Expression of the Formin Daam1 in Pyramidal Neurons 
of the Hippocampus Affects Spine Morphology

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>3AT</td>
<td>3-Amino-1,2,4-Traizole</td>
</tr>
<tr>
<td>ABP</td>
<td>AMPAR-binding protein</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>actin-related protein 2/3</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby Hamster Kidney cells</td>
</tr>
<tr>
<td>CC</td>
<td>coiled-coil domain</td>
</tr>
<tr>
<td>Daaml</td>
<td>Dishevelled-associated activator of morphogenesis</td>
</tr>
<tr>
<td>DAD</td>
<td>Diaphanous autoregulatory domain</td>
</tr>
<tr>
<td>DD</td>
<td>dimerization domain</td>
</tr>
<tr>
<td>DEP</td>
<td>Dishevelled, egl-10, pleckstrin domain</td>
</tr>
<tr>
<td>DID</td>
<td>Diaphanous inhibitory domain</td>
</tr>
<tr>
<td>DIX</td>
<td>Dishevelled and Axin domain</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DOC</td>
<td>deoxycholate</td>
</tr>
<tr>
<td>Drf</td>
<td>Diaphanous-related Formin</td>
</tr>
<tr>
<td>Dvl</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(2-aminoethyl)ether-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>FH</td>
<td>Formin Homology</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GBD</td>
<td>GTPase-binding domain</td>
</tr>
<tr>
<td>GKAP</td>
<td>guanylate kinase associated protein</td>
</tr>
<tr>
<td>GRIP1</td>
<td>glutamate-receptor interacting protein</td>
</tr>
<tr>
<td>HC media</td>
<td>Hartwell’s complete media</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney 293T cells</td>
</tr>
<tr>
<td>IB</td>
<td>immunoblot</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LIMK</td>
<td>Lin11, Isl-1, and Mec-3 kinase</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>mDia</td>
<td>mammalian diaphanous</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptors</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RFPf</td>
<td>farnesylated version of red fluorescent protein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3 domain</td>
</tr>
<tr>
<td>SV2</td>
<td>synaptic vesicle 2</td>
</tr>
<tr>
<td>WW domains</td>
<td>two fully conserved tryptophans (WW) and a proline</td>
</tr>
</tbody>
</table>
Abstract

Formins, also known as formin homology (FH) proteins, are involved in a wide range of actin-mediated processes. The Diaphanous-related formin Daam1 (Dishevelled-associated activator of morphogenesis) interacts with the PDZ domain protein Dishevelled, and is required to establish planar cell polarity in Xenopus. Through a yeast two-hybrid screen, I characterized a PDZ-mediated interaction between the C-terminus of Daam1 and the PDZ domains 456 of GRIP1. In dissociated rat hippocampal cultures, Daam1 expression was seen throughout the soma and dendrites in a punctate pattern. Furthermore, co-staining with a synaptic marker suggests that Daam1 could be associated with post-synaptic specializations. Dendritic spines are enriched with actin filaments, and based on the subcellular localization of Daam1 and the evidence that formins are involved in regulating actin polymerization, I hypothesized that Daam1 might play a role in dendritic spine morphology. In order to investigate the functional roles for Daam1, viral vectors were developed using the Semliki-Forest defective viral vector to over-express the full-length Daam1 protein and a Daam1 lacking the PDZ-binding motif. The over-expression of the full-length Daam1 in organotypic hippocampal slices showed a punctate distribution throughout the dendritic shaft, with the occasional appearance in spines, resulting in an overall increase in dendritic spine length. This suggests that formins, such as Daam1, could potentially regulate spine morphology.
Résumé

Les formines, aussi connues sous le nom de protéines d’homologie de formin (FH), sont impliquées dans un large éventail de procédés facilités par l’actine. La formine connexe Diaphane Daam1 (l’activateur de morphogénèse associé à Dishevelled), interagit avec la protéine formée de domaine PDZ Dishevelled, et elle est aussi requise pour établir la polarité cellulaire planaire chez le Xénopus. Par un criblage double-hybride chez la levure, j’ai caractérisé l’interaction médiane par PDZ entre le carboxy-terminus de Daam1 et les domaines PDZ 456 de GRIP1. Dans des cultures dissociées de neurones hippocampiques chez le rat, l’expression de Daam1, d’apparence ponctuée, était visible partout sur le soma et les dendrites. De plus, une co-coloration avec un marqueur synaptique a indiqué que Daam1 est associé avec les spécialisations post-synaptiques. Les épines dendritiques sont enrichies de filaments d’actine et, en se basant sur la localisation sub-cellulaire de Daam1 et sur l’évidence selon laquelle les formines sont impliquées dans la régulation de la polymérisation d’actine, j’ai émis l’hypothèse que Daam1 joue un rôle dans la morphologie des épines. Afin d’investiguer le rôle fonctionnel de Daam1, j’ai développé, utilisant le virus Semliki-Forest, des vecteurs viraux afin de surexprimer la protéine complète Daam1, ainsi qu’une Daam1 sans le domaine se liant au PDZ. La surexpression de Daam1 pleine longueur dans des tranches organotypiques de l’hippocampe a démontré une distribution ponctuée partout sur l’arbre dendritique, avec une apparence occasionnelle dans les épines, résultant en une augmentation générale de la longueur des épines dendritiques. Ceci suggère que les formines, telles que Daam1, pourraient potentiellement réguler la morphologie des épines.
Chapter I:

Introduction and Literature Review
Introduction

It is widely known that forms of synaptic plasticity are underlined mainly by the insertion/removal of AMPARs at the synapse, and by changes in the morphology of postsynaptic dendritic protrusions known as spines. The molecular mechanisms regulating these pathways are continuously being unravelled, and additional regulatory proteins are currently being identified. It is becoming increasingly apparent that PDZ domain-containing proteins and actin-regulatory proteins within the shafts and spines of dendrites play a role in establishing and regulating these pathways.

For my Master’s thesis research, I characterized a PDZ-mediated interaction between the formin homology protein Daam1 and the glutamate receptor-interacting protein GRIP1. In addition, I conducted experiments to establish a functional role for this PDZ interaction between Daam1 and GRIP1 in the trafficking of Daam1 into spines. Furthermore, I characterized whether Daam1, a known regulator of actin nucleation and polymerization, affects the shape and morphology of dendritic spines.

This introduction will focus on how PDZ proteins, such as GRIP1, are involved in the trafficking and anchoring of AMPARs to the post-synaptic density. I will then discuss how these formin homology proteins are involved in the process of actin nucleation and polymerization. Then, I will elaborate on how PDZ proteins and actin regulatory proteins function in regulating spine morphology.
1.1 **PDZ Proteins in AMPAR-mediated Transmission**

Communication between neurons occurs at a specialized area, called the synapse. The synapse is composed of a pre-synaptic terminal, which sends neuronal signals mainly via neurotransmitter release, and a post-synaptic membrane, which receives these signals through receptors on the surface. One of these receptors, the AMPA-type glutamate receptors (AMPARs), mediates the majority of excitatory synaptic transmission in the brain. Therefore, these AMPARs are ideal post-synaptic receptors to control the strength of synaptic transmission. Changes in strength of synaptic transmission underlie several forms of synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD). In hippocampal neurons, the induction of LTP through activation of another type of synaptic glutamate receptor, called NMDARs, leads to the appearance or "unsilencing" of functional AMPARs in previously silent synapses, thereby potentiating synaptic transmission (Malenka and Nicoll, 1999). Therefore, regulated trafficking of AMPARs to and from the post-synaptic membrane provides a mechanism for changes in synaptic efficacy associated with LTP and LTD (Malinow et al., 2000). Induction of LTP has been shown to increase AMPAR function at silent synapses, with the addition of AMPARs into the post-synaptic membrane (Shi et al., 1999).

1.1.1 **AMPA Receptors and their Function in Synaptic Plasticity**

AMPARs are formed from heteromeric combinations of subunits GluR1-4 to form tetramers. The cytoplasmic carboxyl termini of these subunits classify them into two groups. The GluR1 and GluR4 subunits have a long cytoplasmic tail, whereas the GluR2 and GluR3 subunits have a short cytoplasmic tail. In the adult hippocampus, endogenous AMPARs are composed mainly of GluR1/GluR2 and GluR2/GluR3 heteromers (Wenthold et al., 1996).

The first direct evidence for movement of AMPARs came from the rapid lateral translocation of AMPARs from non-synaptic to synaptic regions. Following a strong synaptic stimulation and NMDAR activation, the GluR1 subunit translocated from the main shaft of the dendrite into the post-synaptic spine (Shi et al., 1999). Furthermore, it was determined that homomeric GluR1 receptors (Hayashi et al., 2000), and not
homomeric GluR2 receptors (Shi et al., 2001), are delivered to silent synapses upon NMDAR activation. Instead, the synaptic delivery of homomeric GluR2 receptors in the absence of activity suggests that the GluR2 receptors are responsible for replacing existing synaptic AMPARs and maintaining the expression of LTP (Shi et al., 2001). Therefore, GluR1 is the key subunit that drives AMPARs to the synaptic surface in response to NMDAR activation, resulting in synaptic potentiation, whereas GluR2 is delivered constitutively to synapses, replacing existing receptors. In the hippocampus, GluR1/2 heteromers are delivered to synapses during activity-dependent synaptic potentiation, such as LTP, whereas GluR2/3 heteromers cycle continuously between the post-synaptic membrane and intracellular compartments (Lee et al., 2004; Shi et al., 2001).

The existence of two distinct groups of AMPAR subunits suggests a model for subunit-specific trafficking of AMPARs, involving a two pathway model for synaptic delivery (Malinow et al., 2000). In the first pathway, receptors containing GluR1/GluR2 subunits are delivered to synapses through a constructive (regulated) pathway and require a calcium stimulus, most likely through activation of NMDARs. This provides additional receptors following induction of LTP to synaptically silent synapses. The second pathway, known as the maintenance (constitutive) pathway, involves GluR2/GluR3 receptors, which continuously replace synaptic receptors, and act to preserve plastic changes despite protein recycling (Shi et al., 2001).

The insertion of AMPARs into the post-synaptic membrane, mainly through exocytosis, is a key component in strengthening the synaptic transmission (LTP). Therefore, a depression in synaptic transmission (LTD) most likely occurs by removal of post-synaptic AMPARs. It has been characterized that AMPARs undergo clathrin-dependent endocytosis, and that this regulated process induces LTD (Man et al., 2000). In addition, inhibiting clathrin-dependent endocytosis blocked internalization of AMPARs (Carroll et al., 1999) and prevented stimulated-induced LTD in hippocampal slices (Man et al., 2000). Presumably, there are locations for this clathrin-mediated endocytosis within post-synaptic compartments. The expression of a clathrin-GFP construct helped to identify specialized endocytic zones immediately adjacent to the synaptic membrane in dendritic spines (Blanpied et al., 2002). This regulated internalization of AMPARs seems
to be a subunit-dependent mechanism, reducing the surface expression of GluR2, and not GluR1. This subunit specificity in internalization was further shown to be mediated by the C-terminus of GluR2 (Man et al., 2000).

The cytoplasmic tails of AMPARs seem to be the key mediators of subunit trafficking, since the long versus short tailed subunits are differentially delivered to synapses (Passafaro et al., 2001), and since they also undergo regulated redistribution to intracellular compartments (Lee et al., 2004). For synaptic delivery of AMPARs, the GluR1 C-terminus mediates regulated delivery, whereas the GluR2 C-terminus is required for continuous delivery (Shi et al., 2001). A depression in AMPAR-mediated synaptic transmission by expressing an interfering GluR2 C-terminal construct, demonstrated the importance of the GluR2 C-terminus in synaptic AMPAR trafficking (Shi et al., 2001). Therefore, this differential trafficking of subunits to and from the synapse is presumably controlled by interactions between the C-terminus of AMPAR subunits with specific intracellular proteins (Passafaro et al., 2001; Shi et al., 2001).

1.1.2 PDZ Proteins: Their Role in Clustering and Targeting Synaptic Proteins

The C-terminal cytoplasmic tails of GluR subunits, which are thought to be involved in receptor trafficking, contain consensus sequences that bind to specific proteins that possess PDZ domains. PDZ domains were named after identification of a 90 amino acid long repeated sequence in three PDZ domain-containing proteins. These include the post-synaptic density protein PSD-95/SAP90, the Drosophila septate junction protein Discs-large (DLG), and the tight junction protein Zona Occludens-1 (ZO-1) (Reviewed by Hung and Sheng, 2002).

Since many channels and receptors are present on the post-synaptic side of the synapse, it was proposed that a post-synaptic specialized area, known as the post-synaptic density (PSD), is the site for clustering of post-synaptic receptors and ion channels (reviewed by Garner et al., 2000). The identification of PDZ domain–containing proteins helped to substantiate the function of the PSD. The PSD-95 protein was the first PDZ domain-containing protein to be identified at the vertebrate CNS synapse and was found enriched in the PSD fraction from rat brain (Cho et al., 1992).
There is strong evidence that these PDZ proteins play a role in the localization and clustering of ion channels and receptors at the post-synaptic membrane, by interacting with the cytoplasmic C-terminus of the receptor. For example, the first two PDZ domains of PSD-95 interact with the carboxy-terminal cytoplasmic tail of the Kv1.4 ion channel, and are responsible for cell surface clustering of these Shaker family K⁺ channels (Kim et al., 1995). The importance of this C-terminal region of Kv1.4 was further revealed by mutations in the C-terminal sequence (EDTV) which abolished their interaction with PSD-95 and prevented their co-clustering in heterologous cells (Kim and Sheng, 1996) and at the Drosophila neuromuscular junction (Tejedor et al., 1997). In addition, PSD-95 co-localizes and interacts with the cytoplasmic tail of the NR2 subunit of the NMDAR in cultured hippocampal neurons (Kornau et al., 1995). These results suggest that PDZ domain-containing proteins, such as PSD-95, play a role in the clustering and localization of receptors at the PSD. However, separate studies have shown that PSD-95-mediated clustering suppresses internalization of Kv1.4 (Jugloff et al., 2000) and NR2B receptors (Roche et al., 2001), suggesting that PSD-95 more likely functions in stabilization of proteins at the PSD, and acts as an anchor instead. In addition, PSD-95 also functions by providing a link between receptors at the surface and intracellular signalling pathways. For example, PSD-95 is required to couple NMDAR activation to intracellular neurotoxic signalling through nitric oxide (Sattler et al, 1999). The role of PDZ proteins to act as a scaffold/anchor for receptors at the PSD has been suggested for the AMPAR subunit GluR2, where its cytoplasmic tail interacts with the post-synaptic AMPAR-binding protein (ABP) (Srivastava et al., 1998).

It is currently recognized that PDZ domains are specialized for binding to short peptide motifs at the extreme carboxyl termini of proteins, although they can also have other modes of interactions by binding to internal sequences. PDZ domains are classified into three types on the basis of the sequence of their preferred C-terminal ligands (Sheng and Sala, 2001). The PSD-95 protein interacts with the C-terminal sequence of the NMDAR NR2 subunit (Kornau et al., 1995) and the Shaker K⁺ channel (Kim et al., 1995), through a class I C-terminal PDZ binding motif, represented by an X-S/T-X-V (where X is any amino acid). However, the PDZ protein ABP recognizes a different C-terminal sequence in GluR2, and is represented as a class II PDZ binding motif X-Φ-X-Φ.
where $\Phi$ is a hydrophobic amino acid) (Srivastava et al., 1998). Interestingly, some PDZ binding proteins require an interaction with multiple PDZ domains, instead of only one, from the same protein. For example, the C-terminus of the $K^+$ channel requires the first two PDZ domains of PSD-95 for a successful interaction (Kim et al., 1995), whereas the NR2 subunit only requires the second PDZ domain of PSD-95 (Kornau et al., 1995). The requirement for multiple PDZ domains is also seen with ABP binding to GluR2, where the PDZ domains 3, 5, and 6 of ABP are required (Srivastava et al., 1998). This requirement for multiple PDZ domains is likely explained by one PDZ domain stabilizing another, allowing for a higher affinity interaction (Feng et al., 2003).

1.1.3 GRIP-family Proteins

ABP is a related member of the GRIP (glutamate receptor-interacting protein) family. GRIP1 is a seven PDZ domain-containing protein, whereas ABP is a six PDZ domain-containing protein and is derived from a separate gene (Srivastava et al., 1998; Dong et al., 1999b). Alternatively, GRIP2 is derived from the same gene as ABP and shares 100% identity, except for an N-terminal extension of 52 amino acids, an internal deletion of 41 amino acids between PDZ3 and PDZ4, and a C-terminal extension that contains a seventh PDZ domain. Over its entire length, GRIP2 is 56% identical and 68% similar to GRIP1 at the amino acid level (Wyszynski et al., 1999). There are two splice forms of GRIP1 (GRIP1a and GRIP1b), in which the difference lies with a short N-terminal peptide motif that can be palmitoylated (GRIP1b) (Yamazaki et al., 2001). Recently, another splice form of GRIP1 (GRIP1c4-7) has been identified, which lacks PDZ domains 1-3 of GRIP1, and also contains N-terminal and C-terminal sequence differences (Charych et al., 2004).

From the overwhelming evidence that the C-terminus of AMPARs play a role in their trafficking, it became interesting to determine which proteins interact with the cytoplasmic tail. Before the isolation of ABP, GRIP1 was the first PDZ domain-containing protein identified through a yeast two-hybrid screen, as interacting with the C-terminus of the GluR2 subunit (Dong et al., 1997). From the original yeast two-hybrid screen, the C-terminal 50 amino acids of GluR2 interacted with a clone encoding PDZ domains 4-6 of GRIP1. The yeast two-hybrid system was further used to establish that
PDZ domains 45 and the link between PDZ 3 and 4, were required for the interaction with the C-terminus of GluR2. It became evident that the extreme C-terminus was responsible for the interaction since deletion of the last three amino acids completely abolished its interaction with GRIP1 (Dong et al., 1997). It was further suggested that GRIP1 may play a role in synaptic targeting and clustering of AMPARs, since GRIP1 was found to be clustered and co-localized with AMPARs in hippocampal cultures, and that disruption of the GRIP1-GluR2 interaction resulted in fewer synaptic AMPAR clusters (Dong et al., 1997). This clustering of AMPARs is not only specific to interactions with GRIP family proteins, since another PDZ protein PICK1 (protein interacting with C kinase 1) also interacts with the cytoplasmic tail of GluR2 and causes AMPAR clustering (Xia et al., 1999).

Biochemical studies have shown that GRIP1 is enriched in synaptic plasma membrane and PSD fractions (Dong et al., 1999b). In cultured hippocampal neurons, GRIP1 is expressed in a somatodendritic staining pattern, and co-localizes with both AMPARs and glutamic acid decarboxylase, suggesting it may play a functional role at both glutamatergic excitatory synapses and GABAergic inhibitory synapses (Dong et al., 1999b). The enrichment of GRIP1 at synaptic sites is seen from its co-localization with the synaptic marker synaptophysin (Wyszynski et al., 1999), and from its punctate distribution that co-localizes with GluR2 (Dong et al., 1999b) and GluR1 at dendritic spines (Dong et al., 1999a). Despite an extensive overlap of GRIP1 and GluR2 in the cell body and the dendritic shafts, one study showed a limited co-localization of GRIP1 and GluR2 in dendritic puncta along the membrane (Wyszynski et al., 1999), contradicting subsequent results of GRIP1 and GluR2 co-localization at dendritic spines (Dong et al., 1999b). Overall, most of the evidence agrees with a co-localization of GRIP1 and GluR2 in dendritic spines.

