we performed pull-down assays from brain lysates using purified full-length RILPL2 fused to GST. We found that GST-RILPL2, but not the control GST alone, was able to associate with myosin Va expressed in brain (Figure 3.2B). These results suggest that RILPL2 and myosin Va interact in the brain.

A

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<tr>
<th>COS-7 lysates</th>
<th>IP: anti-GFP</th>
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<td>GFP-MyoVa CT</td>
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<td>GFP-MyoVb CT</td>
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<td>HA-RILPL2 FL</td>
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Blot: anti-GFP

Blot: anti-HA

B

Interaction of myosin Va with RILPL2 using pull-down assays. Myosin Va from rat brain tissue extract is pulled-down by purified RILPL2 fused to GST, but not by GST alone. Duplicate experiments of the pull-down assay are shown (n=3-4 independent experiments).

Figure 3.2. Biochemical analysis of interaction between RILPL2 and myosin Va.

(A) RILPL2 co-immunoprecipitates with myosin Va, but not with myosin Vb. COS-7 cells were transfected with the C-terminal tail domain of either myosin Va (GFP-MyoVa CT) or myosin Vb (GFP-MyoVb CT) fused to GFP, and with HA-tagged full-length RILPL2 (HA-RILPL2 FL). Cells were harvested 24 hours later and either anti-GFP antibody or pre-immune serum (IgG) was used to immunoprecipitate GFP-fused myosin V constructs. Upper panels show expression of the different constructs in starting lysates. Lower panels show co-immunoprecipitates detected with anti-GFP or anti-HA antibodies (n=3-4 independent experiments).

(B) Interaction of myosin Va with RILPL2 using pull-down assays. Myosin Va from rat brain tissue extract is pulled-down by purified RILPL2 fused to GST, but not by GST alone. Duplicate experiments of the pull-down assay are shown (n=3-4 independent experiments).

3.4.2 RILPL2 transcript is present in the brain and other tissues

Previous examination of RILPL2 transcript expression in cDNA panels from multiple human tissues showed the presence RILPL2 in several tissues, including lung, placenta, brain, heart, liver, kidney, and pancreas (Wang et al., 2004b). Our RT-PCR analyses confirmed RILPL2 presence in numerous brain regions from E18 and adult rats, as well as in other tissues, including spleen, liver, kidney and thymus (Figure 3.3A). In cultured cortical neurons, we found RILPL2 transcript expression to be developmentally regulated, with higher levels of expression achieved
between DIV8 and DIV14, a period where neurons undergo intense synapse development (Figure 3.3B) (Rao et al., 1998)).

**Figure 3.3. RILPL2 mRNA is expressed in the brain and cultured neurons.**

(A) RT-PCR shows RILPL2 expression in various brain regions from 18 days rat embryos (E18; left panel) and from adult rat brain and other tissues (right panel).
(B) RILPL2 mRNA expression is developmentally regulated in cultured primary cortical neurons. RT-PCR analysis shows an increased presence RILPL2 transcript at 1, 3, 8 and 14 days in vitro (DIV).

To further explore the regional distribution of RILPL2 during embryonic brain development, we performed *in situ* hybridization in brains of embryonic day 18 (E18) rats. Consistent with our RT-PCR, RILPL2 transcript was detected in both hippocampus and cortex at E18 (Figure 3.4). RILPL2 transcript was found throughout the various layers of the developing neocortex, and was most strongly localized within the marginal zone (MZ) and the transient subplate layer (SPL) (Figure 3.4, A-C). A concentration of RILPL2 expression was also seen in the germinal ventricular zone (VZ), from which newborn neurons initiate their migration into the cortical plate (Figure 3.4A, arrowhead). In the hippocampus, RILPL2 was predominant in the developing dentate gyrus and CA fields (Figure 3.4, D-F). Neurons expressing RILPL2 appeared widely dispersed throughout these differentiating fields, indicating that cells begin to express RILPL2 as they differentiate. The presence of RILPL2 in neuronal cells in the E18 cortex and hippocampus is suggestive of a role of RILPL2 during neuronal development.
Figure 3.4. RILPL2 transcript expression by non-isotopic in situ hybridization.

In situ hybridization for RILPL2 in E18 rat brain. (A) Low magnification image of RILPL2 expression in cortical layers. Red arrowhead indicates expressing cell in VZ. Red box is magnified in C. (B) Sense control for cortical layers. (C) RILPL2 expression predominates in the MZ and the uppermost CP, as well as in the SPL. Expressing cells are relatively sparse in the IZ and most of CP. (D) Sense control for the hippocampus. (E) Hippocampus is outlined in white and the CA1/3 fields and DG in the GCL are labeled. Red box is magnified in F. (F) RILPL2 expression in GCL next to nascent PP and CA3 shown

Abbreviations: MZ, marginal zone; CP, cortical plate; SPL, subplate layer; IZ, intermediate zone; VZ, ventricular zone; DG, dentate gyrus; CA, cornu ammonis; GCL, ganglion cell layer; PP, perforant pathway. Scale bars, 50 μm.

3.4.3 RILPL2 expression alters cellular shape

In contrast to the well-documented role of RILP in regulating late endosomal and lysosomal compartment morphology (Cantalupo et al., 2001; Progida et al., 2007; Wang et al., 2004b), the cellular function of RILPL2 has not been characterized. To obtain insights into RILPL2 intracellular targeting and putative cellular function, we transiently expressed HA-tagged full-length RILPL2 in COS-7 cells. Control COS-7 cells expressing GFP and/or RFP typically displayed a fibroblast-like morphology featuring a flat cell shape and relatively smooth edges and surface (Figure 3.5, A and B, and Figure 3.6A). Phalloidin staining showed the presence of F-actin in stress fibers and in lamellipodia at the edges of GFP-transfected cells (Figure 3.5, A and B). Interestingly, a considerable fraction of full-length RILPL2-transfected cells underwent significant morphological changes, showing an elongated cell body extending one or more long processes, as well as increased numbers of lamellipodia enriched in F-actin (Figure 3.5C). To
characterize the region of RILPL2 necessary for this morphological rearrangement, we expressed truncated forms of RILPL2 lacking the C- or N-terminal region, and monitored their effects on cellular morphology. Both full-length and C-terminal truncated RILPL2 displayed a diffuse cytoplasmic distribution with some targeting to membrane ruffles, as shown by immunostaining with an anti-HA antibody (Figure 3.5, C-E). Similar to full-length RILPL2, expression of the C-terminal truncated form induced process outgrowth (Figure 3.5D). In contrast, the RILPL2 N-terminal truncated form accumulated as intracellular aggregates and membrane patches at the extremities of the cell, and failed to induce the formation of cellular extensions (Figure 3.5E). Taken together, these observations suggest a role for RILPL2 in controlling cellular shape, and that this effect requires the N-terminal portion of RILPL2.
Figure 3.5. Expression of RILPL2 alters cellular morphology.

(A-E) COS-7 cells were transfected with HA-tagged versions of RILPL2 containing either the full-length protein (HA-RILPL2 FL) or various truncated forms (HA-RILPL2 ΔCT and HA-RILPL2 ΔNT), and visualized 48 hours later using anti-HA antibody. F-actin was visualized with phalloidin staining (red). (A-B) Examples of GFP-transfected cell with a characteristic flat and round cellular shape, with F-actin present at membrane ruffles (white arrow) and stress fibres (asterisk). (C) Expression of HA-RILPL2 FL results in morphological remodelling characterized by cell body elongation, process outgrowth (white arrowheads) and F-actin enrichment at membrane ruffles (white arrows). Expression of HA-RILPL2 FL (D) or HA-RILPL2 ΔCT (E) alters cell shape. (F) Expression of HA-RILPL2 ΔNT leads to the formation of abnormal inclusions of unknown nature. Scale bar, 10 µm.

We next asked whether RILPL2-mediated morphological changes could be prevented by expressing truncated forms of myosin Va or Vb lacking the head motor domain, but containing
the C-terminal cargo-binding domain. Myosin V “headless” forms have been shown to have a dominant-negative effect on myosin V-dependant cargo transport in various cell types (Lapierre et al., 2001; Lise et al., 2006; Rodriguez and Cheney, 2002; Volpicelli et al., 2002; Wu et al., 1998b). Overexpression of MyoVa CT or MyoVb CT fused to GFP in COS-7 cells lead to the formation of numerous bright vesicular-structures distributed throughout the cell and accumulating in a perinuclear region (Figure 3.6C). Notably, co-expression of GFP-MyoVa CT with HA-RILPL2-FL resulted in the redistribution of RILPL2 to myosin Va-positive compartment, as well as an apparent reduction of RILPL2 at membrane ruffles (Figure 3.6C). In contrast, MyoVb CT failed to recruit RILPL2 (Figure 3.6D). Morphological changes were quantified in terms of the proportion of transfected cells showing at least one cellular extension, which had a length, at least twice the diameter of the nucleus. A significant percentage of RILPL2 transfected cells (45.3 ± 3.2%) showed altered morphology compared to control GFP and RFP transfected cells (19.7 ± 2.3%). Co-expression with MyoVa CT blocks the RILPL2-mediated protrusive effect, as shown by a return to RFP control levels (26.6 ± 2.9%; Figure 3.6I). This effect was specific for MyoVa CT, as MyoVb CT did not block the RILPL2-induced phenotype (50.4 ± 4.3%; Figure 3.6I). This is consistent with our biochemical analysis showing a specific interaction of RILPL2 with myosin Va, but not myosin Vb. These results suggest that altering proper trafficking of RILPL2 with a DN form of myosin Va can prevent RILPL2-mediated changes in cell shape.
Figure 3.6. RILPL2-induced morphological changes are blocked by a dominant-negative form of myosin Va, but not myosin Vb.

(A-E) COS-7 cells were co-transfected with HA-tagged full-length RILPL2 (HA-RILPL2 FL) and GFP, or either the tail region of myosin Va (GFP-MyoVa CT) or myosin Vb (GFP-MyoVb CT) fused to GFP. Representative cells for control GFP (A) and HA-RILPL2 FL (B) are shown. Results show that GFP-MyoVa CT (C), but not GFP-MyoVb CT (D), induced RILPL2 redistribution into a perinuclear compartment (black arrowhead), and blocked the effects of HA-RILPL2 FL on process outgrowth (white arrowhead). (E) Quantification of the number of co-transfected cells with at least one process at least twice the diameter of the nucleus. Numbers of cells analyzed per group are: GFP+RFP=397, GFP+HA-RILPL2 FL=303, GFP-MyoVaCT + HA-RILPL2 FL=525, GFP-MyoVb CT+ HA-RILPL2 FL=327. **p<0.01; ***p<0.001. Scale bar, 10 μm.
3.4.4 RILPL2 alters dendritic spine development

The presence of RILPL2 in the brain combined with the observed effects of RILPL2 expression in heterologous cells led us to hypothesize that RILPL2 might play a role in neuronal cells, particularly in neuronal morphogenesis. To test this hypothesis, we decided to first evaluate the long-term effects of RILPL2 expression on dendritic spine morphogenesis. Cultured hippocampal neurons were co-transfected at DIV7 with full-length HA-RILPL2 or GFP, together with RFP as an unbiased marker to visualize dendritic morphology. Neurons were fixed 12 days later and the effects of RILPL2 on dendritic spine morphology were evaluated. At this stage (DIV19), neurons displayed mostly spines, with very few or no filopodia, as shown in control cells transfected with GFP and RFP (Figure 3.7A). Notably, dendrites of neurons expressing full-length HA-RILPL2 displayed a significantly greater number of spine-like protrusions per dendritic length (36.4 ± 2.1; Figure 3.7, B and E) than GFP control cells (26.3 ± 3.4; Figure 3.7, A and E). Interestingly, expression of N- or C-terminal truncated RILPL2 mutants (RILPL2ΔNT/ RILPL2ΔCT) did not increase the number of spine-like structures. Expression of the C-terminally truncated RILPL2 mutant resulted in a number of protrusions similar to control levels (29.8±1.8; Figure 3.7, C and E), whereas expression of the N-terminally truncated RILPL2 mutant reduced the number of spine-like structures below control levels (18.0±2.3; Figure 3.7, D and E), suggesting a dominant-negative effect. It should also be noted that approximately 30-50% of neurons transfected with RILPL2ΔNT displayed abnormal inclusions in their soma and dendrites similar to those observed in COS-7 cells, while the remaining cells showed a diffuse pattern of expression (Figure 3.7D and data not shown). To avoid potential problems associated with the health of neurons with abnormal inclusions, we analyzed only RILPL2ΔNT-transfected neurons showing a diffuse distribution. Together these data suggests that RILPL2 plays a role in dendritic spine morphogenesis.
Figure 3.7. Effect of long-term expression of RILPL2 on dendritic spine morphogenesis.

(A-E) Dissociated primary hippocampal neurons (DIV7) were transfected with RFP, and either GFP (A) or HA-tagged full-length RILPL2 (HA-RILPL2 FL) (B) or truncated forms of RILPL2 (HA-RILPL2 ΔCT (C) or 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 the effect of overexpression of different recombinant forms of RILPL2 on the number of dendritic spine-like protrusions per unit dendritic length. Total numbers of cells analyzed per group from two independent experiments are: GFP=14, HA-RILPL2 FL=13, HA-RILPL2 ΔCT=16, HA-RILPL2 ΔNT=13. Data represents mean ± SEM. *p<0.05. Scale bar, 5 μm.
3.4.5 RILPL2 expression results in enlarged pre-synaptic terminals

Besides the presence of RILPL2 in the somatodendritic compartment and its effects on dendritic spine morphogenesis, ectopic RILPL2 is also detected within the axon. Notably, we observed abnormal varicosities along the axons of HA-RILPL2 transfected neurons, compared to control GFP-transfected neurons (Figure 3.8, A and B). These axonal structures were enriched for the presynaptic marker, synaptophysin (Figure 3.8D), indicating an HA-RILPL2-mediated enlargement of presynaptic terminals. This observation suggests the possibility that RILPL2 might control some aspects of presynaptic morphology or function in addition to its involvement in dendritic morphogenesis.

