CONTROL OF NEURONAL CIRCUIT ASSEMBLY BY GTPASE REGULATORS

Julia E. Sommer

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY
2011
ABSTRACT

Control of Neuronal Circuit Assembly by GTPase Regulators

Julia E. Sommer

One of the most remarkable features of the central nervous system is the exquisite specificity of its synaptic connections, which is crucial for the functioning of neuronal circuits. Thus, understanding the cellular and molecular mechanisms leading to the precise assembly of neuronal circuits is a major focus of developmental neurobiology.

The structural organization and specific connectivity of neuronal circuits arises from a series of morphological transformations: neuronal differentiation, migration, axonal guidance, axonal and dendritic arbor growth and, eventually, synapse formation. Changes in neuronal morphology are driven by cell intrinsic programs and by instructive signals from the environment, which are transduced by transmembrane receptors on the neuronal cell surface. Intracellularly, cytoskeletal rearrangements orchestrate the dynamic modification of neuronal morphology. A central question is how the activation of a neuronal cell surface receptor triggers the intracellular cytoskeletal rearrangements that mediate morphological transformations.

A group of proteins linked to the regulation of cytoskeletal dynamics are the small GTPases of the Rho family. Small RhoGTPases are regulated by GTPase exchange factors (GEF) and GTPase activating proteins (GAP), which can switch GTPases into "on or off" states, respectively. It is thought, that GEFs and GAPs function as intracellular mediators between transmembrane receptors and RhoGTPases, to regulate cytoskeletal rearrangements. During my dissertation I identified the GAP α2-chimaerin as an essential downstream effector of the axon guidance receptor EphA4, in the assembly of neuronal locomotor circuits in the mouse. Furthermore, I identified two novel neuronal GAPs, mSYD-1A and mSYD-1B, which interact with components of the presynaptic active zone and which may contribute to presynaptic assembly downstream of synaptic cell surface receptors.
# TABLE OF CONTENTS

## CHAPTER 1

INTRODUCTION: THE ASSEMBLY OF NEURONAL CIRCUITS .................................................. 1

PREFACE ................................................................................................................................. 2

AXON GUIDANCE .................................................................................................................... 3

  A Historical Perspective of Axon Guidance ................................................................. 3
  Principles of Axon Guidance ......................................................................................... 5
  The Role of Eph-receptors and Ephrin Ligands in Axon Guidance ......................... 9
  Steering the Axon Growth Cone .................................................................................. 10

SYNAPSE FORMATION ......................................................................................................... 14

  Initiation of Synapse Formation by trans-synaptic signaling ..................................... 15
  Molecular Mechanisms of Presynaptic Assembly downstream of Trans-Synaptic Adhesion Proteins ......................................................... 21
  SYD-1: A Key Regulator of Presynaptic Assembly in Invertebrates ..................... 26

GAPS: COMMON SIGNALING MODULES IN AXON GUIDANCE AND SYNAPSE FORMATION .......... 28

THE DISSERTATION PROJECT:

CONTROL OF NEURONAL CIRCUIT ASSEMBLY BY GTPASE REGULATORS ................. 29

  \( \alpha \)-2-Chimaerin ........................................................................................................... 29

  Synapse Defective-1 (mSYD-1) ..................................................................................... 30

FIGURES AND LEGENDS ...................................................................................................... 31

## CHAPTER 2

\( \alpha \)-2-CHIMAERIN IS AN ESSENTIAL EPHA4 EFFECTOR IN THE ASSEMBLY OF NEURONAL LOCOMOTOR CIRCUITS ................................................................................................................. 38

PREFACE ................................................................................................................................. 39

INTRODUCTION ..................................................................................................................... 40

  Regulation of Rho GTPase Signaling Downstream of Eph Receptors ....................... 40

  \( \alpha \)-Chimaerins are GAPs Involved in Neural Development ...................................... 41

RESULTS ................................................................................................................................ 42

  \( \alpha \)-2-Chimaerin Interacts with Activated Eph-Receptors ............................................ 42
α2-Chimaerin Mutant Mice Exhibit Locomotor Defects ................................................................. 44
α2-Chimaerin is Required for Ephrin-Induced Growth Cone Collapse ........................................ 45
α2-Chimaerin Mutant Mice Show Aberrant Midline Crossing of Spinal Interneuron Projections .......... 46
Corticospinal Defects in α2-Chimaerin Mutant Mice ..................................................................... 48
α2-Chimaerin Mutants Show Bilateral Hindlimb Movements in Response to Cortical Stimulation ...... 48
Genetic Dissection of the Functional Origin of Hindlimb Synchrony in α2-chimaerin Mutant Mice ...... 49
DISCUSSION AND FUTURE DIRECTIONS .................................................................................... 50
α2-Chimaerin is an Essential EphA4 Effector in the Assembly of Neuronal Locomotor Circuits ........ 50
Why is there no Redundancy in EphA4 Downstream Signaling in the Neurons Controlling Locomotion? ............................................................................................................................................ 51
Is the α2-chimaerin Phenotype Selective for Locomotor Circuits? .................................................. 52
The Creation of an α2-chimaerin Conditional Allele as Tool to Dissect the Function of Neuronal Locomotor Circuits ............................................................................................................... 53
Conclusion ......................................................................................................................................... 54
FIGURES AND LEGENDS .................................................................................................................. 56

CHAPTER 3
A POTENTIAL ROLE FOR MSYD-1 IN PRESYNAPTIC ASSEMBLY ........................................... 68

PREFACE ......................................................................................................................................... 69
INTRODUCTION ................................................................................................................................. 71
Invertebrate SYD-1 in AZ assembly .................................................................................................. 71
Protein Domain Organization and Function in C. elegans SYD-1 ......................................................... 72
C. elegans SYD-1: a Functional GAP? ............................................................................................... 72
SYD-1: a Presynaptic GAP that Links Trans-Synaptic Adhesion with Cytoskeletal Dynamics? ........ 73
The Actin and Microtubule Cytoskeleton in Presynaptic Assembly ................................................ 73
RESULTS ............................................................................................................................................. 75
Identification of Potential Vertebrate Orthologues of C. elegans SYD-1 ........................................... 75
Expression Analysis of mouse SYD-1 ................................................................................................ 77
Subcellular Localization of mSYD-1A ............................................................................................... 80
Functional Analysis of mSYD1A ....................................................................................................... 82
mSYD-1A Interaction partners ........................................................................................................ 88
Candidate Screening Approach for mSYD-1A Interaction Partners ........................................ 88
Yeast Two Hybrid Screen for mSYD-1A Interaction Partners ................................................. 91
A Functional Interaction between Tankyrase 1 and mSYD-1A and -B ........................................ 95
Analysis of an Essential Role for mSYD-1 in AZ Assembly .................................................. 96
DISCUSSION AND FUTURE DIRECTIONS ......................................................................... 103
Is mSYD-1A a True Orthologue of C. elegans SYD-1? ............................................................... 103
How is the GAP Activity of mSYD-1A Regulated? ................................................................. 106
mSYD-1A: GAP Signaling Downstream of Trans-Synaptic Adhesion? ...................................... 108
Does mSYD-1A have the Potential to be a Key Regulator of Presynaptic Assembly? .............. 110
New mSYD-1A Binding Partners ............................................................................................... 112
Conclusion .................................................................................................................................... 113
FIGURES AND LEGENDS ......................................................................................................... 114

CHAPTER 4

GENERAL DISCUSSION AND FUTURE DIRECTIONS ......................................................... 131

GENERAL DISCUSSION .......................................................................................................... 132
GAPs and GEFs as key regulators of neuronal signaling ........................................................... 133
“Multi-Tasking” ....................................................................................................................... 134
FUTURE DIRECTIONS ........................................................................................................... 137
Prospectus ................................................................................................................................... 140

CHAPTER 5

MATERIALS AND METHODS ............................................................................................... 141

CHAPTER 2: MATERIALS AND METHODS ......................................................................... 142
Recombinant Proteins ............................................................................................................... 142
Biochemical Assays ................................................................................................................ 142
Antibodies ................................................................................................................................... 143
Retrograde Tracing .................................................................................................................. 143
Corticospinal Tract Tracing ...................................................................................................... 144
Corticospinal Stimulation and EMG recording ................................................................. 144
Growth Cone Collapse Assay ......................................................................................... 145

CHAPTER 3 : MATERIALS AND METHODS ....................................................................... 145

Expression Constructs .................................................................................................. 145
Liprin-α Constructs for Recombinant Protein Expression .................................................. 147
Origin of other Expression Constructs ........................................................................ 147
Design and Production of mSYD-1A/B Antibodies .......................................................... 147
HEK293 Cell Subcellular Fractionation ......................................................................... 148
Brain Subcellular Fractionation .................................................................................... 148
Synatposome Prep ......................................................................................................... 149
FRET Assay .................................................................................................................. 150
Co-Immunoprecipitation ............................................................................................... 150
Pull Down ....................................................................................................................... 151
msyd-1B Conditional Knock-Out Mice ......................................................................... 153
In Utero Electroporation ............................................................................................... 153
siRNA Mediated Knock-Down in Primary Granule Neuron Cultures ............................. 155
RT-PCRs and qPCRs ..................................................................................................... 157
Cell and Tissue Lysis ..................................................................................................... 158

REFERENCES ................................................................................................................. 159
LIST OF FIGURES

CHAPTER 1

FIGURE 1.1: The Midline: a Choice Point for Axon Guidance throughout the CNS ......................... 31
FIGURE 1.2: Axon Guidance at the Vertebrate Spinal Cord Midline ........................................... 32
FIGURE 1.3: Axonal Guidance defects in EphA4 knock out mice ............................................... 33
FIGURE 1.4: The GTPase Cycle .................................................................................................. 34
FIGURE 1.5: Synaptic Ultrastructute and Molecular Composition ................................................. 35
FIGURE 1.6: Synaptic Transmembrane Receptors as Nucleation Centers of Presynaptic Assembly . 36
FIGURE 1.7: RhoGTPase Regulators: Common Signal Transduction Modules in Axon Guidance and Synapse Formation .......................................................... 37

CHAPTER 2

FIGURE 2.1: The α2-Chimaerin SH2 Domain Interacts with Eph – Tyrosine Kinase Receptors......... 56
FIGURE 2.2: Binding of the α2-Chimaerin SH2 Domain to Eph Receptors is Phosphotyrosine Dependent ............................................................................................................. 57
FIGURE 2.3: α2- and α1-Chimaerin Bind to the Intracellular Domain of EphA4 .............................. 58
FIGURE 2.4: Characterization of α2-Chimaerin Gene Trap mice ..................................................... 59
FIGURE 2.5: α2-Chimaerin Mutant Mice Show Locomotor Abnormalities .................................. 60
FIGURE 2.6: Impaired Ephrin Induced Growth Cone Collapse in α2-Chimaerin Mutant Axons. .... 61
FIGURE 2.7: Spinal Interneurons have Aberrant Projections that Cross the Midline in α2-Chimaerin Mutant Spinal Cord .................................................................................. 62
FIGURE 2.8: Projections of Parvalbumin Positive Proprioceptive Afferents are Unperturbed in α2- Chimaerin Mutant Animals ................................................................. 63
FIGURE 2.9: Aberrant Cortico Spinal Tract Projections in α2-Chimaerin Mutant Mice ................ 64
FIGURE 2.10: Cortical Stimulation Evokes Bilateral Hindlimb Movement in α2-Chimaerin Mutant Mice ................................................................................................................. 65
FIGURE 2.11: Creation of a α2-Chimaerin Conditional Knock-out Mice ........................................ 66
FIGURE 2.12: α2-Chimaerin mediates Growth Cone Repulsion Downstream of EphA4 ............... 67

CHAPTER 3

FIGURE 3.1: Predicted SYD-1 mouse orthologues: mSYD-1A and mSYD-1B ............................ 114
ACKNOWLEDGEMENTS

As a synaptic structure assembles through the convergence of many parallel forces, this dissertation likewise has been assembled through mentorship, intellectual stimulation, companionship, friendship, and family support.

Foremost, I am grateful to my advisor Dr. Peter Scheiffele, who gave me the opportunity to explore some of the most interesting fundamental questions in Neurobiology, the phenomenon of axon guidance and synapse formation. I will never forget how he purified proteins with me at a weekend, which is something not many mentors would do, or even remember how to do. Under his guidance I learned to think critically and strategically about science. His enthusiasm for science is unwavering, and has repeatedly re-motivated me during the years of my PhD. Of course the tradition of our annual lab ski trip has played a rejuvenating role as well.

I am especially thankful to Dr. Asim Beg, a former postdoctoral fellow, who was my mentor and collaborator during the studies presented in Chapter 2. Asim gave me the opportunity to join him on the α2-chimaerin project, a collaboration which was both fruitful and energizing. He taught me persistence in the face of negative results and to me embodies joy through the application of science.

I am fortunate and grateful to have found a talented congenial collaborator in Corinna Wentzel, a PhD student in our lab, who joined us after our move to the Biozentrum in Basel. She has helped to wrest out promise and excitement, from what seemed initially a daunting and untamable project. Our collaboration reminded me that a problem shared is a problem halved, and more importantly that a success shared is a success doubled.

I am also thankful to the members of my thesis/defense committee, Dr. Fiona Doetsch, Dr. Gilbert Di Paolo, Dr. Brian McCabe and my external committee member Dr. Thomas Biederer for their interest in my work, and for reading this manuscript. Their comments and guidance along the way and their flexibility in scheduling committee meetings after our lab moved to Basel was incredibly helpful.
I also want to thank Dr. Amy MacDermott, who was the Director of the Physiology and Cellular Biophysics PhD program when I joined Columbia, for her guidance and mentorship during the early phase of my PhD.

Of course this dissertation has benefited immensely from the continued support of all Scheiffele lab members past and present. Thoughtful and engaging discourse, reagents, technical assistance and many favors in both physical, as chocolate and spiritual form kept me running on a daily basis. I am very thankful for the technical assistance of Adeline Stiefvater, who especially helped with the biochemical experiments presented in Chapter 3 and saved me an enormous amount of time, through the very professional maintenance of our mouse colonies. I was fortunate that my dissertation time in our lab, overlapped with that of Dr. Elaine Budreck’s, and we could share the joy of nightly food runs and late and stimulating scientific discourse. I am also indebted to Dr. Fatiha Boukhtouche, a postdoctoral fellow in our lab, for teaching me the in utero electroporation technique, helpful comments on this manuscript and friendship.

I am also grateful to Dr. Jane Dodd, for taking me into her lab as a rotation student and mentoring me in my early days at Columbia. I was fortunate to have Dr. Jaenette Perron as a mentor during my rotation in Dr. Dodd’s lab, who I am especially thankful to for teaching me to be meticulous and neat in my experiments (despite occasional neglect of these virtues). My rotation in Dr. Dodd’s lab also introduced me to Georgie Nicholl and Beth Shafer (now Dr. Shafer) who became close friends and were a source of support and joy during my time at Columbia.

I am also fortunate that due to our lab crossing the Atlantic to move to the Biozentrum at the University of Basel I have had the chance to experience two fertile, stimulating and friendly work environments, to which our immediate neighbour labs contributed significantly: the MacDermott, Dodd, Gogos and McCabe labs on the 11th floor of the Black Building at Columbia and the Arber, Affolter and Nigg labs on the second floor of the Biozentrum. I am thankful for the companionship I experienced from the members of those labs and the ability to often borrow last minute reagents, if we had run out. Another source of help came from the transgenic mouse core facility at the Biozentrum. Daniela Klewe-Nebenius and her team always made sure that there were enough mice for our experiments, which I am very thankful for.
I am also indebted to Dr. Michael Greenberg, Dr. Tom Jessell, Dr. Kasper Hogenraad, Dr. Toshihisa Ohtsuka, Dr. Nai Wen-Chi, Dr. Olivier Pertz, Dr. Eunjoon Kim and Dr. Mingjie Zhang for providing us with valuable reagents.

My experience as student in the Neurobiology course at Woods Hole in 2007 was unforgettable. I learned incredible amounts about neuroscience and met brilliant people. I want to thank my "Woods Hole friends" Dr. Evanthia Nanou and Dr. Ruben Portugues for making this intense learning experience a fantastic time packed with intellectual stimulation and fun night time swims. At Woods Hole I also met Dr. Yishi Jin, who I am thankful to for the exchange of ideas and the collaboration on the SYD-1 project. On this I also want to thank Dr. Hidenori Taru a former postdoctoral researcher in Dr. Jin’s lab, who performed the rescue experiments in C. elegans. I am also grateful to Dr. Stefan Sigrist and his lab members for sharing data from their studies on DSYD-1 with us before they were published. Another very fruitful collaboration I am thankful for was with Jack Martin on the α2-chimaerin project.

At this point I also want to take the opportunity to thank Dr. Cary Lai and Dr. Serge Belongie for encouraging me to take the long route of an American PhD. Even though at times I cursed my decision (although I don’t know if the European way would have been much faster), I am grateful I chose this path, since in the end the time spent learning is never wasted time. The classes I took during my first years at Columbia were the best neurobiology classes I have ever attended.

I want to thank my family and friends for providing me with infinite support. My friends in New York and Basel have made my time in both cities unforgettable. Their emotional support and experienced advice, at times when things were difficult, is what kept me pursuing my goals. I especially want to thank my parents, Ellen and Peter, for their continued encouragement and care, that helped me through difficult times and motivated me to keep on pushing forward.

Last, but certainly not least, I want to thank Adam, my love, my best friend and mate through thick and thin who has had a big impact on my life, my personal development and indirectly on this work. Without his support, willingness to spend some weekends in the lab with me, endless patience and during difficult times, commiseration, I would not be writing this today.
CHAPTER 1

INTRODUCTION: THE ASSEMBLY OF NEURONAL CIRCUITS
PREFACE

A vast diaspora of behavior, from vital functions to complex movement, emotion and ultimately creativity, are actuated by the human brain. It is our advanced brain functions that uniquely differentiate us from other mammals. Therefore, it is not surprising that for centuries humans have been fascinated by the mind and have been trying to understand its workings. Already in 400 B.C. Hippocrates suggested “the brain exercises the greatest power in the man.”

Indeed the mind is complex; an estimated 100 billion neurons compose the circuitry of the human brain (Williams and Herrup, 1988). Neurons are the brains elementary computational units and are interconnected to form neural circuits, dedicated to the control of specific behaviors and functions. A wide range of neuronal morphologies exists in the brain and even a small structure such as the retina contains about 55 different types of neurons (Masland, 2001). Morphological diversity accommodates the function of single neurons and their wiring stoichiometry among each other (Masland, 2004). Neurons are “wired” via long cellular processes, the axons that carry trains of electrical pulses, or action potentials. The sites where axons contact recipient neurons are known as chemical synapses, which are the “relay stations” of neural circuits. Remarkably synapses are formed only between specific partner neurons, for example the axon of a retinal ganglion cell is in physical contact with 43 other neurons, yet forms synapses with only four of them (Hamos et al., 1987). The connection specificity in the brains is of critical importance for the functioning of neuronal circuits, and even small aberrations in neuronal connectivity can lead to variances in exhibited behavior. How such precise synaptic connectivity is achieved during neural circuit development is a central question in Neurobiology.

The structural organization and specific connectivity of neuronal circuits arises from a series of developmental steps: cell differentiation and migration, the growth of axonal and dendritic arbors, the guidance of axons towards their targets, synapse formation and activity dependent circuit refinement. All those developmental steps are based on the ability of neurons to communicate at a molecular level with their environment and target cells. Understanding the detailed molecular signals and mechanisms that guide those developmental steps therefore, grounds our understanding of how synaptic specificity is ultimately achieved. In the following I will
introduce some of the molecular mechanisms that underlie axonal guidance and synapse formation.

**AXON GUIDANCE**

With axon guidance we refer to the developmental process by which an axon grows along a prescribed trajectory to reach its target neuron. The term “guidance” refers to the fact that an axons trajectory is not alone determined by intrinsic factors but that the axon depends in its trajectory on cues from the the environment that “guide” it towards its target. Generally, such signals are provided by various cells along the axonal trajectory and are interpreted by steering the growth cone at the tip of the growing axon. The challenge of axon guidance ranges from projection neurons that wire the brain over long distances and connect brain regions that are involved in processing the same sensory – motor modality to interneurons that synapse onto cells in their vicinity.

**A Historical Perspective of Axon Guidance**

The hypothesis that all axons contain molecules to interpret molecular signals in their environment, and that this directs targeted axon growth, had been formulated by Roger Sperry as the “chemo affinity hypothesis”. Original findings that axons grow along prescribed trajectories came from eye and retina transplation experiments in newt and fish performed by Sperry and others in the second half of the last century (Sperry, 1963). Sperry observed that rotation of the eye resulted in inappropriate irreversible motor behaviors upon axonal regeneration. From this he postulated that the axons of retinal ganglion cells (RGC) grow back to innervate the same region of the optic tectum as they did before the rotation, giving rise to an inverted visual map. Another of his findings was that if half of the retina was removed, the axons of the remaining RGC did reinnervate the same portion of the tectum as before, rather than expand into the denervated region. From this he concluded that each axon only links to a specific neuron (Sperry, 1963). Axonal tracing studies demonstrated that the retinotectal map forms in an orderly fashion along the anteroposterior and mediolateral axes, which led Sperry to propose that their must exist
gradients of molecules that are “superimposed on the retinal and tectal fields” and thereby “stamp each cell with a latitude and longitude” giving rise to a “chemical code” with “matching values” for neurons that connect to each other (Sperry, 1963). Later it was also shown for other systems that axons follow prescribed pathways en route to their targets. Pioneering work on motorneuron pathway selection in the chick spinal cord, provided some of the earliest evidence of directed guidance behavior in vertebrates (Lance-Jones and Landmesser, 1980). In the spinal cord the axons of individual motor pools present at different segmental levels initially exit the spinal cord mixed in one nerve. In the plexus they reorganize so that axons destined to innervate the same target muscle emerge together. Remarkably motorneuron axons maintain the correct innervation pattern even if a spinal segment is inverted (Lance-Jones and Landmesser, 1980).

Already in the early 20th century it was shown that the growth cones of axons emerging form neuronal explants in culture preferentially grow along surfaces and not in liquids (Harrison, 1910) (Harrison, 1914). Later it was found that axons in culture grow differentially on various substrates and if given the choice they grow along the surface that they can adhere to the best (Letourneau, 1975a, b). These observations fueled an interest in identifying neural adhesion molecules because it was hoped that they could help to explain the specificity in axonal trajectory. The first adhesion molecule to be biochemically purified was N-CAM (neuronal cell adhesion molecule) (Dubois et al., 2005), which belongs to a large family of adhesion molecules the IgCAMs (named after their extracellular immunoglobulin domains – Ig domains). Before adhesion molecules were found to be important for axonal growth, the pioneering work of Hamburger and Levi Montalcini showed that there must be factors that can influence axon growth behavior from a distance. When they placed tissue from a particular mouse sarcoma adjacent to explants from chick sensory ganglia in a culture dish it induced the growth of neurites from the sensory ganglia within hours (Levi-Montalcini et al., 1954). The specific factor acting in this system was later identified as “nerve growth factor” NGF (Bocchini and Angeletti, 1969 1971), which is mainly known for its trophic and growth promoting effects but seems to play a less important role in axon guidance (Davies et al., 1987). Nevertheless, these experiments suggested that there might be factors guiding axons from a distance. Two key studies, one performed in C. elegans and one
using biochemical purification from chick brain, provided the first discoveries of bonifide guidance molecules. Those were UNC-6, a netrin like molecule in *C. elegans* (Ishii et al., 1992) and Collapsin in chick (Luo et al., 1993). The discovery of many diffusible as well as membrane bound axonal guidance cues and their receptors followed. These included the Netrins (Kennedy et al., 1994; Serafini et al., 1994) and their DCC receptors (Keino-Masu et al., 1996), the Semaphorins, with Sema1 (or Fascilin IV) and Sema3A (Kolodkin et al., 1992; Yu et al., 1998) and their neuropilin (Npn) (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997) and plexin (Ohta et al., 1992; Takahashi et al., 1999) receptors as well as the membrane bound ephrins and their Eph receptors (Cheng and Flanagan, 1994; Drescher et al., 1995).