Electron microscopy revealed that dendritic shafts and spines contained GRIP1 staining near small vesicular structures, which were found to be either associated with the PSD or clustered directly underneath the synaptic plasma membrane (Dong et al., 1999b). Despite the overwhelming evidence for the presence of GRIP1 at synaptic sites, there is evidence that GRIP1 is also associated with non-synaptic sites and intracellular compartments of dendritic spines and shafts (Wyszynski et al., 1999). It has been
suggested that some of the non-synaptic GRIP1 is complexed with intracellular pools of AMPARs, and therefore may play a role in trafficking not only at the synapse (Wyszynski et al., 1999). In support of these claims, GRIP1 was found associated with membrane vesicles in dendritic shafts and in the cell soma near the peri-golgi region (Dong et al., 1999b).

**Functional Role in AMPAR Trafficking**

It is well accepted that the PDZ interactions are functionally important for regulating AMPARs in hippocampal neurons, and that the GluR2 interactions with PDZ proteins play a role in their distribution and in synaptic plasticity. Despite earlier suggestions that the PDZ proteins are involved in the clustering (Dong et al., 1997) and trafficking (Piccini and Malinow, 2002) of AMPARs, the precise function of these PDZ interactions is not fully clear, since the organization and number of proteins at the PSD is very complex.

Some of the earlier evidence suggests that these PDZ interactions function as a scaffold for AMPARs to prevent their reinsertion into the plasma membrane, once they are internalized (Daw et al., 2000). To better understand their function, a peptide corresponding to the GluR2 C-terminus (–SVKI) was used to disrupt the PDZ interaction with GluR2. This induced an increase in basal AMPAR-mediated transmission and inhibited LTD in hippocampal slices (Daw et al., 2000; Kim et al., 2001). It was hypothesized that during LTD, AMPARs are internalized and subsequently anchored to the sub-synaptic membrane through interactions with PDZ proteins (GRIP1, ABP or PICK1), preventing any rapid reinsertion (Daw et al., 2000). Due to the lack of specificity of the –SVKI peptide, all three PDZ proteins that bind the C-terminus of GluR2 could indeed play a role in sub-synaptic stabilization and prevention of GluR2 reinsertion for maintenance of LTD. Kim et al (2001) further elaborated on this by specifically inhibiting only the interaction between GluR2 and PICK1. The perfusion of an –EVKI peptide, previously shown to only bind PICK1, and not GRIP1 (Chung et al., 2000) also increased basal synaptic strength and inhibited LTD. This suggested that the PICK1-GluR2 interaction, rather than the GRIP1-GluR2 interaction, plays a more prominent role in LTD expression (Kim et al., 2001) and that GRIP1 likely has a separate function from PICK1.
Instead of GRIP1 anchoring receptors sub-synaptically, its function may be involved in the accumulation of GluR2 at the synaptic surface (Osten et al., 2000). Suggestions that GRIP1 is involved in synaptic anchoring were based on evidence from the reduced accumulation of GluR2 subunits at the synapse from mutant GluR2 subunits lacking the C-terminus (Dong et al., 1997) and from specifically blocking GRIP/ABP binding (Osten et al., 2000). These results indicate that the GluR2 interaction with GRIP1, and not PICK1, is required for synaptic surface accumulation of AMPARs. Further evidence was also seen from sensory neurons in the spinal cord, where serotonin-induced potentiation of AMPAR responses were blocked by specifically inhibiting the GluR2 interaction with GRIP1 (Li et al., 1999). These results led to a proposed model, where the AMPARs on the synaptic plasma membrane are stabilized through interactions between the C-terminus of GluR2 and GRIP1, whereas receptors present in intracellular pools for receptor recycling are stabilized through interactions between the C-terminus of GluR2 and PICK1 (Kim et al., 2001).

The different roles of the PDZ proteins, despite a similar interaction involving the C-terminus of GluR2, suggest that there is a mechanism that differently regulates this interaction with the PDZ proteins. It was demonstrated that phosphorylation on the C-terminus (Ser880) of GluR2 alters its binding abilities to the two PDZ proteins (Chung et al., 2000). Activation of PKC (protein kinase C) phosphorylates GluR2 and disrupts its interaction with GRIP1, but not with PICK1. In addition, PKC activation increases the number of synaptic clusters of phosphorylated GluR2 and PICK1, and diminishes the levels of surface GluR2 (Chung et al., 2000; Perez et al., 2001). The fact that the distribution of GRIP1 did not change suggests that GRIP1 may be anchored to the PSD (Chung et al., 2000). The regulation of the interaction of GluR2 with GRIP1 and PICK1 suggests that the PDZ proteins serve separate functions. GRIP1 may be involved in synaptic insertion or stabilization of AMPARs by anchoring them at the PSD, whereas PICK1 may alternatively be involved in receptor internalization, and the release of GluR2 receptors from the synaptic anchors (Perez et al., 2001).
GRIPI-interacting Proteins

The multiple PDZ domains of GRIPI make it a good candidate for multiple protein interactions and for the formation of a large adaptor complex. In addition to binding to GluR2, GRIPI binds to other proteins that may help to shed some light in understanding the functions of GRIPI in receptor trafficking and anchoring. Another receptor family, known as Ephrin receptors (EphR), have been demonstrated to interact with GRIPI (Torres et al., 1998). From a yeast two-hybrid experiment, the C-terminus of EphB2 requires the PDZ domains 6 and 7 of GRIPI for an interaction. Since EphB2 binds to PDZ67 and GluR2 binds to PDZ45 of GRIPI, it is possible that GRIPI could have multiple interacting proteins that bind simultaneously (Torres et al., 1998). It also suggests that GRIPI forms interactions with multiple receptors, clustering them at the PSD by acting as a scaffold.

The molecular mechanisms underlying AMPAR synaptic trafficking are not yet fully clear. Recent studies attempt to understand what proteins play a role in the GRIPI-dependent AMPAR trafficking. Yeast two-hybrid experiments have isolated at least two potential proteins, GRASP-1 (Ye et al., 2000) and the liprin-α family (Wyszynski et al., 2002) which interact with GRIPI and play a role in GluR2 trafficking. The glutamate receptor-associated protein (GRASP-1) contains RasGEF activity, and forms a complex with GRIPI and GluR2 in vivo (Ye et al., 2000). Over-expression of GRASP1 in cultured neurons significantly reduced the number of synaptic AMPAR clusters, indicating a disruption in AMPAR targeting (Ye et al., 2000). GRIPI was also found to interact with liprin-α, a family of LAR (leukocyte common antigen receptor) transmembrane protein tyrosine phosphatase-interacting proteins, forming a complex with GluR2 in vivo (Wyszynski et al., 2002). This interaction is important for the surface expression of endogenous AMPARs, since a dominant-interfering liprin-α construct reduced the number of AMPAR clusters and eliminated surface expression of endogenous GluR1 and GluR2 along dendrites (Wyszynski et al., 2002). The trafficking of GRIPI may be mediated by interactions with liprin-α, since liprin-α is localized on intracellular vesicles and interacts directly with the motor protein kinesin KIF1A (Shin et al., 2003). Additional evidence in support of kinesins playing a role in GRIPI-GluR2 transport involves the direct interaction between GRIPI and KIF5B (Setou et al., 2002). The expression of a
dominant-negative KIF5 construct reduced the amounts of GRIP1 and GluR2 in the synapse of cultured neurons. Since another kinesin-interacting protein was localized predominantly in axons, it is likely that GRIP1 can play a role in steering kinesins into dendrites, which subsequently functions to transport GRIP1 and GluR2 complexes along the dendrite (Setou et al., 2002). In the first part of the thesis results, I have identified a formin homology protein that binds to GRIP1, and could therefore play a role at the PSD.

1.1.4 Stabilization of PDZ Proteins and the PSD by the Actin Cytoskeleton

Another known role for PDZ proteins, involves the stabilization of the PSD at the synaptic membrane through interactions with the synaptic cytoskeleton (Reviewed by Garner et al., 2000). This interaction may in fact be the scaffolding mechanism necessary for receptor clustering and anchoring at the PSD. For example, the third PDZ domain of PSD-95 was found to interact with the microtubule-associated protein CRIP1 through its C-terminus. CRIP1 is concentrated at the PSD, and acts by bridging an interaction between microtubules, more specifically tubulin, and PSD-95 (Niethammer et al., 1998). In addition to CRIP1, PSD-95 is linked to the synaptic cytoskeletal matrix through various other protein-protein interactions. Shank was identified as a PSD protein that binds, through its PDZ domain, to the guanylate kinase-associated protein (GKAP) family of PSD-95 binding proteins (Naisbitt et al., 1999). Shank also contains a proline-rich region that binds to the actin-binding protein cortactin, and to the metabotropic glutamate receptor-interacting protein Homer (Garner et al., 2000). Clearly, PDZ proteins present at the PSD are involved in the formation of a large protein complex providing linkage between different glutamate receptors, and also linking them to the synaptic cytoskeletal matrix.

Despite the evidence for PSD-95 in linking NMDARs to the cytoskeleton, there is very little evidence that PDZ proteins, such as GRIP1, provide a link between AMPARs and the cytoskeleton. Recently, a PDZ protein was found to interact with the GluR1 subunit, and provide a link to the cytoskeleton by binding to α-actinin. This PDZ protein RIL (reversion-induced LIM protein) contains one PDZ domain and one LIM domain, and interestingly it is the LIM domain that binds to the C-terminus of GluR1 whereas the PDZ domain binds to α-actinin (Schulz et al., 2004). The over-expression of RIL in
hippocampal neurons enhanced the synaptic accumulation of GluR1-containing AMPARs. The LIM and PDZ domains were necessary for the accumulation, suggesting that the link with α-actinin is required for this accumulation in spines (Schulz et al., 2004). Therefore, the actin cytoskeleton and actin-binding proteins could function to stabilize AMPARs through an interaction with PDZ proteins.

Furthermore, actin filaments possibly have a direct affect on the localization of AMPARs. It was previously shown that F-actin is responsible for AMPAR synaptic localization and for maintaining AMPAR clusters (Allison et al., 1998; Shen et al., 2000). This result along with the identification of other cytoskeletal proteins interacting with AMPARs, suggests a regulatory role for the actin cytoskeleton on AMPAR localization and stabilization, regardless of any interactions with PDZ proteins. Some of these cytoskeletal proteins include the 4.1G and 4.1N proteins, homologs of the erythrocyte membrane cytoskeletal protein 4.1, required for surface expression of the GluR1 (Shen et al., 2000) and GluR4 (Coleman et al., 2003) subunits.
1.2 Formin Homology Proteins

1.2.1 Formins Exist in Various Organisms

Formin Homology (FH) proteins, also known as formins, are a family of highly conserved eukaryotic proteins that participate in a wide range of actin-mediated processes. FH proteins were defined based on similarities in sequence and protein organization among two Drosophila proteins, Diaphanous (DIA) and Cappuccino (CAPU), a yeast protein (Bnilp) and a mouse protein, Formin (Wasserman, 1998; Castrillon and Wasserman, 1994). These proteins contain two regions of sequence homology, defining the formin homology regions FH1 and FH2. The mouse limb deformity gene (Id) encodes the first formin identified (Formin), where mice with mutant alleles failed to form proper limbs and kidneys (Kleinebrecht et al., 1982; Mass et al., 1990). Diaphanous was later identified through a failure in cytokinesis during germ cell proliferation in Drosophila, due to a loss of gene function in the dia alleles (Castrillon and Wasserman, 1994). Most FH proteins were subsequently identified through defects in cytokinesis and polarized cell growth, suggesting they play a role in actin-mediated processes. The identification of two FH proteins in yeast (Bni1p and Cdc12p), provided the first direct evidence that FH proteins are involved in organizing the actin cytoskeleton (Frazier and Field, 1997). Bni1p is localized to the tips of mating projections, and cells with a Bni1p mutation showed defects in polarized morphogenesis with randomly distributed actin patches (Evangelista et al., 1997). The second FH protein Cdc12p, is localized to the actin-based cell division ring, and cells with a Cdc12p mutation demonstrated a failure in assembly of the actin ring, indicating its involvement in cytokinesis (Chang et al., 1997).

Formins are multi-domain proteins (Figure 1A), with each domain conserved among different family members. The most highly conserved domains are the FH1 and FH2 domains, which are involved in actin assembly (Evangelista et al., 2003). FH1 domains contain proline rich stretches, which are known to be ligands for SH3- or WW-domain-containing proteins (Wallar and Alberts, 2003). In addition, the FH1 domain binds to the G-actin binding protein profilin (Imamura et al., 1997; Watanabe et al., 1997). The FH2 domain was originally identified as a short conserved stretch of about
130 amino acids, with a central G-N-X-M-N motif (Castrillon and Wasserman, 1994). In addition to the FH1 and FH2 domains, there exists a less highly conserved FH3 domain. The functions of the FH3 domain are not yet well understood, however, it has been shown to be involved in subcellular localization (Kato et al., 2001; Ozaki-Kuroda et al., 2001). Most formins also contain two coiled-coil domains, one adjacent to the FH3 region, and the other in the extreme C-terminal part of FH2. These coiled-coil regions are common among cytoskeletal proteins and often facilitate dimerization (Wallar and Alberts, 2003).

1.2.2 Formins are Key Regulators in Actin Remodelling

FH proteins are important for many actin-mediated processes, such as polarized cell growth, cytokinesis, vesicular trafficking, and embryonic development. The FH1 and FH2 domains have been shown to directly nucleate actin (Li and Higgs, 2003) and to enhance the overall process of actin assembly (Romero et al., 2004), respectively. Actin filaments are polar, having a slow-growing “pointed” end and a fast-growing “barbed” end (Evangelista et al., 2003). Therefore, most of the growth of actin filaments occurs at the barbed end. Prior to the discovery of formins, the Arp2/3 complex represented the cellular mechanism for actin filament nucleation, which also binds to existing filaments to allow filament branching (Reviewed by Machesky and Gould, 1999). The identification of some formins helped to shed some light on their ability to regulate growth of actin filaments. The Bni1p formin, and more specifically its FH1/FH2 domains, was found to stimulate actin nucleation in vitro (Pruyne et al., 2002). It was further proposed that the mechanism for nucleation by the Bni1p FH1FH2 construct involves stabilization of an actin dimer, and that the presence of the FH1 domain can contribute to nucleation through its interaction with profilin (Pring et al., 2003). Therefore, it has been suggested that the FH1 domain binds to and localizes the profilin-actin to the nucleation vicinity, and delivers the actin to the FH2 domain for polymerization into filaments (Figure 1B) (Evangelista et al., 2003). This model establishes how both the FH1 domain and profilin-actin contribute to formin-induced nucleation seen from previous results.

There was also evidence that the Bni1pFH1FH2 remains associated with the growing barbed ends of actin filaments, suggesting that they are also involved in actin elongation (Pruyne et al., 2002). This barbed end association allows for a competition
Figure 1: (A) Domain organization of the formin protein family. Most formins contain an N-terminal GTPase binding domain (G), a formin homology 3 (FH3) domain, previously shown to be important for the localization of some formins, and the formin homology 1 and 2 (FH1 and FH2) domains, which are involved in actin nucleation and elongation, respectively. (B) Schematic diagram for the functions of the FH1 domain, FH2 domain, and profilin-actin interaction, in actin nucleation and elongation. Profilin binds to actin which is then localized to the formin through its interaction with the FH1 domain. The binding of profilin to the FH1 domain might cause the release of the bound actin. This actin is then utilized by the FH2 domain for nucleation. After nucleation, the process continues with the addition of actin monomers while the formin is attached at the barbed-end, resulting in filament elongation. (C) Domain organization of the Diaphanous-related formins (Drfs). Drfs are characterized by their N-terminal GBD and a C-terminal DAD. DAD is known to bind to an N-terminal sequence, originally thought to be the GBD, producing an inactive conformation. The GBD characterizes Drfs as Rho-small GTPase effectors, and binding of activated Rho (Rho-GTP) results in a conformational change rendering the Drf active, and thus capable of actin nucleation and elongation.
Figure 1
with tight capping proteins, known to inhibit polymerization. This binding acts as a leaky capper, by protecting the end from capping but still allowing elongation, albeit at a slower rate (Zigmond et al., 2003).

The FH1 domain binding to profilin, and the FH2 domain interacting with the fast-growing barbed-ends of actin filaments, allows formins to promote actin elongation (Kovar and Pollard, 2004). They can do this by remaining associated with the barbed ends of the actin filaments, while permitting the insertion of new monomers at those same ends, resulting in actin elongation (Figure 1B) (Bindschadler and McGrath, 2004). One study shows how the FH2 domain appears to be a processive cap that walks with the barbed end as it elongates (Zigmond, 2004).

However, it is interesting to note that because these proteins partially act as capping proteins, their effects on elongation differ among various species. For example, the fission yeast Cdc12p protein can cause strong inhibition (Kovar et al., 2003a), whereas the budding yeast protein Bni1p only causes partial inhibition of barbed-end elongation (Zigmond et al., 2003), as indicated by the leaky capping mechanism. Furthermore, the mammalian homologue of the Drosophila Diaphanous (mDia1) does not inhibit barbed-end elongation (Harris et al., 2004), but is instead a more potent actin nucleator than yeast formins (Li and Higgs, 2003).

1.2.3 Diaphanous-related Formins Activate Rho-family GTPases

The Diaphanous-related formins (Drfs) are a distinct subfamily member of formins, and are defined by their ability to interact with activated Rho-family GTPases. Drfs are known to be Rho effectors, and in addition to the FH domains, they contain an N-terminal GTPase-binding domain (GBD) (Figure 1C), which interacts with the GTP-bound form of Rho-GTPases (Watanabe et al., 1997; Kohno et al., 1996). Evidence from truncated N-terminal constructs and Rho binding leading to actin remodelling, suggests that the N-terminal GBD plays a role in inhibiting the function of Drfs (Ishizaki et al., 2001). Currently, there are numerous proteins classified in the Drf family, and these include, amongst others, the Drosophila protein Diaphanous, the yeast protein Bni1p, and the mammalian homologues of Diaphanous, mDia1-3 (Wallar and Alberts, 2003).
1.2.4 The Mammalian Formin mDia

One of the most characterized Drfs is mDia1, which has been shown to act as a potent nucleator that causes actin filament elongation at the barbed-end (Li and Higgs, 2003). Despite the evidence that mDia1 plays a role in formation of actin stress fibers, the individual roles for FH1 and FH2 of mDia1 are not completely clear. The FH2 domain of mDia1 can serve and is sufficient as a potent actin nucleator in vitro (Li and Higgs, 2003), as seen similarly with the FH2 domain of Bni1p (Pruyne et al., 2002). However, the FH1 domain seems to modulate the activity of the FH2 domain by its interaction with profilin (Shimada et al., 2004). Recent evidence suggests that the FH2 domain is sufficient to induce actin polymerization in vivo. However, homooligomerization of FH2 domains was essential, since point mutations in the FH2 domain reduced their ability to form homo-oligomers with themselves or with wild-type mDia1 (Copeland et al., 2004). These results support previous evidence that the yeast Bni1p FH2 domain dimerizes, and that any disruptions in dimerizations abolishes actin assembly (Moseley et al., 2004). Although FH2 dimers seem important, the point mutant mDia1 derivatives do not disrupt polymerization by forming complexes with endogenous mDia1, but instead do so by acting as a capping protein at barbed ends and preventing actin assembly (Copeland et al., 2004).