Figure 3.8. RILPL2 expression results in abnormally large synaptophysin clusters.

(A-D) Dissociated primary hippocampal neurons (DIV7) were transfected with GFP (A) or HA-tagged full-length RILPL2 (HA-RILPL2 FL). At DIV19, neurons were fixed, and recombinant proteins were immunolabeled using anti-GFP or anti-HA antibodies. Synapses were detected with the presynaptic marker, synaptophysin (Syn). (A, B) Expression of HA-RILPL2, but not GFP, resulted in abnormal axonal morphology characterized by ring-like structures (enlarged in panel in B inset). (C, D) RILPL2-induced axonal varicosities correspond to enlarged synaptic terminals that are enriched for Syn (arrowheads in D). Scale bar, 10 μm.
3.4.6 RILPL2 activates the small GTPase Rac

Cellular morphology and protrusive outgrowths in neuronal and non-neuronal cells are known to be controlled by actin cytoskeletal rearrangements (Matus, 2000). Small GTPase proteins, such as the Rho GTPase, Rac, are a major regulator of the actin cytoskeleton in both cell types. In non-neuronal cells, the small GTPase, Rac1, is known to promote the growth of lamellipodia (Ridley et al., 1992), and in some cases, formation of neurite-like processes (Kozma et al., 1997; Miyashita et al., 2004). In neurons, Rac is involved in the formation, maintenance and structural plasticity of dendritic spines, as well in axonal outgrowth (de Curtis, 2008; Luo, 2002; Nakayama and Luo, 2000; Penzes et al., 2001a; Penzes et al., 2001b; Penzes and Jones, 2008). Hence, we hypothesized the effects of RILPL2 on cell morphology may be in part due to activation of the small GTPase, Rac. To test this hypothesis, we expressed full-length or truncated forms of RILPL2 in COS-7 cells and measured endogenous Rac1 activation by affinity isolation of its activated form (Figure 3.9, A and B). We found that full-length RILPL2 expression significantly increased Rac1 activity compared to control untransfected cells (control: 0.3±0.1; RILPL2-FL: 2.3±0.3 fold bound Rac relative to total Rac). Moreover, expression of the C-terminally truncated mutant significantly increased Rac1 activity while the N-terminal truncated form failed to activate Rac (RILPL2ΔCT: 2.0±0.2; RILPL2ΔNT: 0.6±0.3 fold bound Rac relative to total Rac). Rac activation by both full-length or C-terminal truncated forms of RILPL2 was blocked by MyoVa CT, but not with MyoVb CT (MyoVa CT: 0.9 ± 0.2; MyoVb CT: 2.1 ± 0.3 fold bound Rac relative to total Rac). Based on our observations that MyoVa CT altered the subcellular localization of both the full-length RILPL2 and the C-terminal truncated forms (Figure 3.6C and data not shown), these results suggest that proper localization of RILPL2 to the cytoplasm and membrane ruffles is critical for RILPL2-mediated Rac activation.
Figure 3.9. RILPL2 expression activates the GTPase Rac.

(A-B) COS-7 cell were transfected with HA-tagged full-length and truncated forms of RILPL2 lacking the N- or C-terminal region (HA-RILPL2 ΔNT and HA-RILPL2 ΔCT, respectively), with or without the tail region of myosin Va (GFP-MyoVa CT) or myosin Vb (GFP-MyoVb CT) fused to GFP. Cells were harvested 48 hours post-transfection, and activation of endogenous Rac1 was measured by evaluating Rac binding to GST-Pak domain. Total or bound Rac was detected by Western blotting using anti-Rac antibody (lower panels). Expression of RILPL2 or MyoV constructs was detected using anti-HA or anti-GFP antibodies, respectively (top panels). (B) Quantification of relative Rac activation (bound Rac/total Rac) upon expression of various recombinant forms of RILPL2 and MyoV from three to four independent experiments. Data represent mean ± SEM. *p<0.05.
We then asked whether RILPL2 could activate Rac1 in neurons. Pak is a major downstream effector of Rac1 which regulates spine morphogenesis (Bagrodia and Cerione, 1999; Hayashi et al., 2007; Hayashi et al., 2004). Binding between Rac and Pak causes activation and autophosphorylation of Pak, hence, Pak phosphorylation can be used as readout of Rac activation in neurons (Xie et al., 2008). To test whether RILPL2 overexpression activates the Rac-Pak pathway in neurons, we examined the endogenous levels of phosphorylated Pak (P-Pak) in neurons transfected with HA-RILPL2, or GFP as a control. RILPL2 overexpression resulted in a significant increase in dendritic P-Pak immunoreactivity (150.2 ± 14.3%) compared to GFP-transfected neurons (100.0 ± 9.6%), indicating that RILPL2 expression activates the Rac-Pak signaling pathway (Figure 3.10, A and B). We further tested whether RILPL2 could associate with endogenous Rac by conducting pull-down assays from rat brain lysates using GST-RILPL2, or GST as a negative control. We found that Rac1 bound to GST-RILPL2, but not the GST alone (Figure 3.10C). In contrast, the AMPA-type glutamate receptor subunit, GluR2, was absent from the GST-RILPL2-bound fraction. Combined, these data demonstrate that RILPL2 and Rac interact in neuronal cells, and that RILPL2 expression is sufficient to induce Rac and Pak activity in non-neuronal and neuronal cells.
Figure 3.10. RILPL2 increases Pak phosphorylation in cultured neurons.

(A, B) Cultured hippocampal neurons (DIV10) were transfected with either GFP or HA-tagged RILPL2. At DIV15, neurons were fixed and immunolabeled with anti-GFP or anti-HA antibodies, and anti-phospho-Pak (P-Pak). (A) Increased P-Pak intensity in neurons transfected with HA-RILPL2. (B) Graph shows average integrated intensity of P-Pak immunostaining in dendrites from neurons expressing GFP or HA-RILPL2. Total numbers of cells analyzed per group from two independent experiments are: Control GFP=15; HA-RILPL2=16. Data represent mean ± SEM. *p<0.05. Scale bar, 5 μm. (C) Interaction of RILPL2 with endogenous Rac using pull-down assays with rat brain tissue extract. Rac1, but not the AMPA receptor subunit, GluR2, was pulled down by purified full-length RILPL2 fused to GST, but not by GST alone (n=2-3 independent experiments).

3.4.7 RILPL2 loss-of-function blocks spine development

To further confirm the involvement of RILPL2 in spine development, we next examined spine morphogenesis in neurons in which RILPL2 expression is strongly attenuated by RNA interference, using short hairpin RNA (shRNA) specifically targeting rat RILPL2. We first tested the efficiency of our shRNA in COS-7 cells and showed that two independent RILPL2 shRNA sequences significantly reduced the expression levels of rat GFP-RILPL2 (Figure 3.11A). Expression of the rat version of the related protein, RILPL1, or of the mouse version of RILPL2
(HA-RILPL2-res) which has an altered shRNA recognition site to the rat homologue, were unaltered by the RILPL2 shRNAs (Figure 3.11, B and C), indicating specificity of the RILPL2 shRNAs for rat RILPL2. Since RILPL2 shRNA 496 showed a higher knockdown efficiency, we chose this shRNA for subsequent experiments. Lack of RILPL2 antibody prevented us from testing the potency of our RILPL2 shRNAs for depleting endogenous RILPL2 in cultured neurons. As an alternative, we confirmed that ectopic expression of a rat RILPL2 cDNA fused to GFP is efficiently reduced by RILPL2 shRNA in neurons (Figure 3.11D).
Figure 3.11. Characterization of RILPL2 shRNA.

(A) RILPL2 shRNA constructs (RILPL2 shRNA-493 or RILPL2 shRNA-496) were co-transfected with GFP-tagged full-length RILPL2 (GFP-RILPL2) to determine the efficiency of RILPL2 knockdown in COS-7 cells. Western blot analysis shows that both RILPL2 shRNA-493 and -496 efficiently reduces the expression of GFP-RILPL2 as compared to control shRNA. Graph shows quantification of GFP-RILPL2 signal intensity normalized to actin intensity, as compared to control. Blots show that RILPL2 shRNA knockdown has no effect on the expression levels of HA-tagged RILPL1 (B) or mouse RILPL2 (C). (D) Primary hippocampal neurons (DIV7) were co-transfected with GFP-RILPL2, RFP and either control shRNA or RILPL2 shRNA 496. Neurons were fixed at DIV10, images were taken with the same parameters and the level of GFP signal was assessed. Co-expression with RILPL2 shRNA resulted in a visible reduction of exogenous GFP-RILPL2 signal compared to control. Scale bar, 10 μm.
We tested the effects of endogenous RILPL2 knockdown by transfecting young hippocampal neurons (DIV10) and allowing the shRNA constructs to express for 5 days prior to fixation and immunostaining (DIV15). As expected, these younger neurons (DIV15) displayed a lower basal number of spine-like structures compared to older neurons (DIV19; Figure 3.7). Consistent with our results in DIV19 hippocampal neurons, RILPL2 overexpression significantly increased the number of spine-like structures by ~30-40% (Figure 3.12, A, B and E). In contrast, loss of RILPL2 resulted in a significant reduction in the number of spine-like structures, compared with control shRNA (HA-RILPL2 FL, 23.6±1.1; RILPL2 shRNA, 12.3±0.6; Ctrl shRNA 16.3±0.7; Figure 3.12, C and E). A moderate increase in the number of filopodia with the expression of RILPL2 shRNA was also observed, suggesting that some filopodia might be failing to transform into spines and/or spines are destabilized (Figure 3.12F). To demonstrate the specificity of our RILPL2 shRNA, we tested whether expression of a shRNA resistant cDNA plasmid could rescue the shRNA-dependant inhibition of dendritic spine protrusions. Expression of mouse RILPL2 (HA-RILPL2-res) in shRNA-treated neurons restored the number of spine-like structures to control levels (RILPL2 shRNA + HA-RILPL2 res, 18.1±01.3; Figure 3.12, D and E). Overall, these data provide further evidence that RILPL2 plays a role in dendritic spine formation and maintenance, as loss-of-function analysis results in reduced dendritic protrusion numbers.
Figure 3.12. RILPL2 loss-of-function alters spine morphogenesis.

(A-D) Dissociated primary hippocampal neurons (DIV10) were transfected with control shRNA (GFP-pSuper vector) or RILPL2 shRNA (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 on dendritic spine-like protrusions. (E, F) Summary of changes in the number of spine-like protrusions (E) and filopodia (F) per unit dendritic. Total numbers of cells analyzed per group from two 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<0.05. Scale bar, 5 μm.
3.4.8 Expression of RILPL2 in young neurons reduces axonal outgrowth

In addition to its role in dendritic spine development, Rac also controls neuronal morphogenesis early in development (de Curtis, 2008; Koh, 2006). Based on the ability of RILPL2 to activate Rac and the presence of RILPL2 transcript as early as E18, we hypothesized RILPL2 might play a role in neuronal morphology during this period of development. To test this hypothesis, GFP or HA-RILPL2 cDNAs were electroporated into hippocampal neurons immediately after dissociation (DIV0) and neuronal morphology was examined 72 hours later, when neurons have developed easily discernable dendrites and one clear axon. We evaluated the effect of RILPL2 expression by measuring the length of the longest neurite per neuron. This analysis revealed a decrease in the average length of the longest neurite in neurons expressing HA-RILPL2, as compared to GFP (Figure 3.13).

Figure 3.13. RILPL2 expression in young neurons reduces the average length of the longest cellular process.

(A, B) Dissociated primary hippocampal neurons were electroporated at DIV0 with GFP or HA-tagged full-length RILPL2 (HA-RILPL2 FL) and fixed at DIV3 for immunodetection using anti-GFP or anti-HA antibodies. Representative DIV3 neurons expressing (A) GFP or (B) HA-RILPL2 FL. Graphs showing quantitative analysis of the length of the longest process on a given cell reveal a shift toward shorter primary neurite length in RILPL2-expressing cells. Scale bars, 5 μm.
In order to quantify in more detail the morphological changes associated with RILPL2 overexpression or loss-of-function, morphometric analysis was performed on dendrites and axons in neurons expressing control shRNA, control shRNA and HA-RILPL2, or RILPL2 shRNA 496. Antibodies against the axonal protein, Tau, or the dendritic microtubule-associate-protein 2 (MAP2), were used to specifically label axons or dendrites. RILPL2 expression specifically reduced total axonal outgrowth (Ctrl ShRNA: 70.0 ± 4.5; Ctrl ShRNA + HA-RILPL2 FL: 51.7 ± 4.7), with no effect on overall outgrowth or branching of dendrites (Figure 3.14). In contrast to RILPL2 shRNA effect on dendritic spines, expression of RILPL2 shRNA in DIV0 neurons had no significant effect on either axonal or dendritic outgrowth, compared to control cells (Ctrl shRNA: 70.0 ± 4.5; RILPL2 shRNA: 70.5 ± 4.9; Figure 3.14). Combined, these data indicate that although the ectopic expression of RILPL2 impedes axonal outgrowth in young neurons, RILPL2 is not critical for neurite morphogenesis early in development.
Figure 3.14. RILPL2 expression specifically blocks axonal outgrowth.

Dissociated primary neurons (DIV0) were electroporated with control shRNA or RILPL2 shRNA-496 with or without HA-tagged RILPL2. Three days later, neurons were fixed and exogenous proteins were detected by immunofluorescence with anti-GFP or anti-HA antibodies. (A) Axons were labeled using anti-Tau antibody, and are outlined in transfected cells using white arrowheads. Graphs show quantification of (B) the total length and (C) the average number of primary and secondary Tau-positive and Tau-negative processes. In some experiments, MAP2 was used as a marker of dendrites (not shown). At least 50 were cells analyzed per group from two to four independent experiments. Data represent mean ± SEM. **p<0.01. Scale bar, 5 µm.