The picture arising from these studies is that axons are guided towards the correct target area by attractive and repulsive cues in their environment and that specific receptors for the detection of such cues are located on the axonal growth cone. Activation of the receptors by their ligands triggers intracellular cytoskeletal rearrangements (Fan et al., 1993), which give rise to changes in growth direction. In the following, I will review examples of ligand/receptor pairs involved in axon guidance in vertebrates. Further, I will introduce the role of RhoGTPases and their regulators as adaptor proteins that are crucial for the downstream signaling of axon guidance receptors.

**Principles of Axon Guidance**

To accomplish the targeting and growth of axons over long distances the axonal trajectory is broken down into smaller segments by the introduction of intermediate targets or choice points. The cells at such choice points secret axon guidance cues and, thereby, steer approaching growth cones along their proper path. In insects such as the grasshopper or *Drosophila* intermediate targets are often single cells, so called “guidepost cells”, and their ablation can cause axons to misproject (Bentley and Caudy, 1983; Goodman and Spitzer, 1979). In vertebrates, specialized cell populations mediate axon guidance at intermediate targets. The best example of such an intermediate target in vertebrates is the midline of the central nervous system, since the axons of many projection neurons in the brain and spinal cord decussate. In the
hindbrain, midbrain and spinal cord the midline is formed by the floor plate, a transient glial-like structure at the ventral midline (Placzek et al., 1990). In the forebrain there is no floor plate but several other transient glial structures that direct axons, as e.g. the “palisade” at the optic chiasm, the “tunnel” at the anterior commissure and the “wedge” at the corpus callosum (Chédotal and Richards, 2010) (Figure 1.1). Over the years the guidance molecules secreted by those glial populations have been identified and their actions on axon steering has been analyzed. The first guidance molecule that was identified to be secreted by guidepost cells or intermediate targets was UNC-6 in C. elegans, which is expressed by epidermal cells and necessary for the appropriate guidance of touch receptor neurons (Ishii et al., 1992). An area where substantial progress has been made in understanding midline guidance is the spinal cord. Therefore, I will use the vertebrate spinal cord as a model system to illustrate some of the cellular and molecular principles governing axon guidance.

**Axon Guidance at the Spinal Cord Midline in Vertebrates**

The interneurons of the rodent spinal cord are especially well suited for the study of axon guidance, since their cell bodies sit within the spinal cord and their axons undergo several guidance decisions within an anatomically restricted and experimentally accessible area. The axon trajectory with regard to the midline, is one basic anatomical criterion for the classification of spinal interneurons: the axons of so called ipsilateral projecting interneurons do not cross the midline and innervate target neurons on the same side of the body, whereas the axons of commissural interneurons extend across the midline to innervate target neurons on the opposite side of the body. Longitudinal axon tracts run parallel to the midline and include axons from ipsilateral projecting neurons, ascending and decending axons from the brain and axons of commissural neurons after they have crossed the midline. At the ventral and dorsal midline of the developing spinal cord sits the floor plate and roof plate, respectively. Those are transient structures composed of ependymal cells that express and secrete morphogens as well as axon guidance cues. The morphogens, Bone Morphogenic Protein (BMP) and Sonic Hedgehog (Shh) are important signaling factors during early embryonic patterning and the specification of dorsal
and ventral neuron types in the spinal cord (Chiang et al., 1996; Godsaver and Slack, 1989; Hemmati-Brivanlou and Melton, 1994; Martí et al., 1995). Interestingly, the same molecules later in development function as axonal guidance cues (Butler and Dodd, 2003; Charron et al., 2003). Within the short axonal trajectory of ipsilateral and commissural interneurons several principles of axon guidance have been discovered: initial repulsion (from the roof plate), attraction towards an intermediate target (the floor plate), repulsion from an intermediate target, permissive crossing of an intermediate target and the loss of attraction after interaction with the intermediate target.

Ipsilateral projecting axons are prevented from crossing the midline by Slits that are expressed by cells of the floor plate. The approaching axon growth cones express three variants of the Slit receptor Robo. Binding of Slit to Robo results in repulsion from the midline (Long et al., 2004; Zou et al., 2000) (Figure 1.2B).

Commissural neuron cell bodies are located in the dorsal half of the spinal cord and their axons take a circumferential route, until they turn medial towards the floor plate (Figure 1.2A). The initial ventral trajectory of commissural axons is induced by repulsive factors secreted by the roof plate (Figure 1.2A). Among those are roof plate secreted BMPs, which in addition to their role in neural differentiation, have been found to be potent repellents necessary to orient axons to grow ventrally (Butler and Dodd, 2003; Dickson and Zou, 2010). The floor plate secreted attractant Netrin-1, forms a ventral - dorsal concentration gradient, which promotes the turning of axons towards the midline (Tessier-Lavigne et al., 1988); (Serafini et al., 1994); (Kennedy et al., 1994). The role of netrins in midline attraction is evolutionarily conserved in C. elegans as well as Drosophila (Harris et al., 1996; Ishii et al., 1992). Another cue, attracting commissural axons towards the midline is Shh, which during earlier development functions as morphogen. Shh, similar to netrin-1, is secreted by the floor plate and forms a ventral – dorsal gradient (Charron et al., 2003). Surprisingly, commissural axons also express the Slit receptor Robo, which in ipsilateral projecting axons mediates their repulsion from the midline. Therefore a mechanism to suppress Slit/Robo signaling exists in commissural neurons. Rig-1 or Robo-3 present in commissural axons interferes with Slit/Robo downstream singalling and thereby inhibits repulsion (Sabatier et al., 2004).
Eventually, commissural axons grow underneath the floor plate and emerge on the contralateral side. After commissural axons cross the midline the former attraction to the midline is turned off and the sensitivity to Slit/Robo repulsion is turned on (Figure 1.2). Experiments in *Xenopus* spinal neurons suggest a competition among attractive and repulsive guidance signals. Slit bound Robo receptor can interact with the Netrin-1 receptor DCC in trans and thereby inactivate it. Thus, the responsiveness of the axon growth cone to the attractant is progressively downregulated while crossing the midline, and by the time it reaches the other side repulsion dominates (Stein, 2001). Two additional repellents that pre-crossing growth cones are unresponsive to, are Semaphorin 3B (Sema3B) and Sema3F (Figure 1.2). Here, a hierarchical interaction regulates the balance between attraction and repulsion and it seems that interaction with the attractant Shh can confer the sensitivity to Sema3B and 3F (Parra and Zou, 2010). After crossing the midline axons have to turn anterior or posterior. Another set of attractive guidance cues influences this choice. Several Wnt proteins are expressed in an anterior–posterior decreasing gradient along the ventral spinal cord midline. Wnts function as attractants and cause post-crossing commissural axons to turn anterior (Lyuksyutova, 2003).

The principles of repulsion, attraction and modification of attraction at intermediate targets are central principles to the guidance of all axons in the central as well as peripheral nervous system (CNS / PNS). The principles and guidance cues described here are instructive to the guidance of many other CNS axons. For example Netrin-1 also acts as an attractant in the corticofugal (Métil et al., 1997; Richards et al., 1997) and thalamocortical pathways (Braisted et al., 2000). In mice mutant for Netrin-1 or its receptor DCC corticofugal, thalamocortical and the commissural pathways of the spinal cord are disrupted (Fazeli et al., 1997; Serafini et al., 1996).

Many additional signals have been identified to play instructive roles in axon guidance. One especially well studied family of axon guidance ligands and receptors are the ephrins and their Eph receptors. Since one part of my thesis was concerned with understanding the role of a specific downstream effector of EphA4 receptor signaling during axon guidance, I will introduce the role of Eph and ephrins in axon guidance in the following in more detail.
The Role of Eph-receptors and Ephrin Ligands in Axon Guidance

The Eph-receptors (originally isolated from the Erythropoietin – producing hepatocellular carcinoma cell line) (Hirai et al., 1987) are a family of receptor tyrosine kinases (RTK) with 16 members, complemented by 9 ligands the ephrins (Eph family receptor interacting proteins). Receptors and ligands are membrane bound and thus require cell-cell contact in order to be activated. Ephrins fall into two groups, the glycosylphosphatidylinositol (GPI) – anchored ephrin As and the transmembrane domain containing ephrin Bs. The receptors are grouped according to their binding affinity for the ligands, EphA receptors bind preferentially to ephrin As and EphB receptors have highest affinity for ephrin Bs. There are some exceptions to this rule and one to be mentioned here is the EphA4 receptor that can bind both ephrin As and Bs (Gale et al., 1996).

The first role that was discovered for Eph receptors and their ephrin ligands in axon guidance was in the topographic mapping of retinal axons to specific locations within the optic tectum/colliculum in the visual system (Cheng and Flanagan, 1994; Drescher et al., 1995). The establishment of this topographic map was shown to depend on a gradient of Eph receptor expression on RGCs and ephrin expression by cells in the environment (Birgbauer et al., 2000; Braisted et al., 1997; Cheng and Flanagan, 1994; Hindges et al., 2002; Mann et al., 2002).

Mapping of RGC axons along the anterior – posterior axis in the tectum depends on the repulsion mediated by EphA receptors and their ephrin A ligands (Grunwald et al., 2001). Later a second role for ephrins, as axon guidance cues expressed by the midline cells of the visual system, the optic chiasm, was found. In animals with binocular vision axons of the nasal hemiretinas cross the midline at the optic chiasm and project to the contralateral tectum, whereas axons of the temporal hemiretinas avoid the chiasm midline and project to the ipsilateral tectum. Glia cells at the optic chiasm in the mouse express ephrin B2 which repells EphB1 expressing ipsilateral axons (Williams et al., 2003).

Ephrins and their Eph receptors have been found to play additional roles in axon guidance outside of the visual system as well. Since part of my thesis was concerned with EphA4 downstream signaling I will briefly review what is known about EphA4’s role in axon guidance.
The EphA4 Receptor in Axon Guidance at the Spinal Cord Midline

Genetic studies in mice have implicated EphA4 in controlling multiple axon guidance steps, most prominently in the locomotor circuits (Kiehn, 2006). The EphA4 ligand ephrinB3 is expressed by cells of the spinal cord midline and prevents EphA4 expressing growth cones from crossing (Kullander et al., 2001a). Mice lacking EphA4 exhibit defects in hindlimb posture, as well as a pronounced locomotor defect characterized by synchronous movement of their hindlimbs, which results in a rabbit-like hopping gait (Helmbacher et al., 2000) (Dottori et al., 1998; Kullander et al., 2003). The behavioral phenotype is thought to result from aberrant midline crossing of corticospinal tract (CST) and spinal interneuron axons due to the inability to respond to the repulsive ephrin B3 signal at the midline (Figure 1.3). The aberrant midline crossing results in the inappropriate innervation of contralateral spinal cord hemi-segments and thereby gives rise to the hopping gait (Dottori et al., 1998; Kullander et al., 2001a; Yokoyama et al., 2001). The aberrant hindlimb posture has its origin in a defect in hindlimb innervation stemming from a misrouting of motoraxons (Helmbacher et al., 2000). It has been well described that the expression of EphA4 by motorneurons is necessary for dorsoventral axon pathway selection in the hindlimb. Motoraxons innervating the dorsal muscle, are prevented from innervating ventral muscles by a repulsive ephrin A signal (Eberhart et al., 2002); (Kania and Jessell, 2003). In EphA4 knock-out mice, dorsal motor axons fail to enter the dorsal muscle and join the ventral nerve (Helmbacher et al., 2000).

In chapter 2 of this thesis I describe the identification of α2-chimaerin as an essential downstream effector of EphA4 signaling in neuronal locomotor circuits. An important finding from our study is that mice lacking α2-chimaerin show the same behavioral and anatomical phenotypes, concerning the CST and spinal interneuron projections, as mice lacking EphA4. This suggests that specific signal transduction molecules downstream of axon guidance receptors are essential for the correct wiring of neuronal circuits.

Steering the Axon Growth Cone

Growth cone turning in response to repulsive or attractive cues respectively is based on complex
rearrangements of the actin/microtubule cytoskeleton and local membrane addition (Fan et al., 1993; Igarashi et al., 1996; Tojima et al., 2007). Therefore, most of the signaling induced by axon guidance receptors converges on the cytoskeleton (Bashaw and Klein, 2010). Growth cone motility is mainly characterized by the extension and retraction of filopodia, the long finger-like protrusions at the very tip of the axon (Goldberg and Burmeister, 1986). The growth cone filopodia are the first part of the axon to be in contact with guidance signals from the environment. Upon encountering an attractive cue filopodia detecting the cue are stabilized and extended, whereas filopodia in contact with a repulsive cue are retracted (destabilized) (Fan et al., 1993). Thus, through the dynamic and local regulation of the growth cone cytoskeleton extracellular guidance cues are translated into motile behavior. Filopodia have been suggested to steer the growth cone by differential adhesion, which generates mechanical forces (Letourneau, 1975a).

Actin filaments are found in two different forms within the growth cone, as a branched network of filaments in lamelipodia and as parallel bundles in filopodia (Lewis and Bridgman, 1992). The fast growing barbed end of the parallel bundles points toward the tip of the filopodia (Lewis and Bridgman, 1992). Extension and retraction of filopodia reflects a balance between polymerization at the barbed end and the retrograde flow of the entire filament (Lin et al., 1996; Mallavarapu and Mitchison, 1999; Okabe and Hirokawa, 1991). Microtubules are mainly found in the axon shaft, where they form cross-linked bundles, single microtubules can extend into filopodia (Schnapp and Reese, 1982). Microtubules preferentially grow along the parallel actin bundles in filopodia, and extend and retract as they explore the periphery of the growth cone (Tanaka et al., 1995). Stabilization of a microtubule in a filopodium is thought to be a critical event for growth cone turning (Schaefer et al., 2002; Tanaka et al., 1995). But how is the signal of an extracellular guidance cue translated into intracellular changes in actin and microtubule dynamics?

**Small RhoGTPases: Translation of Extracellular Signals into Intracellular Cytoskeletal Dynamics**

Although there are numerous signal transduction molecules that convey guidance information received by guidance receptors, the most comprehensive picture has emerged for the Rho family of small GTPases (RhoGTPases), a class of molecules that control cytoskeletal dynamics.
downstream of almost all axon guidance receptors. Through the interaction with specific downstream effectors small RhoGTPases can regulate cytoskeletal dynamics (Hall and Lalli, 2010). Their activity is regulated in response to the activation of several axon guidance receptors and functions as integration platform of different axon guidance pathways (Govek et al., 2005). Small RhoGTPases are found in an inactive GDP bound or an active GTP bound state (Figure 1.4). In order to cycle between both states they require accessory proteins, GTP exchange factors (GEFs) – to exchange GDP for GTP and GTPase activating proteins (GAPs) to catalyze the hydrolysis of GTP to GDP (Figure 1.4). In the GTP bound state small RhoGTPases interact with downstream effectors to induce actin nucleation, actin and tubulin polymerization as well as actin contractility (Amano et al., 1996; Kimura et al., 1996; Miki et al., 1998; Rohatgi et al., 1999) (Figure 1.4). Small GTPases thus can be considered switches that are being turned on or off in response to a ligand binding to a receptor (Hall et al., 1993). Most studies of Rho GTPases in axon guidance have focused on the three RhoGTPase family members, Rac1, Cdc42 and RhoA. Several lines of experiments strongly support key functions of RhoGTPases in axon guidance. In *Drosophila* the expression of constitutively active and dominant negative Rac1 or Cdc42 in the nervous system, has distinct effects on motor axon guidance, hinting at a role for Rac1 and Cdc42 in executing specific guidance decisions (Luo et al., 1994). A targeted knock-out approach for Rac1 in mouse ventral telencephalic neurons, prevented corpus callosal as well as hippocampal commissural axons from crossing the midline, in addition, thalamocortical as well as corticothalamic axons showed defasciculation and projection defects (Chen et al., 2007). Also the manipulation of RhoA activity by knocking-out one if its regulators, p190RhoGAP, has a strong impact on axonal guidance. In p190RhoGAP knock-out mice specific axons of the anterior commissure display guidance defects (Brouns et al., 2001).

How RhoGTPases are linked to axon guidance receptors is subject of ongoing studies. However, GEFs and GAPs often interact directly with the receptor and the GTPase and can thereby function as translators of the extracellular signal into intracellular RhoGTPase activation or inactivation, respectively. In the following several examples of such interactions are discussed.
GEFs and GAPs: Linking small RhoGTPases to Axon Guidance Receptors

The GEF Trio interacts with DCC (Briançon-Marjollet et al., 2008), and stimulation of cortical neurons from Trio knock-out mice with Netrin fails to activate Rac as it usually does. Furthermore, Trio knock-out mice show commissural axon guidance defects (Briançon-Marjollet et al., 2008), similar to but not as pronounced as the defects observed in DCC or Netrin knock-out mice. Trio seems to be necessary for DCC mediated attraction but not essential. Another GEF implicated in Netrin/DCC downstream signaling is DOCK180. DOCK180 is required for commissural axon turning in response to Netrin in explant assays (Li et al., 2008). Both Trio and DOCK180 can interact with DCC and it is unclear whether they act together or in parallel.

The repulsive response to Sema3A binding to its receptor Plexin-A/Npn-1, is mediated by Rac1 activation (Jin and Strittmatter, 1997). In hippocampal culture it has been shown that the GEF, FARP2, is associated with the Plexin-A receptor complex; upon binding of Sema3A to the receptor it dissociates and activates Rac1 (Toyofuku et al., 2005). The role of FARP2 in Sema3A induced axon turning in vivo remains to be investigated.

The GEF ephexin1 is bound to the EphA4 receptor in the absence of ephrin stimulation. In this state ephexin activates RhoA, Rac1 and Cdc42 to equal levels. Upon binding of ephrin to EphA4, ephexin1 becomes phosphorylated and switches its GEF activity towards RhoA, which in turn induces actin depolymerization causing a repulsive growth cone response (Egea et al., 2005). Downstream of EphA4 another GEF, Vav2, has been implicated in repulsive growth cone behavior (Cowan et al., 2005). Vav2 is necessary for Rac dependent endocytosis of the ephrin/EphA4 complex, which converts the initially adhesive interaction into a repulsive event (Cowan et al., 2005).

Overall it still remains to be clarified what is the relative contribution of RhoGTPases to axon guidance downstream of specific guidance receptors. It is possible that different GAPs and GEFs are dedicated to specific guidance receptors. This could determine a specific RhoGTPase activity pattern downstream of different receptors, thereby giving rise to differential growth cone behaviors in response to different guidance cues. In chapter 2 of this thesis I will describe our findings that support that the GAP α2-chimaerin is dedicated to translating axon guidance cues
received by the EphA4 receptor into Rac inactivation thereby eliciting a repulsive growth cone response.

SYNAPSE FORMATION

Once axons have reached their target area they form synapses with a view specific neurons in their target field. Structurally, synapses are asymmetric cell–cell junctions consisting of the presynaptic bouton filled with synaptic vesicles, a synaptic cleft and the postsynaptic membrane with densely clustered neurotransmitter receptors (Figure 1.5). Upon stimulation synaptic vesicles fuse with the presynaptic plasma membrane in a highly specialized area referred to as the active zone (AZ). In electron micrographs the AZ is tightly associated with an electron dense protein rich matrix also known as the cytomatrix at the active zone (CAZ) (Figure 1.5). The AZ is precisely aligned with an electron density on the postsynaptic membrane, which is referred to as the postsynaptic density (PSD). The PSD is composed of neurotransmitter receptors and proteins involved in their clustering and regulation (Figure 1.5).

Synapse formation is the process during which the CAZ and synaptic vesicles cluster in the presynaptic bouton, and the protein components of the PSD localize to a stretch of the postsynaptic membrane in direct apposition. The precise alignment of the AZ and the PSD is crucial for efficient synaptic transmission. Therefore the differentiation of the presynaptic bouton and the PSD need to be temporally and spatially coordinated, which is achieved by molecular cross talk between the presynaptic and the postsynaptic compartments.

Positive selection cues present at the membrane of the pre- and postsynaptic neuron, can locally induce the assembly of pre- and postsynaptic specializations. Such cues can be adhesion molecules or secreted anterograde or retrograde signals. Indeed, some synaptic adhesion molecules have been shown to be sufficient when expressed in heterologous cells to induce pre- or postsynaptic assembly in contacting neurons (Biederer et al., 2002; De Wit et al., 2009; Linhoff et al., 2009; Scheiffele et al., 2000). In general signals inducing synapse formation are referred to as “synaptogenic”. A less established concept are negative selection cues, that
can be present on neurites or released from cells in the local environment, and are thought to act similar to repellents in axon guidance, thereby preventing synapses from forming. Indeed some axon guidance cues and their receptors can act locally during synapse formation and influence synaptic partner choices (Kalinovsky A, 2011). It is important to distinguish between two steps leading to synapse formation: the synaptic partner choice, that determines which neurons connect to each other and the induction of pre- and postsynaptic assembly, which takes place after the synaptic partner has been chosen. Several transmembrane receptors and their ligands are thought to be involved in the partner choice, as well as the synapse assembly process. I will focus here specifically on their role in synapse assembly.

A similar challenge during axon guidance and synapse formation is to translate extracellular protein interactions into intracellular structural changes or the recruitment of specific synaptic components. Receptors at the synaptic plasma membranes, as axon guidance receptors, bind upon activation to intracellular adaptors that mediate further downstream signaling. In the following I will give an overview over trans – synaptic “receptor/ligand” pairs and intracellular singaling events downstream of presynaptic transmembrane receptors.

**Initiation of Synapse Formation by trans-synaptic signaling**

The signals regulating the coordinated assembly of the presynaptic CAZ and the PSD can be divided into three subcategories. 1) Anterograde signals, which are diffusible molecules released by the presynaptic bouton influencing the development of the PSD, 2) Retrograde signals, which are diffusible molecules released by the postsynaptic neuron influencing differentiation of the presynaptic terminal 3) trans-synaptic adhesion proteins, which span the synaptic cleft and induce bidirectional signaling (Shen and Scheiffele, 2010). Together all those signals ensure the timely and spatially concise formation of synapses.

**Anterograde Organizers**

A major part of our knowledge about synaptic assembly comes from the study of the vertebrate neuromuscular junction (NMJ). The NMJ is a large and easily accessible synapse of the
peripheral nervous system (PNS) that is formed between a motorneuron (MN) and a muscle fiber. Each muscle fiber is innervated by only one Motorneuron (MN), which combined with the large size of the NMJ makes it easy to identify single synapses. Agrin one of the first “synaptogenic” signals to be discovered, is released by the MN and can induce AChR clustering on the muscle membrane, directly apposing the MN-terminal (Niktin et al., 1987). The clustering of AChR is dependent on MuSK, a tyrosine kinase on the muscle membrane and rapsyn, a scaffolding protein in the myocyte cytosol, that links MuSK with AChRs (Apel et al., 1997; Gillespie et al., 1996). Interestingly, MuSK is not the direct receptor for agrin, but agrin binds to Lrp4, an LDL receptor, which upon binding to agrin associates with MuSK (Kim et al., 2008; Zhang et al., 2008). The neurotransmitter at the NMJ, Acetylcholine (ACh) can itself act as negative anterograde signal of synapse formation, causing the dispersal of AChR across the muscle membrane (Misgeld et al., 2005). At first it seems counterintuitive that the MN releases a positive as well as a negative regulator of NMJ formation, however it could be that the negative effect of ACh can be overcome by agrin (Misgeld et al., 2005) which would ensure that the AChR field is restricted to a space on the muscle membrane that is directly apposed to the site of highest agrin concentration. This would result in the perfect alignment of the presynaptic release site with the postsynaptic AChR field.

Agrin is not a major regulator of synapse formation in the CNS (Kröger and Schröder, 2002), but several other anterograde synaptogenic signals have been identified to function in the assembly of CNS synapses. One that is similar to agrin, in its ability to cluster neurotransmitter receptors on the postsynaptic membrane is NARP (neuronal activity regulated pentraxin). NARP and a related secreted protein neuronal pentraxin 1 (NP1) associate directly with AMPA receptor subunits (GluA). NARP induces clustering of AMPA receptors on cultured spinal neurons (O’Brien et al., 1999), whereas NP1 released from glutamatergic axons is sufficient to drive clustering of GluA4 containing receptors. In vivo NARP/NP1 localize and act selectively at glutamatergic shaft synapses formed onto GABAergic interneurons (Sia et al., 2007).

Another interesting example of a secreted synaptogenic protein is Drosophila wingless (Wg). Wg is secreted by presynaptic boutons at the Drosophila NMJ. Blocking of Wg secretion
prevents the formation of presynaptic active zones and postsynaptic specializations meaning although Wg is secreted by the presynaptic neuron, it can also act in a retrograde fashion (Packard et al., 2002). Indeed Wg belongs to the Wnt family of signaling molecules, of which some members in vertebrates act as retrograde inducers of synapse formation.