Watanabe et al (1999) determined that an N-terminal deletion of mDia1 caused constitutive formation of well-organized actin stress fibers in cells, leading them to propose a mechanism for auto-inhibition involving the N-terminus (Watanabe et al., 1999). This auto-inhibition mechanism proposes that the N-terminus of mDia1 interacts with the C-terminus, in a manner that is disrupted by binding of Rho to its N-terminal GBD (Figure 1C) (Watanabe et al., 1999).

Intramolecular interaction of Drfs Result in Auto-inhibition

A conserved sequence in the C-terminal region (MDXLEEXL) identified as a diaphanous auto-regulatory domain (DAD) is suggested to be the binding partner for the N-terminus (Alberts, 2001). Evidence confirming this includes, first that a DAD peptide can bind to the N-terminus, and second, over-expression of DAD leads to similar changes in actin remodelling seen by the over-expression of an N-terminal deleted mDia1. These
results suggest that the exogenous DAD relieves auto-inhibition by competing with endogenous DAD for its N-terminal binding partner (Alberts, 2001). These findings indicate that mDia1 is regulated by an intramolecular interaction between the N-terminus and the C-terminus DAD domain, where Rho binding activates mDia1 by disrupting the interaction (Alberts, 2001; Li and Higgs, 2003).

It was originally suggested that the DAD sequence binds to the GBD for the intramolecular interaction (Alberts, 2001). However, recently a sequence downstream from the GBD has been suggested to be involved instead, since it could inhibit nucleation induced by an FH2-DAD construct (Li and Higgs, 2004). This region is currently known as the diaphanous inhibitory domain (DID) and most likely interacts with DAD in an inhibitory manner, since deletion of DAD decreases the inhibitory actions of DID (Li and Higgs, 2004).

This study by Li and Higgs (2004) indicated two interesting points. First, the GBD partially overlaps with the DID, and both domains are required for full affinity to RhoA. Second, the results that RhoA does not fully relieve auto-inhibition (Li and Higgs, 2003), and that DAD-less constructs can still be inhibited by DID, suggest that a second interaction with DID is required for inhibition and that the DID-DAD interaction serves to raise the affinity of this inhibition (Alberts, 2001). It was further suggested that the other interaction involves the DID with the FH2 domain, but this remains to be determined (Li and Higgs, 2004).

There have been conflicting studies indicating which domain(s) is/are involved in dimerization of mDia1. A region between the DID and the FH1 domain (amino acids 369-548) has been shown to mediate dimerization (Li and Higgs, 2004). It was hypothesized that a coiled-coil region, predicted between amino acids 470-550 (Higgs and Peterson, 2005) mediates this dimerization. However, a separate study revealed that a dimerization domain (DD) between amino acids 377-452 is sufficient for dimerization of N-terminal mDia1 fragments, and suggests that the coiled-coil domain immediately follows the DD but is not required for dimerization (Otomo et al., 2005).
Additional Roles for mDia

Microtubule Dynamics

In addition to mDia formins playing a role in actin remodelling, they have also been linked to microtubules. Active mDia1 was shown to bind and induce the formation of stable microtubules (Palazzo et al., 2001), and cause cell elongation through parallel alignment of microtubules along the long axis of the cell (Ishizaki et al., 2001). This alignment of microtubules occurred by recruiting their ends to sites of mDia1 enriched processes located at the cell periphery. The FH2 domain was required, since co-expression with an FH2 fragment prevented microtubule alignment and cell elongation, and since point mutations in the FH2 region of active mDia1 also prevented parallel alignment of microtubules (Ishizaki et al., 2001).

Endosome Motility

The linkage of these formins to actin and microtubules suggests they play roles other than in cytokinesis and polarization. A splice variant of human Diaphanous hDia2C has been linked to endosome motility. The GTPase RhoD binds to hDia2C and targets hDia2C on to early endosomes. It was observed that hDia2C aligns with endosomes along actin filaments and reduces endosome motility, presumably by stabilizing the association with actin stress fibers (Gasman et al., 2003). It has been suggested that RhoD and hDia2C play a role in promoting the transition from long-range motility (microtubule based) to short-range motility (actin based) (Gasman et al., 2003).

Filopodia Formation

Another role for formins involves the formation of microspikes or filopodia from cells (Wallar and Alberts, 2003). Filopodia are specialized actin-rich surface protrusions that consist of bundled parallel filaments of actin, oriented with their fast-growing barbed ends at the tips of the protrusion and their slow-growing pointed ends at the base (Mallavarapu and Mitchison, 1999). Since mDia is involved in actin remodelling, it is not unreasonable to predict formation of filopodia through actin polymerization. mDia2 was suggested to do this by either removing capping proteins on pre-existing filaments, binding barbed-end filaments nucleated by Arp2/3, or by initiating new filaments at the cell periphery (Wallar and Alberts, 2003). All these processes result in mDia2-induced proliferation of long actin fibers, generating protrusions, such as filopodia.
The identification of a new Rho-family GTPase Rif (Rho in filopodia) was found to generate filopodial structures when expressed in HeLa cells (Ellis and Mellor, 2000). It was later determined that the formin mDia2 is responsible for mediating Rif-induced filopodia (Pellegrin and Mellor, 2005). Rif expression alone in NIH 3T3 cells, which has no endogenous mDia2, did not lead to filopodia formation. The expression of full-length mDia2 alone was mostly cytoplasmic and had no significant effect on actin morphology. However, the co-expression of active Rif and mDia2 resulted in the translocation of mDia2 to the plasma membrane, and the induction of filopodial projections (Pellegrin and Mellor, 2005). It was further confirmed that mDia2 is required for Rif induction of filopodia, since expression of a dominant-negative mDia2 in HeLa cells, which express endogenous mDia2, blocked induction of filopodia (Pellegrin and Mellor, 2005). The role of formins in filopodia formation was further confirmed from studies involving the Dictyostelium discoideum dDia2. Active dDia2, shown to increase the rate of actin polymerization in vitro, is localized to filopodial distal tips and is required for the formation of filopodia, since over-expression increased the number and length of filopodia, and since dia2 null cells showed a reduction in the number and length of filopodia (Schirenbeck et al., 2005).

Axon Elongation

In addition to filopodia elongation, mDia has been implicated in axon elongation. During axonogenesis of cerebellar granule cells in vitro, mDia1 expression is enriched at the base of early initiating processes and within its growth cone (Arakawa et al., 2003). An active form of mDia1 was shown to facilitate axon elongation, whereas a dominant-negative interfered with a growth factor-induced axon elongation (Arakawa et al., 2003).

1.2.5 Formin-interacting proteins

In addition to profilin and small GTPases binding to formins, there have only been a few other formin-interacting proteins identified. The non-receptor tyrosine kinase, Src, binds to the FH1 domain of the mouse formin protein (Uetz et al., 1996) and the Drfs mDia1 and mDia2 (Tominaga et al., 2000). The interaction of formin proteins with Src has been suggested to regulate endosome motility (Gasman et al., 2003). In addition, mDia-interacting protein (DIP) binds to the FH1 region through its SH3 domain, and
likely plays a role with mDia1 in inducing stress fiber formation (Satoh and Tominaga, 2001). The FH1 domain also binds to the SH3 domain of the insulin receptor tyrosine kinase substrate p53 (IRSp53). This scaffolding protein is a target of small GTPases and is likely a downstream effector of mDia1, mediating stress fiber formation (Fujiwara et al., 2000). Most interestingly, there is a novel formin protein called Delphilin, which contains a PDZ domain at the N-terminus. Delphilin interacts with the C-terminus of the orphan receptor GluR82 subunit through its PDZ domain (Miyagi et al., 2002). GluR82 is called an orphan receptor since there is no evidence for this receptor to bind glutamate or any other known agonists of glutamate receptors (Heintz and De Jager, 1999). Both Delphilin and GluR82 are localized to the post-synaptic junction of cerebellar Purkinje cells, where GluR82 has been shown to play a role in cerebellar LTD (Kashiwabuchi et al., 1995). Therefore, Delphilin may serve as a post-synaptic scaffold protein and an actin-regulatory protein, providing a link between GluR82 and the actin cytoskeleton (Miyagi et al., 2002). Other studies have also suggested that formins might be involved in transducing signals to the actin cytoskeleton, by acting as a scaffold to recruit other components (Frazier and Field, 1997). Along these lines, the formin Daam1 interacts with Dishevelled (Dvl) and likely functions as a scaffolding complex in a signalling pathway required for Xenopus gastrulation (Habas et al., 2001).

### 1.2.6 The Diaphanous-related formin Daam1

The formin homology protein Daam1 (Dishevelled-associated activator of morphogenesis) was originally identified as being required for the Wnt-Frizzled-Dishevelled-RhoA (Wnt/Fz/Dvl/RhoA) signalling pathway in controlling cell polarity (Habas et al., 2001). During Xenopus development, Dvl is necessary for RhoA activation in the Wnt/Fz signalling pathway and has been suggested to act as a scaffolding protein (Habas et al., 2001). Dvl contains three domains, an N-terminal DIX domain, a PDZ domain, and a C-terminal DEP domain. The activation of RhoA in this signalling pathway requires both the PDZ and DEP domains, as neither alone was sufficient to activate RhoA. Since the PDZ domains appeared to be required for RhoA activation, a yeast two-hybrid screen was carried out to identify proteins associated with the PDZ domain. One positive clone encoded the carboxyl terminal of Daam1 (Habas et al., 2001).
Further analysis in cells revealed that Daaml can interact with either the PDZ domain or DEP domain, and is required to form a complex with Dvl-RhoA (Habas et al., 2001). Overall, Daam1 binds to either the PDZ domain or DEP domain of Dvl, and in addition activates RhoA, possibly through recruitment of a Rho-GEF. Since an N-terminal truncated Daaml, and not a full-length Daaml, activated RhoA (Habas et al., 2001), Daam1 likely contains an auto-regulatory mechanism similar to that found in other proteins. However, instead of RhoA binding to the N-terminus and relieving this auto-inhibition, the binding of Dvl with the C-terminus of Daaml likely releases such an intramolecular interaction, allowing Daam1 to function in activating RhoA (Habas et al., 2001). These results led to the identification that Daam1 is required in the Wnt/Fz/Dvl pathway for the proper morphogenetic movements associated with gastrulation and that this signalling pathway leads to the establishment of cell polarity through cytoskeletal changes (Habas et al., 2001).

Daam1 exists in various species, including human (hDaam1), mouse (mDaam1), and Xenopus (XDaam1). The sequence identity of Daam1 amongst various species is highly conserved, where mDaam1 is 97% identical to hDaam1 and 64% identical to XDaam1 (Nakaya et al., 2004). Sequence comparisons have shown that Daam1 contains several conserved motifs including the FH1, FH2, FH3, and GBD (Nakaya et al., 2004; Kida et al., 2004). Higgs and Peterson (2005) recently suggested a region of similarity between Daam1 and mDial that is located C-terminal to the FH2 domains, and corresponds to the DAD motif (Higgs and Peterson, 2005). This suggests that Daam1 likely has an auto-regulatory mechanism between the GBD and the DAD domain, and proteins binding to Daam1 might relieve this auto-inhibition. In addition to Daam1, a second Daam gene has been identified (Daam2), and the protein is 60% identical to Daam1 at the amino acid level (Nakaya et al., 2004).

Expression patterns of Daam transcripts in mouse embryos reveal that they are present during gastrulation, somitogenesis and organogenesis. Expression for both is seen in the central and peripheral nervous system, where at embryonic day 10, mDaam1 transcripts are enriched in the hindbrain (Nakaya et al., 2004). It was also noted that in the adult cerebellum, the Daam1 transcript was expressed in Purkinje cells, where the Daam2 transcript was expressed in the internal germinal layer (Kida et al., 2004).
1.3 Activity-dependent Regulation of Spines

Forms of synaptic plasticity have been associated with morphological changes in spines. The induction of LTP has been shown to result in the appearance of new spine-like protrusions (Engert and Bonhoeffer, 1999), the formation of multiple spine synapses (perforated synapses) on a single pre-synaptic terminal (Toni et al., 1999), the enhanced growth of filopodial protrusions (Maletic-Savatic et al., 1999), and an increase in spine length (Hosokawa et al., 1995). Therefore, these morphological changes seem to be important for synaptic function and contribute to synaptic strength. It is believed that these changes in spine shape and number reflect the reorganization of the spine cytoskeleton (Fischer et al., 1998; Halpain et al., 1998).

Dendritic spines have a high enrichment of actin when compared to the dendritic shaft (Fischer et al., 1998). Spines essentially consist of a bulbous head, containing the PSD, connected to the dendritic shaft by a narrow spine neck, generating a mushroom-shaped spine. Although mushroom-shaped spines are common, these dendritic protrusions can vary widely in shape, including stubby, thin, filopodial, and cup-shaped (Hering and Sheng, 2001). In the spine head, actin filaments are extensively branched and organized into a meshwork of filaments. In the spine neck however, bundles of actin filaments are oriented longitudinally, with their fast-growing barbed ends oriented towards the spine head. It was further noted that the PSD of spines can be associated with the barbed end of actin filaments (Fifkova and Delay, 1982).

Section 1.1 listed ways in which the components of the actin cytoskeleton could regulate receptor localization, clustering, and anchoring through various interactions with PDZ proteins. However, it has been suggested that the elements of the PSD, such as receptors and PDZ proteins, could regulate changes in the actin cytoskeleton (Reviewed by Rao and Craig, 2000). There is evidence that the induction of LTP leads to reorganization of the actin cytoskeleton, mediated by an increase in F-actin content within dendritic spines, suggesting that the persistence of this synaptic strength is controlled by mechanisms regulating the spine actin cytoskeleton (Fukazawa et al., 2003). Therefore, any changes in spine morphology through actin remodelling have the ability to control synaptic strength. For example, larger spine heads resulting from actin remodelling likely
correlates with a larger PSD and more receptors, resulting in an increase in post-synaptic strength (Hering and Sheng, 2001).

There is increasing evidence that small Rho-family GTPases are implicated in regulating actin dynamics and spine morphology. For example, expression of a dominant negative Rac1 in hippocampal slice cultures resulted in the elimination of dendritic spines, suggesting Rac1 promotes spine formation (Nakayama et al., 2000; Tashiro et al., 2000). The blockade of Rac1 also seems to inhibit spine head growth, and forming instead long, thin spines. Inhibition of Rho, on the other hand, has been suggested to increase spine density (Tashiro and Yuste, 2004). In addition, Cdc42 also plays a role in spine morphology, since expression of a dominant negative Cdc42 mutant in hippocampal neurons inhibited spine formation (Irie and Yamaguchi, 2002). It is possible that some of the upstream proteins, including post-synaptic receptors, regulate the activities of these Rho-GTPases and lead to changes in the cytoskeleton.

There is evidence that stimulation of glutamate receptors changes the dynamics of F-actin and leads to changes in spine morphology. The activation of NMDARs through synaptic stimulation resulted in the outgrowth of spine-like protrusions from dendrites (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999) and an increase in the size of spines (Lin et al., 2004). Although AMPAR antagonists decreased spine density and length, much of the evidence for AMPARs suggests that they may play a more prominent role in the maintenance of spines than in the growth of spines (McKinney et al., 1999). However, another study showed that over-expression of the GluR2 subunit in hippocampal neurons increased the length of spines, the width of spine heads and the density of spines (Passafaro et al., 2003), indicating the importance of AMPARs in spine growth. Furthermore, glutamate receptor activation caused an enlargement of dendritic spine heads (Matsuzaki et al., 2004). In general, most of the results provide evidence for an activity-dependent regulation of spine morphology (Hering and Sheng, 2001), where activation of receptors can lead to increased changes in size and density. In support of this model, the activation of NMDARs also induces the targeting of profilin, a key regulator of actin polymerization, to spine heads (Ackermann and Matus, 2003). An increase in F-actin content within spines could change the morphology of spines and the distribution of receptors at the PSD.
The mechanisms currently linking activation of receptors to the actin cytoskeleton are not fully clear, but it is possible that GTPase-activating proteins (GAPs) or guanine nucleotide exchange factors (GEFs) play a role in this regulation, since Rho-family GTPases are known to affect the cytoskeleton. There are currently a few known GAPs and GEFs that are involved in regulating the actin cytoskeleton. These include Kalirin-7, SPAR, Tiam1, and Lfc. The over-expression in cortical neurons of Kalirin-7, a Rac1-GEF, increased the number and size of the spines, and the complexity of the spine shape (Penzes et al., 2001). In support of the activity-dependent regulation of spine morphology, the activation of EphB2 receptors, known to play a role in synaptic plasticity (Henderson et al., 2001), and its subsequent activation of Kalirin-7 and signalling through Rac1 and p21-activated kinase (PAK), are required for these changes in spine morphology (Penzes et al., 2003).

In addition, a GAP for the Rap family of GTPases, SPAR (spine-associated Rap-GAP), has also been identified to regulate spine morphology. SPAR over-expression in hippocampal neurons caused an increase in spine head width, and a change in shape to highly irregular spines (Pak et al., 2001). It was suggested that the function of SPAR is to provide a connection between the PSD-95-NMDAR complex and the actin cytoskeleton, through Rap signalling, further supporting a role for receptors and PDZ proteins in regulating spine morphology (Pak et al., 2001).

There also exists GEFs, such as the Rac1-GEF Tiam1 and the Rho-GEF Lfc (Lbc [lymphoid blast crisis]'s first cousin), that regulate spine morphology. Tiam1 is likely required for spine development and maintenance, since Tiam1 knockdown in hippocampal neurons resulted in reduced spine density and increased spine length (Tolias et al., 2005). Tiam1 was also suggested to play a role in promoting the formation of new spines, since Tiam1 over-expression resulted in an increase in spine density (Tolias et al., 2005). Therefore, the Rac1-GEF Tiam1 is likely part of the molecular mechanism that links NMDAR activation to changes in spine morphology. Furthermore, the over-expression of the Rho-GEF Lfc decreased spine length and spine area, and increased spine density, suggesting Lfc likely functions in the stabilization of spines. It was further determined that spinophilin, an actin-binding protein, binds to Lfc and is a key regulatory in mediating spine morphology (Ryan et al., 2005).
Other actin-binding proteins, in addition to spinophilin, have been implicated in spine morphogenesis, and thus possibly involved in this activity-dependent regulation of spine morphology. For example, cortactin is an F-actin binding protein and has been implicated in the stabilization and branching of actin filaments, through its interaction with the Apr2/3 complex (Weaver et al., 2001). Cortactin definitely plays a role in spine morphology, since hippocampal neurons deficient in cortactin resulted in a decrease in spine density, whereas the over-expression of cortactin caused spines to grow in length (Hering and Sheng, 2003). Cortactin could be involved in these activity-dependent morphological changes, since the SH3 domain of cortactin binds to Shank, a known interactor of the NMDAR-PSD-95 complex, and since glutamate stimulation caused a redistribution of the actin-binding protein (Naisbitt et al., 1999). This interaction with Shank and the fact that Shank over-expression results in the enlargement of spine heads, indicates that PSD scaffold proteins also function in the reorganization of spine morphology (Sala et al., 2001). Another actin-binding protein called Drebrin is also a known regulator of spine morphogenesis. Over-expression studies showed that Drebrin causes elongation of spines, and is dependent on binding to actin filaments for synaptic targeting (Takahashi et al., 2003).