3.5 Discussion

In the present work, we identify RILPL2 as a novel interacting partner for the actin-based molecular motor, myosin Va, and report a novel role for RILPL2 in controlling dendritic morphogenesis. Using a combination of yeast two-hybrid and biochemical assays, we determined that the RILPL2 and myosin Va interaction occurs via the N-terminal region of RILPL2 and the myosin Va globular tail. We found that RILPL2 expression in non-neuronal cells results in change in cell shape. In young and mature, manipulation of RILPL2 expression altered the formation and maintenance of dendritic spines. We also showed that the observed
effects of RILPL2 expression correlates with increased activity of the small Rho GTPase, Rac1, as well as phosphorylation of the Rac downstream substrate, Pak. These combined results uncover a novel role for RILPL2 in the regulation of cellular shape and dendritic spine morphogenesis, likely via the Rac-Pak signaling pathway.

The ability of RILPL2 to trigger changes in cellular morphology and to activate Rac and is particularly intriguing, as it suggests a relatively different function for RILPL2 compared to its related protein RILP, known to regulate trafficking and morphology of late endosomes and lysosomes through distinct interactions with Rab7 and the microtubule motor dynein (Cantalupo et al., 2001; Jordens et al., 2001; Progida et al., 2007). Consistent with differential cellular functions for RILPL2 and RILP, Wang and colleagues (2004) reported RILPL2 inability to affect the morphology and distribution of lysosomes. Here we provide several lines of evidence for a novel role for RILPL2 in the control of some aspects of cellular morphogenesis through Rac signaling that regulates the actin cytoskeleton. First, expression of RILPL2 in non-neuronal cells resulted in the formation of cellular extensions and, to a lesser extent, membrane ruffles. Second, this remodeling was accompanied by increased Rac activity and is consistent with previously reported phenotypes associated with Rac signaling (Kozma et al., 1997; Miyashita et al., 2004; Ridley et al., 1992). Both RILPL2-mediated morphological changes and Rac activation were blocked by the expression of a truncated tail of myosin Va, which sequesters RILPL2 in a vesicular compartment. Third, expression of RILPL2 in developing neurons increased the number of dendritic spine-like structures formed. This is consistent with the reported role of Rac in neurons as a positive regulator of dendritic spine development (de Curtis, 2008; Nakayama and Luo, 2000; Penzes et al., 2008). Fourth, expression of RILPL2 in young neurons increased levels of phosphorylation of the Rac effector Pak, which is also implicated in spine development (Bagrodia and Cerione, 1999; Hayashi et al., 2007; Hayashi et al., 2004). Together, these data suggest RILPL2-mediated effects on cell morphology are mediated, at least in part, by Rac-Pak signaling pathways.

Expression of RILPL2 at early developmental stages (DIV0-3) resulted in defective axonal elongation, with no significant effect on dendritic outgrowth or arborization. This result seems counterintuitive, since Rac1 is generally considered as a positive regulator of neurite outgrowth (Luo, 2000; Luo, 2002; Negishi and Katoh, 2005; Van Aelst and Cline, 2004; Watabe-Uchida et al., 2006). One possibility for the observed phenotype is that RILPL2 sequestered Rac (or a Rac
GEF) that would normally be required for elongation of Tau-positive axons, resulting in a reduction in axonal length. However, other studies have reported the involvement of Rac in growth cone collapse (Jin and Strittmatter, 1997; Vastrik et al., 1999) and selective blockade of axonal outgrowth (both initiation and elongation) without affecting dendrite growth, suggesting Rac can also act as a negative regulator of axon outgrowth (Luo et al., 1996; Luo et al., 1994; Penzes et al., 2001b). Thus, another possibility is that RILPL2 expression during this specific developmental time window locally activated Rac signaling in the growth cone that is responsible for blocking axonal outgrowth. Notably, shRNA-mediated knockdown of RILPL2 at this developmental stage did not facilitate axonal elongation. These results suggest that, although RILPL2 expression is deleterious for axon elongation, endogenous RILPL2 is not essential for normal neuritogenesis at early development stages.

Our analyses of truncated forms of RILPL2 provide insight into the regions important for RILPL2 function. Our data suggest that the N-terminal portion of RILPL2 plays a critical role in its function, since deletion of this portion ablated the ability of RILPL2 to associate with myosin Va, to promote the formation cellular extensions and to activate Rac. However, while expression of RILPL2 N-terminal region alone was sufficient for induction of morphological changes and Rac activation in COS-7 cells, it was not sufficient to increase spine formation, suggesting RILPL2 C-terminal region is also important for RILPL2 function, at least in neurons. In contrast, expression of the RILP2 C-terminal region alone appears to act as a DN by decreasing the number of dendritic spine-like structures formed to below control levels. How the RILPL2 C-terminal constructs acts as a DN is unclear. One intriguing possibility is that RILPL2 self-associate and functions as a dimer, similar to its related coiled-coil protein RILP (Colucci et al., 2005; Marsman et al., 2006; Wu et al., 2005). In this scenario, RILPL2 C-terminal portion would dimerize with endogenous RILPL2 through its remaining coiled-coil region, resulting in the formation of a dysfunctional dimer that failed to effectively activate Rac. Another possibility is that RILPL2 C-terminal region sequestered and compromised the trafficking of some substrate/binding partner of endogenous RILPL2, resulting in reduced RILPL2 activity.

Our pull-down results from brain lysates support a novel interaction between RILPL2 and Rac, which may underlie activation of the Rac/Pak pathway. The mechanism of activation of Rac signaling by RILPL2 remains unclear. RILPL2 does not have a Dbl-homology (DH) domain, the common motif which mediates nucleotide exchange that is shared by the classical GEFs for Rho
GTPases (Cerione and Zheng, 1996). Therefore, it seems unlikely that RILPL2 is a Rac GEF that directly activates Rac. One possible scenario is that RILPL2 and Rac are part of a complex, and that RILPL2 indirectly activates Rac through recruitment of a Rac GEF or another protein that facilitates activation of the small GTPase (see model; Figure 3.15). Many Rac GEFs have been identified in neurons and shown to regulate neuronal morphogenesis through Rac signaling, including Kalirin-7, STEF, Tiam 1, β-PIX, and GEFT (Bryan et al., 2004; Penzes et al., 2008). One particularly attractive candidate is Kalirin-7, which was recently shown to be recruited by the scaffolding protein AF-6/afadin (Ponting, 1995), a PDZ domain-containing protein involved in clustering of receptors (Buchert et al., 1999), to control spine remodeling associated with synapse maturation and plasticity (Xie et al., 2008). The presence of a region of homology with AF-6/afadin within the globular tail of myosin Va (Figure 3.1A) raises the possibility that myosin Va might be implicated in the recruitment of kalirin-7 to RILPL2 for Rac activation. Further analysis will be needed to determine if the recruitment of a specific Rac GEF is involved in RILPL2-mediated regulation of Rac1 activity.
Figure 3.15. Schematic model for the role of RILPL2 in spine morphogenesis.

(A) RILPL2 overexpression in developing neurons results in increased density of protrusions with a clear head and classified as spines. (B) Ectopic RILPL2 is diffusely localized in dendritic shafts and spines in developing neurons. Here we suggest that RILPL2 present at the dendritic shaft activates Rac, possibly through recruitment of a Rac GEF, and facilitates spinogenesis during development. RILPL2 presence in the spine compartment locally activates Rac and regulates spine morphogenesis. The role of RILPL2/myosin Va interaction in RILPL2 function is not clear. One possibility is that myosin Va is required for delivery of RILPL2 into the spine compartment. Another possibility is that myosin Va activates RILPL2, which in turn activates Rac signaling, representing a feed-forward mechanism to create more F-actin tracks for myosin Va-mediated cargo transport during spine development.
The molecular mechanisms that underlie cellular morphogenesis include both cytoskeletal remodeling and membrane trafficking. While RILPL2 ability to activate Rac1 suggests a function in cytoskeletal remodeling, we cannot rule out the possibility that RILPL2 might be implicated in membrane trafficking events as well. Consistent with this idea is our finding that RILPL2 interacts with the motor protein myosin Va, which has been implicated in trafficking of membrane organelles in several cells types (Bridgman, 2004; Desnos et al., 2007a). In neurons, there is increasing evidence that myosin Va act as a calcium-sensor to control the activity-dependent delivery of cargoes from the dendritic shaft to the spine compartment (Correia et al., 2008; Dekker-Ohno et al., 1996; Petralia et al., 2001; Yoshimura et al., 2006). The recently reported association of myosin Va with Rab11 (Correia et al., 2008; Roland et al., 2009), a small GTPase associated with recycling endosomes and implicated in neurite formation and plasticity-dependent dendritic spine growth (Park et al., 2004; Park et al., 2006; Shirane and Nakayama, 2006; Wang et al., 2008), may provide a mechanistic link between RILPL2, myosin Va and membrane remodeling associated with cell shape and spine morphology.

From our study, the functional relevance of RILPL2-myosin Va interaction remains unclear. It is possible that myosin Va capture and dynamically tether RILPL2 (or a membrane-associated complex containing RILPL2) in the actin periphery underneath the plasma membrane, and eventually, regulate the translocation of RILPL2 into spines where it can activate Rac signaling (see model; Figure 3.15). The aberrant mislocalization of RILPL2 truncated form lacking the portion interacting with myosin Va in some cells suggests association with myosin Va may be critical for proper localization of RILPL2 (Figure 3.5). Further analysis in neurons lacking a functional myosin Va should clarify whether RILPL2 localization and function in dendritic spine morphogenesis relies on myosin Va. Regardless of the requirement for myosin Va in the normal functioning of RILPL2, our results elucidate a novel role for the previously uncharacterized RILPL2 protein in regulating dendritic spine morphogenesis through Rac signaling, and provide a new insights into the machinery that regulates dendritic spine development and maintenance. Notably, alteration in Rac-related GTPase-signalling pathways that result in defects in spine development, maturation and function, have been associated with several forms of mental retardation including Down, Rett, Fragile X, and fetal alcohol syndromes (Newey et al., 2005; Ramakers, 2002; Zoghbi, 2003). It would be of interest for future studies to investigate whether RILPL2 localization and function may be altered in these disease states, and thus contribute to their etiology.
3.6 References


CHAPTER 4

4.1 Discussion

4.1.1 Summary of findings

The objective of this thesis was to investigate the functions of class V myosins in neurons, particularly their potential involvement in neuronal trafficking through the identification and characterization of novel cargoes for myosin Va and Vb.

The first specific goal was to identify novel myosin Va and Vb cargoes in neuronal cells using a DN approach. This strategy consists of the expression of the tail fragment of myosin V that contains the CBD which interferes with the function the endogenous myosin V. Combined with immunolocalization, this strategy allows one to assess whether the distribution of several candidates proteins is altered upon disruption of myosin Va or Vb function. In developing hippocampal neurons, we found that expression of the tail fragment of myosin Vb, but not myosin Va, enhances accumulation of the AMPA-type glutamate receptor subunit GluR1 in the soma and reduces its surface expression. These changes were accompanied by reduced GluR1 clustering and diminished frequency of excitatory but not inhibitory synaptic currents. Similar effects are observed upon expression of a full-length mutant form of myosin Vb lacking a C-terminal region required for binding to the small GTPase Rab11. Notably, DN myosin Vb did not change the localization of several other neurotransmitter receptors, including the NMDA-type glutamate receptor subunit NR1. This study is the first report of a role of myosin Vb in neuronal trafficking. Collectively, these results uncover a novel mechanism for the dendritic transport of AMPAR in neurons mediated by a member of the actin-based myosin V family and the GTPase Rab11.

As an alternative approach to identify binding partners for myosin Va in the brain, we conducted a yeast two-hybrid screen with the tail fragment of myosin Va. This approach was successfully used by others to identify several myosin V-binding partners, including conventional kinesin (Huang et al., 1999), DLC (Naisbitt et al., 2000), Rab11a (Lapierre et al., 2001), Rab8a (Roland et al., 2007), and BERP (El-Husseini and Vincent, 1999). Among the proteins identified in our screen, I selected a protein of unknown function previously identified as Rab-lysosomal-
interacting protein like 2 (RILPL2) for further characterization (Wang et al., 2004b). I showed that RILPL2 is present in the brain and interacts with myosin Va from brain lysates. In non-neuronal cells, expression of RILPL2 results in the formation of cellular extensions and activation of the small GTPase Rac1. In developing neurons, overexpression or knock-down of RILPL2 alters the density of dendritic spine protrusions. Moreover, expression of RILPL2 increases phosphorylation of the Rac effector, Pak1. These findings reveal a novel role for the myosin Va-interacting protein, RILPL2, in regulating cellular morphology and dendritic spine development, potentially through Rac signalling.

The significance of this thesis lies in several aspects. As AMPARs mediate most of the fast excitatory neurotransmission in the brain and are essential for the expression of several forms of synaptic plasticity associated with learning and memory, it is critical to understand how they are delivered from their site of synthesis to the synapse. MT-based motors have been implicated in AMPAR transport along dendrites (Hirokawa and Takemura, 2005), however, whether actin-based motors also participate in this delivery process is poorly understood. Our findings that myosin Vb and Rab11 are implicated in trafficking of AMPARs confirm that an actin-based motor of the myosin V family contributes to this process. Moreover, our finding that RILPL2 activates Rac and regulates dendritic spine morphogenesis provides new insights into the molecular players that control development and maturation of dendritic spines. Defects in the molecular machinery that controls development and structural plasticity of dendritic spines have been associated with several forms of mental retardation, including Down, Rett, Fragile X, and fetal alcohol syndromes (Newey et al., 2005; Ramakers, 2002; Zoghbi, 2003). Thus, our findings contribute to our global understanding of the potential mechanisms underlying these neurological disorders.