**Retrograde Organizers**

Retrograde organizers are factors secreted by the postsynaptic cell that influence the assembly of presynaptic terminals. Interestingly, several growth factors (morphogens) that regulate early embryonic patterning and later function as important axon guidance cues, e.g. BMPs and Shh (Butler and Dodd, 2003; Charron et al., 2003), also function as retrograde synaptic organizing molecules. Four examples for morphogens turned retrograde synaptic organizers are discussed below.

In the cerebellum the formation of synapses between mossy fiber axons and granule neurons is partially regulated by the release of Wnt7a from granule neurons (Lucas and Salinas, 1997). In Wnt7a knock-out mice synapsin clustering in mossy fiber terminals is reduced (Hall et al., 2000). Further double mutant mice deficient in Wnt7a and Disheveled (Dvl), a scaffolding protein acting presynaptically downstream of the Wnt7a receptor Frizzled (Fz), show an enhanced defect in the localization of the presynaptic proteins synapsin, synaptobrevin/VAMP2, SV2 and synaptophysin, indicating the importance of Wnt7a signaling for the efficient formation of the presynaptic terminal (Ahmad-Annuar et al., 2006).

Fibroblast Groth Factor 22 (FGF22) plays a role in the development of the same synapse. FGF22 as Wnt7a is released by granule neurons and induces the accumulation of synaptic vesicles in mossy fiber terminals (Umemori et al., 2004). Blocking of FGF22 signaling or knock-out of the FGF22 receptor result in a decrease in synaptic vesicle accumulation.

A third example for a growth factor acting as retrograde signal in synapse formation is *Drosophila* Glass bottom boat (gbb). Gbb belongs to the Transforming Growth Factor beta family (TGFβ), is secreted by the muscle at the *Drosophila* NMJ and binds to its receptor Wishful Thinking (Wit) on the membrane of the MN. Genetic deletion of Gbb as well as of its receptor Wit
results in a reduction of the number of presynaptic boutons (McCabe et al., 2003); (Marqués et al., 2002). Also observed in Wit mutants are floating T-bars (T shaped electrondense structures in the presynaptic bouton of the *Drosophila* NMJ), which indicates a role of Wit in the recruitment or clustering of presynaptic components. All examples above are retrograde factors that promote presynaptic assembly. An example for a retrograde negative regulator of synapse formation is BMP4, which negatively regulates synapse formation between mossy fiber axons and Purkinje neurons in the cerebellum and thereby contributes to the specificity of synaptic connectivity in the cerebellum (Kalinovsky A, 2011).

**Bi-Directional Organizers**
Best suited to coordinate pre – and postsynaptic differentiation, are transsynaptic adhesion proteins due to their ability to induce bidirectional signaling. A single adhesion pair often can recruit further molecules via homophilic interactions in trans and thereby form large clusters. Through their intracellular domains these clusters function as nucleation platforms for pre- and postsynaptic components. Examples of heterophilic and homophilic trans-synaptic adhesion pairs are the EphBs /ephrinBs, Neuroligins / Neurexins and Cadherins and SynCAMs, respectively.

Hints for Eph receptor function during synapse formation came from studies that found that Ephs can interact and colocalize with PDZ domain containing proteins at synapses (Buchert et al., 1999; Torres et al., 1998). Soon after it was found that activated (ephrinB1 bound) EphB receptors can interact with NMDA receptors and that stimulation of hippocampal neurons with recombinant ephrinB1-FC results in EphB receptor activity dependent clustering of postsynaptic NMDA receptors (Dalva et al., 2000). Activated EphBs can directly interact with GEFs that regulate the postsynaptic cytoskeleton through small RhoGTPases, and thereby regulate the morphogenesis and maturation of dendritic spines (Irie and Yamaguchi, 2002; Penzes et al., 2003; Tolias et al., 2007). Mice lacking EphB receptors 1,2 and 3 have a reduction in synapse number, which can be rescued with postsynaptic expression of EphB2. The different effects of EphB signaling on post- and presynaptic assembly as well as spine morphology are attributed to the independent activities of particular EphB domains. EphB2 controls AMPA type glutamate
receptor localization via its PDZ domain, and triggers presynaptic differentiation via its extracellular ephrin binding domain (Kayser et al., 2006). Whereas downstream signaling to RhoGTPases through specific GEFs is dependent on its kinase domain (Penzes et al., 2003; Tolias et al., 2007).

That Neuroligins (NLs) can induce the formation of presynaptic AZs, was discovered in a mixed co-culture assay in which neurons form functional presynaptic boutons onto NL overexpressing HEK cells (Scheiffele et al., 2000). The involvement of Neurexin-α (Nrx) in the clustering of AZ components is in part based on the observation that recombinant exogenously applied NL is able to induce Nrx and synaptic vesicle clustering in cultured neurons, and further that the overexpression of a Nrx deletion construct, lacking the intracellular domain inhibits the clustering of synaptic vesicles (Dean et al., 2003). The NL/Nrx signal is bidirectional, and NL1 expression in hippocampal neurons induces the recruitment of NMDA receptors and postsynaptic scaffolding proteins to the postsynaptic membrane (Chih et al., 2005; Varoqueaux, 2004; Chubykin, 2007). NL2 localizes exclusively to the postsynaptic side of inhibitory synapses (Varoqueaux et al., 2004). Surprisingly mice lacking the expression of all NL isoforms (1,2 and 3) have defects in synaptic transmission but the density of synaptic contacts remains unaltered in most areas of the brain, with a small (20%) reduction in excitatory synapse number in the brainstem, suggesting that NLs might be necessary for synapse maturation and function but not for their initial formation (Varoqueaux et al., 2006). Single knock-outs for α-Neurexin-1, -2 or -3 show no major defects in synaptic function but the α-Neurexin-1, -2, -3 triple knock-out mice die at P1 due to defects in brainstem circuits that underly respiratory function (Missler et al., 2003).

Importantly, the number of inhibitory synapses in α-Nrx triple knock-outs in the neocortex as well as the brainstem is reduced by 50%, but excitatory synapse numbers remain unchanged. Since all α-Nrx isoforms are expressed in GABAergic as well as glutamatergic synapses (Ullrich et al., 1995) it is difficult to explain the selective effect on inhibitory synaptic assembly. One possible explanation is that Nrx may have a general function in synaptic assembly that is selectively redundant in glutamatergic but not in GABAergic synapses. The findings from the NL and Nrx knock-out studies could mean that they are not required for synapse assembly but only for
synapse maturation and modulation. Another interpretation is that they play roles in both, assembly and function, but that they function in parallel with other trans-synaptic adhesion molecules to induce redundant synapse assembly programs.

The co-culture assay described above was further applied to screen for additional adhesion proteins with synaptogenic activity. While most proteins tested show no synaptogenic activity in this assay, it did lead to the identification of SynCAM (Biederer et al., 2002) and more recently LRRTM1, LRRTM2 (Linhoff et al., 2009); (De Wit et al., 2009) as synapse inducing molecules. Knock-out or overexpression of SynCAM in vivo reduces or increases excitatory synapse number respectively (Robbins et al., 2010). In addition SynCAM is involved in the regulation of synaptic plasticity (Robbins et al., 2010). LRRTM2 exerts its synapse inducing activity through binding to Nrx-1 (De Wit et al., 2009); (Ko et al., 2009).

A group of heterophilic trans-synaptic adhesion proteins that has emerged more recently to play a role in synapse formation are the receptor-protein-tyrosine-phosphatases (RPTP) of the LAR family. LAR is found to localize to the post – as well as the presynaptic membrane (Dunah et al., 2005); (Kaufmann et al., 2002). Postsynaptically LAR is involved in AMPA receptor targeting (Wyszynski et al., 2002), presynaptically LAR can induce the assembly of presynaptic terminals through postsynaptic netrin-G ligand 3 (NGL-3) in primary hippocampal neuron cultures (Woo et al., 2009). A role for LAR in presynaptic assembly is further supported by the finding in Drosophila that the loss of DLAR results in a decreased number of presynaptic boutons (Kaufmann et al., 2002). Just recently another heterophilic adhesion pair involving a RPTP was discovered to induce synapse formation in the co-culture assay. TrkC overexpressed in fibroblasts can induce the formation of presynaptic boutons through an interaction with PTPσ on axons (Takahashi et al., 2011). TrkC and PTPσ co-localize at excitatory synapses and clustering of TrkC by PTPσ or vice versa induces clustering of post- and presynaptic components respectively (Takahashi et al., 2011).

How trans-synaptic signaling molecules drive the pre – or postsynaptic assembly program, respectively is still not understood. NLs and Nrx contain C-terminal PDZ binding motifs through which they can interact with post – or presynaptic scaffolding proteins, respectively (Irie
et al., 1997) (Hata et al., 1996; Biederer, 2000). Therefore a common model is that trans-
synaptic adhesion proteins couple adhesion to the intracellular nucleation of a pre – or 
postsynaptic scaffold that in turn recruits and retains ion-channels and neurotransmitter 
receptors. In the following I will focus on the details of presynaptic assembly. In particular, I will 
discuss how presynaptic surface signals link to the recruitment of cytoplasmic synaptic 
components.

Molecular Mechanisms of Presynaptic Assembly downstream of Trans-Synaptic Adhesion 
Proteins

Recent studies have begun to dissect the dense protein network of the CAZ using genetic and 
biochemical approaches. Individual CAZ protein components are interconnected in a complex 
array of protein-protein interactions. Many of those interactions are involved in the regulation of 
synaptic transmission but are also thought to be necessary for CAZ assembly. Trans-synaptic 
adhesion proteins seem to function as anchors for the nucleation of this protein network. One 
model for presynaptic assembly is that of a linear assembly process, in which the trans-synaptic 
adhesion proteins link to a few intracellular adaptors, which in turn are responsible for the 
recruitment of other CAZ components. During the past years work on vertebrate presynapse 
assembly has provided important insights into the biochemical interactions between the CAZ 
components, but only little is known about their specific contributions to the assembly of synapses 
in vivo. This lack of knowledge is partly due to the lack of dramatic presynaptic assembly defects 
in knock-out mouse models for CAZ components (Atasoy et al., 2007; Dick et al., 2003; Kaeser et 
al., 2009; Lanore et al., 2010; Mukherjee et al., 2010; Olsen et al., 2005; Schoch et al., 2002a). 
This could mean that all CAZ components that have been tested in knock-out models so far are 
simply not key mediators of presynaptic assembly. Meaning that the intracellular regulators 
functioning directly downstream of trans-synaptic adhesion molecules still remain to be 
discovered. Another explanation could be that due to the high degree of parallel biochemical 
interactions within the CAZ, a strictly linear protein recruitment process downstream of adhesion 
molecules does not exist, and that synapses can form through multiple redundant signaling
routes. In the following I will discuss some of the central findings that underlie the two models. First, I will introduce the biochemical network at the CAZ and later follow with an explanation of what we have learned about the contribution of single CAZ components to presynaptic assembly in vivo from knock-out mouse models.

Trans-Synaptic Adhesion Proteins as “Nucleation Centers”

Several of the trans-synaptic signaling proteins described above engage in biochemical interactions that link them directly to CAZ components, thereby coupling adhesion with intracellular presynaptic assembly (Figure 1.6).

Nrx (Neurexin) can bind directly via a PDZ binding motif on its C-terminus to the PDZ domain of Lin-2/CASK and XIIα/Lin-10/Mint1 (Hata et al., 1996; Biederer, 2000) (Figure 1.6). Therefore Nrx could function as nucleation center for presynaptic assembly and recruit additional proteins indirectly via those primary interactions.

The receptor protein tyrosine phosphatase (RPTP) LAR, which induces presynaptic assembly in response to postsynaptic NGL-3 (Woo et al., 2009) directly interacts with the CAZ component Liprin-α/SYD-2 (Serra-Pagès et al., 1995) (Figure 1.6).

Another example of a transmembrane protein that does not fall into the group of trans-synaptic adhesion proteins but nevertheless links to a host of CAZ components is the voltage gated Ca²⁺ channel (VGCC). VGCC like Nrx can directly interact with Lin-2/CASK and XIIα/Lin-10/Mint1 (Maximov et al., 1999; Maximov and Bezprozvanny, 2002). In addition VGCCs bind to RIM (Rab3a interacting protein) and RBPs (RIM binding proteins) (Coppola et al., 2001; Hibino et al., 2002; Kiyonaka et al., 2007; Wang et al., 2000). It is possible, considering the absolute essential role of VGCCs in synaptic transmission, that the many interactions with CAZ components are supposed to guarantee their localization at the presynaptic terminal. On the other hand it has been suggested the Ca²⁺-channel could be an essential signal for the presynaptic assembly process itself.

It is important to note that Nrx and VGCCs engage in parallel biochemical interactions, as both interact with Lin-2/CASK and XIIα/Lin-10/Mint1 (Figure 1.6).
I will now move away from the presynaptic membrane to describe the protein interactions between different CAZ components. It will become apparent that the dense protein network at the CAZ contains many more parallel interaction pathways.

*Interactions among CAZ components*

Lin-2/CASK and Xllα/Lin-10/Mint1 form together with Lin-7/Veli a tripartite complex (Butz et al., 1998). Xllα/Lin-10/Mint1 engages in an interaction with Munc-18-1, (Okamoto and Südhof, 1997) an AZ protein that is necessary for synaptic vesicle fusion (Hata et al., 1993). Since the tripartite complex interacts with Nrx and Munc-18, it is thought to couple adhesion with the recruitment of proteins involved in synaptic vesicle exocytosis (Butz et al., 1998). Lin-2/CASK in addition interacts with the neuronal Protein 4.1N (Cohen et al.), which has been proposed to play a role in the localized assembly of F-actin - spectrin filaments (Biederer and Sudhof, 2001). This leads to a model in which Nrx serves as nucleation center for several CAZ components and presynaptic F-actin assembly through its direct interaction with Lin-2/CASK and Xllα/Lin-10/Mint1 (Figure 1.6). The role of F-actin in presynaptic assembly will be discussed in more detail in the introduction to chapter 3.

Downstream of LAR, Liprin-α/SYD2 engages in interactions with RIMs (Schoch et al., 2002a) and ELKS2 (also knowns as ERC2/CAST1) (Ko et al., 2003b). RIM also interacts with ELKS2, independently from Liprin-α/SYD-2 (Ohtsuka et al., 2002)). In addition ELKS and RIM both interact with the large presynaptic scaffolding proteins Bassoon and Piccolo, which are specific for mammalian synapses (Takao-Rikitsu et al., 2004)). RIMs also engages in protein interactions with Rab3A and Munc-13 (Andrews-Zwilling et al., 2006). With this LAR through its direct interaction with Liprin-α/SYD-2 presents another nucleation center for multiple CAZ components (Figure 1.6).

It is remarkable that there are several links between the nucleation centers: through Lin-2/CASK and Xllα/Lin-10/Mint1 which both interact with Nrx as well as VGCCs, through RIMs which interact directly with VGCCs and indirectly via Liprin-α/SYD-2 with LAR and through the
direct interaction between Liprin-α/SYD-2 and Lin-2/CASK, linking the Nrx and LAR nucleation centers (Figure 1.6) (Olsen et al., 2005).

Lin-2/CASK, XIια/Lin-10/Mint1 and Liprin-α/SYD-2 all link directly to trans-synaptic adhesion proteins (Nrx and LAR) that have been shown to mediate presynaptic assembly after binding to their postsynaptic partners in vitro (Dean et al., 2003; Woo et al., 2009). Therefore all three proteins are likely candidates to be essential downstream effectors in a linear presynaptic assembly process. However, if presynaptic assembly indeed is based on the parallel biochemical interactions described above (Figure 1.6), neither Liprin-α/SYD-2 nor CASK alone should be essential for presynaptic assembly downstream of trans-synaptic adhesion.

The Role of Individual CAZ Components in Presynaptic Assembly at the Vertebrate Synapse

To address the question whether single CAZ components are essential for the presynaptic assembly process and which role they play for synaptic function, knock-out mouse models were created for almost all the CAZ components mentioned above. However, knock-out studies in mice have not been particularly informative about the mechanisms of presynaptic assembly. Loss of function studies in mice are inherently difficult due to many CAZ proteins having several isoforms encoded by different genes (e.g. ELKS1/2 or RIM1/2, Liprin-α1, -2, -3, -4). The isoforms often show a high degree of functional redundancy making the creation of double or even “multiple” knock-outs necessary to uncover their function. Here, I will briefly summarize what we have learned from the knock-outs created so far.

Lin-2/CASK knock-out mice form ultrastructurally normal synapses and show only minor changes in spontaneous neurotransmitter release (Atasoy et al., 2007). Therefore, a critical function in presynaptic assembly downstream of Nrx appears unlikely. XIια/Lin-10/Mint1/2 knock-out mice show impaired excitatory and inhibitory synaptic transmission (Ho et al., 2006; Ho et al., 2003), an effect on presynaptic structure has not been analysed so far.

ELKS according to dominant negative studies is involved in the localization of Liprin-α/SYD-2 as well as RIM to the AZ (Ko et al., 2003a; Ohtsuka et al., 2002). Knock-out of ELKS2α
(the predominant splice variant of ELKS in the mammalian brain) results in no major changes in synaptic ultrastructure but in an enhancement of inhibitory synaptic transmission which further complicates interpretation of the results (Kaeser et al., 2009). However, it should be noted that ELKS1b/ERC1b is expressed at normal levels in ELKS2α knock-outs and therefore, could function redundantly.

Synapses of RIM1α knock-out mice also exhibit changes in synaptic transmission without major ultrastructural changes (synaptic density, active zone size and number of docked vesicles are unchanged) (Schoch et al., 2002b). A difficulty in the study of RIM knock-outs was the early lethality and the existence of multiple RIM isoforms. Recent studies have overcome this problem, by using conditional knock-outs of both RIM isoforms (RIM1 and 2) (Deng et al., 2011; Han et al., 2011; Kaeser et al., 2011). The picture emerging from those studies is that RIMs (via their PDZ domain) can specifically interact with P/Q and N-type Ca2+ channels (the VGCC at the AZ) and that this interaction is necessary for VGCC recruitment to the AZ (Kaeser et al., 2011). Loss of RIM1/2 also results in a reduction of the number of docked vesicles suggesting that RIMs couple Ca2+ channel localization with the docking of synaptic vesicles at the AZ thereby enabling efficient neurotransmitter release (Han et al., 2011; Kaeser et al., 2011). In contrast RIMs interaction with Munc-13 seems to be less essential for CAZ recruitment and rather involved in the regulation of synaptic vesicle priming (Deng et al., 2011).

Other CAZ components for which knock-out mice have been created are Lin-7/Veli and Piccolo/Bassoon. Veli triple (Veli isoforms 1,2 and 3) knock-out mice die perinatally due to breathing defects and impaired excitatory synaptic transmission. Presynaptic and postsynaptic areas are enlarged but synaptic vesicles still localize to synapses (Olsen et al., 2005).

Piccolo and Bassoon are some of the first CAZ components to localize to the nascent presynaptic bouton and are highly homologous. Importantly, Bassoon has been found essential for the anchoring of the photoreceptor ribbon to the AZ in photoreceptors of the mammalian retina (Dick et al., 2003). Due to this, Bassoon was expected to play a major role in the formation of synapses in the brain. Recently hippocampal mossy fiber synapses in Bassoon knock-out animals were reported to show defects in synaptic maturation, as shown by defects in synaptic
transmission. However, no major structural defects were found (Lanore et al., 2010). For knock-out mice of Piccolo, no effect on synaptic ultrastructure or transmission was found (Mukherjee et al., 2010). Dual loss of function of Piccolo and Bassoon in neuronal cultures, revealed a reduction in vesicle clustering but no changes in synaptic transmission (Mukherjee et al., 2010), which suggests a rather small impact of Piccolo and Bassoon on the overall recruitment of presynaptic components.

The picture arising from the loss of function studies mentioned here is that a complex protein network at the presynapse might function as a guarantee for presynaptic assembly even in the absence of single components. It seems that single CAZ components as e.g. RIMs are essential for the recruitment of specific proteins to the AZ, but that due to parallel biochemical interactions their loss does not result in a complete failure to form synapses (Deng et al., 2011; Han et al., 2011; Kaeser et al., 2011). Therefore, a more detailed analysis of the molecular composition and organization of the presynaptic active zone in knock-outs of CAZ components might be necessary to reveal their specific functions.

**SYD-1: A Key Regulator of Presynaptic Assembly in Invertebrates**

The hypothesis that presynaptic assembly is a linear process with a few or one protein acting as essential regulator, comes mainly from loss of function studies in the invertebrates C. elegans and Drosophila. Most presynaptic proteins are evolutionary conserved between the three commonly used model organisms C. elegans, Drosophila and mouse. Therefore, insight gained from genetic studies in C. elegans and Drosophila is often transferable to the mammalian synapse. A series of studies in C. elegans have led to a model in which the two presynaptic proteins synapse defective-2 (SYD-2, the C. elegans homologue of Liprin-α) and synapse defective-1 (SYD-1), arise as regulators of presynaptic assembly directly downstream of transmembrane adhesion proteins. In C. elegans, similar to the mouse, loss of function of several CAZ components does not affect the recruitment of most other presynaptic proteins (Patel et al., 2006). For example, loss of function mutations in the CAZ components: elks-1, unc-10/RIM, unc-
13/Munc-13, unc-18/Munc-18 and the Ca^{2+} channels unc-2 and unc-36 do not affect the localization of SYD-2 (Liprin-α), SYD-1 and synaptic vesicles (Patel et al., 2006). However, a loss of function in syd-2, depending on the neuron type, can result in a diffuse localization of synaptic vesicles at presynaptic sites or in the mislocalization of synaptic vesicles to non-synaptic sites (Patel et al., 2006; Zhen and Jin, 1999). Even more pronounced is the effect of loss of syd-1 on presynaptic assembly, which causes the mislocalization of synaptic vesicles, ELKS-1 and other presynaptic proteins to non-synaptic regions (Hallam et al., 2002); (Patel et al., 2006).

Interestingly, the mislocalization of synaptic vesicles and other CAZ components in syd-1 loss of function mutants can be rescued by a gain of function mutation in syd-2 (Dai et al., 2006). Further SYD-2 also physically interacts with ELKS-1 (possibly an analogous interaction to Liprin-α and ELKS at the mammalian synapse) (Dai et al., 2006). The gain of function mutation in syd-2 fails to rescue presynaptic phenotypes in an elks-1 mutant background, which implies that SYD-2 and ELKS function together (Dai et al., 2006). SYD-1 as well might physically interact with ELKS and it has been suggested that SYD-1 promotes the interaction between ELKS and SYD-2 (Patel and Shen, 2009).

In C. elegans HSN neurons, a loss of function mutation in synaptogenesis abnormal-1 (syg-1) causes the mis-localization of SYD-1 and SYD-2 to nonsynaptic areas. SYG-1 is a transmembrane protein of the Immunoglobulin superfamily (IgSF) that interacts with synaptogenesis abnormal-2 (SYG-2) (expressed by epithelial guidepost cells) and specifies the subcellular localization of presynaptic sites along the HSN motoneuron in C. elegans (Shen et al., 2004).

These studies place SYD-1 and SYD-2 as essential recruiters of other CAZ proteins directly downstream of the synaptic adhesion protein SYG-1 at the HSN synapse in C. elegans. It will require further studies to investigate if SYD-1 and SYD-2 have a similar dominant role in presynaptic assembly downstream of other adhesion proteins at other synapses.

Support for an evolutionary conserved role for syd-1 and syd-2/Liprin-α in presynaptic assembly comes from Drosophila loss of function studies. The loss of Liprin-α in Drosophila (DLiprin) results in dispersed synaptic vesicles and lengthened active zones (Kaufmann et al.,
Furthermore, DLiprin mutants show a reduced intensity for the staining of synaptobrevin/VAMP2 (a synaptic vesicle associated protein) and the presynaptic marker Bruchpilot in the motor neuron terminal (Miller et al., 2005). A mammalian Liprin-α knock-out has not been reported so far and thus Liprin-α still has the potential to be an essential in vivo regulator of presynaptic assembly in vertebrates. Interestingly, the Drosophila homologue of syd-1 (Dsyd-1) has been identified and flies with a loss of function in Dsyd-1 have defects in pre- and postsynaptic maturation (Owald et al., 2010).