Overall, there is considerable evidence supporting activity-dependent regulation of spine morphology. Although not much is currently known on pathways connecting the activation of AMPARs to the actin cytoskeleton, there is considerable information regarding the signalling pathways involving NMDARs and Eph receptors (summarized in Figure 2). Eph receptors seem to play a major role in spine morphology, because in addition to signalling through the Rac1-GEF Kalirin-7, EphB2 receptors bind to the protein intersectin, a GEF for Cdc42, which activates N-WASP (neural Wiskott-Aldrich syndrome protein) (Irie and Yamaguchi, 2002), a known regulator of Arp2/3-mediated actin polymerization (Takenawa and Miki, 2001). Their involvement in spine morphology is based on the over-expression of a mutant form of intersectin or a dominant negative form of Cdc42 disrupting spine formation (Irie and Yamaguchi, 2002). These results indicate that EphB2 receptor signalling through intersectin and the activation of Cdc42 and N-WASP, are involved in spine morphology through Arp2/3-mediated actin-polymerization (Irie and Yamaguchi, 2002). Therefore, the activity-dependent regulation
of spine morphology is likely mediated through GAPs and GEFs of Rho-GTPases, and proteins involved in actin polymerization or depolymerization.
Figure 2: Signalling pathways through the activation of EphB and NMDA receptors that regulate actin-binding proteins and spine morphology. The activation of EphB receptors is widely known to affect spine morphology. This occurs through signalling pathways involving the Cdc42-GEF Intersectin and the Rac-GEF Kalirin-7. Intersectin signals through Cdc42 and N-WASP, a known regulator of ARP2/3, resulting in F-actin nucleation, branching, and polymerization. Kalirin-7 signals through the Rac effector protein PAK, which activates LIMK that subsequently inactivates cofilin, a known actin depolymerizing agent. The activation of NMDARs has been linked to the actin cytoskeleton through PDZ proteins PSD-95 and Shank. NMDARs bind to the Rac1-GEF Tiam1, which signals through Rac and PAK to promote actin polymerization. Shank binds to PSD-95 and the actin-binding protein cortactin. Cortactin is known to regulate the ARP2/3 complex. The dotted line represents NMDAR signalling through PSD-95, SPAR and Rap, not yet shown to affect actin remodelling. However, SPAR has been shown to increase the size of the spine head. Highlighted in grey and green are the Rho-family GTPases and actin-regulatory proteins, respectively.
Intersectin

EphB receptors

Kalirin-7

Tiam1

NMDA receptors

PSD95

Shank

Cdc42

Rac

Rap

N-WASP

PAK

LIMK

Cortactin

F-actin nucleation/polymerization

F-actin polymerization

F-actin nucleation/polymerization

Spine Morphology

Figure 2
1.4 **Hypotheses**

From the previously identified interaction between Daam1 and GRIP1, I further characterized that this interaction is PDZ-mediated via the C-terminus of Daam1 and PDZ domains 456 of GRIP1. From this interaction, I hypothesized that Daam1 is enriched in the heads of actin-rich dendritic spines, and that its interaction with GRIP1 is involved in localizing Daam1 into spines. Previous studies have shown that GRIP1 is involved in the trafficking of various proteins, such as GluR2. Therefore, the interaction and localization of Daam1 and GRIP1 in dendritic spines might suggest that they function cooperatively in the localization of the GluR2 subunit of AMPA receptors. In addition, since formin homology proteins are key regulators in actin polymerization, I hypothesized that Daam1 is involved in regulating spine morphology, and that Daam1 over-expression in hippocampal slices results in the enlargement of spines.
Chapter II:

Experimental Procedures
2.1 Yeast Two-Hybrid

2.1.1 Yeast Two-Hybrid DNA constructs

The C-terminus of human Daam1, encoding the last 132 amino acids, was amplified by PCR and subcloned by Ami Tsuchida into a destination vector (pDEST32), generating the "bait" plasmid (DB-X). The C-terminus was inserted immediately after and in-frame with the GAL4 DNA binding domain (GAL4-DB). Except for the PDZ456 construct, Ami Tsuchida generated the various PDZ domain-containing constructs. PCR was used to amplify the PDZ domains 456 from the original clone isolated from the yeast two-hybrid experiment (pGEM7.4).

PCR was performed using PfuUltra high fidelity DNA polymerase (Stratagene) by incubation at 94°C for 2min; 30 cycles of 94°C for 1min, 51-65°C gradient for 1min, 72°C for 2min; and 72°C for 10min. The PCR reaction mixture included 10x Pfu buffer, 10μM dNTPs, 10μM forward primer PDZ-4-F (5'-cag tcg act ggc ggc tat ggt gcc ctc atc ttc t-3'), 10μM reverse primer PDZ-6-R (5'-cag cgg ceg cga tca agt tgg gga cat gat etc ctt tat-3'), 5x Q solution (Qiagen), Pfu ultra, and 2 μl of plasmid DNA (pGEM7.4). The amplified PCR product of PDZ456 was ligated into the digested blunt-end SmaI site of pGEM7Z, and subsequently digested with SalI and NotI and ligated into the pENTR2B plasmid (Invitrogen). The pENTR2B vector, already linearized with SalI and NotI, was generously given by Keith Murai.

Gateway technology (Invitrogen) allowed easy cloning of PDZ456 into the pDEST22 plasmid through an LR reaction. For the LR reaction, mixed 2μl of entry clone (PDZ456-pENTR2B – 1/10 dilution of wizard DNA), 2μl of destination vector (pDEST22 – 150ng/μl), 2μl of 5x LR reaction buffer, 2μl of 1x TE buffer (pH 8.0), and 2μl of LR clonase. Reaction was incubated at 25°C for 2 hours, and then used in DH5α transformation.

2.1.2 Yeast Two-Hybrid Small scale transformation

The yeast two-hybrid screen was performed using MaV203 yeast strain according to the protocol from the Proquest Two-Hybrid System with Gateway Technology (Invitrogen). The MaV203 yeast was grown on an HC-minimal media plate for 48 hrs at
30°C. Colonies were picked and streaked on leucine- and tryptophan-deficient plates (leu’ tryp’) to test for their absence in growth.

A Ma V203 colony was inoculated in 5ml liquid YPAD (1% bacto-yeast extract, 2% bacto-peptone, 2% glucose, 0.01% adenine 2% bacto-agar) and grown at 30°C overnight. Cells (1ml) were transferred to 50ml of YPAD for an OD at 660nm of approximately 0.1. Cells were grown at 30°C for 5 hours, where the OD was 0.72. Cells were centrifuged at 2700 rpm for 10 minutes, and then washed with 10ml sterile dH2O, and centrifuged at 3000rpm for 10 minutes. Cells were resuspended in 5ml 0.1M Lithium acetate (LiAc) in 10x TE (100mM Tris pH8 and 10mM EDTA), and incubated at 30°C for 15 minutes. Then centrifuged at 4000rpm for 8 minutes, and resuspended in 250μl 0.1M LiAc/TE, with 1.2ml PEG (50% w/v), 150μl 1M LiAc, and 150μl 10x TE. Then, 150μl of this mixture was added to each of the co-transformation reactions containing the bait plasmid, prey plasmid, and boiled salmon sperm DNA. Cells were incubated at 30°C for 30 minutes, then heat shocked at 42°C for 20 minutes. Cells were centrifuged at 4000 rpm for 3 minutes, resuspended in 300μl dH2O, and then one-third was used on leu’tryp’ plates, and incubated at 30°C overnight. After 90 hours, three colonies for each co-transformation were picked and streaked on leu’tryp’ plates, and incubated at 30°C for 48 hours. For the Lac Z assay, a nitrocellulose membrane was placed on an YPAD plate, and each of the three colonies on the leu’tryp’ plates were streaked in a line on the nitrocellulose membrane, and then streaked on 20mM 3AT plates deficient in leucine, tryptophan and histidine, for growth analysis after 48 hours. If both proteins interact, than the HIS3 transcript should be activated, resulting in growth on plates deficient in histidine. Colony streaks on the nitrocellulose membrane were grown for about 48 hours, and then the membrane was frozen on dry ice for 20 minutes. Stacked two round 110mm Whatman filters in a 15cm cell culture dish, and saturated them with an X-gal solution, containing 50μl of X-gal (100mg/ml), 5ml Z buffer (16.1g Na₂HPO₄·7H₂O, 5.5g NaH₂PO₄·H₂O, 0.75g KCl, 0.246g MgSO₄·H₂O, dissolved in 1L dH₂O and adjusted to pH7.0), and 30μl β-mercaptoethanol. Frozen membrane was placed on Whatman filters, with the colony side facing up, and incubated at 37°C overnight.
2.2 Cloning of full-length GRIP1

The amplification of full-length GRIP1 proved to be difficult from a cDNA library. Therefore, I generated GRIP1 by amplifying two halves separately and combined them in a plasmid. These separate halves are referred to as head GRIP1 (H-GRIP1) and tail GRIP1 (T-GRIP1).

2.2.1 RNA isolation and Reverse Transcription

The RNA from mouse brain was isolated using the TRIzol RNA isolation (GIBCOBRL Life Technologies) protocol. The RNA was reverse-transcribed using the Superscript first-strand synthesis system for RT-PCR (Invitrogen). For the reverse transcription (RT) reaction, 4.32 µg of total RNA was mixed with 0.5 µg Oligo (dT) primer, and 0.2 µg random hexamer primer. The reaction mixture was incubated for 5 minutes at 70°C, and then kept on ice. Then added 4 µl of 5x reaction buffer, 1 µl of ribonuclease inhibitor (20 u/µl), and 2 µl of 10 mM dNTP mix, and incubated for 5 minutes at 25°C, then for 5 minutes at 37°C. After, 2 µl of M-MuLV Reverse transcriptase (20 u/µl) was added, and reaction was incubated for 10 minutes at 25°C, then for 60 minutes at 37°C. The reaction was stopped by heating for 10 minutes at 70°C.

2.2.2 Full-length GRIP1 construct

The DNA for H-GRIP1 was amplified by PCR using the cDNA isolated from the RT experiment. PCR was performed using *PfuUltra* by incubation at 94°C for 2 min; 35 cycles of 94°C for 1 min, 50-70°C gradient for 1 min, 72°C for 4 min; and 72°C for 10 min. The PCR reaction mixture included 10x *Pfu* buffer, 10 µM dNTPs, 10 µM forward primer GRIP-H-F (5'--aca tga tag ctg tct ctt tta aat gcc g-3'), 10 µM reverse primer GRIP-H-R (5'--acg atc gct cca gtc ctt tca g-3'), 4% DMSO, 2 µl *Pfu* ultra, and 5 µl of cDNA in a final volume of 100 µl. The DNA for H-GRIP1 proved to be difficult to obtain, and a PCR product of 1986 bp only appeared at a temperature of 57.5°C. H-GRIP1 was ligated into the blunt-end SmaI site of the pGEM7Z plasmid.

The DNA for T-GRIP1 was amplified by PCR using the original isolated clone from the yeast two-hybrid experiment. PCR was performed using *taq* DNA polymerase (MBI) by incubation at 94°C for 2 min; 35 cycles of 94°C for 1 min, 61°C for 1 min, 72°C
for 4 min; and 72°C for 10 min. The PCR reaction mixture included 10x Pfu buffer, 5 µM dNTPs, 10 µM forward primer GRIP-T-F (5'‐caag ata act cag acg agc aag aga-3'), 10 µM reverse primer GRIP-T-R (5'‐cag ttc gtc ttt tgt cct gta taa aa -3'), 5x Q solution, 1 µl of taq, and 2 µl of plasmid DNA (pGEM7.4) in a final volume of 50 µl. The 1461 bp PCR product of T-GRIP1 was ligated into pGEMT.

For cloning H-GRIP1 into the T-GRIP1-pGEMT plasmid, a BglII site was needed at the 5’end of the forward primer of H-GRIP1. Therefore, a new H-GRIP1-F primer with a BglII site was designed to amplify H-GRIP1 by PCR from the H-GRIP1-pGEM7Z plasmid, since this new primer failed to generate an amplified product from cDNA. PCR was performed using PfuUltra by incubation at 94°C for 2 min; 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 6 min; and 72°C for 10 min. The PCR reaction mixture included 10x Pfu buffer, 10 µM dNTPs, 10 µM forward primer GRIP-H-F-BglII (5'-aca gat cta tga tag ctc tct tta aat gcc g-3'), 10 µM reverse primer GRIP-H-R (5'- acg atc gct cca gtc ctt tca g -3'), 5x Q-solution, 1 µl Pfu ultra, and 2 µl of H-GRIP-pGEM7Z plasmid DNA (1/20 dilution) in a final volume of 50 µl. The H-GRIP1 PCR product was digested with BglII and Xhol, and ligated into T-GRIP1-pGEMT, already digested with BglII and Xhol. The full-length GRIP1 in the pGEMT plasmid was digested with BglIII and SalI and cloned into the BamHI and SalI sites of pCMVTAG3B.

2.3 Heterologous cell experiments

2.3.1 Immunohistochemistry

Transfected BHK cells on 35 mm dishes containing cover-slips with 3 µg of either myc-Daaml and/or FLAG-GRIP1 using 10 µl polyfect (Qiagen), according to the manuals protocol. Transfected HEK 293T cells using the calcium phosphate method with 2 µg of myc-Daaml and FLAG-GRIP1 with 100 µl of 0.1x TE, 12 µl 2M CaCl₂, and 100 µl 2x HBS. Cells were fixed after 20 hours in 2% paraformaldehyde for 20 minutes at room temperature, then washed three times 5 minutes in PBS, and incubated in blocking solution (0.2% TritonX-100, 5% goat serum, and 3% BSA in PBS) for 2 hours at room temperature. Cells were then immunostained with the primary antibodies anti-GRIP1 (1:300) and/or anti-Daaml (1:750) in blocking solution overnight at 4°C. Cells were washed three times 5 minutes in PBS, and then incubated with goat-anti-mouse-
Rhodamine (1:1000) and/or goat-anti-rabbit-FITC (1:500) in blocking solution for 2
hours at room temperature. Cells were washed three times 5 minutes in PBS, and then
mounted on slides using SlowFade Antifade kit (Molecular Probes). Images were
obtained using the Axioskop2 plus microscope (Zeiss) under the 63x oil lens.

2.3.2 Transfection of HEK293T cells

HEK 293T cells were cultured in DMEM medium (Invitrogen) supplemented with
10% FBS, and 1% penicillin/streptomycin, and were transfected at 50-60% confluency in
100mm plates using calcium phosphate. For transfection, 500μl of 0.1x TE was mixed
with 10μg myc-Daam1, 10μg FLAG-GRIP1, or 10μg of both, along with 60μl 2M CaCl2
and 500μl 2x HBS. This transfection mixture was added to HEK 293T cells in 100mm
dishes already containing 8ml of medium. Cells were incubated at 37°C overnight, and
the medium was changed after 24 hours. After 48 hours, cells were washed in PBS and
scraped off the plates and lysed in 400μl extraction buffer (50mM Tris-HCl pH7.4,
150mM NaCl, 1mM EDTA, 1mM EGTA, 1% TritonX-100, 1mM PMSF, and 1x protease
inhibitor mix (Roche protease pill)). Lysates were kept on ice for 10 minutes while
vortexing occasionally, and then centrifuged for 10 minutes at 13 000 rpm at 4°C. The
insoluble pellets were resuspended in 5% SDS lyses buffer (50mM Tris-HCl pH7.4,
150mM NaCl, 1mM EDTA, 1mM EGTA, 5% SDS, 1mM PMSF, and 1x protease
inhibitor mix). Lysates were sonicated (200w) for 5 seconds and then kept on ice for 10
minutes, while vortexing occasionally. Lysates were centrifuged at 13 000 rpm for 10
minutes at 4°C, and the supernatant was used as the insoluble fraction. The Bio-Rad
protein assay kit was used to determine the protein concentration.

2.3.3 Western blot analysis

For the soluble and insoluble protein fractions, additional SDS was added to the
loading dye to make sure that each sample had the same percentage of SDS when added
with 100μg of proteins. The samples were heated for 5 min at 80°C and were loaded on a
7.5% polyacrylamide gel (SDS-PAGE) along with a pre-stained protein ladder, and
electrophoresed in running buffer containing 25mM Tris-Cl (pH 8.0), 200mM glycine
and 0.05% SDS. The separated proteins were then transferred to an PVDF Immobilon-P
transfer membrane (Millipore) in transfer buffer containing 10% methanol, 10mM Tris-Cl (pH 8.0), and 2.5mM glycine. After transferring, the membranes were then blocked with 5% non-fat dry milk (blotto) in Tris-buffered saline (TBS), and incubated for 1 hour at room temperature. Subsequently, the membranes were incubated with the primary antibody overnight at 4°C. Primary antibodies used were anti-Daam1 (Protein A purified polyclonal-1:250), polyclonal anti-Daam1 serum (1:500), anti-FLAG (M5 monoclonal 1:1000, Sigma), and anti-myc (monoclonal 9E10B, 1:100). Membranes were washed four times 10 minutes in TBS 0.1% Tween-20 and then incubated with an HRP-conjugated secondary antibody, including goat anti-rabbit (1:1000) for Daam1 and goat-anti mouse for FLAG and 9E10B, in 1% milk-TBS for 1 hour at room temperature. Membranes were washed four times ten minutes in TBS-0.1% Tween-20. Signals were detected by enhanced chemiluminescence (ECL–Perkin Elmer) or ECL plus (Amersham), and visualized by autoradiography.

2.3.4 Immunoprecipitations

For immunoprecipitations, HEK 293T cells were lysed in 1% triton extraction buffer as described previously. Approximately 1mg of protein lysate from transfected cells were incubated with either 5μg of anti-FLAG, 5μl of anti-GRIP1, or 5μl of antisera Daam1 antibodies for 2 hours at 4°C on an orbital shaker. Immune complexes were collected by binding to 60μl of protein A or Protein G agarose (1:1 slurry) for each sample and rocking at 4°C overnight. Immunoprecipitations were then centrifuged at 2000rpm for 5 minutes at 4°C, and were washed 4 times with lysis buffer. Immunoprecipitated proteins were boiled and eluted in SDS sample buffer, resolved by SDS-PAGE, and transferred to a PVDF membrane for western blot analysis.

2.4 Mouse whole brain lysates

Two mouse brains (A and B) were dissected from P12 mice with the help of Sarah Martinez, and homogenized in two different lysis buffers. Brain A was homogenized 4 x 30sec in lysis buffer containing 250mM sucrose, 50mM Tris-HCl pH7.5, 25mM KCl, 5mM MgCl₂ and 1x protease inhibitor mix, using a mechanical homogenizer. Lysates were incubated on ice for 30 minutes, and then centrifuged at 14 000 rpm for 10 minutes.
Lysates were subsequently passed through a 25 gauge needle. Brain B was homogenized 4 x 30sec in lysis buffer containing 50mM Tris-HCl pH7.4, 150mM NaCl, 1% TrionX-100, 0.5% deoxycholate (DOC), 1mM EDTA, 1mM PMSF, 1mM Na3VO4, 1mM NaF and 1x protease inhibitor mix, using a mechanical homogenizer. Lysates were incubated on ice for 30 minutes, and then centrifuged at 1800 rpm twice for 15 minutes, and then again for 60 minutes at 4000 rpm. Supernatants were used as soluble fractions, whereas the pellets were resuspended in their respective lysis buffers for use as the insoluble fractions. 30μg of protein were loaded on an SDS-PAGE and transferred to a PVDF membrane for western blot analysis. Western blots were performed as described above, except a polyclonal anti-Daam1 serum (1:500) was used for the primary antibody stain.

