Chapter 7

Discussion

In this thesis we investigated the role of liprin-α proteins in neuronal development and synapse function using primary hippocampal neuron cultures. By examining the expression patterns of the four liprin-α proteins in the brain using specific polyclonal antibodies, we determined that while all liprins show punctate staining throughout the brain, they each have unique distribution over the different regions of the brain. While liprin-α2 is enriched in the hippocampus, liprin-α3 is most highly expressed in the cerebral cortex and liprin-α4 in the cerebellum. Our studies focused on liprin-α1 and liprin-α2, however, further studies of the unique features of liprin-α3 and liprin-α4 will provide insight into the relevance of their different distribution patterns, while the generation of liprin knockout mice could yield interesting tools to study behaviors related to those particular regions of the brains.

7.1. Liprin-α in Axon Growth, Guidance, and Targeting

Liprin-α2 is enriched in mouse brain from late embryonic stages through maturity and expressed at similar levels throughout the development of hippocampal neurons in culture (Figure 7.1), implying that it is involved in multiple stages of neuronal development and function. The first of these to occur in cultured hippocampal neurons is the differentiation of the axon and its subsequent rapid growth and branching. In *Drosophila*, the liprin-α homolog *dliprin*-α is necessary for the proper targeting of R1-R6 photoreceptor axons in a LAR-dependent manner[91] and R7 photoreceptor axons independently of LAR[92]. In Chapter 3, we studied the importance of the LAR-liprin-α signaling pathway in axon growth and branching in mammalian neurons.

In immature hippocampal neurons, considerable amounts of endogenous liprin-α2 are found in the axonal growth cone (Figure 3.2), the structure at the tip of each axon branch that is responsible for promoting the directed growth of the axon[21]. Liprin-α2 is distributed throughout the growth cone in both the actin containing peripheral domain and the microtubule containing central domain (Figures 1.2 and 3.2). The experiments described in Chapter 3 suggest that liprin-α2 functions in the growth cone primarily as an integral member of a potential protein complex between LAR-RPTP, p140Cap, and cortactin that relays signals found in the extracellular matrix surrounding the cell to the actin and microtubule cytoskeleton within it, thereby contributing to axon outgrowth.

In this respect, we found that LAR acts as a positive regulator of axon growth

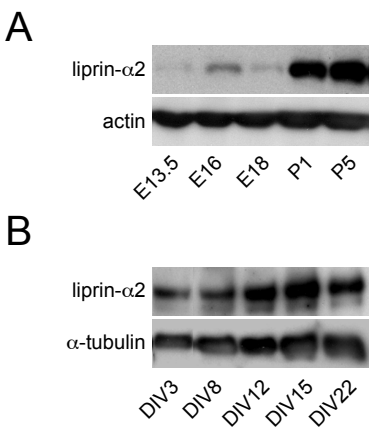

Figure 7.1. *Liprin-α2 is expressed in developing neurons* in vivo *and* in vitro.
(A) Western blots of liprin-α2 protein expression in extracts from developing mouse brains. Actin was used as a loading control.
(B) Western blots of liprin-α2 protein expression in extracts from hippocampal neuron cultures. α-tubulin was used as a loading control.

and a negative regulator of axon branching by two molecular pathways. First, LAR promotes axon growth by signaling through liprin-α2 and p140Cap, likely due to regulation of MT growth and dynamics via EB3. It was previously shown that EB3 acts through p140Cap to control dendritic spine morphology[114]. We propose that this interaction could be important for controlling microtubule dynamics in the growth cones of developing axons, but further experiments are needed to verify this hypothesis. Second, LAR inhibits axon branching by negatively regulating cortactin. Local upregulation of cortactin is known to result in actin polymerization and the subsequent formation of new axon branches[115] and knocking down LAR enhances the increased branching seen with cortactin overexpression. In contrast to the role of LAR in axon growth, its control of axon branching seems to occur independently of liprin-α2. Interestingly, p140Cap is also capable of binding to and regulating cortactin[114], indicating that there may be some cross-talk between these pathways. Future experiments involving the direct manipulation of actin and microtubule dynamics as well as the role of LAR phosphatase activity are needed to foster a more complete understanding of the intracellular signaling mechanisms mediated by LAR to control axon development in hippocampal neurons.