Considering the parallels in presynaptic assembly between C. elegans, Drosophila and mouse and the essential role that SYD-1 plays in C. elegans and Drosophila presynaptic assembly it is of major interest to identify its possible mammalian orthologues.

GAPS: COMMON SIGNALING MODULES IN AXON GUIDANCE AND SYNAPSE FORMATION

During axon guidance, as well as synapse formation, neurons have to respond to extracellular signals with intracellular structural changes. In both cases those signals can be diffusible or membrane bound and are received by receptors on the membrane of the axon growth cone or synapse. During axon guidance the challenge is to translate axon guidance cues into cytoskeletal dynamics, whereas during synapse formation it is to recruit and cluster synaptic components (Figure 1.7). Although the requirements downstream of axon guidance and synaptic receptors seem different we found that RhoGTPase activating proteins (GAPs) seem to be involved in both processes. In chapter 2 I will describe our investigations that lead to the identification of a GAP necessary for the translation of an axon guidance signal into intracellular cytoskeletal changes. Our findings that suggest the involvement of a GAP in presynaptic assembly are discussed in Chapter 3.
THE DISSERTATION PROJECT:

CONTROL OF NEURONAL CIRCUIT ASSEMBLY BY GTPASE REGULATORS

A molecular challenge present during all stages of neural development is the translation of extracellular signals received by receptors on the neuronal cell surface, into intracellular structural changes. More specifically, during axon guidance the activation of transmembrane receptors needs to be relayed into changes of cytoskeletal dynamics and converted into axonal repulsion or attraction. During synapse formation, interaction of trans-synaptic adhesion proteins triggers cytoskeletal changes giving rise to spine and synaptic bouton growth as well as the recruitment of pre- and postsynaptic protein components. Small GTPases of the Rho family and their regulators have been implicated in the signaling downstream of axon guidance receptors as well as trans-synaptic adhesion proteins. For my dissertation I investigated the role of the two GTPase activating proteins (GAPs) α2-chimaerin and msyd-1 in axon guidance and synapse formation, respectively.

α2-Chimaerin

The Chn1 gene encodes two isoforms of the so called chimaerins, α1- and α2-chimaerin. Earlier studies in our laboratory showed that α1-chimaerin overexpression in organotypic slice cultures resulted in pruning of dendritic branches and spines of cerebellar Purkinje neurons, whereas inactivation by RNA interference resulted in over-growth (Buttery et al., 2006). However the same manipulations targeted at α2-chimaerin did not show comparable activities. The α2-chimaerin isoform differs from α1-chimaerin in an N-terminal SH2 domain. I hypothesized that this domain could underlie a specific regulation of the α2-chimaerin GAP activity and pursued the following two goals in this project:

1) Identification of Interaction Partners for the SH2 domain of α2-chimaerin

2) Analysis of the necessity of α2-chimaerin for neural development in vivo
Synapse Defective-1 (mSYD-1)

The GTPase activating protein, synapse defective-1 (SYD-1) was originally identified in a forward genetic screen for synapse formation in *C. elegans* (Hallam et al., 2002). SYD-1 is the only CAZ protein in *C. elegans* that is essential for the localization of synaptic vesicles as well as several other presynaptic proteins *in vivo* (Dai et al., 2006; Patel et al., 2006). In *C. elegans* SYD-1 functions downstream of the synaptic specificity protein SYG-1 (Patel et al., 2006). Given its essential function in *C. elegans* synapse development, it is of major interest to analyze a role for potential orthologues of *syd-1* in vertebrate synapse formation. Therefore, we formulated following goals for this project:

1) Identification of mammalian orthologues of *C. elegans* SYD-1
2) Basic characterization of mouse SYD-1 Expression in the rodent brain
3) Analysis of functional properties of mouse SYD-1
4) Testing the requirement of mouse SYD-1 for AZ Assembly *in vitro* and *in vivo*
FIGURE 1.1: The Midline: a Choice Point for Axon Guidance throughout the CNS

The “wedge” at the corpus callosum, the glial “tunnel” at the anterior commissure, the “palisade” at the optic chiasm are axon guidance choice points in the CNS. Axons cross the midline at several locations in the CNS. (A – C) Depict commissural tracts in schematics of horizontal sections from dorsal to ventral. The axons of projection neurons in the cortex and hippocampus form the corpus callosum (blue) and hippocampal commissure (purple) respectively. Further ventral the anterior commissure (green) connects the two temporal lobes. The axons of retinal ganglion cells crossing the midline form the optic chiasm (red). At each of those commissures glial cells serve as guidance choicepoints. They express or secrete guidance molecules to instruct the approaching axons to cross or avoid the midline (modified from Chédotal and Richards, 2010).
FIGURE 1.2: Axon Guidance at the Vertebrate Spinal Cord Midline

(A) Cross sectional schematic view of the developing spinal cord. Commissural axons are repelled by BMPs secreted by roofplate cells (RF) and attracted by Sonic Hedgehog (Shh) and netrin-1 secreted by floorplate (FP) cells. (B) “Open book” schematic of the vertebrate spinal cord. Commissural axons are drawn to the midline by netrin-1 and Shh (1). Commissural axons are unsensitive to Slit until after they cross the midline. This is due to the expression of Rig-1/Robo-3, which prevents Slit from activating Robo1 expressed by commissural axons. Rig-1 is downregulated upon crossing (2) making axons sensitive to Slit repulsion on the contralateral side. Multiple Slits and semaphorins are expressed at the midline. Semaphorins help to constrain the axons post-crossing in their longitudinal tracts. ephrinBs and their Eph-receptors are involved in preventing axons from re-crossing the midline after they have crossed it once. Ipsilateral projecting axons are repelled by Slits but in general little is known about what other factors guide them (modified from (Williams et al., 2004)).
FIGURE 1.3: Axonal Guidance defects in EphA4 knock out mice

(A) Usually corticospinal tract (CST) axons originate from pyramidal neurons in the sensorimotor cortex (green) cross the midline once at the pyramidal decusation in the hindbrain and are prevented from recrossing the midline in the spinal cord by ephrinB3 (blue) expressed by midline cells. In EphA4 knock-out mice, CST axons recross the midline, since due to the lack of EphA4 expression they cannot detect the repulsive ephrinB3 signal. (B) EphA4 positive spinal cord interneurons usually innervate ipsilateral motorneurons (MN) and are prevented from crossing the midline by ephrinB3 (blue) expressed by midline cells. In EphA4 knock-out mice their axons do not react to the repulsive ephrinB3 signal and therefore, cross the midline aberrantly (Kiehn, 2006).
FIGURE 1.4: The GTPase Cycle
Small GTPases cycle between a GTP bound “active” state and a GDP bound “inactive” state. Only in their GTP bound state small GTPases interact with downstream effector proteins to initiate cytoskeletal rearrangements. Since the intrinsic catalytic activity for the hydrolysis of GTP of small GTPases is low they require accessory factors that promote the hydrolysis of GTP. Those factors are the GTPase activating proteins (GAPs). To exchange GDP for GTP, GTPases depend on GTP exchange factors (GEFs). GAPs and GEFs can be activated by extracellular signals received by axon guidance receptors.
FIGURE 1.5: Synaptic Ultrastructure and Molecular Composition

Left: electron micrograph of an excitatory synapse in the central nervous system (De Camilli et al.). The presynaptic bouton is filled with synaptic vesicles that are clustered around and embedded within an electron dense matrix the cytomatrix at the active zone (CAZ). On the postsynaptic membrane electron dense material, the postsynaptic density (PSD) can be detected immediately beneath the membrane. Right: The CAZ and the PSD are both dense protein rich matrices and their protein components engage in many biochemical interactions among each other. Note: not all CAZ and PSD proteins are depicted in this cartoon. Presynaptic compartment (PRE), Postsynaptic compartment (POST), cytomatrix at the active zone (CAZ), postsynaptic density (PSD). (Ziv and Garner, 2004)
FIGURE 1.6: Synaptic Transmembrane Receptors as Nucleation Centers of Presynaptic Assembly

The presynaptically localized trans-synaptic adhesion proteins LAR and NRX as well as the VGCC interact directly with several proteins of the cytomatrix at the active zone (CAZ). NRX and VGCC both interact with LIN-2/CASK and Mint1. The VGCC in addition interacts directly with RIM. LAR interacts directly with Liprin which in turn binds to LIN-2/CASK and RIM, linking LAR indirectly with Nrx and VGCCs. LIN-2/CASK also interacts with Protein 4.1N, thereby linking Nrx to presynaptic actin assembly. This network of parallel protein interactions could result in redundant signaling pathways all leading to presynaptic assembly. LAR, Leukocyte common Antigen Receptor; Nrx, Neurexin; VGCC, Voltage Dependent Calcium Channel; Liprin, Liprin-α; RIM, Rab3 interacting molecule; LIN-2/CASK, Lin-2/CASK; Mint, Mint1; ELKS, ELKS2/ERC2/CAST1; RBP, RIM binding protein, 4.1N, Protein 4.1N. Black lines: direct interactions between presynaptic transmembrane receptors and CAZ proteins, grey lines: direct interactions among CAZ proteins.
FIGURE 1.7: RhoGTPase Regulators: Common Signal Transduction Modules in Axon Guidance and Synapse Formation.

In axon guidance, as well as synapse formation, cellular responses are induced by the binding of extracellular molecules (membrane bound or diffusible) to transmembrane receptors. In the case of axon guidance extracellular signals are often expressed by guidepost cells along the route of the growing axon. During synapse formation those signals can be trans-synaptic adhesion molecules that trigger cellular responses in the pre- as well as postsynaptic cell. The binding of the transmembrane receptor to its ligand activates the receptor, which can result in the recruitment of adaptor molecules. Such adaptor molecules can e.g. be GTPase activating proteins (GAP). The GAP is activated through the interaction and conveys the signal to downstream effectors as e.g. RhoGTPases (GTPase). The GAP might also function as direct recruiter of other proteins, as e.g. cytomatrix active zone (CAZ) components during presynaptic assembly. Responses downstream of the GTPase result in cytoskeletal rearrangements and give rise to morphological changes, as growth cone turning or synaptic bouton growth. Cytoskeletal dynamics in the presynaptic bouton might also be involved in clustering proteins of the cytomatrix at the active zone (CAZ) (dashed arrow).
CHAPTER 2

α2-CHIMAERIN IS AN ESSENTIAL EPHA4 EFFECTOR IN THE ASSEMBLY OF NEURONAL LOCOMOTOR CIRCUITS
Several years ago a microarray screen was performed in our laboratory, to elucidate gene expression patterns during development of the pontocerebellar system (Díaz et al., 2002). The screen identified a group of genes whose expression was specifically changed during axonal growth and axon target contact formation between mossy fibers and granule neurons. Among those genes were the α-chimaerins, two Rac GAPs that are encoded in the same gene but transcribed from alternative promoters. Studies on α1-chimaerin, which contains a C1 and GAP domain, in our lab and others found that it is involved in the regulation of dendritic spine density (Buttery et al., 2006; Van de Ven et al., 2005). Its overexpression in cerebellar Purkinje neurons in organotypic slice cultures, results in the pruning of spines and dendritic branches. The overexpression of α2-chimaerin, which contains a SH2, C1 and GAP domain, did not have the same effect on neuronal morphology. We hypothesized that it is the SH2 domain that mediates α2-chimaerin’s differential function. Therefore, I initially started this project with a biochemical screen, trying to identify specific interaction partners of the α2-chimaerin SH2 domain. From this screen the EphA4 receptor emerged as specific interaction partner of the SH2 domain, which hinted at a role for α2-chimaerin in axonal guidance.

The results described in the following arose from the joined effort of Asim Beg a former postdoctoral researcher in our laboratory and myself. Whereas I focused on the biochemical and cellbiological characterization of the α2-chimaerin – EphA4 interaction, he characterized the α2-chimaerin mutant mice.
INTRODUCTION

Regulation of Rho GTPase Signaling Downstream of Eph Receptors

Eph receptors mediate repulsive axon guidance in response to their ephrin ligands (Cheng and Flanagan, 1994; Drescher et al., 1995). Downstream of Eph receptors Rho GTPases and their regulator GEFs and GAPs play a central role in translating the extracellular guidance signal into cytoskeletal rearrangements (Hall and Lalli, 2010). Several GEFs have been identified that interact with Ephs and are activated by Eph/ephrin signaling (Cowan et al., 2005; Shamah et al., 2001).

In addition to Ephs other activated axon guidance receptors couple to GAPs and GEFs that drive local inactivation or activation of Rho family GTPases, respectively, and thereby ultimately control cytoskeletal and membrane dynamics in growth cones (Luo, 2002; Shamah et al., 2001; Wong et al., 2001; Yang and Bashaw, 2006). The logic by which GAP and GEF effector proteins transduce surface receptor signals has remained unclear. Analysis of the human genome sequence predicts 69 GEFs and up to 70 GAPs for Rho-family GTPases; however, the biological relevance of this molecular diversity is not well understood (Peck et al., 2002; Schiller, 2006; Tcherkezian and Lamarche-Vane, 2007). Biochemical studies suggest that a single surface receptor can interact with multiple GAPs and GEFs and that a single GAP or GEF can interact with multiple different surface receptors. This poses the question whether there are dedicated Rho-regulators that are absolutely required for one class of receptors in vivo or whether receptor signals are transduced through multiple GAPs and GEFs that act through partially redundant pathways and are associated with many different surface receptors.

In a search for mediators of Eph-receptor forward signaling multiple Rho-family GAPs and GEFs have been isolated, including the EphA4 effectors ephexin-1, Vsm, and Vav-2/-3 (Cowan et al., 2005; Irie and Yamaguchi, 2002; Ogita et al., 2003; Penzes et al., 2003; Shamah et al., 2001; Tanaka et al., 2004). How these multiple EphA4 effectors cooperate in vivo has remained unclear. While knock-out studies for ephexin-1 and Vav-2/-3 support important functions in axonal guidance (for Vav-2/-3 in the retinotectal system), neither one of these knock-out models reproduces the EphA4 mutant phenotype in the locomotor system (Cowan et al.,
2005). This could be explained if EphA4 downstream signaling is mediated through a complex signaling network composed of multiple effectors with redundant functions. Alternatively, EphA4 signaling in the locomotor circuits may depend on highly dedicated effector proteins that have not yet been identified.

**α-Chimaerins are GAPs Involved in Neural Development**

One new family of candidate effector proteins for axonal guidance are the chimaerins which were recovered previously in our laboratory in a microarray screen for genes involved in axonal growth and synapse formation (Díaz et al., 2002). Two α-chimaerin isoforms (α1 and α2) expressed from a single genomic locus under control of two promoters were originally identified by Lim and colleagues (Dong et al., 1995; Hall et al., 1990; Hall et al., 1993). Both isoforms share identical diacylglycerol-binding (C1) and Rho-GAP-domains, but α2-chimaerin contains an additional Src-homology-2 (SH2) domain (Figure 2.1A) (Hall et al., 1990; Hall et al., 1993). The GAP domain down-regulates Rac activity *in vitro* and in transfected cells (Ahmed et al., 1990; Hall et al., 2001). Further over-expression and knockdown studies suggest that α-chimaerins inactivate Rac and that the α1-chimaerin isoform contributes to dendritic retraction events in cultured neurons (Buttery et al., 2006; Leskow et al., 2006; Van de Ven et al., 2005).

Two other proteins β1- and β2-chimaerin share the principal protein domain organization with the α-chimaerins. The crystal structure of β2-chimaerin suggests that the N-terminus protrudes into the active site of the GAP domain, thereby blocking it from interacting with Rac. The Diacylglycerol binding site (DAG) in the C1 domain is covered by the SH2 domain and several other intramolecular contacts thus, unable to bind DAG. This structure suggests an activation mechanism for β2-chimaerin in which binding of the SH2 domain to a phosphotyrosine residue in another protein could open up the protein making the C1 and GAP domains accessible.

Our search for novel interaction partners of the α2-chimaerin SH2 domain identified activated Eph-receptors. Characterization of the behavioral, anatomical and functional abnormalities in the locomotor circuits of α2-chimaerin-deficient mice lead to the discovery that
they closely resemble defects of mice lacking EphA4 kinase-dependent functions. These findings suggest that ablation of a single cytoplasmic effector protein results in a severe but selective loss of EphA4-receptor function.

RESULTS

**α2-Chimaerin Interacts with Activated Eph-Receptors**

We hypothesized that α2-chimaerin might interact via its SH2 domain with tyrosine kinase receptors. To identify such receptors, we performed affinity chromatography of solubilized membrane proteins on an immobilized recombinant protein of the SH2 domain fused to Gluthathione-S-transferase (GST). The proteins that precipitated with the SH2 domain were analyzed on Western Blots. Anti-phospho-tyrosine antibodies revealed several candidate binding proteins. Based on its size and reactivity with anti-EphA4 antibodies one of these proteins was identified as EphA4 (Figure 2.1B). Under the same conditions TrkA, another axon guidance receptor, did not interact with the recombinant SH2 domain.

The selectivity of this biochemical interaction was examined in pull-down assays with lysates from HEK293T cells over-expressing full-length EphA4, EphB1, EphB2, TrkA, Neurexin-1β, or SHPS1. In this assay, all of the tested Eph-receptors were efficiently recovered on GST-SH2 beads, but not on beads containing a GST control protein. No significant binding was observed for TrkA, another tyrosine kinase receptor, or the transmembrane adhesion molecules Neurexin-1β and SHPS1 (Figure 2.1C).

We next examined whether α2-chimaerin preferentially interacts with activated, tyrosine-phosphorylated EphA4 receptors. In pull-down assays with the recombinant α2-chimaerin SH2 domain as bait, no full-length kinase dead receptors were recovered (KD-A4, V635M, Figure 2.2A). In the same assay, an EphA4 mutant that maintains kinase activity but has the two juxta-membrane tyrosine-phosphorylation sites mutated to glutamate (EE-A4; Y596E/Y602E), which mimics the negative charge added by phosphorylation, (Holland et al., 1997) (Binns et al., 2000); (Zisch et al., 2000) showed greatly reduced interaction with the α2-chimaerin SH2 domain. Finally, binding of this double glutamate mutant was not altered when combined with the kinase-
dead mutation (EE/KD). Therefore, the phosphorylated juxta-membrane tyrosine residues Y596 and Y602 in EphA4 are likely to make an important contribution to the interaction with the α2-chimaerin SH2-domain (Figure 2.2). Similar results were obtained in binding assays with mutants of EphB1 (Figure 2.2C). No full-length kinase dead EphB1 receptors were recovered (K651Q, KD-B1). A mutant of EphB1 with one of the juxtamembrane Tyrosine Y919 mutated to a Phenylalanine (Y919F) which results in the prevention of phosphorylation at this Y residue, showed reduced binding, which could mean that Y919 is involved in binding the α2-chimaerin SH2 domain (Figure 2.2C). An EphB1 mutant that maintains kinase activity but has the two juxtamembrane tyrosine-phosphorylation sites mutated to glutamate (YE/YE-B1, Y588E/Y594E) showed reduced binding, as compared to the WT receptor or the Y919F mutant (Figure 2.2C). A possible explanation for this observation is that α2-chimaerin’s main binding sites in EphB1 are the phosphorylated Tyrosine residues Y588 and Y594, but that it also binds in a weaker fashion to Y919. This would mean if Y919 cannot be phosphorylated any longer as in the case of the Y919F mutation, binding to EphB1 is only slightly reduced. It is possible that although the Y588E/Y594E mutations are supposed to mimic phosphorylation at those sites, they are not able to retain full α2-chimaerin binding ability. Additionally, we tested the phosphotyrosine dependence of the α2-chimaerin – EphA4 interaction in a competition assay (Figure 2.2B). For this we added Phorbol 12-Myristate 13 Acetate “PMA” to the pull down incubation mixture. PMA binds to phosphotyrosine binding sites, as SH2 domains, and thereby inhibits their interaction with the target phosphotyrosine. Addition of PMA but not of DMSO alone prevented binding of EphA4 to the α2-chimaerin SH2 domain (Figure 2.2C).

In a reverse pull down it was tested if α2-chimaerin binds to EphA4 in a kinase activity-dependent manner. α2-chimaerin expressed in HEK293 cells bound efficiently to beads coated with the recombinant intracellular domain of the EphA4 receptor (A4) fused to maltose-binding protein (MBP) but not to a MBP control protein (Figure 2.3A). Binding was severely reduced after de-phosphorylation of EphA4 with lambda-phosphatase (A4-λ) and not detectable for EphA4 kinase-dead receptors (A4-KD, Figure 2.3A). This data suggests that α2-chimaerin selectively interacts with activated Eph-receptors through a phosphotyrosine-dependent interaction. Notably,
α1-chimaerin, which contains instead of an N-terminal SH2-domain a short unique N-terminal sequence, still showed binding to recombinant EphA4 receptors, indicating that there are multiple EphA4 binding sites on the α-chimaerin protein. However this interaction seems to be phosphotyrosine independent, since α1-chimaerin binds to lambda-phosphatase treated EphA4 as well as the kinase dead receptor (Figure 2.3A).

Attempts to co-immunoprecipitate α2-chimaerin and EphA4 either from brain lysates or from transfected HEK293 cells failed suggesting that binding might be transient or shows a high off-rate. However, in co-transfected HEK293 cells EphA4-dependent tyrosine phosphorylation of α2-chimaerin could be detected, providing further evidence for functional coupling between the two proteins (Figure 2.3B). In summary, these biochemical studies identify α2-chimaerin as a novel signaling effector for activated Eph-receptors that might be required for transduction of Eph-receptor signaling.

**α2-Chimaerin Mutant Mice Exhibit Locomotor Defects**

To examine the relevance of α2-chimaerin for Eph-receptor signaling we obtained mice with a gene trap insertion in the α-chimaerin gene (Figure 2.4A). The gene trapping cassette consists of a promoterless lacZ reporter gene and is flanked by an upstream 3’ splice site (splice acceptor; SA) and a downstream transcriptional termination sequence (polyadenylation sequence; polyA). The gene trap cassette is transcribed from the endogenous α2-chimaerin promoter in the form of a fusion transcript in which the six exons upstream of the insertion site are spliced in frame to the lacZ gene. Since transcription is terminated prematurely at the inserted polyadenylation site, the processed fusion transcript encodes a truncated and non-functional version of α2-chimaerin. The genomic locus of α-chimaerin contains two independent promoters (Figure 2.4A) that drive transcription of the α1- and α2-chimaerin isoforms. The trap insertion is located upstream of the putative α1-chimaerin promoter (Dong et al., 1995) and therefore selectively suppresses α2-chimaerin full-length mRNA and protein levels, without detectable effect on the α1-chimaerin isoform (Figure 2.4B). Heterozygous gene trap mice did not show any overt anatomical or behavioral phenotype, and we used the lacZ reporter, inserted within the gene-trap cassette to
characterize the pattern of α2-chimaerin expression (Figure 2.4C). This analysis revealed widespread neuronal expression consistent with previous in situ hybridization studies (Hall et al., 1993). In postnatal day 5 cortex, α2-chimaerin expression was detected in multiple layers including CTIP2-positive layer V neurons, which are the source of corticospinal tract axons (Arlotta et al., 2005) (Figure 2.4C). High expression was also observed in the retinal ganglion cells, dorsal root ganglia (data not shown) and the spinal cord, where the lacZ reporter was detected in NeuN-positive interneuron populations, including cells in the ventro-medial zone as well as presumptive motor neurons (Figure 2.4C).

Homozygous α2-chimaerin mutant mice are born at Mendelian frequencies and are viable and fertile. Overall brain architecture including the anterior commissure in α2-chimaerin mutant mice did not show any apparent defects (data not shown and Figure 2.5A). However, the mutant mice exhibit a pronounced locomotor deficit during walking. While wild type mice move their hindlimbs in alternation, α2-chimaerin mutants show synchronous hindlimb movement, observed as a rabbit-like hopping-gait (Figure 2.5B). Importantly this behavioral defect phenocopies locomotor abnormalities observed in mice lacking the expression of the EphA4 tyrosine kinase receptor (Dottori et al., 1998; Kullander et al., 2001b) suggesting that loss of α2-chimaerin may impair EphA4 function.