2.5 **Dissociated hippocampal cultures and immunocytochemistry**

Rat hippocampal primary neuronal cultures (E17) were already prepared and fixed after 3 weeks in culture and generously given by Huashan Peng. Fixed neurons were permeabilized with 0.5% TritonX-100 in PBS for 20 minutes at room temperature. Neurons were washed with PBS, and then incubated with blocking solution (10% goat serum and 0.2% TritonX-100) for 1 hour. Neurons were then incubated with primary antibodies, including Daam1 (anti-serum, 1:500), SV2 (1:100, Developmental Studies Hybridomas Bank), GRIP1 (1:200, BD Transduction Laboratories), and GluR2 (1:200, Chemicon) in 5% goat serum and 0.2% TritonX-100 at 4°C overnight. Neurons were washed in PBS, and then incubated with secondary antibodies, including Alexa Fluor 488 goat-anti-rabbit (1:300, Molecular Probes) and Alexa Fluor 568 goat-anti-mouse (1:300, Molecular Probes) for 2 hours. Neurons were washed with PBS and then mounted on slides using SlowFade Antifade kit (Molecular Probes). Images were obtained using a Zeiss LSM 510 confocal microscope, using a 63x objective with a 1024x1024 pixel resolution.

2.6 **Subcellular fractionation of mouse brain**

Subcellular fractionation was performed by Mathieu Lachance, and carried out by differential centrifugation as described previously (Huttner et al, 1983) and analyzed by western blotting. Briefly, a mouse brain was homogenized in 20ml of H buffer (10mM
Hepes, pH 7.4, 0.3M sucrose, protease inhibitors) with 9 strokes at 900 rpm. The resulting pellet (P1) contains the nuclei and cell debris, while the supernatant (S1) was collected and centrifuged at 10 000 rpm for 15 minutes. The supernatant (S2, cytosol) was removed and centrifuged at 45 000 rpm for 2 hours in T865 rotor, whereas the pellet (P2) was resuspended in 20 ml H buffer and centrifuged at 11 000 rpm for 15 minutes to yield S2' and P2'. The pellet (P2') was resuspended in 9 volumes of 4 C dH2O quickly, with 3 strokes at 2000 rpm. This P2' lysate was added to 1 ml of 1M HEPES- buffer pH 7.4 and kept on ice for 30 minutes. It was then centrifuged at 16 000 rpm for 20 minutes to yield a lysate pellet (LP1), containing the synaptic membrane fraction, and a lysate supernatant (LS1), containing the nerve terminal fraction. The supernatant was collected and centrifuged at 50 000 rpm for 2 hours. This yielded a supernatant (LS2), containing cytosol from nerve terminal, and a pellet (LP2), containing the synaptic vesicle fraction. LP2 was resuspended in 6 ml of 40 mM sucrose.

2.7 Viral Daam1 constructs

The full length sequence of Daam1 was digested out of the pBSIISK plasmid (from Rob Dunn) with the restriction enzymes XhoI and SacII, and then blunt-end ligated into the HindIII site of the pEGFP-C1 vector (Clontech). The Daam1-pEGFP plasmid was digested with NheI, StuI and AclI, and the fragment between NheI and AclI was ligated into the pENTR2B-SGp-RFPf vector (from Keith Murai).

To construct the Daam1 sequence lacking the PDZ-binding motif (Daam1-ΔC), I performed PCR on the Daam1-pCMVTAG plasmid for a 772 base pair fragment at the C-terminus with a deletion of the last three amino acids in the reverse primer. PCR was performed using Pfu DNA polymerase (Stratagene) by incubation at 94°C for 4 min; 30 cycles of 94°C for 45 s, 57°C for 1 min, 72°C for 1 min 45 sec; and 72°C for 10 min. The PCR mixture contained 10x Pfu buffer, 10 μM dNTPs, 10 μM forward primer Daam-ΔC-term (5' – gaa taa agg tca aag agg gaa tg-3'), 10 μM reverse primer Daam-ΔC-term (5' – cag ata tgg gec tca ttt tgt gat tgg tct ctc tc-3'), 5x Q solution, Pfu ultra, and 40 ng of Daam1-pCMVTAG2B plasmid DNA. The PCR product was ligated into a blunt-end SmaI digested pGEM7Z plasmid. The ΔC-terminal tail was digested from the pGEM7Z plasmid with EcoRV, and ligated into the EcoRV site of Daam1-pEGFP plasmid. Then,
the Daam1ΔC-pEGFP plasmid was digested with NheI, BamHI and StuI, and the fragment between the NheI and BamHI sites was then ligated into the blunt-end XmnI site of the pENTR-SGp-RFPf plasmid.

Gateway technology (Invitrogen) allowed cloning of the EGFP-Daam1, and EGFP-Daam1ΔC sequences from the pENTR2B plasmid into the viral vector PD-RFA (from Keith Murai) through an LR reaction. For the LR reaction, mixed 2μl of entry clone (1/5 dilution of EGFP-Daam1-pENTR2B or EGFP-Daam1ΔC-pENTR2B DNA from wizard prep), 2μl of destination vector (PD-RFA – 150ng/μl), 2μl of 5x LR reaction buffer, 2μl of 1x TE buffer (pH 8.0), and 2μl of LR clonase. Reaction was incubated at 25°C for 2 hours. After, added 0.5μl of proteinase K to 5μl of reaction and incubated for 10 minutes at 37°C. Then added 50μl DH5α to LR reaction, and continued with transformation procedure. Both viral Daam1 DNA plasmids preparations were performed by plasmid DNA maxi-preps, as described below.

2.8 Virus production, concentration, and activation

Baby hamster kidney (BHK) cells were seeded at 1.6x10⁵ cells/ml in 60mm dishes and maintained in the Iscove's Modified Dulbecco's Medium (IMDM) with L-glutamine and 25 mM HEPES buffer supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (GIBCOBRL, Life technologies) overnight at 37°C. The following day, six dishes of BHK cells (60-70% confluency) were transfected with each viral Daam1 DNA construct. 18μg DNA with 24μg pHelper and 858μl IMDM (serum-free) were transfected with 90μl polyfect (Qiagen). Transfections were mixed with 6ml IMDM, where 1.1ml was added to each of the six dishes of BHK cells already in 3ml IMDM. Transfections were incubated overnight at 37°C, and changed media (5ml) after 24 hours, and incubated at 31°C for another 48 hours.

In a Beckman ultracentrifuge tube, 6 ml of 20% sucrose in TNE buffer (50mM Tris-Cl pH7.4, 100mM NaCl, 0.5mM EDTA) was added on top of 3 ml of 55% sucrose also in TNE buffer. For each transfection, the medium containing the virus particles was collected from the BHK dishes 72 hour post-transfection, where 30 ml was laid on top of the sucrose gradient and subsequently centrifuged for 90 min at 28,000 rpm using a
swinging bucket. After spinning, 800μl was removed from the bottom and discarded, and collected the next 4ml from the bottom for concentration.

To concentrate the virus, centrifugal filter devices (Centricon YM100-Millipore) were used to remove sucrose and obtain a relatively high titer of the virus particles. The 4ml viral mixture was added to the centricon tube and centrifuged at 2000rpm for 20 minutes. PBS was added to dilute sucrose and the centricon was centrifuged multiple times at 2000rpm for 10 minutes, until all of the sucrose and PBS, except roughly 500μl, passed through the filter. The centricon tube was inverted, and virus particles were collected by centrifugation at 1000rpm for 2 minutes.

For virus activation, 5 μl of α-chymotrypsin (10 mg/ml) was added into 100 μl of concentrated virus and was incubated for 45 min at room temperature. To stop the protease activity of α-chymotrypsin, 7.5 μl of aprotinin (10 mg/ml) was added and kept at room temperature for 15 min.

2.9 Organotypic hippocampal slices – Image analysis and quantification

With the help from Lei Zhou and Michael Haber from Keith Murai’s lab, P6 mice were sacrificed, and the hippocampus was carefully dissected from the brain. The hippocampus was cut in slices of thickness of 300μm using the McIlwain tissue chopper, and incubated on 0.4μm culture plate inserts (Millipore) already in a 35mm dish with 1 ml culture media (50ml of MEM, 25ml 1x HBSS, 25ml horse serum, 0.65g dextrose and 500 μl Penicillin/Streptomycin). Slices were kept on inserts at 37°C for about 9 days. Both Daam1 viral preparations, along with a control RFPf, were injected using a picospritzer in the CA1 region of the hippocampal slice. Slices were then kept at 37°C for approximately 20 hours, before being fixed in 4% paraformaldehyde for 30 minutes. Slices were imaged using a confocal microscope. Images are maximal projections using MetaMorph from z-series stacks of 0.3 um in thickness, using a 63 x objective (with 1.5 zoom) with a 1344x1024 pixel resolution.

Spines measurements were done blindly and manually traced using the Reconstruct program (developed by J. C. Fiala and K. M. Harris, free download from www.synapses.bu.edu). For spine quantification, at least 1000 spines were counted per construct (from 20-23 neurons). For cumulative distributions plots, statistical significance
was determined by Kolmogorov-smironov test. For histograms, statistical significance between two means was calculated using Student’s t test.

2.10 Additional DNA protocols

All DNA constructs were verified through sequencing done by the McGill genome center. All DNA transformations involved XTRAcells-A *E.Coli* competent cells (Bio S&T), also called DH5α. Mixed 50 µl of competent cells with half of ligation reaction, and kept on ice for 1 hour. Cells were heat shocked at 42°C for 90 seconds, gently shaking, and then put back on ice for 10 minutes. Added 300 µl Luria Broth (LB) medium to the cells, and grew at 37°C for 1 hour. After, 150 µl cells were plated on LB plates containing ampicillin (75µg/ml) or Kanamycin (25µg/ml). When plasmids allowed for blue/white colony selection, 50mg/ml X-gal and 100mM IPTG were used on LB plates. The plates were incubated at 37°C overnight, and colonies were selected the following day and grown in 5ml of LB with ampicillin or kanamycin at 37°C overnight. The plasmid DNA was isolated using the DNA wizard mini-prep (Promega), and analysed by restriction enzyme digestion.

2.10.1 Plasmid DNA maxi-prep

*E.coli* containing the plasmid of interest were grown in 1 L of 2-YT (16g tryptone, 10g yeast extract, and 5g NaCl, with appropriate antibiotic) and grown overnight at 37°C. The cells from the 1 L culture were collected by centrifugation at 6000rpm for 14 minutes at 4°C using the JA-14 rotor. The cell pellet was resuspended in 36ml of solution I (50mM glucose, 25mM Tris pH8, 10mM EDTA pH8), and lysed with 2ml of lysozyme (10mg/ml) for 10 minutes at room temperature. Then 80ml of solution II (0.2M NaOH, 1% SDS) were added, followed by 40ml of solution III (5M KoAc, pH4.8). Solutions were kept on ice for 15 minutes and mixed by swirling every 5 minutes. This solution was centrifuged at 6000rpm for 10 minutes at 4°C (JA-14 rotor). The supernatant was strained through a cheese cloth and 0.6 volumes of isopropanol was added, and stored at -20°C for 4 hours. The DNA was collected by centrifugation at 6000rpm for 10 minutes at 4°C (JA 7.5 rotor). The pellet was resuspended in 10ml TE (10mM Tris pH8.0, 10mM EDTA pH8.0) and 500µl 1M Tris pH8.0. The solution was placed in SS34 tubes for
centrifugation, along with 1.1 g of CsCl per 1 ml of solution. Solutions were then mixed with 250 μl of ethidium bromide (10 mg/ml) and incubated on ice for 30 minutes. The solution was centrifuged at 10,000 rpm for 30 minutes at 4°C (JA 20 rotor). The supernatant was transferred to a Beckman quickseal ultracentrifuge tube and centrifuged at 55,000 rpm in a Beckman 70.1ti rotor for approximately 40 hours at 17°C. After centrifugation, the DNA plasmid band was removed from the ultracentrifuge tube using a 18 gauge needle, and mixed with H₂O-saturated butanol three times to remove the ethidium bromide. The DNA was then passed over an A-50M (Bio-Rad Laboratories) agarose bead column in column buffer (25 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 300 mM NaCl) and collected in fractions of 80 drops using a fractionation column. The fractions were measured with a spectrophotometer at OD260 to identify those fractions containing the DNA. These fractions were pooled, and the DNA was precipitated with three times the volume of ethanol.
Chapter III: Results Part A

Characterization of the Daam1-GRIP1 interaction
3.1 Yeast Two-Hybrid Interaction between Daam1 and GRIP1

Through a yeast two-hybrid screen, our lab previously identified an interaction between GRIP1 and the C-terminus of Daam1. The Two-hybrid system is an in vivo yeast-based system that identifies the interaction between two proteins by reconstituting an active transcription factor. Using this yeast two-hybrid method, the last 132 amino acids of the C-terminus of hDaam1, named Daam1(c132_KLNF) (Figure 3a), was used to probe a mouse brain cDNA library. The Daam1 C-terminus of 132 amino acids included the last four amino acids KLNF, which resembles a PDZ-binding motif. The Daam1(c132_KLNF) construct was cloned into the “bait” plasmid, immediately after and in-frame with the GAL4 DNA binding domain (GAL4-DB) (Ami Tsuchida, unpublished). Ami Tsuchida conducted a screen with the Daam1(c132_KLNF) bait plasmid against pre-made cDNA library “prey” plasmids, containing the activation domain (AD). In the yeast-based system, chromosomally-integrated reporter genes are activated by a reconstituted transcription factor generated by the bait:prey (DB:AD) interaction. Positive clones were identified through induction of these various reporter genes (HIS3, URA3, and LacZ), where one of the positive clones corresponded to a portion of the protein GRIP1. This isolated clone included the PDZ domains four to seven of the seven PDZ domain-containing protein GRIP1 (Figure 3B).

The yeast two-hybrid system was further used to determine which of the PDZ domains of GRIP1 are responsible for the interaction with the C-terminus of Daam1. I tested the C-terminus of Daam1 against different combinations of PDZ domains, made from the original GRIP1 isolate (PDZ4567) (Figure 3C). Based on induction of reporter genes HIS3 (Figure 4A) and LacZ (Figure 4B), the co-expression of Daam1(c132_KLNF) with the PDZ45, PDZ4567, and PDZ456 constructs resulted in a fairly weak, intermediate and strong interaction, respectively (summarized in Figure 4C). The growth of yeast by induction of the HIS3 gene was only seen when Daam1(c132_KLNF) was co-expressed with PDZ4567, PDZ456, or PDZ45 (Figure 4A, numbers 1, 3, and 5). The co-expression of Daam1(c132_KLNF) with the constructs PDZ4, PDZ5, PDZ6, PDZ56, or PDZ67 resulted in no induction of the HIS3 reporter gene, as seen by the lack of growth in Figure 4A. Although in Figure 4A there appears to be similar growth in yeast co-transformed
with PDZ45 (Figure 4A, number 5) compared to those with PDZ4, PDZ5, PDZ6, PDZ56, or PDZ67 (Figure 4A, numbers 6-10), the lines on the plates are just empty streaks reflected by the light, with no yeast growth. The X-gal stain showed induction of the LacZ gene when colonies were co-transformed with Daam1 (C132_KLNF) and PDZ4567 (moderately blue) or PDZ456 (strongly blue). Although not dramatically apparent in the picture of the X-gal stain (Figure 4B), colonies co-expressing Daam1(c132_KLNF) with PDZ45 turned a slightly faint blue. As a control, the PDZ456 construct expressed alone (without Daam1(c132_KLNF)), did not generate any positive results from the reporter assays (data not shown). Since the co-expression of Daam1(c132_KLNF) with the constructs PDZ4, PDZ5, PDZ6, PDZ56, or PDZ67 resulted in no induction of reporter genes (Figure 4A and B), they are therefore not sufficient for mediating an interaction with Daam1. Furthermore, the PDZ7 domain is not necessary, and probably hinders the interaction, since PDZ456 produced a stronger induction of the reporter genes than PDZ4567 (Figure 4C). Therefore, it seems that PDZ456 is required for a strong interaction with the C-terminus of Daam1.

Many proteins that interact with GRIP1 occur through a PDZ-mediated interaction. PDZ domains are specialized for binding to short peptide motifs at the extreme carboxy termini of other proteins, although they can also have other modes of interaction. PDZ domains are classified into 3 types on the basis of the sequence of their preferred C-terminal ligands (Sheng and Sala, 2001). Therefore, I wanted to determine whether the Daam1-GRIP1 interaction involves a PDZ-mediated interaction. Interestingly, the C-terminus of Daam1 ends in amino acids KLNF (Figure 3A), which represents a type II PDZ binding motif (XΦXΦ-where X is any amino acid, and Φ is a hydrophobic residue). Therefore, two of the positive interactors (PDZ 4567 and PDZ 456) were re-screened in the yeast two-hybrid system against the C-terminus of hDaam1 lacking the last three amino acids (Daam1(c129_K) ) (Figure 4). Deletion of the last three amino acids now completely abolished the interaction between Daam1 and PDZ4567 or PDZ456. This result suggests that these last three amino acids of Daam1 are required for the interaction with the PDZ domains 456 of GRIP1, further supporting a type II PDZ-mediated interaction.
Figure 3: The domain structure of the formin homology protein Daam1 (A) and the glutamate receptor-interacting protein GRIP1 (B). (A) Daam1 contains an N-terminal GTPase binding domain (GBD, indicated in figure as a G), a formin homology 1, 2, and 3 domain (FH1, FH2 and FH3), and a C-terminal diaphanous auto-regulatory domain (DAD, indicated in figure as a D). The C-terminal 132 residues of Daam1 were used as “bait” (Daam1 (c132_KLNF)) in the original yeast two-hybrid screen against a rat brain cDNA library. The putative PDZ domain recognition motif is shown (KLNF). (B) Schematic representation of the seven PDZ domain-containing protein GRIP1, and the region of GRIP1 that was isolated in the yeast two-hybrid screen for an interaction with the C-terminus of Daam1 (isolated clone). (C) Additional GRIP1 constructs used in the yeast two-hybrid screen to determine which PDZ domains of GRIP1 are required for the interaction with Daam1.
Figure 3
Figure 4: The C-terminus tail of Daam1 interacts with PDZ domains 456 of GRIP1 in the yeast two-hybrid system. Each co-transformation (1-10) was tested with three separate colonies for induction of reporter genes HIS3 and LacZ, by yeast growth on leu' trp' his' 3AT plates (A) and by yeast colonies turning blue on filters supplemented with X-gal (B), respectively. The co-expression of Daam1 (c132_KLNF) with either PDZ4567 or PDZ456 resulted in strong induction of the HIS3 reporter gene (A) and the LacZ reporter gene (B). In addition, the co-expression of Daam1 (c132_KLNF) with PDZ45 resulted in a weak induction of reporter genes (5). A deletion in the putative PDZ-binding motif (KLNF to K__) completely abolished the induction of reporter genes HIS3 and LacZ (A and B) when co-expressed with PDZ4567, PDZ456, or PDZ45. This resulted in no growth on leu' trp' his' 3AT plates, and no colonies turning blue with X-gal. (C) Summary of the yeast two-hybrid interactions based on the degree of induction of reporter genes HIS3 and LacZ. The number of plus signs indicates the strength of the interaction, whereas a negative sign indicates no interaction. The PDZ45 has a positive sign in brackets, indicating the colonies turned only a slightly faint blue.
1: pDEST32-Daam1(C132_KLNF) & pPC86-GRIP1(PDZ-4567)
2: pDEST32-Daam1(C129_K) & pPC86-GRIP1(PDZ-4567)
3: pDEST32-Daam1(C132_KLNF) & pDEST22-GRIP1(PDZ-456)
4: pDEST32-Daam1(C129_K) & pDEST22-GRIP1(PDZ-456)
5: pDEST32-Daam1(C132_KLNF) & pDEST22-GRIP1(PDZ-45)
6: pDEST32-Daam1(C132_KLNF) & pDEST22-GRIP1(PDZ-56)
7: pDEST32-Daam1(C132_KLNF) & pDEST22-GRIP1(PDZ-67)
8: pDEST32-Daam1(C132_KLNF) & pDEST22-GRIP1(PDZ-4)
9: pDEST32-Daam1(C132_KLNF) & pDEST22-GRIP1(PDZ-5)
10: pDEST32-Daam1(C132_KLNF) & pDEST22-GRIP1(PDZ-6)

Figure 4
C) Bait: Daam1(C132_KLFN) Daam1(C129_K)

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Figure 4
3.2 Co-localization of Daam1 and GRIP1 Clusters in Heterologous Cells

To investigate the interaction between Daam1 and GRIP1 in mammalian cells, an N-terminal myc-tagged Daam1 was co-transfected with an N-terminal FLAG-tagged GRIP1 into heterologous cells. These tags at the N-terminus provide easy identification of these proteins by using antibodies against myc or FLAG. These constructs were tagged at the N-terminus, instead of at the C-terminus, since the GRIP1 interaction occurs at the C-terminus of Daam1 and therefore we wanted to make sure that the myc-tag would not hinder the interaction. I first wanted to determine whether co-expression of the two proteins in cells results in co-localization, since this would be consistent with two proteins interacting in vivo.