While we clearly show in Chapter 3 that LAR is a key molecule in controlling the intracellular mechanisms of axon growth and branching in cultured neurons, these experiments largely ignore another important function of LAR in the hippocampus *in vivo*. In addition to its intracellular phosphatase domain, LAR contains an extensive extracellular region consisting of three Ig-like domain and 4-8 FNIII domains (see Figure 2.2) that allows it to function as a cell adhesion molecule[81]. *In vivo*, this likely

means that LAR not only controls axon growth and branching in general, but plays a critical role in the targeting of axons to their proper locations. The importance of the LAR extracellular domain in LAR clustering and phosphatase activity remains largely unknown, but LAR knockout mice do show disrupted innervation of the dentate gyrus, with some axons stopping prematurely and others growing past their intended targets[112, 113]. The importance of liprin-α2 in axon growth and guidance *in vivo* is also unknown, as to date there is no liprin-α2 knockout mouse. Future analysis of a liprin-α2 knockout mouse and comparison of the liprin and LAR knockouts will lead to greater understanding of the relevance of the liprin-LAR interaction in axon growth in the hippocampus.

7.2. Liprin-α in Dendrite Development

Following a period of rapid axonal outgrowth, axon growth slows and dendrite growth and branching commences[13]. During this time, the dendrites of pyramidal neurons take on a characteristic tapered shape, with each primary dendrite branching off into numerous secondary and tertiary dendrites, though they often lack the distinct apical and basal dendrites seen *in vivo*. In the later stages, dendritic filopodia and spines are also formed and become the sites of excitatory postsynaptic receptor clustering. Importantly, liprins are expressed throughout the neuron, both in axons and dendrites[94], and interact with multiple proteins that are important for dendrite development, including GRIP1[94, 134], LAR, and CaMKII (Ch. 4).

In Chapter 4 we show that liprin-α1 functions as a downstream target of CaMKII signaling in dendrites to control dendritic growth and branching. Activation of CaMKII due to synaptic activity causes the specific degradation of liprin-α1, which is necessary for the establishment of normal dendrite morphology. We observed that CaMKII-dependent liprin-α1 degradation is necessary for the dendritic targeting of LAR, loss of which resulted in a decrease in total dendrites, dendrite length, and dendrite branches. Our data suggests that LAR is transported into dendrites in a liprin-α1-dependent fashion, and that local degradation of liprin-α1 results in the unloading of LAR at synaptically active sites within the dendrite, resulting in activity-dependent growth at that particular site. This hypothesis is supported by the finding that liprin-α1 levels are indeed regulated on a local level by synaptic activity, and that liprins

interact with a variety of kinesin proteins (Table 7.1), including KIF1A[109], KIF5, and KIF21 (Figure 7.2 and data not shown). In *Drosophila*, the liprin-KIF1A interaction is important for synaptic vesicle trafficking, but the significance of that and the other liprin-KIF interactions is unclear in mammalian cells. Future experiments that further characterize the association between liprins and KIFs could greatly improve our knowledge of how liprins and other synaptic proteins are differentially trafficked to pre- and postsynaptic sites, and how the selection of different motor proteins affects the efficiency and specificity of MT-based transport.

7.3. Liprin-α in Formation and Function of Synapses

Thus far, liprin-αs have been best studied in the context of synaptogenesis and synaptic function. Invertebrate liprin mutants display notable presynaptic defects (see Ch. 2), and liprins are frequently identified in mass spectrometry analysis of biochemical synaptic fractions[178]. Furthermore, liprins have been shown to interact with a wide variety of pre- and postsynaptic proteins in invertebrates and mammals (see Ch. 2), and manipulation of those interactions has consequences for the trafficking of numerous synaptic proteins.