**α2-Chimaerin is Required for Ephrin-Induced Growth Cone Collapse**

A possible explanation why α2-chimaerin mutants show the same locomotor phenotype as EphA4-receptor knock-outs, is that α2-chimaerin is an EphA4 effector and involved in EphA4 specific downstream signal transduction. Since it is known that the repulsive response of corticospinal axon growth cones at the spinal cord midline depends on EphA4 signal transduction in response to its ligand ephrinB3 (Kullander et al., 2001a), we hypothesized that such repulsive EphA4-receptor signaling could be disturbed in the axons of α2-chimaerin mutant mice.

Repulsive EphA4-receptor signaling responses can be visualized in vitro as ephrin ligand-induced growth cone collapse. To test whether α2-chimaerin deficient axons had lost their ability to respond to ephrins we cultured cortical explants from the presumptive motor cortex area,
which expresses high levels of EphA4 and α2-chimaerin and then documented the behavior of the outgrowing axons in response to ephrin ligand. As observed by time-lapse imaging, basal growth rates of axons extending from wild type and α2-chimaerin mutant explants were not significantly different over 15 minutes of imaging (Figure 2.6C) (distance traveled: wt = 3.70 ± 1.6 mm; a2 mutant = 3.33 ± 1.1 mm; n >27, p > 0.05, t-test). Application of recombinant ephrinA1-Fc fusion protein, a high-affinity ligand specific for A-class Eph-receptors, induced robust growth cone collapse in wild type axons within 30 minutes of application (Figure 2.6B). In contrast, axons from α2-chimaerin mutant explants were largely resistant to ephrinA1-induced collapse (Figure 2.6B, p = 0.004, t-test). Additional measurements of individual growth cone position before and 30 minutes after ephrinA1-addition revealed that these mutant axons continued to advance in the presence of the repulsive cue (Figure 2.6C). Importantly, EphA4 protein levels in cortex of α2-chimaerin mutant mice were unchanged and EphA4 staining on wild type and mutant axons were not noticeably different (Figure 2.6D and E). This suggests that α2-chimaerin is essential for the transduction of repulsive ephrin-induced signaling in motor cortex axons in vitro.

α2-Chimaerin Mutant Mice Show Aberrant Midline Crossing of Spinal Interneuron

Projections

In the lumbar spinal cord, repulsive EphA4 signaling prevents inappropriate midline crossing of spinal interneuron axons, and the locomotor abnormalities in EphA4 mutant mice are attributed at least in part to aberrant innervation of the contralateral central pattern generator (CPG) (Dottori et al., 1998; Kullander et al., 2003; Kullander et al., 2001a; Yokoyama et al., 2001). Since α2-chimaerin mutant mice exhibit similar locomotor defects as EphA4 mutants and show reduced Eph-receptor-mediated repulsion, we analyzed whether loss of α2-chimaerin causes defects in spinal interneuron axonal pathfinding. We performed unilateral injections of rhodamine-dextran into the lumbar spinal cord segment L4 and examined retrogradely labeled axonal projections in transverse sections at L2 (Figure 2.7A and B). In α2-chimaerin mutant animals we observed an increase in rhodamine positive fibers crossing the midline as compared to wild type. A significant increase in midline crossing was further confirmed in quantitative analysis of the label density
near the midline on the contralateral side (Figure 2.7C, fraction of 10 x 100 µm region of interest that is dextran positive on contralateral side: 3.7% ± 1.8% in wild-type and 33.4% ± 5.1% in α2-chimaerin mutant spinal cords, n = 12 wild type and n = 15 α2-chimaerin mutant spinal cords, >4 consecutive sections per animal, p = 0.004, t-test). In contrast, ipsilateral labeling was similar between the two genotypes (p = 0.42, t test). Despite the severe defect in midline crossing, other axonal projections in the spinal cord of α2-chimaerin mutants were apparently unperturbed. We examined the central projections of proprioceptive sensory afferents, which depend on plexinA1-signaling for axon positioning within the dorsal spinal cord (Yoshida et al., 2006). Immunostaining with antibodies to parvalbumin, a marker of proprioceptive afferents, revealed that parvalbumin-positive axons were similarly excluded from the superficial dorsal horn in wild-type and mutant spinal cords (Figure 2.8). Therefore, α2-chimaerin is required for restricting midline crossing of interneurons in the lumbar spinal cord but not for the positioning of proprioceptive sensory afferents.

To test whether EphA4 and α2-chimaerin genetically interact in restricting midline crossing of spinal interneurons, we examined single and double heterozygous EphA4 and α2-chimaerin mutant mice. While single heterozygous mutants did not show any overt phenotype, α2-chimaerin/EphA4 double heterozygotes had a hindlimb locomotor phenotype characterized by perturbed hindlimb coordination and occasional synchronous hindlimb movements. Rhodamine dextran tracing revealed increased midline crossing of fibers as compared to the wild-type mice (Figure 2.7C). A genetic interaction between EphA4 and α2-chimaerin was also observed at the level of spinal cord morphology. The dorsal funiculus in EphA4 mutant mice is broader and shallower than in wild type or heterozygous mutant mice (Dottori et al., 1998; Kullander et al., 2001a; Kullander et al., 2001b). This is also observed in α2-chimaerin mutants and α2-chimaerin / EphA4 double heterozygotes show a similar, though somewhat less severe defect (Figure 2.7B). These findings provide genetic evidence supporting the a2-chimaerin – EphA4 interactions observed in our biochemical studies and strongly suggest that both proteins act in a common signaling pathway in vivo.
**Corticospinal Defects in α2-Chimaerin Mutant Mice**

Axons of the CST that originate from layer V pyramidal neurons in the motor cortex and cross the midline ones at the pyramidal decussation in the hindbrain, are prevented from recrossing the midline in the spinal cord by repulsive ephrinB3 – EphA4 signaling (Yokoyama et al., 2001) (Kullander et al., 2001a). In EphA4 mutant mice the midline repulsion is lost and the CST axons show aberrant recrossing. Therefore we next examined CST projections in α2-chimaerin mutant mice. The anterograde tracer biotin-dextran amine was injected into one side of the motor cortex of adult mice, and descending CST axonal projections in the spinal cord were observed 10-12 days later (Figure 2.9A). Wild type animals exhibited strictly contralateral labeling within the dorsal funiculus and predominantly contralateral terminations within the spinal grey matter (Figure 2.9B and C). In α2 chimaerin mutant mice, CST axons were also restricted to the contralateral dorsal funiculus. However, there were extensive bilateral terminations within the spinal grey matter, indicating that α2-chimaerin mutant CST axons fail to respect the repulsive ephrin-barrier along the midline (Figure 2.9B and C; n = 5 animals/genotype, 4 sections/animal, p = 0.008, t test).

Taken together, our anatomical studies demonstrate that α2-chimaerin mutant mice selectively phenocopy defects resulting from impaired EphA4 forward signaling in spinal and cortical motor circuits *in vivo*.

**α2-Chimaerin Mutants Show Bilateral Hindlimb Movements in Response to Cortical Stimulation**

The anatomical studies reveal inappropriate midline crossing for the axons of corticospinal motor neurons and spinal interneurons. To directly test whether the loss of bilateral segregation of these axonal pathways was responsible for the functional defects in α2-chimaerin mice, we performed extracellular microstimulation (EMG) within the hindlimb representation of the motor cortex (Li and Waters, 1991) and observed hindlimb movements and muscle activation. In wild type animals, a threshold stimulus (18.75 ± 0.75 mA, n = 4 animals) applied in motor cortex of one cortical hemisphere resulted in exclusively contralateral EMGs and hindlimb flexion (Figure 2.10A...
Weak ipsilateral hindlimb responses in wild type animals could be recruited only with supra maximal stimuli (66 ± 11 mA, n = 4 animals). In contrast, unilateral stimulation of motor cortex in α2-chimaerin mutant mice evoked exclusively bilateral EMGs and hindlimb flexion at threshold intensity (21 ± 4.02 mA, n = 4; Figure 2.10A and B). These bilateral responses in α2-chimaerin mutant mice might result from bilateral CST projections that trigger local spinal motor circuits on both sides of the spinal cord. Alternatively, bilateral stimulus-evoked responses might be due to contralateral CST projections that activate interneurons of spinal motor circuits with aberrant bi-lateral connections to motor neurons. In either scenario, our data suggest that the wiring defects in spinal motor circuits are responsible for hindlimb synchrony in the α2-chimaerin mutant mice.

**Genetic Dissection of the Functional Origin of Hindlimb Synchrony in α2-chimaerin Mutant Mice**

As mentioned above the hindlimb synchrony in α2-chimaerin mutant mice could have its origin in aberrant bilateral projecting CST axons or misprojecting interneurons in the spinal motor circuits.

To dissect between the possible anatomical origins of the hindlimb synchrony in α2-chimaerin mutant mice, we decided to create mice with the possibility of conditional (Cre recombinase induced) α2-chimaerin (α2-flox) knock-out (Figure 2.11). This enabled us to breed α2-flox animals with mouse lines that express Cre-recombinase under the control of promoters that restrict α2-chimaerin ablation to either the cerebral cortex of the forebrain (Emx1-Cre) or to neurons in the spinal cord (Hb9-Cre) (Guo et al., 2000); (Arber et al., 1999). To confirm loss of function for the conditional allele, we induced germline ablation of α2-chimaerin using a mouse line that ubiquitously expresses Cre recombinase under control of the CMV promoter (Cre-del) (Schwenk et al., 1995). Resulting α2-chimaerin knock-out animals (α2−/−) showed the characteristic hopping gait phenotype, as described for the α2-chimaerin mutant mice above, confirming the functionality of the conditional allele. However, neither animals with α2-chimaerin expression abolished in the forebrain (α2Emx−/−) nor animals that lost expression of α2-chimaerin in the spinal cord (α2Hb9−/−), exhibited a hopping gait. The preliminary observation that α2Emx−/− mice
do not show a hopping gait suggests that in α2<sup>−/−</sup> and α2-chimaerin gene-trap mutant mice, the misrouting of corticospinal tract axons is not sufficient to induce this behavior. However, confirmation of complete gene ablation and an anatomical analysis of the α2<sup>Emx<sup>−/−</sup> will be necessary to confirm that their corticospinal tract axons show the same aberrant midline recrossing, as it is observed in α2 gene trap mutants. With regards to the absence of a hopping gait in α2<sup>Hb9<sup>−/−</sup> mice it is more difficult to draw a conclusion, since the Hb9 promoter only exhibits transient activity in neurons of the spinal cord, followed by persistent activity in MNs. As in the case of the α2<sup>Emx<sup>−/−</sup> mice, confirmation of the complete ablation of the α2-chimaerin gene in spinal cord interneurons and an anatomical analysis will be necessary, to draw further conclusions about the contribution of aberrant spinal cord interneuron projections to the hopping gait. The fact that the conditional allele results in a successful ablation of α2-chimaerin after combination with Cre-recombinase in germline cells (Cre-del), makes us confident that also the combination with Emx-Cre as well as Hb9-Cre resulted in complete ablations of the gene.

**DISCUSSION AND FUTURE DIRECTIONS**

*α2-Chimaerin is an Essential EphA4 Effector in the Assembly of Neuronal Locomotor Circuits*

Our findings provide several important new insights into the mechanisms of Eph-receptor function. We demonstrate that α2-chimaerin is a novel downstream effector for EphA4 signaling *in vivo*. Loss of α2-chimaerin results in a close phenocopy of anatomical and locomotor defects in EphA4 knock-in mice expressing a kinase-dead receptor (Kullander et al., 2001b). Together with our biochemical studies, these findings demonstrate that α2-chimaerin is a dedicated signaling effector that is essential for EphA4-receptor forward signaling.

Several lines of evidence support the conclusion that abnormalities in the α2-chimaerin mutant mice are due to an impairment of downstream signaling and not simply lack of EphA4-receptor expression. Our biochemical studies suggest an interaction with activated EphA4 and EphA4-dependent tyrosine phosphorylation of α2-chimaerin. Moreover, EphA4 protein levels in tissue lysates and in axonal growth cones are unaltered in the α2-chimaerin mutant mice and,
most importantly, formation of the anterior commissure, which depends on EphA4 reverse signaling, is normal in α2-chimaerin mutants. Therefore, the phenocopy of the EphA4-receptor mutant in the corticospinal tract and spinal interneuron projections of α2-chimaerin mutant mice is due to the loss of an indispensable downstream signaling function of the α2-chimaerin protein.

Why is there no Redundancy in EphA4 Downstream Signaling in the Neurons Controlling Locomotion?

Besides α2-chimaerin, the α1- and β2-chimaerin isoforms are broadly expressed in the nervous system (Buttery et al., 2006); (Hall et al., 2001); (Leung et al., 1994). Interestingly, a recent proteomic analysis of an EphB2-overexpressing cell line identified β2-chimaerin in phosphotyrosine-containing protein complexes (Zhang et al., 2006). Therefore, β2-chimaerin may also be recruited by Eph-receptor activation. However, our data suggest that neither α1- or β2-chimaerin isoforms can compensate for the loss of α2-chimaerin in vivo. The severe impairment of EphA4 forward signaling in α2-chimaerin mutant mice suggests that also with respect to other effectors there is no redundancy in the signaling pathways downstream of EphA4. How can this be explained considering that EphA4 interacts with multiple Rho-GTPase GAPs and GEFs? Ablation of ephexin-1 or Vav-2/Vav-3, that couple to activated EphA4 receptors does not phenocopy the locomotor defects observed in EphA4 and α2-chimaerin mutant mice. Rather, these mutants show defects in retinal ganglion cell axon guidance (Cowan et al., 2005). Therefore, dedicated Eph-receptor effectors might act in a cell type-specific manner. The significant number of proteins with predicted GAP or GEF domains encoded in mammalian genomes would make such a scenario possible. Alternatively, multiple Rho-GTPase regulators might act sequentially within the same cell type. Studies in retinal axons reported ephrin-induced down-regulation of Rac activity within minutes followed by an increase of active Rac levels over the next 30 minutes (Jurney et al., 2002). Therefore, α2-chimaerin-mediated Rac-inactivation might represent an early step in EphA4-mediated responses followed by an increase in active Rac through the Rac-GEF activity of Vav-2 and/or Vav-3, which might result in Eph-receptor endocytosis and termination of signaling (Cowan et al., 2005). In this latter scenario, only the first
α2-chimaerin-dependent step would exclusively rely on a single effector protein; whereas, the subsequent steps might be controlled through multiple overlapping pathways. Another explanation for why α2-chimaerin might be functioning non-redundantly with other GAPs is, due to its domain composition, which allows it to integrate different signals. It can bind DAG via its C1 domain and phospho-tyrosine with its SH2 domain. The crystal structure of β2-chimaerin, which is very similar in its primary amino acid sequence to α2-chimaerin, suggests that the GAP domain is covered by the SH2 and C1 domain and therefore cannot bind a GTPase (Canagarajah et al., 2004). It requires binding of the SH2 to phospho-tyrosine residues and binding of the C1 domain to DAG to make the GAP domain accessible and thereby activate it. This means it is possible that α2-chimaerin functions as an integrator of different upstream signals and that therefore a loss of α2-chimaerin has a more drastic effect than the loss of other RhoGTPase regulators that do not integrate multiple upstream signals. It would be interesting to know which extracellular signal is responsible for the increase in DAG levels that possibly activate α2-chimaerin. It is however possible that the rise in DAG levels has its origin in the activation of the EphA4 receptor, since it can bind and activate Phospholipase-C-γ (PLCγ) itself (Zhou et al., 2007). Regardless of the exact mechanism, our data demonstrate that α2-chimaerin is essential for EphA4 signaling in spinal locomotor circuits.

**Is the α2-chimaerin Phenotype Selective for Locomotor Circuits?**

The second important observation in our study is the selectivity of the α2-chimaerin phenotype. Previous studies highlighted axon guidance defects in several central regulators of the cytoskeleton including the Rho-GTPases, PI3-kinase, Abelson kinase, and Ena/VASP family proteins which are common signaling mediators for multiple neuronal surface receptors (Ng and Luo, 2004); (Chang et al., 2006); (Lebrand et al., 2004); (Bashaw et al., 2000); (Wills et al., 1999). In contrast, defects in α2-chimaerin appear to be rather selective for EphA4 as the α2-chimaerin mutant mice do not show any overt abnormalities besides the locomotor defects and have overall normal brain morphology. Since our study on EphA4- α2-chimaerin signaling three other studies have been published, which agree with our findings in almost all points (Iwasato et al., 2007; Shi
et al., 2007; Wegmeyer et al., 2007). Remarkably the α-chimaerin knock-out mice created by Iwasato et al. and Wegemeyer et al., respectively show the same selectivity for the axonal guidance phenotype in locomotor circuits. Previous work suggested a function for α2-chimaerin in semaphorin-induced growth responses in cultured DRG neurons (Brown et al., 2004). We found that plexinA1-dependent central projections of proprioceptive afferents were not perturbed in α2-chimaerin mutants. Therefore, α2-chimaerin is either not involved in plexin signaling in vivo or other Rho-regulators, including other chimaerin isoforms, act redundantly with α2-chimaerin in this pathway. Interestingly mutations leading to a hyperactivation of α2-chimaerin in humans are linked to Duane’s retraction syndrome a condition in which the lateral rectus muscle, an extraocular muscle, fails to be innervated by its natural afferent, the cranial nerve and instead is aberrantly innervated by a branch of the occulomotor nerve (Miyake et al., 2008). Eph receptors and ephrins are expressed in the developing cranial motor nuclei in rodents and therefore it is possible that mutant α2-chimaerin could be hyperactivated by Eph signaling during occulomotor axon guidance (Miyake et al., 2008). It is striking how two mutations with opposing effects in the same widely expressed molecule, one leading to inactivation the other to hyperactivation, can result in anatomically restricted and none overlapping phenotypes. Although overall brain morphology in α2-chimaerin mutant mice seemed to be unaffected, we did not analyse other EphA or EphB dependent axonal projections in detail. Our biochemical studies also suggest an interaction of α2-chimaerin with EphB receptors in vitro. This warrants future studies on the role of α2-chimaerin to test wether α2 chimaerin is a dedicated effector for EphA4 or wether it also transduces EphB receptor signaling in vivo.

**The Creation of an α2-chimaerin Conditional Allele as Tool to Dissect the Function of Neuronal Locomotor Circuits**

The synchronous hindlimb movement, exhibited by α2-chimaerin gene-trap mutants, could have its origin in the aberrant projections of CST neurons or spinal cord interneurons. To dissect between the contribution of the miswiring of CST and spinal interneuron axons to the hopping gait, we specifically ablated α2-chimaerin in neurons of the forebrain, (which includes the neurons
of the motor cortex giving rise to the CST) or the spinal cord, using mouse lines expressing Cre-recombinase under control of the Emx1 or Hb9 promoter, respectively (Arber et al., 1999; Guo et al., 2000). Neither the mice with a forebrain specific knock-out of α2-chimaerin (α2Emx1−/−), nor the animals with a spinal cord specific knock-out (α2Hb9−/−) show the characteristic hopping gait. However the complete ablation of the conditional allele in both mouse lines awaits confirmation.

If α2-chimaerin is indeed ablated under those conditions, it would suggest that neither the aberrant bilateral projections of CST axons nor the aberrant midline crossing of spinal interneuron axons alone, is sufficient to induce synchronous hindlimb movements. It is possible that Emx1-Cre or Hb9-Cre is not expressed in the neurons of the CST or spinal cord that are responsible for the control of alternating hindlimb movements. To exclude this possibility, it is possible to breed α2Emx1−/− mice with α2Hb9−/−, to create mice with an α2-chimaerin ablation in the forebrain as well as spinal cord (α2Emx1−/−/Hb9−/−). If the combined conditional knock-out with Emx-Cre and Hb9-Cre, indeed targets the neuronal populations that work together to induce synchronous hindlimb movements in α2−/− mice, the α2Emx1−/−/Hb9−/− mice should exhibit the hopping gait. If the α2Emx1−/−/Hb9−/− mice do not exhibit synchronous hindlimb movements it is likely that the misprojection of a population of neurons that does not express Emx1 or Hb9 is responsible for the hopping gait. In this case it could help to cross α2Emx1−/− and α2Hb9−/− with a GFP reporter, containing a conditional Cre inducible GFP transgene. That way the neurons expressing Emx-Cre and Hb9-Cre could be identified and excluded as potential source of the hopping gait. These studies could improve our understanding of the importance of different neuronal populations in the control of hindlimb movements.

**Conclusion**

Our studies identified α2-chimaerin as essential downstream effector of EphA4/ephrinB3 signaling during the formation of CST and spinal interneuron projections in the mouse. Lack of α2-chimaerin expression phenocopies the behavioral as well as anatomical abnormalities observed in EphA4-receptor knock-out mice. Furthermore α2-chimaerin binds via its SH2 domain to activated/phosphorylated EphA4 receptors. This suggests a signaling pathway in growing
axons, in which EphA4 phosphorylation upon binding to ephrinB3 at the surface of spinal cord midline cells, recruits and activates α2-chimaerin. α2-chimaerin downstream inactivates Rac1 (Iwasato et al., 2007), which would contribute to a reduction in F-actin polymerization and thereby promote axonal growth cone repulsion from the midline (Figure 2.12).
FIGURES AND LEGENDS

FIGURE 2.1: The α2-Chimaerin SH2 Domain Interacts with Eph – Tyrosine Kinase Receptors.

(A) Schematic diagram of α1- and α2-chimaerin isoform domain structure. Both isoforms share identical diacylglycerol-binding C1 and GTPase-activating protein (GAP) domains; α2-chimaerin contains an additional unique N-terminal Src-homology-2 (SH2) domain. (B) Pull-down assays from cortical lysates with GST control beads (GST), or GST-SH2 beads carrying the purified recombinant α2-chimaerin SH2 domain (SH2). Input lysates (input) and bound proteins (GST or SH2) were probed in western blots for EphA4 and TrkA. (C) EphA4, EphB1, or TrkA were over expressed in HEK293T cells and incubated with GST control beads or GST-SH2 beads. Blots show an aliquot of the total cell lysate (input) or proteins bound to GST beads or GST-SH2 beads probed with anti-EphA4 (A4), anti-EphB1 (B1), anti-TrkA, or anti-phosphotyrosine antibodies (p-Tyr).
FIGURE 2.2: Binding of the α2-Chimaerin SH2 Domain to Eph Receptors is Phosphotyrosine Dependent.

(A) GST pull-down with GST control beads or GST-SH2 beads from HEK293T cell lysates expressing full-length wild-type EphA4 (A4), EphA4-V635M (KD), EphA4-Y596E/Y602E (EE), or EphA4-Y596E/Y602E/V635M (EE/KD). Bound proteins were probed with anti-EphA4 antibodies, and an aliquot of the cell lysate was probed with anti-EphA4 and anti-phosphotyrosine antibodies (p-Tyr).

(B) GST pull-down with GST control beads or GST-SH2 beads from HEK293T cell lysates expressing full-length wild-type EphB1 (B1), EphB1-K651Q (KD) EphB1-Y919F (Pinto et al.), EphB1-Y588E/Y594E (EE). (C) GST pull-down with GST control beads or GST-SH2 beads from HEK293T cell lysates expressing full-length wild-type EphA4 (A4). Beads and lysates were incubated in the presence of DMSO or Phorbol 12-Myristate 13 Acetate / PMA or EphA4 was dephosphorylated by preincubation with λ-Phosphatase (λ).
FIGURE 2.3: α2- and α1-Chimaerin Bind to the Intracellular Domain of EphA4

(A) HEK293T cells overexpressing full-length HA-tagged α2-chimaerin or α1-chimaerin were incubated with beads carrying purified recombinant maltose-binding protein (MBP), MBP fused to the intracellular domain of EphA4 (A4), MBP fused to the intracellular domain of EphA4 treated with λ-phosphatase (A4-λ), MBP fused to the intracellular domain of EphA4-Y596E/Y602E (A4-EE), or MBP fused to a kinase-dead mutant (V635M) of the intracellular domain of EphA4 (A4-KD). Bound proteins were detected with anti-HA antibodies. (B) α2-chimaerin is phosphorylated upon co-expression with EphA4 in HEK293T cells. α2-Chimaerin was immunoprecipitated from lysates of control HEK293 cells or cells expressing α2-chimaerin, α2-chimaerin and EphA4, or α2-chimaerin and kinase-dead EphA4 (A4-KD). Immunoprecipitates were probed with antibodies to phosphotyrosine antibodies (p-Tyr) or α2-chimaerin (α2). Expression of EphA4 in cell lysates was confirmed with anti-EphA4 antibodies (EphA4).
FIGURE 2.4: Characterization of α2-Chimaerin Gene Trap mice.