Approximately 24 hours after transfection of myc-Daam1 and/or FLAG-GRIP1, cells were stained and visualized by immunofluorescence microscopy. The transfection of FLAG-GRIP1 alone in BHK cells resulted in the localization of GRIP1 expression in clusters (Figure 5A). Other studies have shown that transfected GRIP1 localizes in the cytoplasm, and frequently forms intracellular aggregates (Charych et al., 2004). Untransfected cells showed no endogenous expression of GRIP1 (Figure 5A, arrowhead). FLAG staining on FLAG-GRIP1 transfected cells supported the clustered appearance of GRIP1 (Figure 5C). BHK cells transfected with myc-Daam1 showed a distribution of Daam1 in small puncta (Figure 5B). Untransfected cells also showed no endogenous expression of Daam1 (Figure 5B, arrowhead). Myc staining on myc-Daam1 transfected cells supported the punctate expression pattern of Daam1 (Figure 5D). These stains revealed transfected Daam1 in small puncta throughout the cytoplasm. Control secondary antibody stains revealed that the immunofluorescence pattern seen from the GRIP1 and Daam1 stains were not due to non-specific binding of the secondary antibodies (Figure 5, C & D, bottom panels). When myc-Daam1 and FLAG-GRIP1 were co-expressed in BHK cells, they continued to form clusters, with evidence of co-localization (Figure 6, arrows). Interestingly, the Daam1 puncta appeared larger when co-transfected with GRIP1 compared to when expressed alone, suggesting the interaction with GRIP1 might affect Daam1 distribution. Therefore, these results are consistent for a Daam1-GRIP1 interaction in vivo.
To further prove this interaction, a co-immunoprecipitation would demonstrate an \textit{in vivo} complex between Daam1 and GRIP1. The transfections involving BHK cells were not very efficient, and therefore HEK 293T cells were required for the co-immunoprecipitations. Prior to performing a co-immunoprecipitation, I sought to determine whether expression of both proteins in HEK cells resulted in the same outcome as in the BHK cells. First, immunofluorescence of Daam1 and GRIP1 revealed that untransfected cells likely contain no endogenous Daam1 or GRIP1 (Figure 7, asterisk). Second, HEK cells are circular in shape, with a small cytoplasmic area, and were therefore difficult to analyze the immunofluorescent co-localization of transfected Daam1 and GRIP1. However, immunofluorescence on the transfected HEK cells revealed a partial co-localization of GRIP1 and Daam1 (Figure 7, arrows). Interestingly, some of the transfected cells showed a dense aggregate of Daam1 and GRIP1 at a particular place in the cell near the surface (Figure 7, arrowheads). With the involvement of formins in actin-mediated processes such as cytokinesis and cell division, these dense aggregates of Daam1 could represent areas where these processes might occur. Although only some of the transfected cells contained these dense aggregate, GRIP1 co-localized with Daam1 in these clusters. Therefore, these cells could be in the stages of cytokinesis, preparing for cell division, and Daam1 along with GRIP1 might play a role in this process.
Figure 5: Transient expression of GRIP1 or Daam1 in BHK cells. (A) BHK cells transfected with a FLAG-GRIP1 construct. GRIP1 immunostaining revealed expression of GRIP1 localized in clusters throughout the cytoplasm. Arrowhead indicates untransfected cell with no staining of endogenous GRIP1. (B) BHK cells transfected with a myc-Daam1 construct. Daam1 immunostaining revealed expression of transfected Daam1 in small puncta throughout the cytoplasm. Arrowhead indicates untransfected cell with no staining of endogenous Daam1. (C) FLAG immunofluorescence confirms the clustered appearance of GRIP1 in transfected cells. Below is a secondary antibody control, using goat-anti-mouse-rhodamine on FLAG-GRIP1 transfected cells. (D) Myc immunofluorescence also confirmed the appearance of Daam1 in small puncta in transfected cells. Below is a secondary antibody control, using goat-anti-rabbit-FITC on myc-Daam1 transfected cells. Scale bar, 20μm.
Figure 5
Figure 6: Co-localization of GRIP1 and Daam1 in transfected BHK cells. Immunofluorescence using anti-GRIP1 and anti-Daam1 antibodies revealed a partial co-localization of GRIP1 and Daam1 clusters (arrows) in BHK cells transfected with myc-Daam1 and FLAG-GRIP1 (A, B, & C). Scale bar, 20μm.
Figure 6
Figure 7: Transient co-expression of GRIP1 and Daam1 in HEK 293T cells. Immunofluorescence revealed a partial co-localization of Daam1 and GRIP1 in cells co-transfected with myc-Daam1 and FLAG-GRIP1 (arrows). Some transfected cells appeared to have a dense aggregate of Daam1 and GRIP1 near the cell membrane (arrowheads). Immunofluorescence also revealed that untransfected cells did not appear to express any endogenous GRIP1 or Daam1 (asterisk). Scale bar, 20μm.
Figure 7
3.3 Protein Analysis of myc-Daam1 and FLAG-GRIP1 Transfected Cells

To confirm the yeast interaction data and to further investigate the interaction between GRIP1 and Daam1 in vivo, I examined whether transfected FLAG-GRIP1 and myc-Daam1 form a complex in mammalian heterologous cells. The constructs were expressed in HEK 293T cells separately and together to first examine the solubility of both proteins, since it has been shown that GRIP1 is partially insoluble in 1% TritonX-100 (Dong et al., 1999a).

From western blot analysis, immunoblotting for Daam1 revealed a band at the expected weight of 119 kDa in lysates from myc-Daam1 transfected cells. (Figure 8A, upper panel, lanes 1 & 2, arrow). First, lysates from myc-Daam1 transfected cells showed a significant proportion of insoluble Daam1 when lysed in 1% TritonX-100 (Figure 8A). From the western blot (Figure 8A), the amount of soluble Daam1 in 100ug of total protein represents approximately 2% of total cell lysate (lane 1), whereas the amount of insoluble Daam1 is approximately 4% of total cell lysate (lane 2). Therefore, there is roughly a 50:50 ratio of soluble to insoluble Daam1. Second, the Daam1 immunoblot revealed a higher molecular weight band just above 130 kDa in all the cell lysates, including from untransfected cells (Figure 8A, arrowhead). This band was originally thought to be endogenous Daam1, however it is significantly higher than the expected size of Daam1. The higher molecular weight band could be due to phosphorylation or some other post-translational modification of Daam1. It could also possibly represent endogenous expression of Daam2, since the antibodies used in these experiments are directed against the C-terminus of Daam1, which contain sequence similarities with Daam2. However, the human Daam2 protein is similar in size to human Daam1, and is likely not above 130 kDa. Overall, it appears that these HEK cells endogenously express a Daam1-like protein. Third, Daam1 also appeared highly insoluble in 1% TritonX-100, from cells co-transfected with FLAG-GRIP1 (Figure 8A, lanes 5 and 6). From the co-transfected cell lysates, both the soluble and insoluble Daam1 seen on the blot represents approximately 2% of total cell lysate. Therefore, the western blot suggests that Daam1 is more insoluble in the presence of GRIP1. In these co-transfected cells, it is possible that GRIP1 is preventing Daam1 to solubilize as much as in the Daam1-transfected cells.
However, there was also a reduction in the amount of insoluble Daam1 protein, suggesting that the overall expression levels of Daam1 were low in the co-transfected cells compared to the Daam1-transfected cells. In addition, the myc antibody further confirmed the general insolubility of Daam1 from myc-Daam1 transfected cells, and also recognized less soluble Daam1 in the co-transfected cells (Figure 8A, middle panel). I speculated that the reduced levels of soluble Daam1 in co-transfected cells might suggest that GRIP1 is preventing soluble extraction of Daam1, through their interaction. Therefore, the reduced amount of soluble Daam1 should conversely indicate an increase in the amount of insoluble Daam1 in co-transfected cells compared to Daam1-transfected cells. From the myc blot, the amount of insoluble Daam1 showed only a slight increase in the co-transfected lysates (2% of total extracts) compared to Daam1 transfected lysates (4% of total extracts).

In agreement with other articles using lysates from cells transfected with GRIP1, it has proven difficult to fully solubilize GRIP1 using 1% TritonX-100 (Dong et al., 1999a). The FLAG antibody used for immunoblotting indicated a high level of expression of the FLAG-GRIP1 protein in the insoluble fraction at the corresponding molecular weight of 135 kDa (Figure 8A, bottom panel). Of the small amount of FLAG-GRIP1 that was solubilized by 1% TritonX-100, the antibody recognized a doublet in the soluble fraction. It has been shown that GRIP1 is predominantly expressed as a 135 kDa doublet in brain lysates (Dong et al., 1999b).

Despite the difficulties in fully solubilizing Daam1 and GRIP1 from transfected cells using the non-denaturing detergent 1% TritonX-100, I wanted to test whether Daam1 binds to GRIP1 when expressed in heterologous cells by performing co-immunoprecipitations. The western blots indicated that some of Daam1 and GRIP1 are soluble in HEK cells, and so I wanted to test whether these solubilized proteins interact with one another in vivo. I first performed immunoprecipitations for the myc-Daam1 and FLAG-GRIP1 constructs from transfected cell lysates to verify the expression level of immunoprecipitated proteins (Figure 8B i,ii,iii). From the Daam1 immunoprecipitation, Daam1 antibodies stained for roughly similar amounts of Daam1 from myc-Daam1 transfected cells compared to co-transfected cells (Figure 8B i). However, from the FLAG immunoprecipitation, the FLAG antibodies indicated a reduced amount of GRIP1 protein
Figure 8: Western blot analysis (A) and co-immunoprecipitation analysis (B) on lysates from HEK 293T cells transfected with Daam1 and GRIP1. (A) HEK 293T cells were transfected with either myc-Daaml (lanes 1 and 2), FLAG-GRIP (lanes 3 and 4), or both (lanes 5 and 6). The soluble (S) and insoluble (I) lysates were analysed by immunoblotting with either anti-Daaml (upper panel), anti-myc (middle panel), or anti-FLAG antibodies (bottom panel). The immunoblots (IB) for Daam1 and myc revealed the expected band at 119 kDa (arrow), whereas the immunoblot for FLAG revealed a band, possibly a doublet, at 135 kDa (arrow). The Daam1 immunoblot also revealed the expression of a higher molecular weight protein (arrowhead), further referred to as a Daam1-like protein. (B) HEK 293T cells transfected with myc-Daaml or co-transfected with myc-Daaml and FLAG-GRIP1 were lysed and immunoprecipitated with anti-Daaml (i), anti-FLAG (ii), or anti-GRIP1 (iii) antibodies. Immunoprecipitations (IP) were analyzed by 7.5% SDS-PAGE and immunoblotted with anti-Daaml (i), anti-FLAG (ii), or anti-GRIP1 (iii) antibodies to determine efficacy of IP. The Daam1 (i) and GRIP1 (ii) antibodies were able to IP similar amounts of protein from single transfections (i-lane 4, iii-lane 4) compared to co-transfections (i-lane 3, iii-lane 3), respectively. (iv) IB from (i) was stripped and re-probed with anti-FLAG antibodies to visualize co-immunoprecipitation (lane 4). An additional IB (v) revealed that the co-immunoprecipitation of FLAG-GRIP1 by Daam1 antibodies was also seen in immunoprecipitations from only FLAG-GRIP1 transfected cell lysates. This co-immunoprecipitation is most likely due to the interaction between GRIP1 and the endogenous Daam1-like protein. Inputs represent 10% of the lysates used in the IP.
Figure 8
immunoprecipitated from lysates of co-transfected cells compared to only FLAG-GRIP1 transfected cells (Figure 8B ii). These results either suggest that co-expression of GRIP1 with Daam1 results in a reduced solubility of GRIP1, or that less FLAG-GRIP1 is immunoprecipitated in co-transfected cells because Daam1 binding to GRIP1 is blocking the FLAG epitope from being recognized by its antibodies. The latter seems more plausible since there are roughly similar expression levels of FLAG-GRIP1 between cells transfected with FLAG-GRIP1 alone or cells co-transfected (Figure 8A, bottom panel, compare lanes 3 & 4 to 5 & 6). It is possible that less FLAG is immunoprecipitated from co-transfected cell lysates because the overall expression levels of FLAG-GRIP1 in co-transfected cells are reduced compared to single transfected cells. However, immunoblotting with GRIP1 antibodies on lysates immunoprecipitated with GRIP1 indicated similar levels of GRIP1 in co-transfected cells compared to GRIP1 transfected cells (Figure 8B iii). This indicated that the expression levels are the same in the cell lysates and therefore it is possible that the Daam1 interaction with GRIP1 is blocking only the FLAG, and not the GRIP1, epitope.

In the co-immunoprecipitation (Figure 8B iv), the Daam1 antibodies were able to immunoprecipitate Daam1 along with the GRIP1 protein (shown from the FLAG stain in lane 3). A second attempt in the co-immunoprecipitation also indicated an interaction between Daam1 and GRIP1 in co-transfected cells, since the immunoblot revealed that GRIP1 was immunoprecipitated with Daam1 (Figure 8Bv, lane 3). Lysates from cells transfected with FLAG-GRIP1 served as a control for the co-immunoprecipitation. The immunoblot revealed that GRIP1 immunoprecipitated with Daam1 in lysates from cells not transfected with myc-Daam1. This first appears to suggest that the co-immunoprecipitation generated a false positive. However, it is possible that GRIP1 is interacting with the endogenous Daam1-like protein of HEK cells, and that is why GRIP1 immunoprecipitated in FLAG-GRIP1 transfected cells. One way around this problem concerning the endogenous Daam1-like protein would be to perform co-immunoprecipitations using the myc antibody, instead of Daam1. However, immunoprecipitations failed using the myc antibody on lysates from cells transfected with myc-Daam1 (data not shown). This myc antibody, like so many others, may not be well suited for immunoprecipitations.
I sought to confirm whether the Daam1 antibodies are indeed recognizing this upper molecular weight band in HEK cells. Since the Daam1 antibodies, previously purified by a Protein A method, were appearing weak on further immunoblots, I used the serum collected from the rabbit to immunoblot for Daam1 on transfected cells (Figure 9A). The Daam1 immunoblot revealed a strong stain for transfected myc-Daam1 at 119 kDa and also the higher molecular weight band, in both the soluble and insoluble fractions (Figure 9A, lanes 1 & 2). This Daam1-like protein was also seen in untransfected cells (Figure 9A, lane 4), which contained no myc-Daam1 protein.

To further confirm expression of the Daam1-like protein in HEK cells, I immunoblotted lysates from cells and brain (Figure 9B) for comparison. Two brains (A and B) were lysed in either extraction buffer containing sucrose (A), or in a modified RIPA buffer (B). Immunoblotting revealed Daam1 expression in lysates from myc-Daam1 transfected cells (Figure 9B, lane 2) in the insoluble fraction of brain A (Figure 9B, lane 3), and in the soluble and insoluble fractions of brain B (Figure 9B, lanes 4 & 5). Furthermore, the Daam1-like protein was only present in lysates from HEK cells, and not in any of the brain lysates (Figure 9B). Although there appears to be a faint band in the insoluble fraction of brain B (Figure 9B, lane 5), there was a lot of background staining in this lane, and is likely not an endogenous Daam1-like protein. These immunoblots further confirmed that a Daam1-like protein is endogenously expressed in HEK cells. Due the appearance of a single band on the immunoblot of brain lysates, I further used the antibody serum for any additional stainings of Daam1.
Figure 9: Western blots showed expression of Daam1 in mouse brain, and expression of a Daam1-like protein in HEK 293T cells. (A) Lysates from myc-Daam1 transfected cells and untransfected cells were immunoblotted (IB) with the Daam1 antibody serum. The 119 kDa protein is seen in the soluble (S) fraction (lane 1) and insoluble (I) fraction (lane 2) of lysates from myc-Daam1 transfected cells (arrow). These fractions also contained the Daam1-like protein, located above 130 kDa (arrowhead). The untransfected HEK cells also contained the Daam1-like protein, without any expression of the myc-Daam1 (lane 4). (B) Lysates from FLAG-GRIP1 or myc-Daam1 transfected cells, and from mouse brain were immunoblotted with the Daam1 antibody serum. The control FLAG-GRIP1 transfected cells showed no expression of the 119 kDa myc-Daam1 protein in the soluble lysate (lane 1), whereas myc-Daam1 is expressed (arrow) in the soluble fraction from myc-Daam1 transfected cells (lane 2). Both transfected cells (lanes 1 and 2) showed expression of a Daam1-like protein (arrowhead). Immunoblotting revealed Daam1 expression (arrow) in the insoluble fraction of brain A (lane 3), and the soluble and insoluble fractions of brain B (lanes 4 and 5). These brain lysates showed no expression of the Daam1-like protein. The soluble fraction from brain A contained very little Daam1 protein (data not shown).
Figure 9
Chapter IV: Results Part B

The effects of Daam1 on spine morphology
4.1 Immunolocalization of Daam1 in Hippocampal Dissociated Cultures

To evaluate the subcellular distribution of Daam1 in neurons, hippocampal primary cultures were immunostained (Figure 10), and subcellular fractions of mouse brain lysates were immunoblotted (Figure 11) with Daam1 antibodies. The expression of Daam1 in dissociated hippocampal neurons was seen as a punctate distribution throughout the soma and dendrites (Figure 10A). Since many PSD proteins, such as PSD-95, appear in a punctate expression pattern, this punctate pattern for Daam1 is a good indication that Daam1 could be present post-synaptically. Although many of the clusters were present in the dendritic shaft, some of the puncta showed partial co-localization with the pre-synaptic marker SV2 (Figure 10B, arrows). This partial overlap, or close apposition, between Daam1 and SV2 is consistent with the presence of Daam1 on the post-synaptic side of the synapse. While not all Daam1 puncta partially co-localized with SV2, it appeared that most of the SV2 puncta partially overlapped with the Daam1 stain (Figure 10B), suggesting that most synapses were positive for Daam1.