Cell adhesion molecules are of key importance not just in axon pathfinding, but in the establishment and stabilization of nascent synaptic sites. They are responsible for bridging the gap between the pre- and postsynaptic membranes and inducing the clustering of other major synaptic components. Our protein binding and mass spectrometry experiments, combined with previously published data, indicated that multiple cell adhesion molecules are associated with liprins in the brain, including LAR, β-catenin and N-cadherin (through LAR[96]), and neurexin 2 and 3 (likely through CASK), suggesting that liprins are closely associated with cell adhesion molecules and likely to be important in the early assembly of synaptic specializations. This is known to be true in *C. elegans*, where the liprin-α homolog SYD-2 is one of the first proteins to arrive at a newly formed synapse and is necessary for the hierarchical assembly of other synaptic proteins[87, 88]. Our data described in Chapter 5 also supports this conclusion, as the synaptic localization of liprin-α2 is not dependent on other major synaptic scaffolding proteins.

In Chapter 5, we show that liprin-α1 and liprin-α2 are capable of differentially regulating the composition of individual presynapses, due to their different binding

affinities for other synaptic scaffolding proteins. We found that liprin-α2 interacts preferentially with CASK and is both necessary for its synaptic localization and sufficient to recruit it to presynaptic sites, while liprin-α1 binds more strongly to CAST. The upregulation of liprin-α1 at presynapses due to low activity results in the specific loss of bassoon and piccolo at those individual synapses as a result of competition for binding to CAST. These data suggest that liprin-α1 and liprin-α2 control presynaptic composition in opposite directions, with liprin-α2 supporting synapse formation and liprin-α1 facilitating synapse elimination.

Moreover, we show that depletion of liprin-α2 causes a defect in synaptic vesicle release. Additionally, overexpressing liprin-α2 is sufficient to cause increased SV release, while overexpressing liprin-α1 results in decreased SV exocytosis. Therefore, we believe that liprins play critical roles in the control of synaptic transmission on both sides of the synapse, likely due to their ability to control the protein composition of individual synaptic sites.

7.4. Liprin-α in Maintenance and Plasticity of Synapses

Even in maturity, synapses in the hippocampus remain tremendously plastic. That is, the molecular composition and the strength of synaptic transmission at an individual

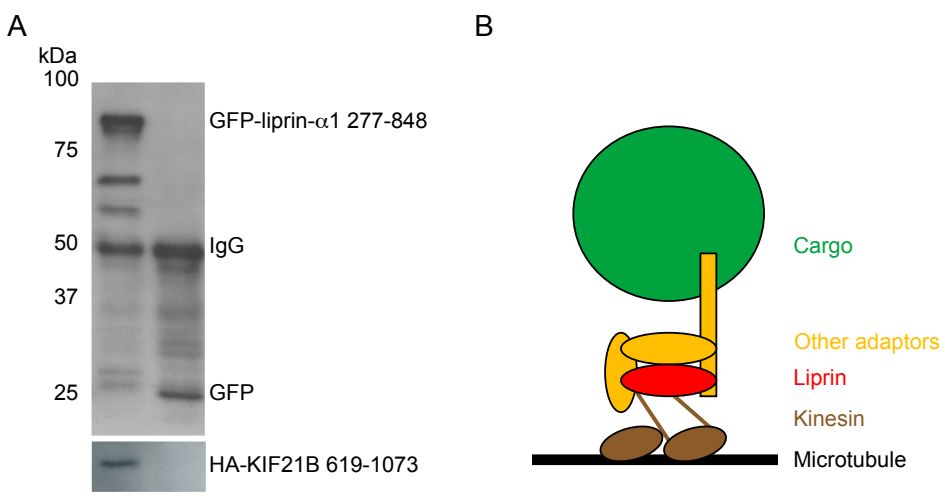

Figure 7.2. *Liprins interact with kinesins.*
(A) Western blots of immunoprecipitation of GFP-liprin-α1 aa 277-848 and coimmunoprecipitation of HA-KIF21B aa 619-1073 from HeLa cell extracts.
(B) Schematic diagram of interaction between liprins and kinesins to traffic cargo along microtubules.