(A) The genomic locus of α-chimaerin contains two independent promoters (arrows) that drive transcription of the α1- and α2-chimaerin isoforms. The gene-trap cassette containing a lacZ reporter is inserted upstream of the putative α1-chimaerin promoter. (B) Western blots and RT-PCR of brain and spinal cord lysates of adult wild-type (+/+), heterozygous (+/-), and homozygous (-/-) gene trap animals. α1- and α2-chimaerin isoform-specific antibody blots and RT-PCR demonstrate that gene-trap homozygous mutants have a specific reduction of the α2-chimaerin protein and mRNA in both brain and spinal cord lysates, respectively. (C) Analysis of α2-chimaerin expression using the lacZ reporter inserted within the gene trap cassette. Wild-type animals (upper panels) do not show any detectable β-galactosidase activity or significant lacZ staining. In mice heterozygous for the gene trap insertion (lower panel), β-galactosidase activity is detected throughout the cortex of P5 animals except for layer I (left panel). Immunostaining with anti-LacZ antibodies (punctate red staining) of P5 cortex reveals overlap with the cortical layer V specific marker CTIP2 - positive cells (green), and widespread neuronal nuclei, identified by immunoreactivity for NeuN (blue). In the lumbar spinal cord, expression was observed throughout the cord, including motor neurons (identified by their large cell bodies located in the ventral part of the spinal cord), and in neurons in the ventral area shown at higher magnification on the right. Scale bar is 100 µm.
FIGURE 2.5: α2-Chimaerin Mutant Mice Show Locomotor Abnormalities

(A) Horizontal brain sections from adult wild type and α2-chimaerin mutants visualizing the anterior / aAC and posterior / pAC sections of the anterior commissure. Scale bar is 1 mm. (B) Images and footprints of wild-type animals display an alternating hindlimb gait, while α2 gene trap mutants display a synchronous rabbit-like hopping gait. Direction of animal movement is from left to right. Blue represents the left forelimb, red is right forelimb, green is left hindlimb, and yellow is right hindlimb. Scale bar is 1 cm.
FIGURE 2.6: Impaired Ephrin Induced Growth Cone Collapse in α2-Chimaerin Mutant Axons.

(A) Representative examples of time-lapse imaged growth cones from wild type and α2 mutants before and after application of ephrinA1-Fc (1 mg/ml). Scale bar is 10 μm. (B) Quantification of the percentage of growth cones collapsing after application of recombinant Fc protein or ephrinA1-Fc for explants from wild type and α2-chimaerin mutant mice. The number of growth cones imaged per condition was 34 (wild-type + Fc), 85 (wild-type + ephrinA1), 27 (mutant + Fc), 113 (mutant + ephrinA1). Error bars are SEM. (C) Growth rate before and after addition of EphrinA1 measured for 15 min before and 30 min after addition of ephrinA1-Fc. Growth cone advancement is shown as positive, retraction as negative values. Error bars are SEM. (D) The expression level of EphA4 is similar at the growth cones of axons from cultured cortical neurons of wild type and α2-chimaerin mutant mice. Staining for the EphA4 receptor (red) and phalloidin (green). Scale bar is 10 μm. (E) Western blots of lysates of the cortical explants used in the collapse assays from P1 wild type (+/+) and homozygous (-/-) gene trap animals. Detection with EphA4- and α1-chimaerin specific antibodies demonstrates unchanged levels of total EphA4 and α1-chimaerin protein levels. The same blot was stripped and reprobed with an anti-tubulin antibody as a loading control.
**FIGURE 2.7: Spinal Interneurons have Aberrant Projections that Cross the Midline in α2-Chimaerin Mutant Spinal Cord.**

(A) Schematic diagram of the lumbar spinal cord highlighting the site of unilateral rhodamine-dextran application (red rectangle at L4) and the labeling observed in the contralateral L2 segment (blue dashed box). (B) Retrograde labeling using rhodamine dextran amine to determine the contralateral projections in wild-type mice, α2-chimaerin -/- mutant mice, and α2 chimaerin +/-; EphA4 +/- double heterozygous mice. The boxed area is enlarged in the lower panel. Many fibers cross the midline below and above the central canal (white circle) in the mutant spinal cords. Scale bar is 100 μm. (C) α2-chimaerin -/- mutants and α2-chimaerin +/-; EphA4 +/- double heterozygous mutant mice exhibit a more shallow and widened dorsal funiculus (DF) and an expansion of the dorsal gray matter above the central canal as compared to wild-type animals. The quantitation shows the ratio of height/width measurements for the dorsal funiculus (n > 4 mice per genotype, 3 sections per animal). Compared to wild-type mice, the DF morphology was most severely altered in α2-chimaerin -/- mutant mice (p < 0.01, Bonferroni’s multiple comparisons test). No significant differences were observed when comparing wild-type and single heterozygous mutant mice (p > 0.05), whereas α2-chimaerin+/--; EphA4+/− double heterozygous mutants were significantly different from each single heterozygous mutant as well as from wild type and α2-chimaerin -/- homozygous mutants (p < 0.01). The central canal is marked by a white circle; the dashed lines represent measures of height and width of the dorsal funiculus.
FIGURE 2.8: Projections of Parvalbumin Positive Proprioceptive Afferents are Unperturbed in \( \alpha_2 \)-Chimaerin Mutant Animals.

Schematic diagram of parvalbumin-positive proprioceptive sensory neuron trajectory in the dorsal spinal cord (green). The red dotted lines illustrate trajectory observed in plexinA1 mutant mice (Yoshida et al., 2006). The parvalbumin-positive axon projections in spinal cords from postnatal day 0 animals (green) do not show any significant differences between wild type and \( \alpha_2 \)-chimaerin mutants. White asterik marks the dorsal funiculus. Scale bar is 100 \( \mu \)m.
FIGURE 2.9: Aberrant Cortico Spinal Tract Projections in α2-Chimaerin Mutant Mice.
(A) Schematic diagram of injection site and anterograde labeling of corticospinal tract axons. (B) Darkfield micrographs of anterogradely labeled CST projections in the spinal cord of wild type and α2-chimaerin mutant animals shown in horizontal (left) and transverse (right) sections of the cervical spinal cord. The column on the right shows an enlargement of the midline area below the DF in the transverse section. Scale bar is 100 µm; error bars are SEM. (C) Quantification of axonal labeling was performed by placing a 100 x 3 x 100 µm region of interest in the ipsilateral or contralateral gray matter and measuring tracer positive pixels to estimate axon density on each spinal hemisegment (n = 5 animals per genotype, 4 sections per animal).
FIGURE 2.10: Cortical Stimulation Evokes Bilateral Hindlimb Movement in α2-Chimaerin Mutant Mice.
(A) Representative EMG data from contralateral and ipsilateral hindlimb muscles in response to threshold cortical stimulation (20 mA) in wild type and α2-chimaerin mutant animals. Scale bar is 5 mV. (B) Average stimulus intensity measurements for eliciting contralateral or ipsilateral hindlimb flexion in wild type and α2-chimaerin mutant animals. Error bars are SEM.
FIGURE 2.11: Creation of a α2-Chimaerin Conditional Knock-out Mice

loxP sites were inserted in intron 1 and intron 3 of the Chn1 gene. This specifically labels only α2-chimaerin (green, exons 1-6 are α2-chimaerin specific exons) for CRE recombinase mediated excision since the loxP sites are inserted upstream of the promoter giving rise to the α1-chimaerin transcript (blue, exons 7-13). An FRTneoFRT selection cassette encoding a neomycin resistance gene was inserted into intron 3 to allow for the positive selection of ES cell clones containing the targeted Chn1 locus. The FRTneoFRT cassette was removed by crossing the mice with the targeted locus to mice carrying a FLIP recombinase transgene. Mice carrying the knock-in locus where crossed to different mouse lines expressing the CRE recombinase under control of cell type specific promoters Emx (forebrain) or Hb9 (spinal cord). Splicing of exon 1 into exon 4 is out of frame.
FIGURE 2.12: α2-Chimaerin mediates Growth Cone Repulsion Downstream of EphA4

left: During Axonal Growth α2-chimaerin resides in an inactive state in the cytoplasm and F-actin is assembled downstream of Rac1 signaling. Upon encountering the midline, the EphA4-receptor clusters and autophosphorylates (P) due to binding to its ephrinB3 ligand. α2-chimaerin binds to phosphotyrosine residues in EphA4, which induces conformational changes that un-block the GAP domain. α2-chimaerin catalyzes the GTP hydrolysis by Rac1, und thereby inactivates it. Rac1 does not induce F-actin assembly anylonger, which contributes to cytoskeletal instability and promotes axonal growth cone repulsion from the midline.
CHAPTER 3

A POTENTIAL ROLE FOR MSYD-1 IN PRESYNAPTIC ASSEMBLY
PREFACE

Synapses are highly specialized cell-cell junctions, where electrical signals from the presynaptic cell are relayed as chemical signals to the postsynaptic cell. They are composed of a presynaptic and postsynaptic compartment. The presynaptic compartment, also referred to as presynaptic bouton, contains neurotransmitter filled synaptic vesicles that are released in response to an action potential. The postsynaptic membrane is prepped with receptors to detect the neurotransmitter. The fusion of synaptic vesicles with the presynaptic membrane is regulated by a complex protein network at the active zone, the cytomatrix at the active zone (CAZ). The structure of this protein network therefore is essential for synaptic function. Small changes in the composition of the CAZ can lead to disturbances in synaptic function (Schoch and Gundelfinger, 2006). Thus it is of major interest to understand the molecular mechanism that lead to proper CAZ assembly. My second dissertation project was concerned with understanding the molecular mechanisms underlying the assembly of the CAZ with a particular focus on the identification of a novel regulator in this process.

Presynaptic active zone formation is thought to be a stepwise assembly process, in which the localization of active zone proteins to the presynaptic membrane depends on their recruitment through other active zone components. Considering this model it is remarkable that most proteins of the vertebrate CAZ do not seem to be essential for active zone assembly. Knock-out mice for major CAZ components like RIM1, ELKS2 or Nrxx show changes in synaptic transmission but have a surprisingly normal synaptic ultrastructure (Kaeser et al., 2009; Missler et al., 2003; Schoch et al., 2002a). Similarly, in *C. elegans* loss of function mutants for elks-1/ELKS, unc-10/RIM, unc-13/Munc-13 or unc-18/Munc-18 have severe defects in synaptic transmission, although localization of synaptic vesicles and other CAZ components is unaffected (Patel et al., 2006). However, in recent studies the protein synapse defective-1, SYD-1, has emerged as a key factor in presynaptic assembly. In *syd-1* mutants synaptic vesicles and multiple proteins of the CAZ are mislocalized to non-synaptic regions. The SYD-1 homologue in *Drosophila*, DSYD-1, is essential for the assembly of the CAZ at the fly NMJ (Owald et al., 2010). Dsyd-1 mutants form smaller presynaptic terminals with fewer release sites. The remaining active zones are often
structurally abnormal and ectopic accumulations of Bruchpilot/ELKS form in axons.

SYD-1 is the only protein known to date to have such a far-reaching effect on presynaptic structure. No vertebrate orthologue of SYD-1 has been described so far. In this chapter I will describe the identification and characterization of two mouse orthologues of SYD-1.

I started to work on this project while our laboratory was still located at Columbia University in New York. My initial work focused on identifying and cloning two potential mouse syd-1 orthologues (msyd-1A and msyd-1B). From there I went on to raise specific antibodies and to create knock-out mouse models. Using these tools I characterized mSYD-1A protein expression and function in presynaptic assembly in vivo. After our laboratory moved to the Biozentrum at the University of Basel, Corinna Wentzel a new PhD student in our laboratory, joined me on the project. The characterization of mSYD-1A GAP activity as well as the interaction with Liprin-α is her work.
INTRODUCTION

Invertebrate SYD-1 in AZ assembly

SYD-1 was originally identified in a *C. elegans* forward genetic screen designed to identify genes that are necessary for the appropriate localization of synaptic vesicles at the presynaptic active zones of DD motorneurons (Hallam et al., 2002). SYD-1 itself is a protein component of the CAZ in *C. elegans* and localizes exclusively to the presynaptic bouton. In *syd-1* loss of function mutants synaptic vesicles in DD motorneurons partially mislocalize to dendrites; in other neurons like the HSN motorneuron and the ASI sensory neuron they are retained in the cell body (Dai et al., 2006; Hallam et al., 2002). Besides synaptic vesicles many proteins of the CAZ fail to localize to their appropriate presynaptic localization in *syd-1* mutants, among those are: ELKS-1, LIN-7 (Veli), SAD-1 (SAD-kinase), GIT and even SYD-2/Liprin-α (Hallam et al., 2002); (Dai et al., 2006); (Patel et al., 2006). Interestingly a gain of function mutation in *syd-2* can rescue the *syd-1* loss of function phenotype. *Syd-2*’s rescue ability depends on the presence of *elks-1* with which it biochemically interacts (Dai et al., 2006). The interaction between SYD-2/Liprin-α and ELKS is conserved at the mammalian synapse (Ko et al., 2003a). The only protein found to be indispensable for SYD-1’s synaptic localization in *C. elegans* HSN neurons, is the adhesion protein SYG-1 (Patel et al., 2006). SYG-1 is required to specify the localization of presynaptic terminals in the *C. elegans* HSN neuron. In *syg-1* loss of function mutants SYD-1, synaptic vesicles and other presynaptic proteins mislocalize to non-synaptic sites (Shen et al., 2004). This suggests that SYD-1 functions directly downstream of SYG-1 in a hierarchically organized synapse assembly program. The *Drosophila* orthologue of SYD-1, DSYD-1 has been reported to biochemically interact with Bruchpilot/ELKS. Loss of function in *dsyd-1* results in fewer synaptic release sites and the formation of ectopic AZs. This is similar to the *C. elegans* phenotype in DD motorneurons, where AZs form in the dendrite. *In vivo* imaging of the developing fly NMJ reveals that DSYD-1 precedes DLiprin and Bruchpilot/ELKS at the nascent synapse (Owald et al., 2010). Those findings suggest a linear assembly process in which DSYD-1 is required for the recruitment of DLiprin and Bruchpilot/ELKS to the AZ.
Protein Domain Organization and Function in C. elegans SYD-1

The domain structure of SYD-1 gives some hints regarding its cell biological functions. SYD-1 as well as DSYD-1 contain an N-terminal PDZ domain, followed by a C2 domain and a C-terminal Rho GTPase activating (GAP) domain (Figure 3.1). In SYD-1 a Proline rich potential SH3 domain binding motif is found in addition, N-terminal to the C2 domain. PDZ and SH3 domain interactions are a common mode of protein-protein interaction between many proteins of the CAZ. Thus these domains could be involved in the coupling to other CAZ proteins. C2 domains can bind to phospholipids in a Ca^{2+} dependent manner, which offers the possibility that SYD-1 could be recruited to the presynaptic membrane in response to Ca^{2+} influx. RhoGAP domains interact with small RhoGTPases and thereby influence cytoskeletal dynamics. Based on this domain structure SYD-1 could exert its function by recruiting other CAZ proteins through protein-protein interactions and regulation of the stability of the presynaptic cytoskeleton. The SYD-1 PDZ domain was shown to be insufficient for its synaptic localization (Hallam et al., 2002). The C2 domain mediates membrane clustering but does not mediate an exclusively synaptic localization (Hallam et al., 2002). Therefore SYD-1 synaptic localization might depend on a synergy between the C2 domain and protein interactions or might require stoichiometric complexes with additional proteins.

C. elegans SYD-1: a Functional GAP?

Interestingly the RhoGAP domain in C. elegans SYD-1 differs in several key residues from previously characterized Rho-GAP proteins. Functional and crystallographic studies identified key amino acid residues that are essential for binding to RhoGTPases and the stimulation of Rho protein mediated hydrolysis (Scheffzek and Ahmadian, 2005). C. elegans SYD-1 deviates in two key residues from the conserved consensus sequence: instead of an arginine in position 723 SYD-1 contains a valine, and an asparagine in position 839 is replaced by an arginine. In other GAPs the arginine has been shown to be necessary to efficiently catalyse the hydrolysis of GTP to GDP (Graham et al., 1999) and the conserved asparagine has been suggested to be important for the stability of the RhoGTPase-GAP complex (Rittinger et al., 1997). For SYD-1 the functional
consequences of these amino acid alterations compared to the characterized consensus sequence have not been tested. However, it has been hypothesized that SYD-1 may function as an interaction platform for RhoGTPases and additional proteins (Hallam et al., 2002). Physiological importance of the GAP domain was demonstrated by expression of a GAP deletion construct (GAPΔ). GAPΔ expressed in C. elegans DD motorneurons in a wildtype background, resulted in morphological defects like longitudinal axon defasciculation, premature axon stop and neurite outgrowth arrest, presumably representing a dominant negative. The GAP activity of C. elegans SYD-1 has never been analysed directly, therefore the possibility of SYD-1 functioning as a conventional GAP should not be excluded.

**SYD-1: a Presynaptic GAP that Links Trans-Synaptic Adhesion with Cytoskeletal Dynamics?**

In C. elegans SYG-1 is a heterophilic adhesion protein that through the interaction with its binding partner SYG-2, on vulval epithelial cells, specifies the localization of presynaptic boutons in HSN neurons (Shen et al., 2004). SYD-1 mislocalizes to non-synaptic sites in the HSN neuron of syg-1 mutants. Interestingly in syd-1 mutants CAZ components are mislocalized albeit the presence of SYG-1 at synaptic sites (Patel et al., 2006). Those observations suggest that SYD-1 can link trans-synaptic adhesion with presynaptic assembly. This raises the question of how it regulates presynaptic assembly? One possibility is by recruiting CAZ proteins through protein-protein interactions the other is by influencing the stability of the presynaptic actin cytoskeleton. The first hypothesis is supported by the observation that C. elegans as well as Drosophila SYD-1 can directly interact with Bruchpilot/ELKS (Owald et al., 2010; Patel and Shen, 2009). The second hypothesis is supported by SYD-1 containing a GAP domain via which it could signal to Rho GTPases. It is also possible that both mechanisms co-exist.

**The Actin and Microtubule Cytoskeleton in Presynaptic Assembly**

Actin dynamics are required for almost every step during neural development. During neural migration, actin polymerizing at the leading edge of the cell and disassembling at the opposite
end moves the cell in a targeted direction (Mogilner and Keren, 2009). Similarly during axon guidance F-actin assembly and disassembly is responsible for growth cone dynamics, enabling the growth cone to explore its environment for attractive or repulsive cues and to respond to them in an intricate manner (Geraldo and Gordon-Weeks, 2009). The growth of elaborate dendritic and axonal arbors requires actin dynamics as well as the growth of dendritic spines during development and structural plasticity (Matus, 2000). Comparably little is known about the role of actin in presynaptic assembly. Early ultrastructural studies show that a dense actin network is present in the presynaptic bouton and connects to synaptic vesicles (Hirokawa et al., 1989). The role of F-actin at the presynaptic terminal seems to be that of a scaffold retaining presynaptic proteins like synapsin (Sankaranarayanan et al., 2003). F-actin is concentrated at developing presynaptic terminals in 5 day in vitro (DIV) hippocampal neuron cultures and inhibition of actin polymerization (with lantrunculin) results in the dispersal of synaptic vesicles as well as the CAZ protein Bassoon, suggesting that early synaptic vesicle clusters and presynaptic proteins are tethered by actin (Zhang and Benson, 2001). However, in cultures at 10-13DIV synaptic vesicles and Bassoon localization remain unchanged after treatment with lantrunculin, indicating that actin is necessary for the development but not the maintenance of presynaptic structures (Zhang and Benson, 2001). In Drosophila motorneurons the knock-down of alpha-spectrin (an actin crosslinking protein) results in a severe disruption of the synaptic MT cytoskeleton and in the disappearance of synapsin (synaptic vesicles), Bruchpilot (T-bars) and Fascilin II (a homophilic adhesion protein) from the presynaptic bouton, which eventually results in the retraction of the entire bouton (Pielage et al., 2005). All of the studies above suggest that a disruption of the presynaptic actin or tubulin cytoskeleton either directly or indirectly result in defects of presynaptic assembly or lead to synaptic instability.

The formation of release competent presynaptic terminals containing synaptic vesicles, bassoon, RIM, F-Actin, Cav2.2 and N-cadherin in hippocampal neurons in culture, can be triggered in axons contacting Poly-D-Lysine (PDL) coated beads (Lucido et al., 2009). If cultures are treated with lantrunculin, the clustering of synaptic vesicles in the axon touching a bead is abolished, therefore F-actin reorganization seems to be a critical step in nascent presynaptic
assembly in response to extracellular stimuli (Lucido et al., 2009). Several adhesion molecules involved in synapse formation have been found to link to the actin cytoskeleton. Most is known about the link between N – Cadherin and the actin cytoskeleton via α-, β- and p120 catenin (Hülsken et al., 1994). In non-neuronal cells E-Cadherin, signals upon homo-dimerization via activated Rac1 to the Arp2/3 actin nucleation complex and thereby induces actin polymerization at sites of adhesive contacts (Kovacs et al., 2002; Kraemer et al., 2007). Downstream of Nrx a role for a LIN-2/CASK - neuronal Protein 4.1N interaction has been proposed to play a role in the localized assembly of F-actin - spectrin filaments (Biederer and Sudhof, 2001; Cohen et al., 1998).

Although the function of the actin cytoskeleton is crucial for presynaptic assembly we still know little about how changes in intracellular presynaptic actin dynamics are triggered by extracellular signals. As described in previous chapters, the small GTPases of the Rho family and their GEF and GAP regulators, play a central role in linking transmembrane receptors with intracellular actin dynamics.

Considering the critical role that SYD-1 plays in invertebrate synapse assembly we hypothesized that its function might be conserved at vertebrate synapses. In order to investigate a role for SYD-1 in presynaptic assembly in the mouse, we decided to focus on following points: 1) Does an orthologue of syd-1 exist in the mouse? 2) Is it expressed in neurons, and does it localize to synapses? 3) Is its GAP domain functional? 4) Does it interact with other CAZ components as e.g. ELKS or Liprin-α? And finally 5) Is it essential for presynaptic assembly?

RESULTS

Identification of Potential Vertebrate Orthologues of C. elegans SYD-1

No human or mouse orthologues of C. elegans SYD-1 have been described so far. Therefore we employed a bioinformatics based sequence analysis to identify candidate orthologues of C. elegans syd-1. We used a “protein Blast” search from NCBI with the primary amino acid
sequence of *C. elegans* SYD-1. The search led to the identification of two potential mouse and human orthologues: Syde1 (mSYD1A and hSYD1A; NP_082151.1) and Syde2 (mSYD1B and hSYD-1B; NP_001159536) based on their similarity to *C. elegans* SYD-1 in the primary amino acid sequence of the GAP domain. In zebra fish and chick a single protein each was identified as potential orthologue. The next closest mouse proteins identified with the Blast search were isoforms 1-3 of the active break point cluster region protein (Bcr) and isoforms 1-3 of myosin-IXb. None of these proteins are good candidates to be orthologues of SYD-1 since their predicted domain structure is very different from that of *C. elegans* SYD-1. Bcr contains in addition to a RhoGAP and a C2 domain also a RhoGEF and PH domain. Myosin-IXb contains a Ras association domain, an ATPase domain, a calmodulin binding domain, a C1 domain and a RhoGAP domain. Indeed no known mouse or human proteins show the exact same domain composition and arrangement as *C. elegans* SYD-1 as assessed with a “conserved domain architecture” (cdart) BLAST search on the NCBI webpage. But of all identified mouse proteins mSYD-1A and mSYD-1B resemble the SYD-1 structure the closest.

The genes of the predicted mouse orthologues SYD1A and SYD1B are found on chromosomes 10 and 3, respectively. Analysis of their protein structure with the protein domain prediction tool “SMART” predicted a C2 (Ca²⁺ dependent lipid binding domain) and a Rho-GAP domain for SYD1A as well as SYD1B. However, for neither protein a PDZ domain was predicted, which presents a significant difference to the predicted protein structures of *C. elegans* and *Drosophila* SYD-1 (DSYD-1) (Figure 3.1). The same is the case for the predicted *danio rerio* (zebrafish) and chick orthologues. Despite the absence of a PDZ domain, mSYD-1A and mSYD-1B both have a long N-terminal domain (NTD), which may serve an analogous function. Although no known protein domains are predicted for the NTD it could be involved in regulating inter- or intramolecular protein interactions. It is also important to keep in mind, that the PDZ domain of *C. elegans* SYD-1 alone cannot localize to presynaptic sites, therefore the absence of a PDZ domain from the SYD-1 vertebrate orthologues does not preclude a synaptic localization.