4.1.2 Role of myosin V in AMPAR trafficking

How does myosin Vb involvement in AMPAR transport fit in with the current understanding of 1) myosin Vb cellular functions and 2) AMPAR trafficking from the soma to the synapse?
4.1.2.1 Myosin Vb is associated with plasma membrane recycling systems in neurons

Overexpression of tail fragments of unconventional myosins has been a standard technique used to inhibit their function. In their initial study, Lapierre and colleagues (2001) showed that overexpression of a tail fragment of myosin Vb in HeLa cells causes accumulation of transferrin and Rab11-positive endosomes in perinuclear compartments, suggesting that myosin-Vb functions in the point-to-point transport of vesicles between the perinuclear recycling endosomes and the plasma membrane (Lapierre et al., 2001). This DN strategy has been widely used in the past eight years to confirm a specific role for myosin Vb in transport of various cargoes that traffic through the plasma recycling systems (Table 1.2). Although the DN strategy is a very useful tool to disrupt myosin Vb activity and identify cargoes, the abnormal accumulation of the tail fragment to perinuclear compartments does not reflect the endogenous location of myosin Vb, which has been detected along dendritic processes (Lise et al., 2006). Therefore, the DN strategy is not informative of the role of endogenous myosin Vb in peripheral compartments.

An alternative approach to overexpression of DN tail fragments was recently developed to block myosin V function at its endogenous location. This elegant approach, termed chemical-genetic inhibition strategy, was originally designed for kinases (Knight and Shokat, 2007; Specht and Shokat, 2002) and recently adapted to unconventional myosins, allowing one to acutely and specifically induce tight binding of a sensitized mutant myosin to actin by microinjection or dialysis of an ADP analog (Gillespie et al., 1999; Holt et al., 2002; Karcher et al., 2007; Stauffer et al., 2005). The generation of a sensitized mutant form of myosin Vb with a mutation (Y119G) that enlarges the ATP/ADP-binding pocket of myosin Vb, renders it accessible to a bulky ADP analog, N6-2-phenylethyl-ADP (PE-ADP) (Provance et al., 2004). In the absence of PE-ADP, MyoVb-Y119G still hydrolyzes ATP and exhibits actin mobility indistinguishable from wild-type myosin Vb (Provance et al., 2008; Provance et al., 2004). Upon binding the nonhydrolyzable PE-ADP, MyoVb-Y119G tightly associates with F-actin and motor processivity ceases, thereby locking myosin Vb in place.

Provance and colleagues (2004, 2008) used this chemical-genetic inhibition technique to provide evidence that full-length myosin Vb is not required for transport between perinuclear compartments and the plasma membrane, but functions as a dynamic tether in the actin-rich periphery (Provance et al., 2008; Provance et al., 2004). In contrast to overexpression of myosin
Vb tail fragment which results in a perinuclear accumulation of transferrin (Lapierre et al., 2001), inhibition of the myosin Vb sensitized mutant prevents accumulation of transferrin-positive vesicles in the perinuclear region (Provance et al., 2004). These apparently contradictory results can be reconciled in a model where myosin Vb acts peripherally as a dynamic tether that antagonizes the retrograde transport of transferrin to perinuclear compartments, possibly by holding the parental organelle in the periphery during fission (Figure 4.1A-C) (Provance et al., 2008). Thus, in cells overexpressing the motorless tail fragment, peripheral endocytic compartments cannot be tethered to actin at the periphery, resulting in the entire peripheral endosomes being retrogradely transported to the perinuclear region (Figure 4.1D). This role of myosin Vb as a dynamic tether at the actin-rich periphery is reminiscent of the proposed model of myosin Va function in pigmented cells (Desnos et al., 2007a; Provance and Mercer, 1999; Rogers and Gelfand, 2000).

Figure 4.1. Myosin Vb as a dynamic tether for peripheral endocytic compartments.

(A) Peripheral endosomes are retained in the periphery by multiple myosin-Vb motors whose heads periodically detach from actin as they go through the ATPase cycle, but usually reattach. (B) Dynein (or a minus-end-directed kinesin) attaches to a microtubule and exerts retrograde force. Occasionally, adjacent myosin-Vb detaches from actin (dotted circle), allowing dynein to pull it away from the actin filament. (C) Following fission, the daughter vesicle moves retrogradely, carrying myosin-Vb as a passenger. (D) Diagram depicting displacement of peripheral endosomes in cells transfected with a myosin Vb tail construct. Two different means of binding myosin Vb to the endosome as cyan and purple circles, since two different means have been demonstrated experimentally: Rab11a (Lapierre et al., 2001) and the CART complex (Yan et al., 2005). Adapted from (Provance et al., 2008), with permission.
In our study, we show that myosin Vb is also associated with plasma membrane recycling systems in neurons, as overexpression of tail fragment of myosin Vb results in the accumulation of Rab11-positive endosomes and GluR1-containing AMPARs in a perinuclear compartment (Lise et al., 2006). Our initial interpretation of this observation was that wild-type myosin Vb is likely to be involved in trafficking AMPARs from a Rab-11 positive perinuclear recycling compartment in the soma to the plasma membrane. The enrichment of endogenous myosin Vb in the soma is consistent with this interpretation (Lise et al., 2006). However, the presence of endogenous myosin Vb in dendritic spines in mature neurons suggests myosin Vb could also be implicated in the local delivery of AMPAR into dendritic spines. Very recently, Wang and colleagues (2008) used the acute chemical-genetic inhibition approach described above to show that myosin Vb functions to capture and translocate AMPAR-containing REs into dendritic spines during synaptic plasticity (see model Figure 4.2C). In light of these recent findings using the chemical-genetic inhibition that support a major role for myosin Vb as a dynamic tether at the periphery and active motor into dendritic spines, I must raise the possibility that endogenous myosin Vb does not primarily function at the perinuclear region and suggest an alternative interpretation of DN tail fragment data. Shortly after their internalization, a population of AMPARs is known to be targeted for recycling and therefore traffic through a Rab11-positive endocytic compartment located at the dendritic shaft (Brown et al., 2007; Park et al., 2004). In neurons overexpressing myosin Vb tail fragments, this endocytic compartment might not be retained at the periphery due to the inability of myosin Vb mutants to bind to actin and antagonize retrograde transport. Thus, it is likely the loss of peripheral tethering that caused the observed collapse of the Rab11- and AMPAR-associated endocytic compartments to the perinuclear region and resulted in defective delivery of AMPAR to the membrane.
Figure 4.2. Myosin Vb mobilizes recycling endosomes and AMPARs for postsynaptic plasticity.

(A) Schematic diagram of myosin Vb (MyoVb) and its association with REs via Rab11/Rab11-FIP2.
(B) Model diagram indicating the proposed Ca^{2+}-regulated conformational switch and cargo binding by MyoVb. Wild-type MyoVb is folded into an inactive structure at low Ca^{2+} levels. High Ca switches MyoVb to an extended structure that binds to Rab11/Rab11-FIP2, resulting in membrane recruitment.
(C) Schematic model for spine mobilization of AMPAR-containing recycling endosomes by Ca^{2+}-regulated MyoVb during LTP. LTP stimulation triggers the rapid Ca^{2+}-dependent extension of MyoVb, allowing binding to Rab11-FIP2 adaptors on REs and thereby recruiting endosomes into spines to supply AMPARs and membrane for functional and structural plasticity. Adapted from (Wang et al., 2008), with permission.
4.1.2.2 Different roles of myosin V family members in AMPAR trafficking

In our study, we contrasted the effect of overexpressing DN forms of myosin Va and Vb on the distribution of various neuronal proteins. I found that DN myosin Vb, but not DN myosin Va, specifically alters the distribution of GluR1-containing AMPARs and reduces their clustering and surface expression. The lack of effect on glutamate receptor distribution upon myosin Va inhibition is consistent with the results from myosin Va-null mice, as they show a normal postsynaptic glutamate receptor distribution, excitatory synaptic transmission, short-term plasticity, and LTP (Petralia et al., 2001; Schnell and Nicoll, 2001). This is also consistent with recent results from Wang and colleagues (2008) showing that in contrast to myosin Vb, myosin Va does not co-traffic into spines with transferrin receptor (TfR)-positive REs that contain AMPA receptors (Park et al., 2004; Wang et al., 2008). Together, these studies indicate that while myosin Va is not essential for AMPAR trafficking, myosin Vb function is critical for AMPARs trafficking.

It is worth mentioning that a recent study also published in the course of my PhD (in which I participated by generating myosin Va cDNA and siRNA constructs), did report a role for myosin Va in activity-dependant delivery of AMPAR into dendritic spines (see model Figure 4.3) (Correia et al., 2008). In this study, DN and siRNA strategies were used to block myosin Va function in CA1 neurons in organotypic hippocampal slices. Consistent my results, loss-of-function myosin Va did not affect trafficking of AMPARs into distal apical dendrites or basal AMPAR-mediated neurotransmission. However, myosin Va loss-of-function abolished LTP, suggesting a specific role for myosin Va in the regulated mobilization of AMPARs to synapses. The reasons for the discrepancies between the electrophysiological results obtained in the study from Correira and colleagues (2008) and results obtained in dilute-lethal mice (Schnell and Nicoll, 2001) are not clear, but may be related to the acute blockade of myosin Va function in their system (15 h for dominant-negative expression or 3 d for RNA interference) versus the chronic absence of myosin Va during development and postnatal life in the dilute lethal mice.

Similar to our study, Correira and colleagues (2008) tested the effects of DN forms of myosin Va and myosin Vb in parallel. In contrast to our findings that expression of myosin Vb tail fragment blocks dendritic delivery of endogenous GluR1 and AMPAR-mediated mEPSC (Lise et al., 2006), their myosin Vb tail construct, which is shorter than the one used in our study, failed to
alter dendritic transport of exogenous GFP-GluR1 or AMPAR-mediated synaptic transmission (Correia et al., 2008). The lack of effect of their myosin Vb tail fragment on GluR1 trafficking into spines is likely due to the absence of a region required for Rab11/Rab11-FIP2 binding (Hales et al., 2002; Lapierre et al., 2001; Swiatecka-Urban et al., 2007). Indeed, we have demonstrated that deletion of a C-terminal region that encompasses the Rab11 binding site eliminates the DN effects of myosin Vb tail on GluR1 trafficking (Lise et al., 2006).

4.1.2.3 Rab11 as an adaptor protein for myosin V-mediated trafficking events.

Mounting evidence indicates that Rab11/Rab11-FIP2 form an adaptor complex that recruits myosin Vb to endosomes (Hales et al., 2002; Lapierre and Goldenring, 2005; Lapierre et al., 2001). Consistent with this, our study presented several lines of evidence that coupling of myosin Vb to Rab11 is required for proper GluR1 trafficking (Lise et al., 2006). First, expression of the DN truncated form of myosin Vb interferes with the normal distribution of both Rab11 and GluR1. Second, expression of a full-length mutant form of myosin Vb, which contains the motor domain and is expected to associate with F-actin, but lacks amino acids required for binding to Rab11 (Hales et al., 2002; Lapierre et al., 2001; Swiatecka-Urban et al., 2007), interferes with myosin Vb and Rab11 interaction and results in a significant decrease in GluR1 clustering and surface expression. Third, expression of a DN form of Rab11 (Rab11-S25N) results in redistribution of GluR1 and myosin Vb in the soma and reduction of GluR1 surface expression. Together, this evidence indicates that binding to Rab11 is necessary for myosin Vb mediated effects on GluR1. In their recent study, Wang and colleagues (2008) confirmed that Rab11/Rab11-FIP2 form the adaptor complex that allows the recruitment of myosin Vb to REs (Figure 4.2A). My inability to co-immunoprecipitate Rab11 with myosin Vb is likely due to differences in experimental procedures, for instance the presence of Ca$^{2+}$ which promotes a conformational switch of myosin Vb and binding to Rab11-FIP2 (Figure 4.2B) (Wang et al., 2008). Overall, this evidence indicates that Rab11 is the organelle acceptor for myosin Vb.

The commonly cited organelle acceptor for myosin Va is Rab27a. However, the low levels of Rab27a detected in the brain (Barral et al., 2002; Desnos et al., 2007a), indicate that another unknown adaptor protein is likely to mediate myosin Va association with cargo in neurons. Surprisingly, Correira and colleagues (2008) suggested that Rab11 is the organelle acceptor that
catalyzes the directional transport by myosin Va of AMPARs into spines during activity-dependent synaptic plasticity. This is unexpected since another study reported that contrary to myosin Vb, myosin Va does not interact with Rab11 in a yeast two-hybrid assay (Lapierre and Goldenring, 2005). Moreover, the myosin Va tail does not colocalize with Rab11-positive REs, has no effect on recycling of transferrin receptor in either HeLa or MDCK cells (Lapierre and Goldenring, 2005), and full-length myosin Va fails to co-traffic with REs (Wang et al., 2008). Nevertheless, Correira and colleagues (2008) provided evidence that Rab11 can couple myosin Va to AMPARs. First, they showed that exogenous Rab11 and GluR1 can be pulled-down with the myosin Va globular tail region in non-neuronal cells. Second, they reported that both endogenous Rab11 and Myosin Va are co-immunoprecipitated with GluR1 or GluR2 from brain tissue lysates. Third, their imaging data revealed that a DN form of myosin Va impairs the translocation of both GluR1 and Rab11 from the dendritic shaft into spines. Finally, the active GTP-bound form of Rab11 appeared to facilitate the interaction of GluR1 with the myosin Va globular tail, since the addition of Rab11 in the presence of nonhydrolyzable GTP enhanced myosin Va binding to GluR1 in co-immunoprecipitation experiments. Taken together, these results suggest that Rab11 may act as the vesicular link that couples AMPARs and myosin Va for their activity-dependant delivery into spines (see model Figure 4.3) (Correia et al., 2008). The shared ability of myosin Va and Vb to associate with Rab11 may underly a possible functional redundancy between these isoforms in regulating the activity-dependant delivery of AMPARs. Further analysis will be required to assess whether association to Rab11-positive organelles is differentially regulated in time and space between myosin Va and Vb.
Figure 4.3. Schematic model for the role of myosin Va and Rab11 in the regulated delivery of AMPA receptors into spines.