Identified protein	α1		α2		α3		α4		β1		β2		
	H	B	H	B	H	B	H	B	H	B	H	B	
liprin family proteins													input
α-subfamily													
liprin-α1 (PPFIA1)	#	#	13	7	7	5	13	5	2	-	-	-	50
liprin-α2 (PPFIA2)	13	10	#	#	6	-	15	12	-	3	-	-	53
liprin-α3 (PPFIA3)	10	8	8	5	#	#	7	5	-	-	-	-	58
liprin-α4 (PPFIA4)	-	4	17	8	4	-	#	#	-	-	-	-	52
β-*subfamily*													
liprin-β1 (PPFIBP1)	-	2	11	8	6	5	7	5	#	#	5	4	79
liprin-β2 (PPFIBP2)	-	-	-	-	-	-	3	-	5	4	#	#	57
kinesin motor protein family													function
kinesin-1													
KIF5A	-	5	2	-	3	-	-	-	-	-	-	-	organelle
KIF5B	-	-	3	-	-	-	-	-	-	-	-	-	transport
KIF5C	3	-	2	-	-	-	-	-	-	-	-	-	
kinesin-2													
KIF3A	-	-	-	3	3	-	-	-	-	-	-	-	organelle
KIF3B	-	-	8	-	-	-	-	-	-	-	-	3	transport
kinesin-3													
KIF1A	-	2	-	3	3	3	-	3	-	3	-	-	
KIF13A	-	-	6	6	-	3	-	-	-	-	-	-	organelle
KIF13B	3	-	-	6	-	-	-	4	3	2	4	-	transport
KIF16B	-	-	-	-	-	3	-	-	-	-	-	2	
kinesin-4													
KIF7	12	5	12	9	2	-	5	12	-	3	-	-	
KIF21A	4	-	6	-	-	-	-	-	3	2	-	-	unknown/
KIF21B	-	-	2	2	3	-	2	-	-	-	-	-	multiple
KIF27	8	2	-	-	3	-	-	-	-	-	-	-	
kinesin-9													
KIF6	-	-	-	2	3	2	-	2	-	2	-	-	unknown
KIF9	-	-	-	-	-	-	-	3	-	4	-	-	
kinesin-11													
KIF26A	-	-	2	-	3	-	-	-	-	-	-	-	signal
KIF26B	3	-	-	-	6	-	-	-	-	-	6	-	trans.
kinesin-13													
KIF2A	3	-	-	-	-	-	-	-	-	-	-	-	MT depo-
KIF2B	-	-	3	-	-	-	-	-	-	-	2	-	lymeriza-
KIF2C (MCAK)	-	3	-	-	-	-	-	3	-	-	-	-	tion

Table 7.1. *Binding partners of bio-GFP-liprin proteins from HEK293 cells and brain P2 extracts identified by mass spectrometry.*
The table shows liprin binding proteins identified with a significant Mascot score in the pull down with streptavidin beads from extracts of HEK293 cells co-expressing each of the bio-GFP-liprin and biotin ligase BirA (column H). The number of unique peptides identified from bio-GFP-liprin pull downs using the P2 fraction from total rat brain is indicated (column B). For each identified protein, the list is filtered for duplicates and shows the number of unique peptides. The list is corrected for background proteins which were identified in a control pull-down from HEK293 cells expressing bio-GFP and BirA. Only proteins with at least 3 unique peptide hits in one of the liprin samples are shown.

synapse are constantly being modified in response to activity, even as its neighbors remain unchanged. In spite of the fact that liprins are known to be important for synaptogenesis and trafficking of synaptic proteins, their role in synaptic plasticity has not previously been explored. As in invertebrates, mammalian liprins are major components of the active zone and interact with and influence the distribution of numerous other active zone proteins. The findings in Chapters 4 and 5 represent exciting new knowledge about how liprins are regulated globally and locally, and provide a basis for the further study of liprins in synaptic plasticity.