Subcellular fractionation of mouse brain lysates showed Daam1 enriched in the synaptic membrane and synaptic vesicle fractions (Figure 11, LP1 and LP2). Interestingly, GRIP1 showed a similar distribution to Daam1 among the subcellular fractions, with highest expressions in the LP1 and LP2 fractions (Figure 11). Daam1 also co-fractionated with the post-synaptic NMDAR subunit NR1 in the synaptic membrane fraction (LP1). Furthermore, the pre-synaptic potassium channel Kv3.1b is present in the LP1 and LP2 fractions, and its co-fractionation with Daam1 in the synaptic membrane fraction further supports a synaptic presence for Daam1. Overall, the subcellular fractionation reveals a consistent pattern for synaptic expression of Daam1. These results along with the partial overlap of Daam1 with SV2 indicate a post-synaptic expression of Daam1. Furthermore, the presence of Daam1 in the synaptic vesicle fraction (LP2) suggests that Daam1 may be present in, or associated with, synaptic vesicles.

The subcellular fractionation of Daam1 in mouse brain lysates partially resembles that of GRIP1, with a strong level in the synaptic membrane fraction (Lee et al., 2001). Therefore, I wanted to identify the co-localization of Daam1 and GRIP1 in hippocampal neurons. As shown in Figure 12A, Daam1 appears to have limited co-localization with
GRIP1. Most of the GRIP1 staining along the dendrite near the soma appeared concentrated at or near the surface of the dendrite (arrowhead), whereas Daam1 puncta were more localized in the dendritic shaft. However, there were instances where Daam1 could be seen to partially co-localize with GRIP1 (Figure 12B, arrows). Although there is evidence that GRIP1 is localized within spines and in dendritic shafts (Dong et al., 1999b), it was difficult to determine from these hippocampal stains where partial co-localization with Daam1 actually occurred.

Previous studies have shown that GRIP1 interacts with the GluR2 subunit of AMPARs. Therefore, I wanted to establish whether Daam1, possibly through its interaction with GRIP1, co-localizes with GluR2 in hippocampal cultures. Immunostainings demonstrated some co-localization (Figure 12C, arrows), but in general Daam1 and GluR2 showed very little overlap. However, there were some clusters of Daam1 that appeared next to, or apposed to, clusters of GluR2 (Figure 12C, arrowheads). This close apposition could very well be due to their interactions with GRIP1, where GRIP1 serves as a link between GluR2 and Daam1. Overall, despite the partial co-localization of Daam1 with the pre-synaptic marker SV2, there appears to be limited co-localization between Daam1 and either GRIP1 or GluR2.
Figure 10: Immunolocalization of Daam1 and SV2 in dissociated hippocampal cultures. (A) Hippocampal primary cultures (21 DIV) were fixed and stained with a polyclonal anti-Daam1 serum. The distribution of endogenous Daam1 was punctate throughout the soma and dendrites. Scale bars, upper panel 20μm, lower panel 10μm. (B) Hippocampal primary cultures (21 DIV) were fixed and co-stained with an anti-Daam1 serum and anti-SV2 antibodies. Arrows indicate partial co-localization of Daam1 and SV2. Scale bars, left panel 20μm, right panels 10μm.
Figure 10
Figure 11: Subcellular fractionation of Daam1 from mouse brain. 20μg of brain lysates (per lane) were separated by PAGE. Immunoblotting with anti-Daam1 antibodies revealed that Daam1 is present in the nuclear fraction (P1), the microsomal pellet fraction (P3), the synaptic membrane fraction (LP1) and the synaptic vesicle fraction (LP2) (upper panel). Daam1 is not present in the soluble protein fraction (S3) or the synaptosomal cytosolic fraction (LS2). Daam1 has a similar subcellular distribution compared to GRIP1. GRIP1 expression was highest in the LP1 and LP2 fractions, and almost absent in the S3 and LS2 fractions (the lane containing the LS2 fraction was only loaded with 10μg of brain lysate). Daam1 is also present in the same LP1 fraction as the NMDAR subunit NR1 and in the same LP1 and LP2 fractions as the potassium channel Kv3.1b.
Figure 11
Figure 12: Immunolocalization of Daam1, GRIP1, and GluR2 in dissociated hippocampal cultures. Hippocampal primary cultures (21 DIV) were fixed and co-stained with a polyclonal anti-Daam1 serum and anti-GRIP1 antibodies (A and B), or anti-Daam1 serum and anti-GluR2 antibodies (C). (A) Most of the GRIP1 clusters were located near the dendritic membrane (arrowhead), whereas Daam1 puncta were more localized in the dendritic shaft. (B) However, there were some examples of co-localization of Daam1 and GRIP1 (arrows). (C) Stainings revealed limited co-localization of Daam1 and GluR2 (arrows), where most of the Daam1 and GluR2 clusters were in close proximity, or apposed with one another (arrowheads). Scale bars, 20μm for first panels in A, B, and C and 10μm for enlarged images.
4.2 Over-expression of Daam1 in Hippocampal Slices

Considering the role of formins in actin remodelling, and since Daam1 appears to be located both post-synaptically and within dendrites, I hypothesized that it may have a function in regulating the morphology of actin-rich dendritic spines. To test this hypothesis and to evaluate the function of Daam1 in neurons, I set out to over-express a full-length Daam1 in CA1 organotypic hippocampal slices using the Semliki-Forest virus. In addition, I also wanted to determine whether the C-terminal PDZ binding motif of Daam1 has a role in its localization and function in dendritic spines, by expressing a Daam1 construct lacking the last three amino acids of the C-terminal PDZ-binding motif (Daam1ΔC).

The Daam1 and Daam1ΔC constructs were subcloned in-frame with an N-terminal EGFP, where each construct was then followed by a sub-genomic promoter and a farnesylated version of RFP (RFPf) (Figure 13). The farnesylated version anchors the cytosolic RFP protein to the cytosolic face of membranes, allowing for visualization of the spines under confocal microscopy. These constructs were then cloned into the Semliki Forest virus (SFV) vector, and injected in the CA1 region of hippocampal slices for efficient delivery into neurons. The SFV is an efficient method for recombinant protein expression. However, alphaviral vectors, such as the SFV, inhibit host cell protein synthesis, and eventually lead to cell death (Lundstrom et al, 2003). In light of this problem, two point mutations have been found in one of the four non-structural proteins (nsP2) of the SFV vector, which are required to form the cytoplasmic RNA replicase complex (Lundstrom et al, 2003). These two point mutations (S259P, R650D) generated a SFV(PD) virus which is less cytotoxic and leads to higher transgene expression levels. In addition, the SFV(PD) particles are replication defective, since no structural proteins for proper replication are produced. Therefore, no new particles are generated upon infection (Lundstrom et al, 2003). After cloning the EGFP-Daam1-RFPf into the SFV(PD) viral vector, the resultant PD vector contained two sub-genomic promoters, one upstream from EGFP and the other immediately upstream from RFPf (Figure 13). Both promoters allowed for co-expression of EGFP-Daam1 and RFPf, to visualize the distribution of Daam1 (EGFP) and to visualize the morphology of dendritic spines (RFPf).
4.2.1 Distribution of the Over-expressed Daam1 Constructs

Preliminary results from the over-expression of EGFP-Daam1 in CA1 pyramidal neurons resulted in distinct punctate clusters of Daam1 distributed along the dendritic shaft, but more interestingly the dendritic spine heads were enriched with these Daam1 clusters (Figure 14). However, after multiple repeated experiments and focusing on the primary dendrite (Figure 15A), the over-expression of Daam1 was mostly seen as puncta throughout the dendritic shaft with occasional clusters either in the spine head or neck (Figure 15C). Many of the punctate clusters within the shaft seemed to be localized to the base of the spine, directly underneath the spine neck (Figure 15C). Although the over-expressed Daam1 was not frequently seen in spines, it is possible that these clusters which entered the spines require an interaction with GRIP1 through the C-terminal PDZ-binding motif of Daam1.

To examine the function of the C-terminal PDZ-binding motif, I over-expressed EGFP-Daam1ΔC in CA1 pyramidal neurons. The over-expression of EGFP-Daam1ΔC also resulted in these punctate clusters distributed along the dendritic shaft, often at the base of the spine and occasionally in the spine head and neck (Figure 16). In quantifying the percentage of spines with clusters, it appeared that there was no significant difference between neurons expressing Daam1 or Daam1ΔC (15.3% ± 2.15 for Daam1-expressing neurons and 10.8% ± 1.70 for Daam1ΔC-expressing neurons). Since both the over-expression of Daam1 and Daam1ΔC resulted in the occasional appearance of puncta in spines, the interaction of Daam1 with GRIP1, through its PDZ binding motif, is most likely not required for Daam1 targeting into spines.
Figure 13: The Daam1 and Daam1ΔC SFV-PD viral constructs. Each construct contains an N-terminal EGFP followed by either the full-length Daam1 (Daam1), or Daam1 lacking the last three amino acids (LNF) of the C-terminal PDZ-binding motif (Daam1ΔC). Each viral construct contains a sub-genomic promoter (P) in front of EGFP and one in front of the farnesylated version of RFP (RFPf), to allow co-expression of both fluorescent proteins. Both constructs were subsequently used for injections into the CA1 region of hippocampal slices.
Figure 13

PD-EGFP-Daam1-RFPf

PD-EGFP-Daam1ΔC-RFPf

Figure 13
Figure 14: Over-expression of Daam1 in CA1 pyramidal neurons of hippocampal slices. The PD-EGFP-Daam1-RFPf virus was injected into the CA1 region of the hippocampal slice. The top panel is an example of a pyramidal neuron expressing EGFP-Daam1 and RFPf. Daam1 is expressed as diffusely spaced puncta throughout the dendritic shaft of the primary dendrite, and in spines along the secondary dendrites. Below is a digital zoom of the spines, indicating an enrichment of Daam1 in spine heads.
Figure 15: Over-expression of Daam1 in CA1 pyramidal neurons of hippocampal slices. (A) Schematic representation of a CA1 pyramidal neuron, where the small rectangle represents the region used for imaging and analysis. (B) The top panel is an example of a pyramidal neuron expressing RFPf. Scale bar, 10μm. Below is a digital zoom of the RFPf expressing neuron, showing typical morphology of spines used for spine quantification in figure 17. Scale bar, 5μm. (C) The top panel is a pyramidal neuron expressing EGFP-Daam1 and RFPf. Scale bar, 10μm. Below is a digital zoom indicating expression of Daam1 in puncta along the dendritic shaft, often at the base of spines (arrowheads), and occasionally in spines (arrows). Scale bar, 5μm.
Figure 15
Figure 16: Over-expression of Daam1ΔC in CA1 pyramidal neurons of hippocampal slices. The top panel is a pyramidal neuron expressing EGFP-Daam1ΔC and RFPf. Scale bar, 10μm. Below is a digital zoom of Daam1ΔC expression in puncta. Its distribution is similar to Daam1, with puncta along the dendritic shaft, often at the base of spines (arrowheads), and occasionally in spines (arrows). Scale bar, 5μm.
4.2.2 Changes in Spine Morphology from Over-expression of Daam1 Constructs

The regulatory role Daam1 has on actin polymerization and its apparent post-synaptic expression in dissociated hippocampal cultures, I assessed what effects over-expressing Daam1 and Daam1ΔC have on dendritic spine morphology of CA1 pyramidal neurons.

Upon over-expression of Daam1, I observed a subset of dendritic spines on neurons appearing longer than spines in control neurons infected with RFPf alone. From this observation, I used the expression of RFPf to manually outline the spines from each of the infected neurons, and measured spine parameters including total spine area, spine head area, spine head length and width, spine neck length and width, total spine length, and spine density using the Reconstruct program (Figure 17A, inset). After quantification of spines from neurons expressing RFPf, Daam1, and Daam1ΔC, frequency distribution plots revealed that expression of the full-length EGFP-Daam1 significantly increased total spine area by 30.6%, spine head area by 16.6%, and spine head length by 8.7%, compared to control RFPf neurons (Figure 17A). In addition, the over-expression of the Daam1 C-terminal deletion mutant (Daam1ΔC) also significantly increased total spine area by 20.4%, spine head area by 18.7%, and spine head length by 9.9%, compared to control RFPf neurons (Figure 17A). Since Daam1ΔC can also be found in spines or at the base of spines, similarly to Daam1, it was not unreasonable for the C-terminal mutant, which has the actin-regulatory domains, to alter spine morphology to the same degree as Daam1. These results indicate that both Daam1 and Daam1ΔC increase the length of the spine head, which contributes to the increases seen in spine head area and total spine area.

Frequency distribution plots for the other spine measurements revealed no significant changes (Kolmogorov-Smirnov test) between each construct and the control (Figure 17B). However, the frequency plots for spine density, total spine length and spine neck length, did reveal distributions that appeared different between Daam1 and the control (Figure 17B). Further analysis by plotting means and performing student’s t-test (Figure 17C), revealed that the mean density of spines significantly decreased in Daam1-expressing neurons (0.540 ± 0.039 spines/µm for Daam1 neurons, versus 0.726 ± 0.052 spines/µm for RFPf neurons, p<0.005) and in Daam1ΔC-expressing neurons (0.600 ±
0.046 spines/µm for Daam1ΔC neurons, versus 0.726 ± 0.052 spines/µm for RFPf neurons, p<0.05) compared to controls. In addition, the mean spine length (1.593 ± 0.095µm for Daam1 neurons, versus 1.309 ± 0.063µm for RFPf neurons, p<0.01) and mean neck length (0.791 ± 0.074µm for Daam1 neurons, versus 0.573 ± 0.038µm for RFPf neurons, p<0.01) significantly increased in Daam1-expressing neurons. These results indicate that neurons expressing Daam1 and Daam1ΔC had spines with an overall increase in length compared to control spines. In addition, neurons expressing Daam1 had fewer spines than control neurons.

These changes in length could contribute to changes in the overall shape of spines. Spine shapes are generally categorized into five types, including filopodia-like spines, mushroom spines, stubby spines, thin spines, and others (irregular shaped such as branched spines). The changes in spine size seen by the over-expression of Daam1, was also observed as changes in the spine shape. There was a significant increase in the percentage of thin spines (22.8% ± 3.4 for Daam1 neurons, versus 14.2% ± 2.4 for RFPf neurons, p<0.025) and a decrease in the percentage of stubby spines (12.0% ± 1.5 for Daam1 neurons, versus 17.7% ± 2.3 for RFPf neurons, p<0.025) in Daam1-infected neurons (Figure 18). Overall, it appeared that the over-expression of Daam1 resulted in an increase in the length of the spine head and neck, leading to the appearance of more thin spines, and a reduction in the number of stubby spines.
Figure 17: Spine morphology analysis of neurons infected with Daam1, Daam1ΔC, and control viruses. Spines were manually traced using the Reconstruct program (Inset in A). (A) Cumulative frequency distributions of total spine area, spine head area, spine head length, and spine head width in neurons infected with PD-EGFP-Daam1-RFPf, PD-EGFP-Daam1ΔC-RFPf, and control PD-RFPf. There were significant differences for both the Daam1 and Daam1ΔC distributions compared to control, for total spine area (Kolmogorov-smirnov test, p<0.025, and p<0.05, respectively), spine head area (Kolmogorov-smirnov test, p<0.025 and p<0.05, respectively), and spine head length (Kolmogorov-smirnov test, p<0.025 and p<0.0025, respectively). The distributions for spine head width were not significantly different from that of control RFPf neurons (Kolmogorov-smirnov test). (B) Cumulative frequency distributions of spine density, total spine length, spine neck length, and spine neck width in neurons infected with PD-EGFP-Daam1-RFPf, PD-EGFP-Daam1ΔC-RFPf, and control PD-RFPf. Each frequency distribution revealed no significant difference from that of control RFPf neurons (Kolmogorov-smirnov test). (C) Histograms of average spine density, average total spine length, and average spine neck length. Average spine density was significantly decreased in Daam1-expressing neurons (0.540 ± 0.039 spines/μm for Daam1 neurons, versus 0.726 ± 0.052 spines/μm for RFPf neurons, *p<0.005) and in Daam1ΔC-expressing neurons (0.600 ± 0.046 spines/μm for Daam1ΔC neurons, versus 0.726 ± 0.052 spines/μm for RFPf neurons, **p<0.05) compared to control neurons. In addition, only Daam1-expressing neurons had an increase in average total spine length (1.593 ± 0.095μm for Daam1 neurons, versus 1.309 ± 0.063μm for RFPf neurons, *p<0.01), and an increase in average spine neck length (0.791 ± 0.074μm for Daam1 neurons, versus 0.573 ± 0.038μm for RFPf neurons, *p<0.01) compared to control neurons (Student’s t test, error bars indicate s.e.m).
Figure 17
Figure 17
Figure 18: Percentage of spines in the different morphological categories from neurons infected with control PD-RFPf, PD-EGFP-Daam1-RFPf, and PD-EGFP-Daam1ΔC-RFPf. Stubby spines were significantly decreased in Daam1-expressing neurons compared to control RFPf-expressing neurons (*p<0.0025, Student’s t test, error bars indicate s.e.m). Thin spines were significantly increased in Daam1-expressing neurons compared to control RFPf-expressing neurons (**p<0.0025, Student’s t test, error bars indicate s.e.m). Images below histogram represent examples for each type of spine.
Spine Classification

![Graph showing spine classification]

- Filopodium-like Spines
- Mushroom Spines
- Stubby Spines
- Thin spines
- Other Spines

Figure 18
Chapter V:

Discussion
5.1 Formins: Function in Actin Organization

In this thesis, the main findings were that Daam1 is expressed post-synaptically, and that over-expression of Daam1 results in the elongation of spines. Dendritic spines are enriched with actin filaments throughout the spine neck and head. Therefore, the overall shapes of spines are likely mediated by mechanisms involved in the formation and regulation of the actin cytoskeleton within the spines. Many formins, other than Daam1, have been implicated in controlling cell shape through their effects on the actin cytoskeleton (Review by Wallar and Alberts, 2003). For example, the yeast formins Bni1p and cdc12p are required for cytokinesis (Chang et al., 1997), and are implicated in the formation and organization of the actin contractile cell division ring (Evangelista et al., 1997). These formins are specifically localized to the actin ring, and mutations in Bni1p fail to localize actin patches (Evangelista et al., 1997), where mutations in cdc12p fail to assemble the actin ring (Chang et al., 1997), leading to defects in cytokinesis. In addition, over-expression of Bni1p and Cdc12p in yeast increased the formation of actin cables (Sagot et al., 2002; Kovar et al., 2003b). This function of formins is also conserved in mammalian cells, as mammalian homologs of diaphanous (mDia) are involved in mammalian cytokinesis. The identification of these formins at the actin contractile ring (Tominaga et al., 2000), implies that formins are specifically localized to areas that are rich in actin, and regulate actin-mediated processes. In general, there appears to be a common theme for formins in the parallel alignment and reorganization of actin filaments. The localization of Daam1 in dendritic spines could therefore also play a role in the assembly and maintenance of actin filaments. Therefore, the overall function of Daam1 might be to organize an array of linear actin filaments in the spine, providing the template for the structure of the spine.