Left part (“constitutive”) depicts the continuous (activity-independent) delivery of GluR2/GluR3 receptors, which does not require myosin Va. Right part (“LTP”) represents the activity-dependent translocation of GluR1/GluR2 receptors mediated by Myosin Va and Rab11. In this case, receptor transport may be triggered by myosin Va activation upon NMDAR activation and Ca2+ entry. Alternatively, the transport machinery may stay constitutively active, whereas LTP regulates the competency of GluR1 to interact with it (perhaps through GluR1 phosphorylation and/or binding to adaptor proteins). As recently reported, final synaptic delivery after receptor entry into the spine is thought to be mediated by a different endosomal compartment, controlled by Rab8 (Brown et al., 2007). Myosin Va motor and cargo binding domains are represented in pink and blue, respectively. Reprinted from (Correia et al., 2008), with permission.
4.1.2.4 Subunit specificity of myosin V-mediated AMPAR trafficking

AMPAR subunits are synthesized and assembled as hetero- or homodimers in the ER, and then directed to the Golgi for subsequent post-translational modifications (Shepherd and Huganir, 2007; Vandenberghe and Breit, 2004). The majority of the AMPAR are formed by GluR1/GluR2 or GluR2/GluR3 heterodimers, and AMPA receptor complexes containing homomeric GluR1 are thought to represent only a small pool of total AMPAR (\( \sim 8\% \)) in hippocampal neurons (Wenthold et al., 1996). We were surprised to find that myosin Vb tail fragment specifically altered the clustering of the AMPAR subunit GluR1, without significantly altering GluR2 distribution (Lise et al., 2006). Moreover, our immunoprecipitation experiments from brain lysates failed to detect an association between myosin Vb and GluR2. These results suggest that myosin Vb preferentially regulates a specific vesicular pool containing GluR1 but not GluR2 and that acute overexpression of myosin Vb tail for 3-4 days is sufficient to cause the accumulation over time of GluR1 in the perinuclear region and reduce its surface expression. The partial reduction rather than total loss of GluR1 from the cell surface is consistent with the sequestering of a selective pool of GluR1 homodimers by myosin Vb tail.

Although Rab11-FIP2 is likely implicated in coupling of REs with myosin Vb, the molecular details of myosin Vb selective association with GluR1-containing AMPARs remains unclear. For instance, GluR1 failed to be redistributed with Rab11-positive endosomes in non-neuronal cells expressing a myosin Vb tail fragment, suggesting interaction of myosin Vb with GluR1 might require a neuron-specific adaptor protein in addition to Rab11/Rab11-FIP2. Consistent with this, I could not co-immunoprecipitate GluR1 with myosin Vb in non-neuronal cells. Putative adaptor proteins may include synaptic scaffolding proteins that specifically associate with AMPAR subunits and that have been implicated in AMPAR trafficking events (Bredt and Nicoll, 2003; Fukata et al., 2005; Kim and Sheng, 2004; Kneusse, 2005; Malenka, 2003; Shepherd and Huganir, 2007). For instance, the GluR1 subunit is known to associate with SAP97 (Leonard et al., 1998) and protein 4.1 (Shen et al., 2000), while GluR2 is known to associate with GRIP1, PICK1, NSF (Nishimune et al., 1998; Noel et al., 1999), and AP-2 (Braithwaite et al., 2002). Another possibility is that myosin Vb may be coupled to GluR1 through interaction with a protein complex containing DLC-GKAP-PSD-95-stargazin (Naisbitt et al., 2000). Thus, it is possible that association with a subunit-specific adaptor protein may...
explain the selective effects of myosin Vb on a specific AMPA receptor subunit. Further studies are required to explore these possibilities.

To summarize, independent studies using different experimental approaches to block the function of class V myosins suggest that both myosin Va and Vb are involved in AMPARs trafficking. Figure 4.4 shows a schematic summary of data from the three studies involving myosin V in AMPAR trafficking (Correia et al., 2008; Lise et al., 2006; Wang et al., 2008). My data indicate that myosin Vb, but not myosin Va, regulates trafficking of AMPARs, possibly from the soma to dendrites, but most likely at the periphery where myosin Vb act as a dynamic tether. The study from Wang and colleagues (2008) supports a role for myosin Vb, but not myosin Va, at the periphery where it controls the activity-dependant delivery of AMPAR-containing REs into dendritic spines. The study from Correira et al (2008) implicates myosin Va in the activity-dependant translocation of AMPAR into dendritic spines. At this point, whether both these motors are essential for postsynaptic plasticity in vivo is still a matter of debate.
Figure 4.4. Schematic model for the role of myosin Va, myosin Vb and Rab11 in trafficking of AMPAR.

(1) Myosin Vb in the perinuclear region may act as a dynamic tether to retain the RE mother compartment in the perinuclear region and/or regulate the translocation of GluR1/GluR1-associated REs to microtubules for long-range transport along dendrites. (2) Myosin Vb present at the dendritic shaft may act as a dynamic tether for the RE compartment in the actin-rich periphery. (3) Myosin Vb or myosin Va mediates translocation of GluR1/GluR2-containing REs in response to a LTP-inducing stimulus. For more details on a proposed mechanism, see Figure 4.2 and Figure 4.3. In the case of myosin Va, coupling to AMPAR involves direct interaction of myosin Va with GluR1, and is facilitated by the small GTPase Rab11 (green). Myosin Vb-mediated AMPAR trafficking requires association with Rab11-Rab11-FIP2 (green), however, selective coupling to GluR1 subunit may require an unknown adaptor protein (blue). Myosin Va motor and cargo binding domains are represented in pink and blue, respectively. Myosin Vb motor and cargo binding domains are represented in orange and green, respectively.
4.1.3 RILPL2 function in cellular morphogenesis

How does RILPL2 function in dendritic spine morphogenesis fit in with the current understanding of dendritic spine development?

How does RILPL2 interaction with myosin Va fit in with the current understanding of myosin Va function in neurons?

4.1.3.1 RILPL2 controls cell shape and dendritic morphogenesis

In the second part of my thesis, I identified RILPL2 as a novel and specific partner for myosin Va. RILPL2 is related to RILP, a protein that regulates lysosomal morphology through interaction with Rab7 and Rab34, as well as with the retrograde motor dynein (Cantalupo et al., 2001; Johansson et al., 2007; Jordens et al., 2001). In a previous study, the lack of effect of RILPL2 overexpression on lysosomal morphology suggested that the cellular function of RILPL2 differs from RILP (Wang et al., 2004b). In my study, I found that RILPL2 overexpression alters cell shape and causes the appearance of cellular extensions in non-neuronal cells. Based on its protrusive effect in non-neuronal cells, we suspected that RILPL2 to be implicated in neurite formation in neurons early in their development. While RILPL2 overexpression specifically attenuates axonal outgrowth, the apparently normal neurite formation and growth in the absence of RILPL2 indicates that it may not be essential for normal neuritogenesis early in development. At a later developmental stage, RILPL2 overexpression increased the density of dendritic spines, while siRNA-mediated RILPL2 knockdown blocked their formation. Overall, these observations point to a novel role for RILPL2 in regulating cellular morphogenesis.

4.1.3.2 RILPL2 activates Rac signaling

A change in cellular morphology is often regulated by signalling pathways that control the actin cytoskeleton. Potential candidate signaling molecules that modulate F-actin dynamics are members of the Rho GTPase family, such as Rac1, cdc42 and RhoA (Hall, 1998; Luo, 2002). In the case of RILPL2, the formation of cellular extensions and lamellipodia in non-neuronal cells, as well as increased formation of dendritic spines in neurons is reminiscent of the reported
phenotype of increased Rac signaling in fibroblast and neuronal cells (de Curtis, 2008; Hall, 1998; Nakayama and Luo, 2000; Penzes et al., 2008). In our study, we provide evidence that RILPL2 is likely to function via Rac signaling. First, RILPL2 promotes Rac activation in non-neuronal cells and sequestration of RILPL2 in a perinuclear compartment with DN myosin Va blocked both Rac activation and change in cell shape. Second, RILPL2 expression in neurons enhanced the phosphorylation of the Rac1 effector, Pak. Last, Rac was found associated with RILPL2 in brain lysates, an association that may facilitate activation of Rac controlled by RILPL2.

How RILPL2 activates Rac is not clear. The absence in RILPL2 of the conserved (DH) region characteristic of the GEFs suggests RILPL2 is unlikely to be a GEF that directly activates Rac. An attractive possibility is that RILPL2 recruits a Rac GEF to active Rac. In neurons, possible candidate Rac GEFs implicated in dendritic spine morphogenesis are Kalirin-7, STEF, Tiam 1, β-PIX, and GEFT (Bryan et al., 2004; Penzes et al., 2008). Notably, the CBD of myosin V contains a region of homology with the scaffolding protein AF-6/afadin (Ponting, 1995), a PDZ domain-containing protein involved in clustering of receptors (Buchert et al., 1999) and recruitment of Kalirin-7 to synapses to control the morphology of dendritic spines (Xie et al., 2008). Whether the AF-6 homology domain in myosin Va is implicated in the recruitment of kalirin-7 to RILPL2 for Rac activation remains to be tested.

We have provided evidence that RILPL2 activates the Rac-Pak1 signaling pathway in neurons, Whether RILPL2-mediated effects occur via this specific signaling pathway remains unclear. For instance, other Rho GTPase family members or other Rac downstream signaling pathways have been implicated in dendritic spine morphogenesis. (Bryan et al., 2004; Linseman and Loucks, 2008; Murata et al., 2006; Nishimura et al., 2006; Penzes et al., 2008; Tashiro et al., 2000). Further analysis assessing the activity of cdc42 or RhoA upon RILPL2 expression, as well as activation of other Rac substrates, such as phosphoinositide 3-kinase (PI3K)-Akt and Jun N-terminal kinase (JNK, or mitogen-activated protein kinase 8, also known as MK08), should help clarify this issue.
4.1.3.3 Functional relevance of RILPL2-myosin Va interaction

Analysis of truncated forms of RILPL2 indicates that the N-terminal half of RILPL2 is critical for RILPL2 function. Indeed, the expression of the N-terminal region of RILPL2 is sufficient to activate Rac in heterologous systems. In neurons, expression of RILPL2 ΔCT was not sufficient to increase the density of dendritic spines to levels similar to wild-type RILPL2. However, the lack of this N-terminal region did block the formation of dendritic spines. Our yeast two-hybrid and biochemical data indicate that the N-terminal half of RILPL2 is sufficient for association with myosin Va globular tail. The functional relevance of the RILPL2 and myosin Va interaction remains obscure. There is increasing evidence for a role of myosin Va in the directed delivery of organelles or mRNA-protein complexes from the dendritic shaft to the spine compartment (Correia et al., 2008; Dekker-Ohno et al., 1996; Petralia et al., 2001; Yoshimura et al., 2006). Thus, myosin Va may control translocation of RILPL2 (or a protein complex containing RILPL2) into spines where it can activate Rac signaling. An additional possibility is that myosin Va may be dynamically tethering or capturing RILPL2 in the actin periphery underneath the plasma membrane. The observed mislocalization of RILPL2 ΔNT construct lacking the portion interacting with myosin Va is consistent with the idea that RILPL2 interaction with myosin Va is critical for proper localization of RILPL2. Further analysis in neurons lacking a functional myosin Va should clarify whether RILPL2 localization and function in dendritic spine morphogenesis relies on myosin Va.

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 class V myosins in neurons. The initial finding that the AMPAR is a cargo for myosin Vb provides further insight into the mechanisms that regulate trafficking of this crucial receptor. The findings concerning the myosin Va-interacting protein RILPL2 uncover a role for this previously uncharacterized protein in synapse morphogenesis. Together, these findings relate myosin V family members to process that are critical for proper neuronal function and plasticity.
4.2 Future directions

The data presented in this thesis suggests numerous avenues of future investigation. These include molecular and genetic studies to further our understanding of neuronal trafficking mechanisms and are ultimately aimed at identifying therapeutic targets for treatment of neurological disorders associated with defects in neuronal transport. The understanding molecular and cellular mechanisms that regulate active transport by motor proteins, combined with the development of technology allowing particle tracking with microsecond time-resolution and nanometer spatial precision (Nan et al., 2008; Yildiz and Selvin, 2005), may also eventually serve bio-engineers in the development of nanodevices suitable for a controlled displacement of objects or specific substances with nanometre precision (Bachand et al., 2005; Bohm et al., 2003; Clemmens et al., 2004; Hess, 2006; Hess et al., 2004; Hess and Vogel, 2001; van den Heuvel and Dekker, 2007). For instance, such a technology could increase the accuracy of delivery of therapeutics to specific subcellular localization in the cells.

4.2.1 Mechanisms for myosin V-mediated AMPAR trafficking

The amount and composition of cargoes that are being delivered or recycled to the synapse require tight regulation of mechanoenzymatic activity and cargo interaction of molecular motors. Most of what is known about the regulation of myosin Va structure-function comes from in vitro studies, which strongly suggest that Ca$^{2+}$ play a major role in regulating myosin V activity (Li et al., 2004; Nascimento et al., 1996; Wang et al., 2004a). However, the precise mechanism for Ca$^{2+}$ regulation of myosin V activity in vivo is poorly understood. Recent work has led to a model for the activity-dependant regulation of myosin V-mediated delivery in dendritic spines, where local Ca$^{2+}$ influx through NMDARs triggers a conformational change from the folded inactive state to the extended active state, allowing interaction of myosin V with its cargo and processive transport to occur (Figure 4.2 and Figure 4.3) (Correia et al., 2008; Wang et al., 2008). Remaining questions are whether additional factors regulate recruitment and association of myosin V to AMPAR-containing REs are and how this cargo is released from the myosin V motor after delivery to the spine. Notably, there is evidence that myosin V function could be modified by Ca$^{2+}$-dependant enzymes, such as CaMKII and calpain, which respectively promote...
phosphorylation and proteolytic cleavage of the tail domain of myosin V (Alavez et al., 2004; Coelho and Larson, 1993; Costa et al., 1999). Whether these Ca\textsuperscript{2+}-regulated mechanisms also participate in the activity-dependant regulation of myosin V, possibly by controlling the interaction with the cargo or the amount of functional motor available for transport in vivo, is currently unknown and should be investigated in future studies.