Recently a tremendous amount of work has gone into determining the rates of protein turnover of synaptic scaffolding proteins and what role that plays in synaptic function. While some proteins, such as bassoon[69] are exchanged at relatively low frequencies (on the order of hours), others exchange almost continuously (on the order of minutes). In Chapters 4 and 5 we show that liprin-α1 and liprin-α2 are both highly dynamic and short-lived components of the synaptic machinery. Both mammalian liprin-α1 and liprin-α2 contain two APC destruction box motifs, and disrupting liprin-α degradation by the proteosome affects both total liprin levels and the stability of liprin at presynapses. Those sites are conserved from *Drosophila*, where the APC/C acts through *dliprin*-α to control synapse size[130]. Though we show that liprin-α1 APC- and CaMKII-dependent degradation is important for dendrite development in mammalian neurons, the significance of liprin-α2 degradation by APC is not yet known. The rapid turnover rates of presynaptic liprins, however, likely contribute to their ability to influence the activity levels of individual synapses through the recruitment and/or elimination of other synaptic proteins.

In addition to their general defect in synaptic vesicle release, liprin-α2 deficient hippocampal autapses also show a decrease in paired-pulse depression indicative of an impairment in short-term presynaptic plasticity. This is consistent with the idea that liprins act locally to rapidly influence synaptic composition and function and generally organize active zones to promote synaptic vesicle release. Comprehensive analysis of synaptic transmission and plasticity in the hippocampus liprin-α2 mutant mice would contribute greatly to our knowledge of the importance of liprins in these processes.

Liprin-α2 is expressed at very low levels in non-neuronal tissue, and at particularly high levels in the hippocampus (Ch. 2)[106], raising the possibility that the phenotype of a liprin-α2 knockout mouse may be specific to the hippocampus. Furthermore,

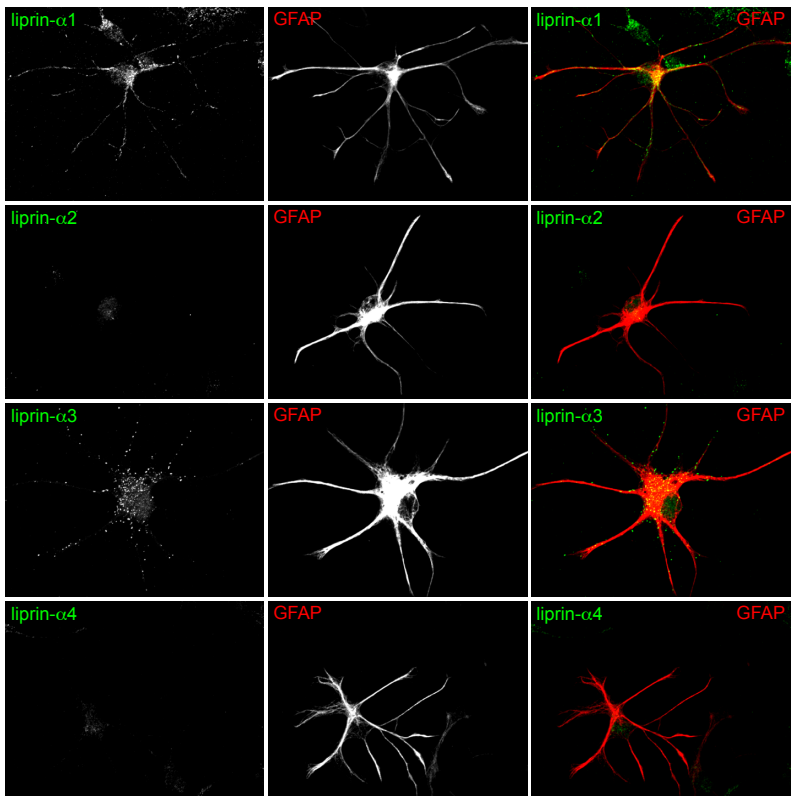

Figure 7.3. *Liprin-α1 and liprin-α3 are present in glial cells.* Representative images of endogenous liprin-α in glial cells. GFAP is used to identify astrocytes.