Daam1 has been implicated in various structural changes associated with planar cell polarity (PCP) signalling in *Xenopus* gastrulation (Habas et al., 2001) and with tracheal development in *Drosophila* (Matusek et al., 2006). During gastrulation in *Xenopus*, the function of Daam1 is implicated in cytoskeletal changes associated with the polarized orientation of cells during PCP signalling. Interfering with Daam1 in the Wnt/Fz/Dvl signalling pathway impaired elongation movements in gastrulation (Habas et
al., 2001), and Daam1 was strongly suggested to mediate these changes through the cytoskeleton. The function of Daam1 on the cytoskeleton and its effects on actin organization has also been implicated in *Drosophila* tracheal development. The tracheal cuticle pattern is organized into parallel-running actin cables or actin rings which run around the lumen of the tracheal tube (Matusek et al., 2006). This parallel alignment of actin filaments by Daam1 is comparable to the function of formins in cytokinesis and proper actin ring formation. Daam1 mutant larvae exhibited an impaired cuticle pattern leading to the collapse and flattening of tracheal tubes (Matusek et al., 2006). The disorganized parallel bundles of actin filaments in tracheal cells of Daam1 mutants indicates that Daam1 is responsible for the organization of linear bundles in an actin ring running perpendicular to the axis of the cell. Therefore, based on these previous studies, the function of Daam1 in dendrites and spines might be to maintain the structure of the spine during development, providing for normal spine functions, such as finding a pre-synaptic partner to form a synapse. In addition, since linear actin filaments are present in the spine neck and protrude from the neck into the spine head (Fifkova and Delay, 1982), Daam1 might function in the proper alignment of actin filaments in dendritic spines. The bundling of linear filaments in the neck most likely requires proteins which cross-link the actin filaments into these bundles. One of these proteins could potentially be Daam1, regulating the organization of these actin filaments into bundles.

The function of formins in actin nucleation and polymerization can explain the increase in spine length seen from the over-expression of Daam1. In contrast to the Arp2/3 complex, which nucleates on linear filaments causing them to branch (Machesky and Gould, 1999), formins nucleate and polymerize only linear actin filaments. Some Diaphanous-related formins have recently been implicated in the formation and maintenance of filopodia (Pellegrin and Mellor, 2005; Schirenbeck et al., 2005). Filopodia are precursors to spines, and therefore the localization and function of formins in filopodia provide strong indications for their potential function in post-synaptic spines. The formin mDia2 mediates the induction of filopodia by Rif (Pellegrin and Mellor, 2005). The co-expression of a full-length mDia2 with Rif in cells resulted in translocation of mDia2, induction of filopodia, and concentration of mDia2 at the filopodial tips. mDia2 clearly functions in filopodial formation, since a dominant-negative mDia2 mutant
blocked Rif-induced filopodia (Pellegrin and Mellor, 2005). Similar experiments found that the *Dictyostelium discoideum* Diaphanous-related formin dDia2 is enriched at the tips of the filopodia, along with F-actin, and is required for the formation and maintenance of filopodia (Schirenbeck et al., 2005).

These formins located in filopodia help to support the evidence for the localization of Daam1 in spines and their role in spine elongation. Although we did not evaluate the endogenous expression of Daam1 in the organotypic slices, the localization of Daam1 in the spine heads could likely be a result of its association with the growing barbed-ends of the linear actin filaments protruding from the spine neck.

Since Daam1 is involved in the organization of actin filaments, and since Daam1 is present in dendritic shafts, Daam1 could also be involved in the morphological characteristics of dendrites. Interestingly in neurons, over-expression of Dvl, one of the binding partners for Daam1, increases dendritic length and arborisation. However, a dominant-negative Daam1 determined that Dvl-induced dendritic development is not mediated by Daam1 (Rosso et al., 2005). Despite the lack of function for Daam1 in dendritic morphology, the expression of Daam1 in neurons, and the high expression of actin in spines compared to the shafts of dendrites (Fischer et al., 1998), suggests Daam1 likely functions instead in actin reorganization in spines, affecting spine morphology.

Most formins are activated by binding to Rho-GTPases through their N-terminal GBD, relieving the auto-inhibition. Although Daam1 was originally found to activate RhoA instead (Habas et al., 2001), there is recent evidence that RhoA acts upstream of Daam1, and is responsible for activating the formin Daam1 (Matusek et al., 2006). As will be discussed later, RhoA might be required for the activation, or even recruitment, of Daam1 into spines for their function in spine development and shape.

Overall, the function of formins in actin organization and actin polymerization suggest that Daam1 might be involved in the structural organization of actin in spines and might also contribute to the growth of spines. Since most actin filaments in spines are linearly oriented towards the head and positioned parallel to the length of spines (Fifkova and Delay, 1982), Daam1 could function in bundling these actin filaments together, providing the framework of a spine. Furthermore, Daam1 could enhance the polymerization of filaments by binding to the barbed-ends of the filaments, and remain
associated while continuously adding new actin monomers, resulting in growth of the spine. The elongation of spines is suggestive of polymerization of actin filaments within the spine.

5.2 Over-expression of Daam1 Results in a Punctate Distribution

The over-expression of Daam1 in organotypic hippocampal slices resulted in a punctate distribution throughout the shaft of the dendrites, with the occasional puncta in spines. The percentage of spines with Daam1 puncta was low at only 15%. The short duration in the expression of Daam1 (20 hours) is a possible explanation for these low levels in spines. Other studies involving proteins which effect spine morphology, such as Kalirin-7, SPAR, and Shank, utilize anywhere from 2 days to 1 week in culture after transfection to establish their effects. Therefore, the over-expression of Daam1 may require a longer expression time for targeting into spines.

The presence of Daam1 in spines and the identification of its interaction with GRIP1, led to the hypothesis that the C-terminus of Daam1 functions in the targeting of Daam1 into spines. However, the over-expression of a Daam1 construct lacking the C-terminal PDZ-binding motif also resulted in puncta located in the dendritic shaft, with the occasional puncta in spines. This suggested that the PDZ binding motif, and its interaction with GRIP1, is likely not involved in spine targeting. Although Daam1ΔC appeared in spines as frequent as the over-expression of Daam1, and since formins are known to dimerize, one cannot exclude the possibility that Daam1ΔC is binding with endogenous Daam1 forming a complex which is targeted to spines. Compared to the over-expression of Daam1, there were similar changes seen in spine morphology upon over-expression of Daam1ΔC. These results are not surprising since Daam1ΔC had a punctate expression pattern similar to Daam1, and that Daam1ΔC also contains the actin-regulatory domains necessary for actin polymerization.

Spine targeting and accumulation for many actin-binding proteins, such as cortactin, neurabin I, spinophilin, and drebrin, require their actin-binding domains (Hering and Sheng, 2003; Zito et al., 2004; Grossman et al., 2002; Hayashi and Shirao, 1999). The expression of most of these proteins usually has a high spine to shaft ratio,
except when the actin-binding domains are deleted. The actin-binding domains of Daam1 (FH1 and FH2) may in fact be the domains necessary for spine targeting or spine accumulation.

It was quite evident that over-expression of Daam1 resulted in a punctate distribution. It remains to be determined why Daam1 forms these punctate clusters. There is evidence that hDia2C, a novel splice variant of human Diaphanous, is recruited onto early endosomes, and regulates their motility along actin filaments (Gasman et al., 2003). We can infer from these results that the Daam1 puncta could be the result of its association with vesicles. It is also possible that Daam1 functions at endocytic hot spots (Blanpied et al., 2002) in spines to regulate clathrin-mediated endocytosis.

5.3 A PDZ Domain-containing Formin: Delphilin

Recently, a formin other than Daam1, was found to be present in dendritic spines (Matsuda and Yuzaki, 2005). This formin, Delphilin, was identified as a multiple domain protein containing an N-terminal PDZ domain along with an FH1 and FH2 domain (Miyagi et al., 2002). Delphilin binds to the C-terminal PDZ-binding motif of the GluR82 subunit and is localized in post-synaptic sites of cerebellar Purkinje cells. Therefore, Delphilin was suggested to serve as a link between GluR82 and the actin cytoskeleton (Miyagi et al., 2002). Furthermore, an alternative splice isoform of Delphilin was identified (L-Delphilin), and contains two N-terminal PDZ domains. The original Delphilin (S-Delphilin) contains only one PDZ domain, but has an N-terminal cysteine residue which can be palmitoylated. This palmitoylation resulted in a different localization pattern of S-Delphilin compared to L-Delphilin. Upon over-expression of both in hippocampal neurons, L-Delphilin was mostly seen as puncta in the dendritic shaft, whereas S-Delphilin was mostly seen in spines (Matsuda and Yuzaki, 2005). Although S-Delphilin was highly expressed in spines, its punctate expression pattern was highly similar to the over-expression of Daam1. The morphology of the spines were not analyzed, but S-Delphilin did enhance the surface clustering of GluR82, suggesting this formin could play a role in receptor localization (Matsuda and Yuzaki, 2005). Therefore
the interaction between Daam1 and GRIP1 raises the question of whether Daam1 functions in the localization or surface clustering of the GluR2 subunit of AMPARs.

5.4 Association of the Daam1 Puncta with the Spine Apparatus

The appearance of Daam1 at the base of spines and in the neck of spines suggests that these puncta could be localized at the spine apparatus. The spine apparatus was identified as a specialization of the smooth endoplasmic reticulum (SER), which extends from the dendrite into the spine compartment, and is composed of stacks of the SER, with electron dense plates (Spacek, 1985). The spine apparatus is primarily found in the spine neck with an F-actin associated protein, synaptotodin (Deller et al., 2000). Interestingly, not all spines contain a spine apparatus. In addition, only spines which are synaptotodin-positive contain a spine apparatus (Deller et al., 2000). Although the function of the spine apparatus is not completely clear, it has been suggested to function in regulating levels of Ca$^{2+}$ since the SER is an internal storage for Ca$^{2+}$ (Fifkova et al., 1983). It was further speculated that the spine apparatus, and its association with synaptotodin and actin filaments, is involved in the elongation or shortening of the spine neck (Deller et al., 2000). The spine apparatus could potentially alter the morphology of spines and play a role in synaptic plasticity. In support of this, mice deficient in synaptotodin also lack spine apparatuses, and show a reduction in hippocampal LTP (Deller et al., 2003).

Overall, the subcellular localization of synaptotodin and its association with α-actinin (Wyszynski et al., 1998), suggests a potential link between the spine apparatus and the cytoskeleton architecture of spines. The localization of Daam1 to regions of the spine known to contain a spine apparatus, indicates that Daam1 could function at the spine apparatus in regulating levels of Ca$^{2+}$ or altering the actin cytoskeleton. In addition, there have been suggestions that the spine apparatus is linked or related to stimulated-induced cluster formation of GluR1-tagged receptors (Shi et al., 1999). This relationship between the spine apparatus and GluR1 clustering could involve the actin cytoskeleton.
5.5 Function of Spines with Increased Length

Most of the morphological changes associated with LTP involve the enlargement of spine heads (Yuste and Bonhoeffer, 2001), thereby providing a larger synaptic area for synaptic transmission. However, spines with increased length due to the dynamics of actin, have a few functions as well. For example, spine motility is an actin-dependent process, and can be blocked with drugs preventing actin polymerization (Fischer et al., 1998). The length of the spines can increase the general motility of the spines, which might allow the spines to be more dynamic, and aid immature spines in increasing their ability to find synaptic targets (Dunaevsky et al., 1999).

Furthermore, the shape of the spines might serve as a compartment for Ca\(^{2+}\) and thus regulate Ca\(^{2+}\) dynamics (Majewska et al., 2000). The levels of Ca\(^{2+}\) in spines acts as a molecular switch in controlling LTP or LTD. LTP is activated by large increases in Ca\(^{2+}\), whereas LTD is generated by moderate increases in Ca\(^{2+}\) (Yang et al., 1999). The induction of LTP occurs by an increase in post-synaptic levels of Ca\(^{2+}\) by entry through the activation of NMDARs. The high levels of Ca\(^{2+}\) associated with the induction of LTP, result in the activation of CaMKII, and the recruitment and anchoring of AMPARs at the post-synaptic membrane (Lisman and Zhabotinsky, 2001). It has been suggested that the geometry of spines regulates the levels of post-synaptic Ca\(^{2+}\) in the spine head (Majewska et al., 2000). The spine neck acts as a passageway for the diffusion of Ca\(^{2+}\) between the spine head and dendritic shaft. Small thin spines have thin necks, and maintain high levels of Ca\(^{2+}\) in the spine head due to a smaller Ca\(^{2+}\) efflux through the thin neck. In contrast, large spines contain wider necks and therefore allow greater efflux of Ca\(^{2+}\) into the dendritic shaft, lowering the amount of Ca\(^{2+}\) in the spine head (Noguchi et al., 2005). Furthermore, it has been suggested that filopodia and thin spines represent post-synaptic spines with components of a silent synapse and are preferential sites for LTP induction due to the accumulation of Ca\(^{2+}\) in the spine head (Matsuzaki et al., 2004).
5.6 Activity-dependent Regulation of Spines

NMDAR activation through induction of LTP causes changes in spine morphology and a redistribution of GluR1 and GluR2 AMPARs at synapses (Lin et al., 2004). The mechanisms regulating these changes are not completely clear, but probably involve the actin cytoskeleton. Interestingly, activation of NMDARs increases the content of F-actin in spines (Fukazawa et al., 2003). Remodelling of the actin cytoskeleton could contribute to synaptic plasticity by inducing changes in spine morphology or by mediating AMPAR trafficking. The impairment of LTP by inhibiting actin assembly highlighted the importance of the actin cytoskeleton in LTP (Kim and Lisman, 1999). In addition, the shape of the spine is tightly correlated with the distribution of AMPARs within the spines. Spines with an increased head size have an increase in the number of AMPARs at the synaptic membrane (Matsuzaki et al., 2001).

Mechanisms regulating spine morphology and receptor distribution through the spine actin cytoskeleton likely contribute to the long-lasting effects of LTP. As previously mentioned, synaptic stimulation is known to alter the morphology of the spine (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999). In some cases, the activity-dependent regulation of spines is seen by the translocation of Rho-family GTPases and actin-binding proteins into spines. The translocation of Rho-GTPases Kalirin-7 (Penzes et al., 2003) and Lfc (Ryan et al., 2005) into spines in response to synaptic stimulation, results in spine morphological changes. In addition, actin-binding proteins profilin (Ackermann and Matus, 2003) and cortactin (Naisbitt et al., 1999) also translocate into spines in response to synaptic stimulation. The fact that synaptic activity changes spine morphology through dynamic remodelling of the actin cytoskeleton, and that Rho-GTPases and actin-binding proteins relocate into spines upon synaptic stimulation, suggests that these proteins provide a link between receptor activation and cytoskeletal changes associated with the shape of the spine. From these studies, we can conjecture that Daam1 might be recruited to spines by activated RhoA, which in turn could recruit profilin into spines. Although Daam1 is known to be activated by RhoA binding, any proteins interacting with the N- or C-terminal ends of Daam1, such as GRIP1, could potentially relieve auto-inhibition and
activate nucleation and polymerization. Therefore, the PDZ protein could have its own role in regulating spine morphology through the activation of Daam1.

Activation of EphRs provides a great example for the activity-dependent regulation of spines, as they are involved in cytoskeletal changes of the spine. These receptors signal through Kalirin-7 and Rae (Penzes et al., 2003) or Intersectin and Cdc42 (Irie and Yamaguchi, 2002), which both affect actin dynamics through the Arp2/3 complex. Currently there is not much known for pathways linking AMPAR activation to changes in spine shape. However, the interaction between Daam1 and GRIP1 may provide the signalling link between AMPARs and the cytoskeleton necessary for structural changes. In fact, synaptic stimulation could possibly translocate Daam1 into spines, as it does to other actin binding proteins. As most synapses appear silent in AMPAR synaptic transmission prior to induction of LTP, it is possible that NMDAR stimulation signals through RhoA and activates Daam1. In addition to Daam1 possibly enhancing actin polymerization in spines, leading to morphological changes, the activation of Daam1, and its association with GRIP1, could cause a redistribution of GluR2 AMPARs to the synapse. There is evidence that the cytoskeleton is involved in enhancing AMPAR-mediated transmission (Kim and Lisman, 1999), stabilizing AMPARs at the synapse (Allison et al., 1998), and trafficking of AMPARs (Shen et al., 2000; Allison et al., 1998). Therefore, cytoskeletal changes associated with Daam1 could regulate stabilization or trafficking of AMPARs at the surface possibly through an interaction with GRIP1.

5.7 Future Directions

To further confirm the interaction between Daam1 and GRIP1, it would be ideal to repeat the co-immunoprecipitation experiments with a cell line not expressing any endogenous Daam1, such as BHK cells. Obviously the next step would be to perform co-immunoprecipitation on brain lysates. However, both proteins would have to be solubilized more efficiently.

Despite the successful immunostaining of Daam1 in a punctate distribution in hippocampal dissociated cultures, I had difficulties in staining for endogenous Daam1 on
the organotypic slices. Over-expression of RFPf to outline the spines and staining for Daam1 would be valuable in establishing the localization of endogenous Daam1 in dendritic spines. In addition, I would like to see if endogenous Daam1 is localized at the spine apparatus by immunohistochemistry, and whether these Daam1 puncta are dynamically moving in the slices along the dendrites or into the spines by using time-lapse imaging. If Daam1 is not highly expressed in spines, it would be interesting to evaluate whether stimulation of slices by KCl or glutamate application causes a redistribution of Daam1 puncta in spines.

The increase in spine length, seen by over-expressing Daam1, suggests that Daam1 enhances the polymerization of the actin filaments. To better understand how Daam1 affects spine morphology, it would be interesting to determine whether there is an increased accumulation of F-actin in spines upon over-expression of Daam1. Clearly, expression of a dominant-negative Daam1 or the use of RNAi to knockdown endogenous Daam1 in dissociated cultures, would help in establishing the functional role of Daam1 in regulating the actin cytoskeleton and thus the morphology of spines.

The hippocampal immunostainings for Daam1 and GRIP1 revealed only a subset of clusters that co-localized. Therefore, their interaction might only occur in a subset of dendritic spines, which could be preferential sites for LTP. Daam1 likely does not directly interact with GluR2, but instead indirectly through GRIP1. This could explain the limited co-localization and apposition of Daam1 and GluR2 clusters. One question that remains is whether the Daam1-GRIP1 interaction plays a role in GluR2 distribution. A co-immunoprecipitation experiment on brain lysates would indicate a complex between Daam1, GRIP1 and GluR2. One could ask whether the GluR2 surface expression is affected by RNAi knockdown of Daam1 in dissociated cultures. In addition, recording EPSCs after infusion of a peptide against the C-terminus of Daam1 in neurons, to disrupt the interaction between GRIP1 and Daam1, would be worth evaluating for any impairment in LTP. The importance of the C-terminal PDZ-binding motif of Daam1 is currently unknown, but its association with GRIP1 suggests that this interaction might be involved in the trafficking of GluR2, or may even play a role directly at the synapse by stabilizing GluR2 receptors at the surface through an interaction between GRIP1 and the
actin cytoskeleton. The answers to these questions could help in further understanding the molecular mechanisms regulating GluR2 targeting and surface expression.
Summary

In this study, Daam1 was identified in the post-synaptic region of dendrites, and found to interact, via its C-terminus, with the PDZ domains 456 of GRIP1 in a yeast two-hybrid experiment. The over-expression of Daam1 in organotypic hippocampal slices using the Semliki-Forest virus, resulted in a punctate expression pattern throughout the dendritic shaft, and occasionally in spines. Furthermore, GRIP1 is likely not responsible for targeting Daam1 into spines, since the over-expression of Daam1ΔC resulted in a similar expression pattern as Daam1. Finally, the over-expression of Daam1 also resulted in elongation of spines. This change in the morphology of spines, and the presence of Daam1 in post-synaptic regions, suggests that the formin Daam1 functions in regulating the actin cytoskeleton in spines. Therefore, formins are likely key mediators of actin reorganization and actin polymerization in dendritic spines, and could be implicated in spine morphological changes associated with LTP.
Reference List


Piccini A, Malinow R (2002) Critical postsynaptic density 95/disc large/zonula occludens-1 interactions by glutamate receptor 1 (GluR1) and GluR2 required at different subcellular sites. J Neurosci 22: 5387-5392.