To understand when during evolution the PDZ domain might have been lost we created a phylogram from the alignment of the primary amino acid sequences of all potential
SYD-1 orthologues from mouse, human, zebrafish and chick (Figure 3.1). The phylogram was created with the “constrained-based multiple alignment tool” COBALT from NCBI. Since the potential zebrafish orthologue of SYD-1 had previously been described as a homologue of β2-chimaerin (Leskow et al., 2006), β2-chimaerin was included in the phylogram as well. The branch lengths in the phylogram are proportional to the amount of inferred evolutionary change. The distances in the phylogram suggest that mSYD-1A and hSYD-1A as well as mSYD-1B and hSYD-1B are closely related. All predicted vertebrate SYD-1 orthologues (chick, zebrafish, mouse and human) segregate together from the invertebrate SYD-1s. β2-chimaerin shows the largest evolutionary distance from SYD-1 and does not co-segregate with the predicted vertebrate orthologues of SYD-1. The phylogram suggests that the PDZ domain was lost at the time of segregation of invertebrates and vertebrates.

In order to test if expression of msyd-1A or msyd-1B in C.elegans can rescue the loss of function phenotype in syd-1 mutants we collaborated with Yishi Jin (USCD) and Hidenori Taru (Hokkaido University, Japan). Neither expression of full length msyd-1A nor msyd-1B cDNA rescued the syd-1 loss of function phenotype in GABAergic (DD) motor neurons in C.elegans (data not shown). This can be interpreted in several ways, one of them being that the PDZ domain of SYD-1 is essential for its synaptic function. However, this would not exclude that SYD-1A/B may play a similar role in presynaptic assembly as C. elegans SYD-1 since it is possible that the function of the PDZ domain at vertebrate synapses has been taken over by a SYD-1A/B interacting protein.

**Expression Analysis of mouse SYD-1**

For mSYD-1A and -1B to contribute to vertebrate synaptogenesis, the protein should be expressed in the developing nervous system. We first used RT-PCR to detect RNA transcripts in various mouse tissues. As a second step we produced mSYD-1A and mSYD-1B specific antibodies, to analyze protein expression.

*mSyd-1A and msyd1B Transcripts are Present in the Brain: RT-PCR Expression Analysis*

RNA was extracted from various adult mouse tissues (muscle, kidney, liver, lung, spleen and
brain) and subjected to RT-PCR. mSyd-1A and -1B RNA products were detected, using primers that span across two exons to avoid the detection of genomic DNA. The RT-PCR analysis showed a widespread expression for msyd-1A as well as msyd-1B RNA (Figure 3.2A). Although the same amount of RNA was used for each tissue specific RT-PCR the method is not sufficiently accurate to make a statement about the relative expression levels between the different tissues. We concluded that msyd-1A and msyd-1B RNA is present in muscle, kidney, liver, lung, spleen and brain and that it is likely that the corresponding proteins are present in those tissues as well. Further we analyzed transcript expression in the brain between embryonic day 14 (E14) and the adult. msyd-1A and msyd-1B transcripts are present as early as E14 and remain to be expressed in the adult (Figure 3.2A). We did not detect major differences in transcript expression levels between different ages. From this we can conclude that msyd-1A and -1B are transcribed in the brain during the developmental timeframe of neuronal migration, neurite outgrowth, axonal and dendritic arbor growth and synapse formation.

Production of mSYD-1A specific Antibodies and Initial Expression Analysis

To obtain specific antibodies for mSYD-1A or mSYD-1B, we raised isoform specific anti-peptide antibodies for mSYD-1A as well as mSYD-1B. All antibodies were raised in rabbit against peptides specific for either protein. We were unsuccessful in obtaining an antibody that recognizes endogenous mSYD-1B in brain lysates. Most anti-sera harvested after the immunizations with mSYD-1A specific peptides showed only a weak immunoreactivity for mSYD-1A overexpressed in HEK293 cells and no immunoreactivity on mouse brain lysates. Only immunization with the N-terminal peptide of mSYD-1A: MAEPLRKTFSRLRGREK (Figure 3.2C), resulted in an anti-serum that detected overexpressed as well as endogenous mSYD-1A. This serum was further purified by affinity chromatography on the antigen. Since we were unable to raise antibodies sensitive enough for the detection of mSYD-1B, we focused our subsequent studies mainly on mSYD-1A.

The purified anti-mSYD-1A antibody detected two proteins of approximately 100kDa and 80kDa, respectively in lysates from HEK293 cells as well as cultured cortical neurons (Figure
3.2B). The molecular weight predicted from the amino acid sequence for mSYD-1A is 80kDa. To confirm whether both bands or one of the bands represent mSYD-1A, we obtained msyd-1A specific siRNAs (Dharmafect) and treated HEK293 cells as well as cultured cortical neurons with msyd-1A siRNA or a control siRNA for two and six days, respectively. In both cases the 100kDa band was significantly reduced after treatment with the siRNAs (Figure 3.2B), whereas the lower band (especially in lysates from cortical neurons) was only weakly affected. The anti-mSYD-1A antibody also detected HA-tagged full-length mSYD-1A overexpressed in HEK293 cells on Western Blot, but did not show reactivity with HA-tagged overexpressed mSYD-1B (Figure 3.2D). It also detected proteins with the same migration pattern as overexpressed HA-mSYD-1A in lysate of untreated HEK cells, cultured cerebellar granule neurons and P5 brain (Figure 3.2D). We therefore concluded that the anti-mSYD-1A antibody specifically recognizes mSYD-1A at 100kDa and that mSYD-1A is expressed in brain, primary granule neurons, primary cortical neurons and HEK293 cells.

**Developmental and Regional Expression of mSYD-1A in the Brain**

The RT-PCR analysis did not reveal any changes in msyd-1A transcript levels in the mouse brain during development. Since the amount of transcript does not necessarily reflect the amount of protein present we asked at what levels mSYD-1A protein is expressed at different developmental stages. Mouse brains and dissected mouse cortices of different embryonic and postnatal (P) stages were lysed and probed on Western Blot for the presence of mSYD-1A (Figure 3.2E). mSYD-1A protein was present in the brain and cortex between E14 and adult, which is in agreement with the results of the RT-PCR analysis. Since mSYD-1A protein expression correlates with the peak period of synaptogenesis in the mouse brain (P0 – P7) (Li et al., 2010) it might play a role in the formation of the presynaptic AZ. At P5 mSYD-1A is expressed at similar levels in the cortex and the cerebellum (Figure 3.2F).
**Subcellular Localization of mSYD-1A**

The detection of a protein by immunostaining is the most accurate and direct way to determine its subcellular localization. However, our mSYD-1A antibody was not suitable for immunostaining applications. Therefore we turned to biochemical subcellular fractionation methods to determine SYD-1As subcellular localization.

*mSYD-1A is Found in the Insoluble Membrane Fraction after Subcellular Fractionation*

Since mSYD-1A is also expressed in HEK293 cells we initially applied the subcellular fractionation to HEK cells (Figure 3.3A). We employed a simple subcellular fractionation method that separates cellular components by differential centrifugation and gives rise to five fractions total. After a low speed centrifugation of the whole cell homogenate (H), a pellet containing nuclei, mitochondria and unbroken cells (P1) and a postnuclear supernatant containing the cytosol and membranes (PNS) are collected. The second high speed centrifugation of the PNS results in an insoluble pellet containing membranes and cytoskeletal proteins (P2) and a soluble fraction mainly consisting of cytosol (S2). mSYD-1A is enriched in the P2 membrane fraction together with the other synaptic proteins, as Munc-18 and LIN-2/CASK, that are also expressed in HEK cells. To assess the quality of the fractionation we probed for the ER intermediate compartment marker ERGIC53, the cytosolic proteins actin and tubulin and the nuclear Histone H3. As expected for a successful fractionation, Histone H3 is mainly found in fraction P1, which contains nuclei, ERGIC53 mainly in the membrane fraction P2 and actin (the antibody recognizes multiple isoforms) mainly in the cytosolic fraction S2 (Figure 3.3A).

To analyze the presence of mSYD-1A in the membrane/insoluble fraction of neurons we applied the same fractionation method to brain homogenate. Many presynaptic proteins are found in the insoluble membrane fraction (P2). This can be explained with the CAZ being a very dense protein structure that renders most of its protein components insoluble. We therefore expected to find mSYD-1A as potential CAZ component in the insoluble pellet P2. We found that in brain similar to HEK cells, mSYD-1A can be found in the membrane insoluble fraction together with the
CAZ proteins Munc-18, LIN-2/CASK and ELKS (Figure 3.3B). As quality control we probed for Neuroligin with a pan-Neuroligin antibody that recognizes all Neuroligin isoforms (neuronal membrane marker). Neuroligins were detected in the membrane fraction P2. Further we probed with an anti-actin antibody that recognizes multiple actin isoforms (cytosol marker, but often also found insoluble), which detected actin in the S2 as well as P2 fraction and an anti - Histone H3 antibody, which detected Histone H3 in fractions H and P1. (Figure 3.3B).

Our finding, that mSYD-1A behaves similar to other CAZ components in a subcellular fractionation from brain, supports the possibility that mSYD-1A is associated with the CAZ.

*mSYD-1A is Present at Synapses and is Enriched in the PSD Fraction after Synaptosomal Fractionation*

To address if mSYD-1A is found at synapses we isolated synaptosomal membranes from brain homogenate of P10 mouse brains. Initially, the whole brain homogenate (H) was subjected to a low speed centrifugation at 1000 x g to yield the supernatant S1, containing brain cytosol and membranes and the pellet P1, containing nuclei and unbroken cells. S1 underwent a high speed centrifugation at 10,000 x g to yield the supernatant S2, containing brain cytosol and the pellet P2, containing crude synaptosomes. The crude synaptosomes (P2) were washed once and subjected to a hypoosmotic shock, which causes them to burst open. The lysed synaptosomes were centrifuged at high speed (25,000 x g) giving rise to the supernatant S3, containing synaptic vesicles and soluble synaptic proteins and the pellet P3, containing synaptosomal membranes and associated proteins. After resuspension of P3 the suspension was subjected to a high speed centrifugation (150,000 x g) across a sucrose gradient (0.8M / 1.0M / 1.2M) which results in the collection of synaptic plasma membranes and associated proteins (SPM) at the interface of 1.0M and 1.2M sucrose. The SPM fraction was than extracted with Triton (0.5%) to yield the supernatant SPM-S1, comprised of Triton soluble, mainly presynaptic proteins and the pellet PSD, containing Triton insoluble protein complexes of the postsynaptic density as well as the CAZ. The protein concentration of each fraction was determined and equal amounts of protein
were loaded per lane on a polyacrylamide gel and probed with mSYD-1A, PSD95, VAMP2, LIN-2/CASK and actin antibodies in Western Blot (Figure 3.3C). mSYD-1A is present in the whole brain homogenate H and it seems to be enriched in the pellet P1 (nuclei, unbroken cells) over the supernatant S1 (brain cytosol) which can be explained with a significant amount of cells remaining intact during the homogenization. After high speed centrifugation of S1 mSYD-1A is enriched in the brain cytosol (S2) over the crude synaptosomes (P2), suggesting that more mSYD-1A in the brain is cytosolic than synaptic. PSD95 (a PSD component) and VAMP2 (a synaptic vesicle protein) in comparison are enriched in the crude synaptosomal fraction (P2) over brain cytosol (S2). After lysis of the synaptosomes equal amounts of mSYD-1A associate with the synaptic vesicles/soluble synaptic proteins fraction S3 and the synaptosomal membrane fraction P3. After further purification of the synaptosomal membranes across the sucrose gradient, only small amounts of mSYD-1A remain associated with the synaptic plasma membrane fraction (SPM), the same is the case for PSD95. However, after extraction of the SPM with Triton-X-100 mSYD-1A is strongly enriched in the Triton insoluble pellet PSD-1 as compared to the soluble supernatant SPM-S1. In this it behaves similar to PSD95 and LIN-2/CASK which also enrich in the PSD-1 fraction over the supernatant SPM-S1.

This fractionation shows that approximately half of mSYD-1A in the brain associates with synaptosomes (P2) and the other half is cytosolic (S2). Of the mSYD-1A associated with synaptosomes (P2) approximately half is found in a soluble pool that co-fractionates with soluble synaptic proteins and synaptic vesicles (S3) and the other half associates with synaptosomal membranes (P3). Almost all of the membrane associated mSYD-1A is found in the Triton insoluble PSD fraction (PSD-1). Since the PSD-1 fraction contains insoluble postsynaptic as well as presynaptic CAZ components, our finding that mSYD-1A is found in the PSD-1 fraction suggests that it can localize to the CAZ and interact with its components.

Functional Analysis of mSYD1A

To understand how mSYD-1A might be involved in intracellular signal transduction we decided to perform a structure-function analysis. Through its C2 domain mSYD-1A might couple to Ca$^{2+}$
signaling events and membrane phospholipids and via its GAP domain it might alter cellular RhoGTPase functions. In addition it has a long NTD predicted to be mainly unstructured, through which it could engage in protein-protein interactions. To predict downstream signaling effects of mSYD-1A it is crucial to know if its GAP domain is functional and ideally which RhoGTPases serve as its substrate. The active (GTP bound) small RhoGTPase, RhoA, has been associated with acto-myosin contractility, whereas the small RhoGTPases Rac1 and Cdc42, in their GTP bound state, seem to be associated with actin polymerization (Hall and Lalli, 2010). Thus inactivation of RhoA through mSYD-1A, could lead to a stabilization of F-actin in the presynaptic bouton. Since stable F-actin has been shown to be necessary for the clustering of synaptic vesicles and AZ proteins mSYD-1A acting on RhoA could promote the clustering of presynaptic components (Zhang and Benson, 2001).

mSYD1A and mSYD1B were identified as SYD-1 orthologues in a BLAST search mainly based on their similarity in the Rho-GAP domain. Notably, the GAP domain of C. elegans SYD-1 is related to the Rho-GAP family but deviates from the consensus Rho-GAP sequence at two crucial positions: it contains a valine at position 723 instead of an arginine (R723V) that has been shown to be essential for the catalytic activity of GAP domains (Graham et al., 1999). And at position 839 it contains an arginine instead of an asparagine (N839R) that has been shown to stabilize the GTPase-GAP complex (Scheffzek and Ahmadian, 2005). Similarly Drosophila DSYD-1 contains an arginine to alanine change in the position important for GTPase binding (Hallam et al., 2002). To analyze if mSYD1A and mSYD1B show the same amino acid deviations from the Rho-GAP consensus sequence the GAP domains of C. elegans SYD-1, DSYD-1, mSYD1A, mSYD1B, α-chimaerin and human Rho-GAP (hRhoGAP) were aligned (Figure 3.4). Neither mSYD1A nor mSYD1B show deviations from the consensus hRhoGAP sequence in any of the residues necessary for catalytic GAP activity (red, Figure 3.4). Therefore, we predicted that mSYD1A and mSYD1B are active GAPs. Interestingly, the GAP domains of mSYD-1A, mSYD-1B, SYD-1 and DSYD-1 show a higher similarity among each other than to hRhoGAP or α-chimaerin's GAP domain.
To test mSYD1A’s GAP activity we used a FRET (Förster Resonance Energy Transfer) based method developed by Itoh et al. (Itoh et al., 2002) (Figure 3.5). GTPases cycle between a GTP and GDP bound state. Small GTPases of the Rho family only have a very low intrinsic GTP hydrolysis activity and therefore require GAPs to catalyze the hydrolysis. GEFs are required to catalyze the exchange of GDP for GTP. In the GTP bound state GTPases interact with downstream effectors. The GTPase dissociates from its effector after the hydrolysis of GTP to GDP. The assay by Itoh et al. uses the biochemical interaction between the GTPase and its effector as a read out for how much of a GTPase is found in a GTP versus GDP bound state. RhoGAP-activity is monitored in heterologous cells using a RhoA sensor, which is a fusion protein consisting of the RhoA GTPase, the fluorescent proteins YFP and CFP and the Rho binding domain of an effector protein (RBD) (Figure 3.5) (Pertz et al., 2006). In the GTP bound state the GTPase interacts with the RBD resulting in CFP and YFP being in close spatial proximity, which gives rise to FRET. We performed the assay in HEK293 cells where the sensor is mainly found in its GTP bound form, due to the high number of GEFs. Therefore initial FRET emission at 530nm is always high. Co-transfection of a protein with GAP activity results in decreased FRET as compared to controls (transfected with an empty vector) since fewer sensor molecules exist in the GTP bound state. FRET was measured in suspended HEK293 cells with a Fluorometer.

*mSYD1A Exhibits GAP Activity Towards RhoA*

In an initial experiment to test if mSYD-1A has any GAP activity towards RhoA we co-transfected HEK293 cells with a plasmid encoding the RhoA sensor and a second plasmid either encoding HA tagged mSYD-1A (HA-mSYD-1A), myc tagged p50RhoGAP as positive control, myc tagged LIN-2/CASK as negative control or HA-mSYD-1A containing a R to A mutation at amino acid position 436 (HA-mSYD-1A R436A) which has been shown to reduce GAP activity in other GAPs (GAP dead) (Graham et al., 1999). We found that co-expression of the RhoA sensor with HA-mSYD-1A significantly reduces FRET (n = 12, p<0.0001) to similar levels as co-expression of
myc-p50RhoGAP (n = 12, p<0.0001) (Figure 3.6A) as compared to control cells transfected with the RhoA sensor plasmid and an empty vector (mock). The co-expression of HA-mSYD-1A R436A (n=12) and LIN-2/CASK (n=12) did not lead to significant changes in FRET compared to control (mock, n=12) (Figure 3.6A). This means that the R436A mutation reduces mSYD-1A’s GAP activity as it does in other GAPs. Therefore we concluded that mSYD-1A is a functional GAP that can inactivate RhoA. Due to the high overexpression of GTPases and GAPs in this assay, we found this assay to be unsuited to determine the GTPase specificity of mSYD-1A. Currently we are using an in vitro assay with purified mSYD-1A and purified GTPases to assess the GTPase specificity of mSYD-1A.

The N-terminal Domain of mSYD-1A Regulates its GAP Activity

The GAP activity of several known GAPs is regulated by their tertiary structure. In the case of β2-chimaerin, a Rac GAP with SH2, C1 and GAP domains, the GAP domain is sterically blocked by the N-terminus of the protein, which prevents it from binding to Rac (Canagarajah et al., 2004). The C1 domain is buried beneath the SH2 domain. The N-terminus dissociates from the GAP domain upon conformational changes induced by binding of the C1 domain to Diacylglycerol (DAG) and the SH2 domain to phospho-tyrosine residues in other proteins. By contrast, the Rap GAP activity of SynGAP requires the presence of its C2 domain, as its isolated GAP domain does not show any GAP activity (Pena et al., 2008). Inspired by those findings we wanted to test if mSYD-1A’s GAP activity is regulated by its tertiary structure. Therefore, we generated several deletion constructs for mSYD-1A and tested them in the FRET assay. Co-expression of the RhoA sensor with the HA tagged N-terminal domain (NTD) and C2 domain (C2) of mSYD-1A (HA-NTD, C2) resulted, as expected, in no reduction (n=12) of the FRET signal as compared to the mock control (n = 12) (Figure 3.7A). However co-expression of the RhoA sensor with the C2 and GAP domain or the GAP domain alone significantly reduced FRET as compared to control and full-length mSYD-1A (n = 12, p<0.0001, anova, tukey) (Figure 3.7A). Since constructs with the NTD deleted (HA-C2,GAP or HA-GAP) show a stronger GAP activity (more significant reduction in the
FRET signal) than the full-length mSYD-1A we hypothesized that mSYD-1A’s NTD might inhibit its GAP activity through an intramolecular interaction, similar to the N-terminus in β2-chimaerin. Thus, we tested if co-expression of the RhoA sensor with the GAP domain and the NTD domain or the NTD, C2 domains in trans (from separate plasmids), would inhibit the reduction in FRET induced by expression of the GAP domain alone. We found that co-expression of the NTD, C2 fragment with the GAP domain significantly increased FRET as compared to expression of the GAP domain alone (n=12, p<0.0001, anova, tukey) (Figure 3.7B). However co-expression of the NTD (without C2) with the GAP domain did not have an effect on GAP activity as compared to expression of the GAP domain alone (Figure 3.7B). Thus it seems that the NTD, C2 construct can inhibit the GAP activity in trans, mimicking the full-length protein. It is difficult to explain why the presence of the C2 domain in the C2, GAP protein fragment (Figure 3.7A) does not have an inhibitory effect on GAP activity, whereas it is required in the NTD, C2 construct to cause full inhibition of GAP activity (Figure 3.7B). RhoA is myristoylated and thus most RhoA sensor molecules localize to the membrane. Possibly the presence of the C2 domain enriches the NTD at membranes, which allows it to specifically inhibit the GAP molecules close to the Rho sensor.

Membrane Targeting of mSYD-1A Stimulates its GAP activity

If the NTD, C2 domain is indeed sterically blocking the access to mSYD-1A’s GAP domain, there must exist a cellular mechanism that induces a conformational change to remove the inhibition. As mentioned above, for β2-chimaerin the inhibition through the N-terminal domain is removed by binding of the SH2 domain to phospho-tyrosine residues and subsequent binding of the C1 domain to DAG at the membrane (Canagarajah et al., 2004). Since mSYD-1A has a C2 domain, which could mediate binding to membrane phospholipids in a Ca^{2+} dependent manner, phospholipid binding to the C2 domain could be involved in the activation mechanism. In addition, binding of proteins to the NTD of mSYD-1A could, similar to the SH2 mediated protein interaction in β2-chimaerin, induce the initial trigger for a conformational change that makes the C2 and GAP domain accessible. In order to bring mSYD-1A into close proximity with membrane phospholipids without having to use any chemical additives in the FRET assay, we generated an expression
construct encoding msyd-1A with N-terminal myristoylation and palmitoylation sites and a C-terminal HA-tag (myr-mSYD-1A-HA). Efficient membrane recruitment of myr-mSYD-1A was confirmed in HEK293 cells by immunostaining with an anti-HA antibody (data not shown).

Remarkably, the expression of myr-mSYD-1A-HA leads to a reduction in the FRET signal that is close to the reduction observed upon expression of the GAP domain alone and stronger than the reduction caused with the regular full-length mSYD-1A (n=12, p<0.0001, anova, tukey) (Figure 3.6B). This finding supports our hypothesis that phospholipid binding to the C2 domain could induce a conformational change that relieves inhibition from the mSYD-1A GAP domain. Future experiments with purified mSYD-1A in vitro should address if addition of phospholipids can increase GAP activity. Another interpretation of this finding is that myr-mSYD-1A-HA enriched at membranes, is closer to the membrane bound Rho sensor and therefore, can inactivate it more efficiently.

C. elegans and Drosophila sequence variations reduce mSYD-1A GAP activity
In C. elegans SYD-1 the R723V and N839R and in Drosophila DSYD-1 the N1106A sequence variations in the GAP domain have led to the hypothesis that their GAP domains might be non-functional. Indeed, the conserved arginine residue mutated in SYD-1 has been shown to be necessary for efficient GAP induced hydrolysis of GTP (Graham et al., 1999) and the conserved asparagine has been found to be necessary to stabilize the interaction with the GTPase (Scheffzek and Ahmadian, 2005). In order to test if those amino acid changes would render mSYD-1A inactive, we introduced mutations at the corresponding positions (R436 and N552) into mSYD-1A’s GAP domain and tested GAP activity of the mutants in the FRET assay. Expression of full-length mSYD-1A containing two mutations (R436V N552A) as SYD-1 or one mutation (N552A) as DSYD-1 as well as the GAP dead mutant (R436A) did not cause a significant reduction in the FRET signal as compared to control transfected cells (n=12) (Figure 3.8), and therefore do not exhibit detectable GAP activity in this assay. However, when we expressed GAP domain fragments with the same mutations, we observed a significant reduction in the FRET
signal \( (n=12, p<0.001, \text{anova tukey}) \) as compared to control, although to a significantly lesser degree than the wild type GAP domain \( (n=12, p<0.0001, \text{anova tukey}) \) (Figure 3.8).