4.2.1.1 Phosphorylation by CaMKII

In Xenopus melanophores, CaMKII-mediated phosphorylation of the CBD of myosin Va resulted in the release of the motor from its organelle-cargo (Karcher et al., 2001). In the brain, myosin Va is phosphorylated by CaMKII (Coelho and Larson, 1993; Costa et al., 1999). Thus, one can envision that a similar mechanism may exist in dendritic spines to reversibly control the association myosin V with AMPAR-containing REs upon changes in neuronal activity. In this scenario, Ca\textsuperscript{2+} influx through NMDARs would locally activate CaMKII, resulting in phosphorylation of myosin V and release of the AMPAR-containing REs. Another possibility is that myosin V attachment to cargo is regulated by the phosphorylation state of the adaptor protein Rab-FIP2 (Ducharme et al., 2006). Finally, phosphorylation of AMPAR subunits by CaMKII or by other kinases such as PKA or PKC, may provide another means to regulate association of myosin V with AMPAR-containing REs. These avenues could be tested using biochemical analysis to monitor association between myosin V, AMPARs and Rab11-RabFIP2 upon mutation of phosphorylation sites or in the presence of kinase inhibitors.

4.2.1.2 Proteolytic cleavage by calpain

Both myosin Va and Vb have a PEST sequence in their C-terminal tail sequence that targets the molecule for proteolytic cleavage (Figure 1.10). In the case of myosin Va, this process is dependant on the entry of Ca\textsuperscript{2+} upon neuronal depolarization and activation of the cysteine protease calpain (Alavez et al., 2004; Casaletti et al., 2003). It is possible that neuronal activity regulate myosin Va or Vb cleavage and/or degradation to control the amount of AMPARs delivered to the synapse. Although this mechanism might not be as economical as conformational change and phosphorylation, this non-reversible mechanism might be used in the
neuron in specific conditions. Time-lapse imaging and biochemical studies using mutant form of myosin Va or Vb resistant to cleavage and in the presence of agents that enhance or suppress neuronal activity would help determine whether activity-dependant cleavage is important for AMPAR delivery to the plasma membrane and at the synapse, and if it regulates coupling of myosin Va or Vb to Rab11/RabFIP2 and AMPARs.

4.2.2 Myosin V knockout and transgenic mice

Cultured neurons and brain slices from dilute-lethal mouse brains have been used to investigate myosin Va involvement in the transport of several cargoes, including ER, mRNA/protein complexes, synaptic proteins, as well as for electrophysiological studies (Desnos et al., 2007a). Dilute-lethal mice die within 3 weeks of age, impeding further studies of myosin Va function in adult animals. Generation of conditional myosin Va-/- knockout mice would allow researchers to bypass the developmental stages and assess myosin Va involvement in maintenance and plasticity processes in the mature brain and to perform behavioural assessment. Compensation might occur between myosin V isoforms, at least in the case of AMPARs delivery to synapses. This could be tested by the generation of myosin Va -/- and Vb -/- double knockout animals and comparison with the single knockout animals. As far as I know, no myosin Vb -/- mice has been generated. Generation of myosin Vb-null mice will likely contribute to the understanding of myosin Vb function in vivo. Generation of myosin Va- or Vb conditional knockout mice, using Cre/lox recombination system, would allow researchers to bypass the developmental stages, to assess myosin Va involvement in maintenance and plasticity processes in the mature brain, and to carry out behavioural assessments in adult animals. Another strategy that would avoid potential effects of chronic disruption of myosin V is the generation of transgenic mice expressing sensitized mutant forms of myosin Va or Vb and allowing for chemical-genetic inhibition of individual isoforms (Provance et al., 2008; Provance et al., 2004). While such transgenic mice have already been generated and successfully used for myosin Vb (Wang et al., 2008), similar mice have not been generated for myosins Va and Vc. These mice will likely advance our current understanding of the respective physiological roles of myosin V isoforms in vivo.
4.2.3 Cooperation between microtubule- and actin-based motors for neuronal transport

The current dual filament model for organelle and vesicle transport suggests that long-range transport occurs along MTs, whereas local, short-range transport occurs along F-actin. This idea of collaboration between MT-based motors and actin-based motors is not new (Langford, 1995), and is supported by growing evidence showing that both MT-based and actin-based motors are found on individual vesicles and organelles (Brown, 1999; Gross et al., 2002; Huang et al., 1999; Kural et al., 2007; Levi et al., 2006; Mallik and Gross, 2004; Stachelek et al., 2001). Several questions can be asked for future investigation of neuronal transport. How does the transition occur between MT-based motors and myosin V-motors when there is a shift between cytoskeletal tracks? Which motors collaborate for the transport of a given cargo and how is the motor matched with appropriate cargo? How does a motor share its cargo with other motors? Can a molecular motor present at the surface of an organelle modulate the function of another motor protein also present on the same cargo?

Can the dual filament transport model be generalized to AMPARs trafficking in neurons? The kinesin family members KIF5 (Setou et al., 2002) and KIF1A (Shin et al., 2003; Wyszynski et al., 2002), as well as the myosin family members myosin Va (Correia et al., 2008) Vb (Lise et al., 2006; Wang et al., 2008) and myosin VI (Osterweil et al., 2005), have all been individually implicated in dendritic and synaptic trafficking of AMPARS. It will be interesting in the future to assess whether these motors collaborate to bring AMPARs to their final destination. For example, it would be interesting to see whether manipulations of myosin V activity can affect kinesin-dependent AMPAR transport or vice versa. The concept of cooperation with MT-based molecular motors, as described by the dual filament model of transport, will be essential for a complete understanding of myosin-V function, and how intracellular transport is co-ordinated and finely-tuned in neurons.

4.2.4 Screen for novel myosin V-binding proteins

As the number and diversity of molecules that need to be delivered to the different neuronal subcompartments largely outnumbers the molecular motors present in neurons, specificity of cargo recognition is likely to rely on protein adaptor complexes that link a particular motor to a
cargo. Although several new cargoes have been identified for MT-based motors (Hirokawa and Noda, 2008), the list of cargoes and adaptor proteins for myosin Va and Vb in neurons remains limited. In the future, identification and characterization of additional transport complexes should help us understand whether specific cargoes travel as passengers of distinct or identical motors. Because of their relatively low abundance, small size and absence of unique morphology, not all of these transport complexes can be initially identified by microscopy. Hence, a more fruitful approach is to use the putative CBDs of motor proteins to identify these transport complexes.

In our study, we have used the yeast two-hybrid approach as a tool to identify novel proteins that bind to the CBD of myosin Va. A list of positives from our screen is presented in Appendix B. Dynein light chain 2 (DLC2) is a candidate adaptor protein that could link myosin Va to cargoes, while DAZ interacting protein 1 (DZIP1) may couple myosin Va to RNA/protein complexes to regulate RNA transport events. A pitfall of the yeast two-hybrid approach is the occurrence in the yeast nucleus of protein-protein interactions that may not be physiologically relevant due to different subcellular locations of the endogenous proteins in mammalian cells. This could be the case for the identified interactions of myosin Va, with nuclear proteins, since myosin Va is thought to localize mainly to the cytoplasm. However, a recent report has located myosin Va in the nucleus (Pranchevicius et al., 2008), and a role for nuclear actin and nuclear myosins in the regulation of gene expression has been reported (de Lanerolle et al., 2005; Grummt, 2006; Hofmann et al., 2006). Further investigation is required to assess the functional relevance of myosin Va association with nuclear proteins.

Another approach to identify novel protein complexes that associate with myosin V consists in using an epitope-tagged myosin V CBD for pull-down assays from brain lysates, followed by chromatography and mass spectrometry to identify individual proteins present in the complexes (DeSelm et al., 2004). Alternatively, generation of transgenic animals overexpressing an epitope-tagged myosin V full-length version would allow co-immunoprecipitation of native myosin V complexes. This powerful strategy has been successfully used in the past to identify native GluR1 complexes (Fukata et al., 2005). Identifying proteins that bind to the CBD of myosin Va or Vb (or other molecular motors) is likely to be a fruitful approach used in the discovery of novel trafficking pathways in the future.
4.2.5 RILPL2 and activity-dependant spine remodeling

In our initial study, we show that RILPL2 overexpression concomitantly increases dendritic spine density and phosphorylation of the Rac effector Pak. Although these observations suggest that the RILPL2-mediated effect on morphogenesis is likely to occur through Rac signaling, the link between RILPL2 and Rac remains mysterious. Future experiments should test whether a specific Rac-GEF is involved in this process.

Mature dendritic spines are subjected to activity-dependant structural and functional remodelling. For instance, increasing evidence points to a convergence between Rac- and Ca\textsuperscript{2+}-dependant signaling in the control of dendritic structure and function (Figure 4.5) (Penzes et al., 2008). Whether RILPL2 has any role in this scheme remains an open question. In the future, it will be interesting to address whether RILPL2 localization and function are regulated by synaptic activity to control dendritic spine remodeling.
Figure 4.5. Schematic of the signaling cascade from the influx of Ca^{2+} via Ca^{2+} channels and NMDARs to the morphogenesis of dendritic spines.

Calcium increases in concentration in the dendritic and spine cytoplasm and in complexes with calmodulin, which activates CaMKs. Active CaMKs then phosphorylates downstream targets, including Rac GEFs (red arrow), which modulate the activity of the small GTPase Rac by enhancing GDP–GTP exchange. GTP-bound Rac, by downstream effectors such as Pak, performs a variety of functions in the dendrite, particularly dendritic spine morphogenesis. Reprinted from (Penzes et al., 2008), with permission.

4.2.6 RILPL2 as a p40-phox-binding protein

In our initial study on RILPL2, we have focused our investigation on the function of RILPL2 in neuronal cells, however the presence of RILPL2 in other tissues, notably in the immune system, suggests a role for RILPL2 in other organs. In this regard, it is worth noting that the human homologue of RILPL2 has been suggested to bind to p40-phox (BAC76826) (Kuribayashi,F., Ago,T. and Sumimoto,H., unpublished observations), a protein that is part of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex, which produces superoxide upon its activation (Wientjes and Segal, 1995). Although this complex has been most studied in
macrophages and neutrophils, it has also been detected at the synapse, where it is suggested to be the source of superoxide production required for LTP and memory function (Kishida et al., 2005; Serrano et al., 2003; Tejada-Simon et al., 2005). Intriguingly, Rac1 is a subunit of the NADPH oxidase and regulates its enzymatic activity (Hordijk, 2006). It is tempting to speculate that RILPL2 might be involved in regulating the NADPH oxidase activity through Rac activation at the synapse. Further studies will be required to test this hypothesis.

4.3 Conclusion

Proper neuronal development and function require precise sorting and delivery of various elements from the soma to the synapse. Candidates for regulating trafficking of synaptic protein are the actin-based motors of class V myosins. Identification and characterization of novel cargoes for myosin Va and Vb in neurons reveal that myosin Vb is required for proper AMPAR trafficking to the synapse, while, the myosin Va-interacting protein, RILPL2, is required for normal dendritic spine development. These findings further our understanding of the respective function of myosin V isoforms and associated proteins in neurons and provide new insights into the molecular players that regulate neuronal trafficking and dendritic morphogenesis.
4.4 References


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APPENDICES

Appendix A  Myosin Vb proteolytic cleavage

Figure A.1. Modulation of myosin Vb cleavage and distribution by neuronal activity.

(A) Location of the PEST sequence present in myosin Vb.

(B) Treatment of cultured neurons (DIV 16-20) with 90 mM KCl for 30 minutes results in myosin Vb cleavage. This effect is blocked with 5 μM calpain inhibitor (Calpain Inh) or 50 μM of proteasome inhibitor MG132, 1 hour prior to KCL treatment.

(C-D) Cultured neurons (DIV 16-20) were treated with 50 mM KCl or 50 μM bicuculline (Bic) for 4 hrs and was then subjected to protein analysis. Detection of a major ~ 75-80 KDa band (arrowhead) with our myosin Vb polyclonal antibody, corresponding to the C-terminal tail fragment of myosin Vb, after KCl (C) or bicuculline (D) treatment.

(E) DIV 28 neurons were incubated overnight with vehicle (control untreated) or 50 μM bicuculline, and changes in endogenous myosin Vb localization were assessed using immunocytochemistry. This treatment resulted in loss of myosin Vb positive puncta from dendritic shafts and spines (arrowheads). Scale bar, 1 μm. Lisé and Husseini, Unpublished data.
### Appendix B  Yeast two-hybrid screen with MyoVa CT

Table B.1. List of positive from a yeast two-hybrid screen of a brain cDNA library with myosin Va CT.

<table>
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<th>Y2H positive Protein ID</th>
<th>Function</th>
<th>Reference</th>
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<td>Dynein light chain 2 (DLC2 also PIN, LC-8)</td>
<td>Cytoplasmic localization</td>
<td>(Espindola et al., 2000; Fuhrmann et al., 2002; Haraguchi et al., 2000; Hodi et al., 2006; Naisbitt et al., 2000)</td>
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<td>DAZ interacting protein 1 (DZIP1)</td>
<td>Nuclear and cytoplasmic localization</td>
<td>(Curry et al., 2006; Moore et al., 2004; Morton et al., 2006)</td>
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<td>Poly (rC) binding protein 2 (PCBP2)</td>
<td>Nuclear localization</td>
<td>(Chkheidze and Liebhaber, 2003; Leffers et al., 1995)</td>
</tr>
<tr>
<td>Phosphodiesterase 4D interacting protein (Pde4dip, Myomegalin)</td>
<td>Cytoplasmic localization</td>
<td>(Verde et al., 2001)</td>
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<tr>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 13B (Ppp1r13b also Apoptosis-stimulating protein of p53 (ASPP1)</td>
<td>Nuclear localization</td>
<td>(Samuels-Lev et al., 2001)</td>
</tr>
<tr>
<td>Proliferating cell nuclear antigen (PCNAR; Pcna/cyclin)</td>
<td>Nuclear localization</td>
<td>(Imai et al., 2002; Matsumoto et al., 1987)</td>
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</table>
B.1 References


Appendix C  Material and methods

C.1 Tissue culture and cell lines

Cell lines, including COS-7 (Green Monkey kidney) and HEK-293 (Human Embryonic kidney) cells, were grown in Dulbecco's modified Eagle's medium (DMEM) or Minimum Essential medium (MEM) (Gibco-Invitrogen) supplemented with 10% fetal bovine serum, penicillin and streptomycin. PC12 (pheochromocytoma from rat adrenal medulla) cells were cultured in RPMI 1640 medium (Gibco-Invitrogen) containing 2 mM L-glutamine, penicillin and streptomycin. All cell lines were maintained at 37°C and 5% CO2.