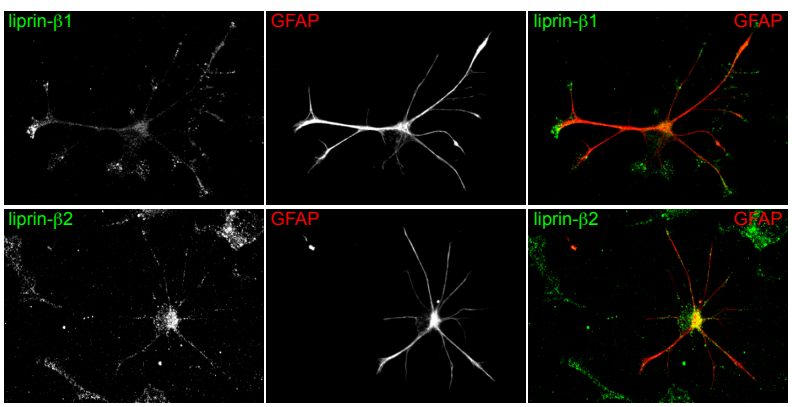

Figure 7.4. *Liprin-β1 and liprin-β2 are present in glial cells.* Representative images of endogenous liprin-β in glial cells. GFAP is used to identify astrocytes.

generation of liprin-α2 knockouts would further clarify the potential of other liprins to compensate for its loss *in vivo*, in spite of the fact that they do not seem to do so *in vitro*. However, since liprin-α2 is likely important for the expression of presynaptic and/or postsynaptic plasticity in the hippocampus, it stands to reason that liprin-α2 conditional knockout mice would also display defects in hippocampal learning and memory. Generation of these mice will be important for the understanding of the contribution of liprin-α2 to learning and memory processes in an *in vivo* setting.

7.5. Liprins in the Rest of the Brain – Future Directions

While the experiments presented in this thesis have focused on the roles of liprin-α1 and liprin-α2 in neuronal development and synaptic function in hippocampal neurons, these and other liprin family members likely function in similar processes in other regions of the brain. We believe that liprin-α1 function is probably conserved throughout the brain, as CaMKII activation is a significant hallmark of synaptic activity at a wide variety of synapses[179, 180]. However, based on general expression patterns and the amino acid sequences of liprin-α2, liprin-α3, and liprin-α4 proteins, it seems likely that they play similar roles in neurons, but in different brain regions. Additionally, the interactions between liprins and GRIP, GIT1, and LAR to control AMPAR surface expression[94, 96, 107] imply that they could play important roles in the expression of postsynaptic long-term potentiation and depression. The distribution of postsynaptic proteins in liprin-α2 knock down neurons has not yet been studied, and will be crucial to determining the complete role of liprins in postsynaptic development and plasticity.

In addition to their expression in neurons, some members of the liprin family are expressed in glial cells (Figure 7.3). Liprin-α2 and liprin-α4 seem to be neuron specific, while the others have moderate to high expression in cultured astrocytes from the hippocampus and the midbrain (Figure 7.3 and data not shown). Interestingly, the liprin-βs, which are less enriched at synapses than liprin-αs, are also found in glial cells (Figure 7.4). Of particular interest is liprin-β1, which is found in large amounts at astrocyte endfeet and can be seen colocalizing with astrocyte markers in clusters near synapses in neuron-glia co-cultures (Figure 7.4). The exploration of this and other liprin functions in different cell types and more *in vivo* model systems will lead to a more comprehensive understanding of the importance of liprin proteins in the mammalian nervous system.