Dissociated primary neuronal cultures were prepared from hippocampi or cortices from E18/E19 rats. The hippocampi or cortices were dissociated by papain enzymatic digestion followed by brief mechanical trituration. Cells were plated at densities ranging from 80,000 to 150,000 cells on poly-D-lysine-(Sigma) treated glass coverslips (12 mm in diameter, Fisher) in 24-well plates (Falcon). Cultures were maintained at 37°C and 5% CO2 in Neurobasal media (NBM) (Gibco-Invitrogen), supplemented with B27, 2mM GlutaMAX, penicillin and streptomycin.
## C.2 Antibodies

Table 5.2 and Table 5.3 list the antibodies used during the course of the thesis work.

### Table C.1. Commercial Antibodies

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<td>R-960-25</td>
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<td>Reference</td>
<td>Working dilution</td>
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<tr>
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<td>aa 1005-1830</td>
<td>(Evans et al., 1997)</td>
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<tr>
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<td>rabbit</td>
<td>aa 899-1830</td>
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<td>From AM Craig lab</td>
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<tr>
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<td>PSD-95</td>
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<td>(Huang et al., 2004)</td>
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C.3 Generation of myosin V antibodies

C.3.1 Preparation of antigen

GST fusion proteins were produced in E. coli (strain BL21) using the pGEX 4T expression system (GE Healthcare). For GST-MyoVb CC and GST-MyoVa MT, induction was performed with 300 µM IPTG at 30°C for a minimum of 5 hours or overnight. After induction, cells were centrifuged at 6,000 rpm for 20 minutes and the supernatant discarded. Pellets were frozen at -80°C. After 1 hour or overnight, pellets were thawed on ice. For 1L of induced bacteria culture, cell pellets were resuspended in 10 mL of ice cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1 mg/mL DNAse I, 1 mg/mL lysozyme, 1 mM PMSF and 1X protease inhibitors (Roche). Lysates were then sonicated on ice for 4 X 15 seconds, followed by incubation at 4°C for an hour. Next, samples were centrifuged at 45,000 rpm at 4°C for 60 minutes to remove the bacteria debris. Previously washed Glutathione Sepharose™ 4B beads (GE Healthcare) were added to the supernatant (80µL of 50/50 beads per 0.5mL lysate) and incubated on rotator at 4°C for 1 hour or overnight. Next, beads were washed 10 times with wash buffer (25mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 1 mM PMSF). Finally, GST fusion proteins were eluted with 20 mM glutathione/50 mM Tris base (pH 8.0). In some cases, purification was done using Glutathione Sepharose 4B prepacked disposable column (GE Healthcare) following manufacturer’s protocol. Purity, degradation and approximate concentration was determined by running the purified GST fusion protein along a BSA standard on a SDS gel, followed by Coomassie blue staining to visualize the proteins. Approximately 10 mg of GST-MyoVb CC and GST-MyoVa MT was purified, concentrated, dialysed in 1X PBS and sent to Affinity BioReagents for immunization of rabbits and guinea pigs, respectively.

C.3.2 Antibody purification

Preparation of the column

The appropriate amount of activated CH Sepharose 4B beads (GE Healthcare) was weighted out (1g gives about 2–3 ml gel volume). Beads were washed and re-swelled on a sintered glass filter or in a column with 1 mM ice-cold HCL. The ligand (GST-fusion protein) was dialysed in coupling buffer (0.1 M NaHCO3, pH8, 0.5 M NaCl) in cold room using a dialysis tubing/bag. The buffer was changed 2 times. During this time, beads were equilibrated in ligand coupling buffer. Ligand and gel suspension were then mixed end over end on a rotator for 1 hour at room temperature or 4 hours at 4°C. The coupling buffer was drained away and the column washed with 15 mL 0.1 M Tris buffer, pH 8.0. Excess active groups were blocked with 0.1M Tris buffer, pH 8.0 for 1 hour in a 50 mL tube. Excess ligand was washed away.
by washing with coupling buffer. Then, the column was washed with 10 mL 0.1M Tris, 0.5 M NaCl (pH 8) followed by 10 mL 0.5 M NaCl (pH 4.0) (from acetic acid and in Milli-Q water), and repeated two more times. Beads were finally equilibrated in PBS. For storage; beads were kept in PBS with 0.1% azide at 4°C.

**Affinity purification**

The column was washed and equilibrated with 5 volumes of 1X PBS. Serum (5 ml) was filtered using a syringe and mixed with the beads in a 50 mL tube, a rotator at room temperature overnight. Beads with serum were then applied back into the column and the serum flow through collected in a clean 50ml Falcon tube. The beads in the column were thoroughly washed with 20 column volumes of PBS. Antibody was eluted with 20 ml of 0.1M glycine, pH 2.5 slowly dripping through the column (1 drop/sec). Samples were collected in 1.5 ml tubes as 0.5 ml fractions and put on ice. Each fraction was neutralized by adding 1M Tris base and checking with a narrowly cut strip of pH paper until pH is in the range of 7-8. Antibody concentration was assessed using the BCA protein assay kit (Pierce).

**C.4 DNA constructs and cloning**

**C.4.1 DNA constructs**

The plasmids containing various cDNA constructs used during the course of the thesis work are listed in Table 5.4. DNA was generated from these constructs in bacteria (usually in DH5α or BL21 bacterial strains) using standard methods.
<table>
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<th>aa</th>
<th>Vector</th>
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<td>pEGFP-C2</td>
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C.4.2 Site-directed mutagenesis

Full-length myosin Vb lacking the Rab11 interacting region identified (Lapiere et al., 2001) was generated by deleting 15 internal residues corresponding to aa 1797-1811 with the following sense oligo 5’-CCTTTATACGAACAATCCAGTCCAAACACATGTTCCTCC-3’ and corresponding antisense oligo using QuickChange site-directed mutagenesis kit (Stratagene) following manufacturer’s protocol.

C.4.3 RT-PCR cloning

RNA from embryonic day 18 (E18) or adult rat tissues, as well as cultured cortical neurons was isolated using the RNeasy Mini Kit (Quiagen) following the manufacturer’s protocol. RNA (1 μg) was used for each RT reaction with oligo (dt) primers (Invitrogen). The specific primers used to clone RILPL2 and RILPL1 full-length cDNAs are listed in Table 5.5.

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<th>Clone ID</th>
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<td>+10AREcor1 (EcoRI)</td>
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<td>RT_MFBPfl_R (ApaI)</td>
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<td>RT-RLP1_R (BamHI)</td>
<td>5’-GGGCCGCCGGATGACGCAGACGCCGG-3’</td>
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C.4.4 ShRNA design and cloning

For designing the small hairpin against RILPL2, I used an online SiRNA Design Software (http://i.cs.hku.hk/~sirna/software/sirna.php). Two target sequences were selected from the ones suggested by the software and a NCBI BLAST for short nt sequences was performed to make sure the these sequences were specific for rat RILPL2 and would not target any other rat DNA. To create RILPL2 shRNA, two complementary 60 bp oligos containing the sense and antisense sequences corresponding to nt 768-786 (shRNA 493) and 771-789 (shRNA 496) of rat RILPL2, were annealed and ligated into BglIII and HindIII sites of pSUPER (OligoEngine) or a modified version of pSUPER, pSUPERneo+GFP (OligoEngine) vector in accordance with OligoEngine’s instructions.
C.5 Cell lysis and tissue preparation

C.5.1 Transient transfections

For most cell lines transfection, Lipofectamine 2000 reagent (Invitrogen) was used based on the manufacturers protocol. For cultured neurons, I used either Lipofectamine, calcium phosphate (CaPO₄) or electroporation transfection methods depending of the experiments.

Lipofectamine
Culture neurons (between DIV 7-10) were transfected using Lipofectamine 2000 reagent (Invitrogen) based on the manufacturer’s protocol. Briefly, for each well of the 24 well plate, 0.5 to 1μg of DNA and 0.5μl of Lipofectamine 2000 reagent were mixed in 50 μl of OPTIMEN (Invitrogen) and incubated at room temperature for 15 minutes. Then, the mixture was added dropwise to the wells and cells were incubated for 1-3 hour at 37°C and 5% CO2 before replacing cell media containing the transfection reagent with fresh pre-warmed NBM media.

Calcium phosphate
Culture neurons (between DIV 7-10) were transfected using calcium phosphate method using the CalPhos kit (Clonetech) following manufacturer protocol and as previously described (Jiang et al., 2004). For 4 wells of the 24 well plate, 4-8 μg of DNA was mixed with 6.2 uL 2M calcium solution and sterile water to a final volume of 100 μl. This pre-transfection mixture was added dropwise while gently vortexing to 100 μl of 2X HBS, and incubated at room temperature for 10 minutes. Then, the calcium/DNA suspension was added dropwise to each well (50uL/well), and cells were incubate for 1-3 hours at 37°C and 5% CO2 before replacing cell media with pre-warmed NBM media.

Electroporation
Freshly dissociated neurons were resuspended in 5ml of pre-warmed DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS) and counted. The required number of cells (2-3 million per nucleofection sample for 6 wells from a 24-well plate) was spun down and the supernatant completely removed. Cells were resuspended in 100 μl of room temperature electroporation solution (120mM KCl; 10mM KH₂PO₄; 2mM EGTA; 25mM HEPES; 5mM MgCl₂; 0.5mM CaCl₂; Mix + adjust pH to 7.5-7.6; add last and freeze immediately: 5mM GSSG; 2mM ATP. 1-3 μg of high quality endotoxin-free DNA (no more than 5μl in TE or H2O) was added to the cell suspension and the mixture was transferred into an electroporation cuvette (Amaxa or Biorad) and electroporated with the Amaxa Nucleofector. Cells were immediately transferred from the cuvette into the necessary amount of DMEM supplemented with

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10% FCS and plated according to the desired density (200,000 to 300,000 per well). Cells were incubate for 2-4 hours at 37°C and 5% CO2 before replacing cell media with pre-warmed NBM media.

C.5.2 Cell lysis

**Cultured cells**

To harvest cells from 6 well plates, 1 mL of cold 1X PBS was added to each well and cells were scraped into a 1.5ml eppendorf tube. After spinning samples at 14,000rpm for 1 minute, supernatants were removed and pellets ressuspended in 500 µL of lysis buffer for soluble protein (50 mM Tris pH 7.5, 150 mM NaCl, 2mM EDTA, 2 mM EGTA, 1% Triton-X100, 1X protease inhibitors (Roche) and 1 mM PMSF)). Samples were incubated for 30 minutes on rotator at 4°C, followed by centrifugation at 14,000 rpm for 15 min at 4°C to remove debris.

**Fresh tissue**

Fresh brains and other organs were obtained from rats exposed to halothane and sacrificed by decapitation. Organs were quickly dissected and weighted. For each 0.5 g of tissue, 5 mL of lysis buffer without detergent was added. Samples were homogenized for 20 times on ice and then sonicated briefly (3 x 10s on ice). Next, detergent was supplemented and samples were rotated at 4°C for 1hour. Samples were then centrifuged for 25 minutes at 50,000 RPM at 4°C. Supernatants were transferred in new tubes and protein concentration was assessed with BCA protein assay kit (Pierce).

**Lysis buffer for myosin Vb and GluR1:**

TEEN buffer (50 mM Tris-HCl (ph 7.4), 1mM EDTA; 1mM EGTA; 150mM NaCl), 2.5 mM sodium vanadate; 10 mM ATP, 10 mM MgCl2, 1X protease inhibitors (Roche); 1mM PMSF

**Lysis buffer for other soluble proteins**

TEEN buffer (50 mM Tris-HCl (ph 7.4); 1mM EDTA; 1mM EGTA; 150mM NaCl); 1X protease inhibitors (Roche); 1mM PMSF

C.6 Immunoprecipitation

C.6.1 From cultured cell lysates

Immunoprecipitations from COS-7 or HEK-293 cells lysates were performed in lysis buffer containing 1% Triton X-100. For each sample, supernatant was collected, 50 µL was set aside (for loads) and 2-10
μg of primary antibody was added before incubation for 1 hour at 4°C on rotator. Then, 20-30 μL of Sepharose A (for polyclonal antibody) or Sepharose G (for monoclonal antibody) beads (GE Healthcare) were added and samples incubated for 1 hour at 4°C or overnight with shaking. Samples were 3X washed with TEEN + 1% Triton X-100 and spun down at 5,000 rpm for 1 min at 4°C. Samples were ressuspended in 2X SDS-PAGE sample buffer supplemented with 10% β-mercaptoethanol or 5mM DTT and processed for immunoblotting.

C.6.2 From brain lysates

Brain lysates were prepared as described above. For myosin Vb immunoprecipitation, cells were lysed in TEEN by the addition of 0.1% SDS, 0.8% Triton X-100. For GluR1 immunoprecipitation, cells were lysed in TEEN containing 0.5% deoxycholate and 1% NP-40. Samples (500 μg to 1 mg of total protein per tube) were incubated for 1 hour at 4°C with 5μg anti-myosin Vb or anti-GluR1 polyclonal antibodies, raised against GST fusion proteins of coiled-coil region of myosin Vb and carboxyl tail of GluR1, respectively. After addition of 40 μl protein A Sepharose 4 Fast Flow beads (GE Healthcare), samples were incubated at 4°C for 1 hour or overnight. Immunoprecipitates were washed 3 times with TEEN buffer containing 1% Triton X-100. Samples were boiled in SDS-PAGE sample buffer with 10% β-mercaptoethanol or 5mM DTT and processed for immunoblotting.

C.7 GST pull down assays

Brain lysates were prepared as described above. Cells were lysed by the addition of 1% Triton X-100 followed by rotation for 1 hour at 4°C. Insoluble material was removed by centrifugation at 50,000 rpm for 25 min at 4°C. Samples (500 μg to 1 mg of total protein per tube) were incubated on a rotator at 4°C overnight with Sepharose 4B beads (GE Healthcare) coupled to 30 μg purified GST-coupled proteins. Beads were washed 3 times with TEEN buffer containing 0.5% Triton X-100. Samples were ressuspended in sample buffer containing 5 mM DTT and processed for immunoblotting.

C.8 Subcellular fractionation

For subcellular fractionation, cerebral cortices from seven adult rats were homogenized and fractionated as previously described (Fleisig et al., 2004). Briefly, rat brain homogenates were centrifuged at 1,000×g to remove nuclei and large debris. The supernatant was centrifuged at 12,000×g to obtain a crude synaptosomal fraction which was subsequently lysed hypo-osmotically and centrifuged at 28,000×g to
yield a pellet of synaptosomal membranes. The supernatant was additionally centrifuged at 251,000×g to obtain presynaptic cytosol and a pellet enriched in synaptic vesicles. Samples were analyzed by SDS-PAGE and immunoblotting.

### C.9 Immunoblotting

Standard procedures were used for most immunoblotting. Briefly, sample preparation involved denaturing the lysates or immunoprecipitates in SDS gel loading buffer and heating for 5 minutes at 90°C-100°C. For proteins of molecular weight between 40 kDa to 200 kDa, 10% polyacrylamide gels were used. For proteins of smaller molecular weight (20–30kDa), 4-12% bis-tris (Invitrogen) or 12% polyacrylamide gels were used. To get a better resolution for proteins of big molecular weight such as myosin V (200kDa), 3-8% Tris-acetate gels (Invitrogen) were used in some cases. The gels were run for 1-2 hours at 150-200V. Rainbow marker (GE health, RPN800) was used for indicating the molecular weight. Transfers were performed onto nitrocellulose membranes (GE Healthcare) at 0.5A for 60-90 minutes using transfer buffer (11.6g Tris, 58g glycine, 400ml MeOH and 2g SDS in 2L H2O). Blocking was performed with 5% milk or 5% BSA depending on the primary antibody. Western blots signals were detected with an Odyssey machine (LiCor) as previously described (Swayze et al., 2004) or ECL (Amersham Pharmacia Biotech).

### C.10 Rac GTPase activation assay

COS-7 cells were grown in 6 well dishes until 60% confluent. Between 1 to3 µg of DNA was transfected using the Lipofectamine 2000 reagent. Two days after transfection, cells were lysed by the addition of ice cold 400 µl MBL with protease inhibitors provided with the Rac assay kit (Millipore). Samples were briefly sonicated, spun down at 14,000 RPM, and the supernatant retained. For input/loading control, 40 µl was removed from the samples, while the rest of the samples was used to perform Rac activation assay as previously described (Xie et al., 2007). Quantification was performed by densitometry as previously described (Srivastava et al., 2005; Xie et al., 2007). Intensities were averaged and a one way ANOVA with Tukey B Post-Hoc was performed using SPSS. Experiments were repeated 3-4 times.

### C.11 Yeast two-hybrid assay

All our yeast protocols, including small scale LiAc yeast transformation procedure, β-galactosidase (β-gal) colony-lift filter assays and preparation of yeast protein extracts were obtained from the Clonetech yeast
protocols handbook (http://www.clontech.com/images/pt/PT3024-1.pdf). Brain myosin Va C-terminal tail (aa 1005-1830) was subcloned into pGBK T7 (GAL4 DNA binding domain (DB) vector) and used to screen a adult rat brain cDNA library subcloned into pGAD T7 (GAL4 activation domain (AD) vector) (MATCHMAKER system; Clonetech, Palo Alto, CA). For assays of specificity and binding domains, desired cDNA fragments were amplified by PCR with specific primers and subcloned into pGBK T7 and pGAD T7. These fusion proteins were tested for interaction using HIS3 and β-gal as reporter genes and induction levels were semi-quantified as previously described (Kim et al., 1995). Briefly, HIS3 activity was measured by the percentage of colonies growing on histidine-lacking medium: +++ : >60% growth; ++ 30-60% growth; +, 10-30% growth; -: no significant growth. β-gal activity was determined from the time taken to colonies to turn blue in X-gal filter lift assays at 30°C +++ : <45 min; ++: 45-75 min; +, 75-120 min; -: no significant growth after 2 hours.

C.12 Immunohistochemistry

Adult female Wister rats were perfused with 4% PFA, pH 7.4. Brain sections (15 µm) were incubated for 1 hour with 2 µg/mL anti-myosin Vb antibodies and the ABC technique (Elite ABC kit; Vector Laboratories) was used for detection.

C.13 In situ hybridization

In situ hybridization was conducted as per (de Lecea et al., 1997), with some modifications. E18 and P5 rat brains were perfused with 4% PFA, and cryoprotected in 30% sucrose in 4% PFA. Brains were then embedded in grade IV-V bovine albumin (Fisher Scientific), cut into blocks and further cryoprotected in the sucrose/PFA solution prior to sectioning on a Leica tabletop cryostat microtome. Rat RILPL2 full-length cDNA was subcloned into pSPT-19 between EcoRI and XmaI. Probes were synthesized with the DIG-RNA labeling kit (Roche). Hybridization with 1µg/ml digoxigenin-labeled RLP2 probes proceeded at 65°C at in a solution of 50% deionized formamide, 10% dextran sulphate, 5x Denhardt’s solution, 0.62 M NaCl, 10mM EDTA, 20 mM PIPES-Na, 0.2% SDS, 250µg/ml heat-denatured salmon sperm DNA and 250µg/ml heat-denatured yeast tRNA.

C.14 RT-PCR

RNA from embryonic day 18 (E18) or adult rat tissues, as well as cultured cortical neurons was isolated using the RNeasy Mini Kit (Quiagen) following the manufacturers protocol. RNA (1 µg) was used for
each reverse transcription reaction with oligo (dt) primers (Invitrogen). The RT reaction was followed by PCR amplification using specific primers for rat RILPL2 or β-actin. 6X DNA sample buffer containing bromophenol blue or xylene cyanol was added to PCR samples before loading on a 2% agarose gel and running at 100V for 15-30 minutes. The 1 kb or 100 pb ladders (Invitrogen) were used to confirm the molecular weight of the amplified fragments.

**C.15 Electrophysiology**

Recording of mEPSCs and mIPSCs were performed 3 days post-transfection. Hippocampal neurons on coverslips were transferred to a recording chamber continuously perfused with extracellular solution [pH 7.4; 320–330 milliosmolar (mosM)] containing 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 1.3 mM CaCl₂, 25 mM Hepes, 33 mM glucose, and 0.0005 mM tetrodotoxin (Alomone, Jerusalem). Transfected cells with GFP signal were identified under a fluorescent upright microscope. Intracellular solution (pH 7.2; 300–310 mOsm) was composed of 115 mM Cs gluconate, 17.5 mM CsCl, 10 mM Hepes, 2 mM MgCl₂, 10 mM EGTA, 4 mM ATP, 0.4 mM GTP, and 0.1% Lucifer yellow (Sigma-Aldrich). A MultiClamp 700A amplifier (Axon Instruments, Foster City, CA) was used for recording. Access resistance was monitored, and recordings where series resistance varied by >10% were rejected. No electronic compensation for series resistance was used. Whole-cell patch-clamp recordings were performed in voltage-clamp mode while maintaining the membrane potential either at the reversal potential for GABA (A) receptor-mediated miniature PSCs (~60 mV) to isolate mEPSCs or at the reversal potential for ionotropic glutamate receptor-mediated miniature PSCs (+10 mV) to isolate mIPSCs. Recorded mEPSCs and mIPSCs were antagonized completely by the ionotropic glutamate receptor antagonist cyano-7-nitroquinoxaline-2, 3-dione (Sigma-Aldrich) and the GABA (A) receptor antagonist bicuculline (Sigma-Aldrich), respectively. Recordings were low-pass-filtered at 2 kHz, sampled at 10 kHz, and stored in a computer by using CLAMPEX 8.0 (Axon Instruments).

**C.16 Immunocytochemistry**

Immunocytochemistry was performed in cultured cells that were grown on coverslips. One of two fixation methods was used depending on the primary antibody: 4% PFA/4% sucrose for 10-15 minutes at room temperature or ice-cold MeOH for 5 minutes at -20°C. Many antibodies produced similar staining with both fixation methods; however some antibodies such as synaptic proteins only stained appropriately with MeOH fixation. Following fixation, cells were 3X washed with PBS containing 0.3% Triton X-100 (PBS-T). Cells were then incubated with the primary antibody in 2% normal goat serum (NGS) in PBS-T for 1h at RT or overnight at 4°C. Cells were washed 3x with PBS-T, and then incubated in the appropriate
secondary antibody (rabbit, mouse, chicken, rat or guinea pig) conjugated with Alexa 568, Alexa 488 or Alexa 350 (1/1000, Molecular Probes) or AMCA (1/50; Jackson ImmunResearch) in 2% NGS in PBS for 1 hour at RT. Cells were washed 3X PBS-T before mounting. Coverslips were mounted on slides (Frost Plus; Fisher) with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL).

For GluR1 surface labeling experiments, cells were incubated with NBM containing antibody against an extracellular region of GluR1 (rabbit: 1:15; Oncogene Research) for 30 minutes at 37°C with 5% CO₂. Neurons were fixed with 4% PFA/4% sucrose for 15 minutes, washed 3X with PBS (non-permeabilized conditions) and incubated with appropriate secondary antibody diluted in PBS for 1 hour at room temperature.

C.17 Imaging, analysis and bioinformatics

Images were taken using a 63X objective affixed to a Zeiss Axiovert M200 motorized microscope with a CCD camera (Princeton Instruments Inc.). Images were captured using AxioVision software and exported as separate 16 bits TIFF files for each channel.

C.17.1 Synaptic protein clustering

For analysis of total and surface cluster density (number), the images were analyzed in Northern Eclipse (Empix Imaging, Mississauga, Canada) by using custom written software routines as described elsewhere (Prange et al., 2004). Briefly, dendrites of cells of interest were manually outlined an experimenter blinded to the treatment conditions using fluorescent signal. Puncta were defined as sites of intensities at least 1.5 times the dendritic background. The average punctate number per dendrite length in transfected cells was compared to either GFP transfected or untransfected cells present in the same field. Statistical analyses were performed using Student-t-test in XLSTAT.

C.17.2 Perinuclear accumulation

For changes in perinuclear accumulation in neuronal and non-neuronal cells, a minimum of 30 transfected cells per group was analyzed from at least 3 independent experiments. Image J 1.33u software (Wayne Rasband, NIH, USA) was used to represent graphically the fluorescence intensity patterns per µm. Statistical analysis were performed using Student-t-test in XLSTAT.
C.17.3 Dentritic protrusions

For dentritic protrusions analysis, images were analyzed using the Northern Eclipse software (Empix Imaging, Mississauga, Canada). Briefly, an experimenter blinded to the treatment conditions used the RFP or GFP fluorescent signal to detect transfected cells and manually outline spines and filopodia present on all dendritic processes in the field of view. Any protrusion with a clear head of a diameter greater than 0.35 μm (as assessed with the polygon tool) was considered as spine-like and counted. Any thin protrusion of a length between 2 and 10 μm and lacking a head was considered as a filopodia. Statistical analyses were performed using unpaired Student-t-test to compare 2 groups. For 3 and more groups, ANOVA with Tukey B Post-Hoc was performed using XLSTAT.

C.17.4 Neurite outgrowth

For quantitative analysis of morphology images of neurons were captured with the 20X objective based on immunoreactivity against GFP. Dendrites and axons were identified based on standard morphological criteria and based on immunoreactivity against the axonal marker TAU-1 or the dendritic marker MAP-2. In our DIV 3 hippocampal cultures, the majority of neurons had one clearly classifiable axon and one or more dendrites. Neurons presenting more than one axon or no classifiable processes were excluded from analyses. The total length of the axon and dendrites, as well as the number of primary and secondary branches were determined manually using Neuron J 1.4.0 (Meijering et al., 2004), a plug-in software for Image J (NIH). All analyses were performed by an experimenter blinded to the identity of the transfected constructs. Statistical analyses were performed using unpaired Student-t-test to compare 2 groups. For 3 and more groups, ANOVA with Tukey B Post-Hoc was performed using XLSTAT.

C.17.5 Pak phosphorylation assay

Quantitative analysis of changes in Pak phosphorylation, cultures that were directly compared were stained simultaneously and imaged with the same acquisition parameters. Neurons were analyzed using the Northern Eclipse software (Empix Imaging, Mississauga, Canada). An experimenter blinded to the treatment conditions used the fluorescent signal to manually outline approximately 50-100 μm of primary dendrites (including dendritic protrusions). In parallel, regions corresponding to areas without cells were outlined to create a background mask. For summary average gray intensities (total immunofluorescence intensity /pixel area), average gray values of background areas were subtracted to average gray values of the outlined dendritic segments. Statistical analyses were performed using unpaired Student-t-test in XLSTAT.
C.17.6 COS-7 cells morphology

For analysis of COS-7 cell morphology, an experimenter blinded to treatment conditions used the 10X objective to randomly select 10-15 fields for analysis. In each field of vision, the total number of co-transfected cells was manually counted and the number of cells showing at least 1 protrusion with a length of 2 times or more the diameter of the nucleus was noted. Statistical analyses were performed using unpaired Student-t-test to compare 2 groups. For 3 and more groups, ANOVA with Tukey B Post-Hoc was performed using XLSTAT.
References