This confirms that the GAP activity of mSYD-1A is reduced if it contains the same amino acid exchanges as \textit{C. elegans} SYD-1 or \textit{Drosophila} DSYD-1 suggesting that SYD-1 and DSYD-1 have a reduced GAP activity. However to conclusively address this question the GAP activity of purified full length SYD-1 and DSYD-1 should be examined in the future.

\textit{mSYD-1A Interaction partners}

We were interested in the identification of protein interaction partners of mSYD-1A for two reasons: 1) To identify endogenous proteins that can bind the NTD and possibly induce the conformational change in mSYD-1A that seems to be required to activate its GAP activity and 2) To identify proteins that could shed light on a role for mSYD-1A in CAZ assembly or a related function.

In order to identify proteins that interact with mSYD-1A we chose two different approaches. The first was a candidate approach with which we specifically asked if mSYD-1A can interact with CAZ proteins, specifically those found to either genetically or biochemically interact with \textit{C. elegans} SYD-1 and \textit{Drosophila} DSYD-1. The other is a Yeast-Two-Hybrid (Y2H) screen with a mouse brain cDNA library, which allowed for the unbiased identification of interaction partners.

\textit{Candidate Screening Approach for mSYD-1A Interaction Partners}

Since a gain of function mutation in \textit{syd-2}/Liprin can overcome the mislocalization of presynaptic components in \textit{C. elegans syd-1} mutants, the two proteins interact functionally. The \textit{syd-2} gain of function depends in its rescue ability on the presence of ELKS with which it interacts biochemically in the worm as well as the mouse (Dai et al., 2006; Ko et al., 2003a). Further in \textit{Drosophila} Bruchpilot/ELKS has been shown to biochemically interact with DSYD-1 (Owald et al., 2010). Therefore we decided to test if mSYD-1A can interact with the mouse homologues of ELKS/Bruchpilot or SYD-2/Liprin.
**Investigation of a Potential mSYD-1A / ELKS2 Interaction**

Two ELKS genes exist in vertebrates, ELKS1 and ELKS2 also called CAST2/ERC1 or CAST/ERC2, respectively. Due to alternative splicing the genes give rise to several transcripts of which ELKS1b (ERC1b) and ELKS2 (ERC2) show brain specific expression (Kaeser et al.; Wang et al.). Of the two brain specific ELKS variants only ELKS2 is exclusively localized at the synapse, ELKS1b is found at synaptic sites as well as in the cytosol (Wang et al., 2002). In a co-immunoprecipitation assay we tested if mSYD-1A can interact with ELKS2. Plasmids encoding N-terminally HA-tagged mSYD-1A (HA-SYD-1A) and N-terminally GFP-tagged ELKS2 were either co- or single-transfected into HEK293 cells. After 48 hours of expression the cells were harvested, lysed and the lysates incubated with either an antibody directed against the HA-tag (anti-HA) or one directed against the GFP-tag (anti-GFP). Following the antibody incubation proteins bound to the antibody were precipitated with agarose or Protein-A conjugated sepharose beads and the precipitates were analysed on Western Blots probed with anti-HA or anti-GFP antibodies (Figure 3.9A). HA-mSYD-1A and GFP-ELKS2 were co-immunoprecipitated when co-expressed in HEK293 cells, however neither protein precipitated when expressed alone and incubated with the antibody recognizing the other protein. To confirm that this interaction is also present in the brain mSYD-1A was precipitated with the anti-mSYD-1A antibody from brain lysates and the precipitates were probed with anti-SYD-1A, anti-ELKS and anti-LIN-2/CASK (negative control) antibodies. We found that small amounts of ELKS but not LIN-2/CASK co-precipitates with mSYD-1A (Figure 3.9B). However, we were unable to detect any mSYD-1A in precipitates collected after the incubation of brain lysate with an anti-ELKS antibody (data not shown). This could mean that the interaction detected after precipitation with the mSYD-1A antibody is a false positive due to recovery of large partially insoluble protein aggregates or that the interaction is weak and therefore requires a more sensitive method to be detected.
**mSYD-1A Interacts with Liprin-α2**

A genetic interaction between *syd-2/Liprin-α* and *syd-1* has been found essential for presynaptic assembly in *C. elegans* and *Drosophila*. But no biochemical interactions between the proteins have been reported. The vertebrate homologue of SYD-2 is called Liprin-α. All members of the Liprin-α family of proteins in the mouse (Liprin-α1, -α2, -α3 and -α4) have the same domain structure with an N-terminal coiled-coil region that mediates homo- and heteromultimerization followed by three sterile alpha motifs (SAM) domains which make up the Liprin homology region (LH) (Figure 3.10). Liprin-αs interact with ELKS through an amino acid stretch within the coiled-coil region (Ko et al., 2003a) and with RIM and LIN-2/CASK through segments in their LH domain (Olsen et al., 2005; Schoch et al., 2002a). Originally Liprin-αs were identified for their interaction with the receptor protein tyrosine phosphatase (RPTP) LAR via their LH region (Serra-Pagès et al., 1995). Interestingly, LAR has recently been shown to induce presynaptic differentiation after binding to the postsynaptic adhesion protein netrin-G ligand-3 (NGL-3) (Woo et al., 2009).

To investigate a possible interaction between mSYD-1A and Liprin-α we used pull down assays with purified recombinant Liprin-α fragments fused to a GST or His tag immobilized on glutathione or Ni²⁺ beads. The fragments tested are containing the CC (Liprin-CC) or SAM domains (Liprin-SAM) of Liprin-α respectively (Figure 3.10C). HEK293 cells were transfected with HA-mSYD-1A and lysed 48 hours after transfection. The lysates were incubated with the glutathione or Ni⁺ beads coated with Liprin-CC or Liprin-SAM fragments, respectively. The beads were precipitated and the bound proteins analyzed by Western Blot. We found that mSYD-1A does not interact with the CC domain fragment of Liprin-α1, 2 or 4 whereas ELKS does (as shown previously) (Ko et al., 2003a) (Figure 3.10A). Since mSYD-1A interacted with ELKS2 in the co-immunoprecipitations, we asked if mSYD-1A can bind to Liprin-α1, 2 or 4 through ELKS2. Therefore we co-transfected HEK cells with HA-mSYD-1A and Myc-ELKS2 and incubated the cell lysate with the Liprin-α1, 2 and 4 recombinant CC domain. We did not detect any HA-mSYD-1A binding to the CC domain in the presence of Myc-ELKS2 (Figure 3.10A).
We did find mSYD-1A to specifically interact with the SAM domain of Liprin-α2 but not with a recombinant control protein (GST) (Figure 3.10B). As a positive control for our assay conditions we used the binding of LIN-2/CASK to Liprin-α2-SAM since this interaction had previously been described (Olsen et al., 2005). To further understand which domain of mSYD-1A is responsible for the interaction with Liprin-α2-SAM, we incubated the affinity matrix with lysates of HEK293 cells expressing different HA tagged deletion variants of mSYD-1A. The N-terminal domain (NTD) showed the strongest interaction with Liprin-α2-SAM (Figure 3.10D), however the presence of the C2 domain seems to weaken the interaction. The C2, GAP construct and the GAP domain show no interaction with Liprin-α2-SAM (Figure 3.10D).

From our candidate approach screen we conclude that mSYD-1A interacts via its NTD with Liprin-α2-SAM. This leads us to two conclusions: 1) Through binding to the NTD Liprin-α2 could induces conformational changes that lead to the opening of mSYD-1A thereby activating it, 2) Since Liprin-α2 is an AZ protein with a known function in presynaptic assembly (Stryker and Johnson, 2007) it supports our hypothesis that mSYD-1A can localize to the CAZ and is involved in presynaptic assembly. In in vitro assays with purified mSYD-1A, we are currently testing if the presence of Liprin-α2 can increase mSYD-1A GAP activity. The interaction of mSYD-1A with Liprin-α2 could place mSYD-1A in a signal transduction pathway downstream of trans-synaptic adhesion through LAR and NGL-3. To confirm the interaction between mSYD-1A and ELKS2, it will require further examination.

**Yeast Two Hybrid Screen for mSYD-1A Interaction Partners**

We commissioned “Hybrigenics” a company specialized in Y2H screens, to perform a screen for mSYD-1A interaction partners. Two overlapping mSYD-1A expression constructs were used as bait to screen an adult mouse brain cDNA library, one encoding the N-terminal half of the protein (amino acids 1-411), containing the NTD and C2 domains and the other encoding the C-terminal half of the protein (amino acids 369-737), containing the GAP domain. By splitting the protein this way, we hoped to identify proteins that are involved in activating mSYD-1A by binding to its NTD, C2 domain or proteins that are effectors of mSYD-1A GAP activity.
The “prey” cDNAs recovered from the screen were evaluated systematically for their likeliness to be a true mSYD-1A interaction partner. For each interaction a predicted biological score (PBS) was calculated to assess the interaction reliability. The score predicts the probability of an interaction to be non-specific. It presents an e-value, primarily based on the comparison between the number of independent prey fragments found for an interaction and the chance of finding them at random (background noise). The value varies between 0 and 1 and several thresholds have been arbitrarily designed in order to rank the results in six categories A through F. In general, prey clones identified with following characteristics were excluded from any further analysis and were not considered as possible mSYD-1A interaction partners: if all the recovered fragments for one prey clone were antisense, if they all lay in the 5’ or 3’ UTR, if they all would give rise to out of frame protein products or if for all fragments the 5’ sequence was missing. We also excluded prey clones falling into category F. As defined by Hybrigenics: Category F contains experimentally proven artifacts of the Y2H technique as e.g. LexA or Gal4 binding partners or proteins known to bind to the DNA sequences upstream of the reporter gene. Category E contains interactions involving prey domains connected at least 10 times in unrelated screens with Human, Mouse, Drosophila and Arabidopsis cDNA libraries and 6 times for all other organisms. Thus highly connected most likely unspecific protein interactions were assigned to category E. We did not consider any prey fragments falling into category E as potential mSYD-1A interaction partners. All proteins we included into our further analysis were ranked category A through D. Category D proteins generally represent interactions identified through one unique prey fragment or multiple identical ones. This can represent interactions hardly detectable by the Y2H technique due to low representation of the mRNA in the library or prey misfolding or toxicity in yeast, or it can represent false-positive interactions. All interactions that do not show any of the characteristics described above fall into category A, B or C. If an interaction falls into category A, B or C at least two overlapping but non identical prey fragments were identified in the screen to interact with the bait. If one of the prey fragments was pulled out of the screen twice, the interaction is categorized as B interaction and if multiple prey fragments were pulled out more than twice the interaction is categorized A. Therefore prey proteins falling into category A present
a “very high” confidence interaction, category B presents “high confidence” interactions, category C “good” confidence interactions and category D “moderate” confidence interactions.

The prey proteins identified to interact with mSYD-1A in the Y2H screen participate in diverse cellular pathways (Figure 3.11A). Here I will focus on the proteins that could explain a role for mSYD-1A at the synapse and the interactions falling into category A.

Y2H Interaction Partners for the NTD, C2 Domain of mSYD-1A

The cDNA clones identified to interact with the N-terminal fragment of mSYD-1A (mSYD-1A-NTD, C2) encode a total of 30 different proteins falling into categories A to D. The identified interactions with Rabaptin-5 (Rabep1) and Tankyrase 2 (tnks2) were categorized as A. For Rabep1, seven overlapping but non-identical cDNA clones were found to encode protein fragments that can interact with mSYD-1A. For tnks2, eight overlapping but non-identical cDNA clones were found to encode protein fragments interacting with the NTD, C2 fragment of mSYD-1A. Rabep1 is known to be an effector of GTP bound Rab5 and is involved in the coordination of endocytic events (Vitale et al., 1998). Tnks2 is a poly (ADP-ribose) polymerase reported to be involved in diverse cellular functions including telomere elongation, E-Cadherin mediated adhesion and Wnt signaling (Hsiao et al., 2006; Huang et al., 2009; Yeh et al., 2006). Other interesting candidate interacting proteins identified for the N-terminal domain were: tnks1 (a homologue of tnks2, ranked category C since only two overlapping cDNAs were identified), kalirin (a Rho-GEF), DOCK3 (a Rho-GEF) and Stxbp1/Munc-18 (a regulator of synaptic vesicle exocytosis). All classified as category D since only one cDNA clone was identified for each of them to encode a protein fragment that can interact with mSYD-1A-NTD, C2.

Y2H Interaction Partners for the C-terminal Fragment / GAP domain of mSYD-1A

For the C-terminal fragment of mSYD-1A (mSYD-1A-GAP) 48 interacting proteins were identified. The four interactions falling into category A were with protein fragments of: Ccnh, a cyclin that regulates CDK7 (Fisher and Morgan, 1994); Nr1d2, a nuclear receptor involved in the repression of transcription (Woo et al., 2007); Sec5l1, a component of the exocyst complex (Spiczka and
Yeaman, 2008) and Smarce 1, part of the SWI/SNF chromatin remodeling complex (Kazantseva et al., 2009). The fact that three out of the four category A proteins are known to have nuclear functions could mean that mSYD-1A has a function in the nucleus. However, in experiments in which we overexpressed mSYD-1A in heterologous cells, we observed only small amounts of mSYD-1A to localize to the nucleus. Furthermore, only small amounts of mSYD-1A were found in the nuclear fraction after subcellular fractionation of HEK cells (Figure 3.3A). Therefore, we think that mSYD-1A is more likely to play a cellular role outside of the nucleus. Other interesting interaction partners identified for the C-terminal fragment were: Plectin 1 (category D), a component of desmosomes (Sonnenberg and Liem, 2007) and MAGI-3 (category D), a member of the membrane-associated-guanylate kinase (MAGUK) family (Adamsky et al., 2003).

Most of the proteins identified to interact with the N- or C-terminal fragment of mSYD-1A are currently not known to play an immediate role in presynaptic assembly or function. It is however remarkable that several of them play roles in membrane trafficking or the regulation of endo- or exocytotic events (Rabep1, Sec5l1). Interestingly, membrane trafficking events regulated by the RabGEF Glo-4 at C. elegans synapses, seem to be involved in axon termination and synapse formation (Grill et al., 2007). An interaction of mSYD-1A with those proteins could help to explain a potential role for mSYD-1A in synaptic vesicle localization. The interactions with the GEFs kalirin and DOCK3 support a role for mSYD-1A in the regulation of Rho-GTPases. Complex formation between the RhoGAP mSYD-1 and GEFs would suggest that activation and inactivation of GTPases, might be a process that is closely coordinated by complex formation between their regulators. Supportive of a role in presynaptic assembly or even function is the interaction with Stxbp1/Munc-18. The potential interaction with Plectin-1 is interesting since it is a major component of desmosomes, that can bind actin and that has recently been shown to bind to Ca^{2+}-channels in torpedo electric organ synapses (Carlson et al., 2010; Sevcik et al., 2004). The MAGUK family member MAGI-3 can interact with the receptor-protein-tyrosine-phosphatase (RPTP) -β (Adamsky et al., 2003). In consideration of our results that mSYD-1A can interact with Liprin-α, which can bind to the RPTP LAR (Serra-Pagès et al., 1995) the Y2H interaction with MAGI-3 gains of importance.
A Functional Interaction between Tankyrase 1 and mSYD-1A and -B

The fact that tankyrase2 is a regulator of Wnt signaling, which has been shown to be implicated in presynaptic differentiation in the cerebellum (Hall et al., 2000), prompted us to investigate a potential functional interaction between mSYD-1A and tankyrase. Tankyrase poly-ADP-ribosylates (PARSylates) target proteins and itself, which stimulates its own degradation, and the degradation of its substrates through the ubiquitin-proteasome pathway (Huang et al., 2009). A tankyrase specific inhibitor XAV939 blocks the PARSylation activity of the enzyme (Huang et al., 2009). Thus we argued, if mSYD-1A interacts with Tankyrase it most likely also is a substrate for PARSylation and, therefore, might be degraded if co-expressed together with Tankyrase in heterologous cells. Moreover, treatment of the co-expressing cells with XAV939 should prevent mSYD-1A as well as tankyrase degradation. We tested the effect of tankyrase co-expression and inhibition on mSYD-1A as well as mSYD-1B protein levels. mSYD-1A or mSYD-1B were expressed either alone or together with tankyrase 1 in HEK293 cells and treated with either DMSO or XAV939 as indicated (Figure 3.11B). Indeed, co-expression of mSYD-1A or -1B and tankyrase 1 resulted in a reduction of their protein levels as compared to their expression alone (Figure 3.11B). Furthermore, the protein level of either mSYD-1 was brought back to single transfection levels when the co-transfected cells were treated with XAV939 (Figure 3.11B). The treatment with XAV939 also resulted in an increase in the tankyrase 1 protein level, since the inhibitor also blocks the auto-PARSylation activity of tankyrase (Figure 3.11B). The observation that co-expression of tankyrase with mSYD-1A/B in heterologous cells reduces mSYD-1A/B protein levels, indirectly supports the possibility of a biochemical interaction between the two proteins and hints at a potential role for PARSylation in the regulation of neuronal proteins.

In general, this result indicates that the protein interactions found with the Y2H screen are most likely physiologically meaningful biochemical interactions and should be explored in future studies.
**Analysis of an Essential Role for mSYD-1 in AZ Assembly**

Our results thus far strongly support a role for mSYD-1A in synapse formation. We were able to show that mSYD-1A protein is expressed in the brain and can localize to synapses. That the CAZ proteins ELKS2, Liprin-α2 (biochemical interactions) and Munc-18 (Y2H interaction) were found to interact with mSYD-1A, further supports a potential role for mSYD-1A in active zone assembly.

In order to analyze if msyd-1A, -1B or both are essential for neural development in vivo, we decided to create conditional knock-out mouse models for both genes. The loss of genes involved in development can potentially result in embryonic lethality. This would prevent an analysis of synapse formation, which mainly occurs after birth. Therefore, we used the Cre inducible Cre/loxP knock-out system that allows for the creation of neuron specific knock-outs to prevent embryonic lethality. We hoped to create knock-outs for both genes to be in the position to breed animals that lack expression of mSYD-1A and mSYD-1B since it is possible that they have a redundant function in synapse formation.

**msyd-1A Conditional Knock Out Mouse Model**

The msyd-1A targeting vector introduces one loxP site in the genomic region upstream of exon 1 and a second loxP site in intron 4 downstream of exon 4 (Figure 3.12A). Further it contains an FRT-neo-FRT resistance/selection cassette in intron 4 upstream of the 3’ loxP site. Cre-recombinase mediated recombination results in the excision of exons 1 through 4, which encode the translational start site, the NTD, C2 domain and half of the GAP domain of mSYD-1A. Therefore even if a truncated RNA containing exons 5 through 8 is transcribed this will most likely not give rise to a functional protein product. Unfortunately, we were unable to create the msyd-1A KO animals, since the electroporation of the targeting construct never produced any ES cell clones that could survive in the neomycin containing selection medium. After a detailed analysis of the nucleotide sequence of the targeting vector, we discovered a point mutation in the neomycin resistance gene, which was already present in the parental vector and the sequence documentation thereof and thus remained undetected up to that timepoint. The mutation renders the protein product of the resistance gene inactive, which explains why no electroporated ES cell
clones could survive in the selection medium (Yenofsky et al., 1990). Thus, instead of a knock-out model we used RNA mediated knock-down of msyd-1A to address the necessity of msyd-1A expression for synapse formation.

*msyd-1B Conditional Knock Out Mouse Model*

For the creation of the *msyd-1B* conditional knock-out construct and the creation of animals with the targeted *msyd-1B* (*msyd-1B* flox) allele, we commissioned the Gene Targeting and Transgenic Facility of the University of Connecticut Health Center. The *msyd1B* targeting construct introduced two loxP sites, one in intron 1 upstream of exon 2 and the second in intron 3 downstream of exon 3 (Figure 3.12B). Cre mediated recombination results in a *msyd-1B* genomic locus lacking exon 2 and 3, but still containing exons 1 and 4 through 7. The knock-out locus could still give rise to the transcription of an mRNA containing exons 1, 4, 5, 6 and 7. However, splicing of exon 1 into exon 4 results in out of frame splicing and therefore translation from the predicted translational start site in exon 1 would result in an out of frame protein product. A translational start codon in exon 5 could give rise to a truncated mSYD-1B protein product containing half of the GAP domain.

To create *msyd-1B* knock-out (*msyd-1B*<sup>−/−</sup>) mice with a ubiquitous deletion of the targeted exons, we crossed the *msyd-1B* flox mice with mice expressing the Cre-recombinase under control of the CMV promotor (Cre-del) (Schwenk et al., 1995). In the Cre-del mouse line Cre is expressed in the cells of the germline, which causes excision of the floxed sequence in the whole organism. Heterozygous (*msyd-1B*<sup>+/−</sup>) and homozygous *msyd-1B*<sup>−/−</sup> animals are viable and fertile and do not show any obvious behavioral abnormalities. The absence of exon 2 and 3 containing *msyd-1B* mRNA was confirmed by RT-PCR, followed by agarose gel analysis, using primers specific for exons 2 and 3 (Figure 3.13A). However, RT-PCR with primers binding in exons 5 and 6 revealed that mRNA transcripts containing at least exons 5 and 6 are still present in the knock-out animals. It is to be expected that the truncated mRNA in the *msyd-1B* knock-outs is subject to nonsense mediated decay, but since a reduction in transcript level was not evident from the agarose gel analysis, we decided to analyze *msyd-1B* mRNA levels in the knock-out with
comparative quantitative PCR (qPCR), using SYBR green. The qPCR analysis confirmed the absence of msyd-1B mRNA containing exon 2 and 3 in mRNA extracts from msyd-1B−/− animals. Levels of msyd-1B mRNAs containing exon 5 and 6 are reduced by 44% in the knock-out as compared to wild type levels (Figure 3.13A). This suggests that the truncated msyd-1B mRNA product remaining in the msyd-1B knock-out is subject to nonsense mediated RNA decay. Unfortunately, the absence of mSYD-1B protein in the knock-out could not be confirmed, since we did not succeed in obtaining an anti-mSYD-1B antibody that is capable of detecting the endogenous protein. Importantly, we found that the level of mSYD-1A protein after knock-out of msyd-1B is unchanged (Figure 3.13B). Overall brain cytoarchitecture as assessed by immunostaining with antibodies recognizing NeuN (general neuron specific nuclear marker) and Calbindin (labeling some interneurons in the cortex and Purkinje neurons in the cerebellum) was unchanged in msyd-1B−/− mice as compared to WT littermate controls (3.13C).

**In Utero Electroporation As a Tool to Analyze Synapse Formation in vivo**

To assess whether mSYD-1A expression is required during neural development we used microRNA (miRNA) mediated knock-down of msyd-1A in vivo. We employed a knock-down vector encoding GFP and a miRNA targeting msyd-1A (miR-1A) or a control miRNA (miR-CNT) under control of the β-actin promoter (Figure 3.14A). The miRNA and GFP are transcribed as one transcript and following transcription the miRNA is cleaved off the GFP mRNA (see Material and Methods for details). This construct design has the advantage that the GFP expression confirms miRNA expression, and results in a cell-fill that enables the analysis of cell morphology. The ability of miR-1A to knock-down msyd-1A was assessed by co-transfection of HA-tagged msyd-1A and miR-1A or miR-CNT in HEK293 cells (Figure 3.14A). Protein levels of HA-mSYD-1A were assessed by Western Blot probed with anti-HA antibody. We concluded that miR-1A efficiently knocks-down msyd-1A.

To employ an assay that allows for the analysis of multiple neurodevelopmental steps, we delivered the miRNA construct by in utero electroporation at E15.5, into neural precursor cells in the cortical subventricular zone of mouse embryos. The targeted precursors give rise to cortical
pyramidal neurons of layer 2/3 (Figure 3.14). After the neurons have migrated to their respective layers, they grow an extensive dendritic and axonal arbor. Their axons branch extensively in layer V, to form synapses onto the dendrites of layer V pyramidal neurons, before they join the axon bundle of the corpus callosum to project to the contralateral cortex (Broser et al., 2008) (Figure 3.14B). Thus manipulation of layer 2/3 precursor neurons through the introduction of miRNA encoding plasmids at E15.5 gives the opportunity to analyse the necessity of the miRNA targeted gene on neural migration, dendritic and axonal growth as well as synapse formation. We found that electroporation at E15.5 more reliably targets layer 2/3 precursors than electroporation at E14.5. The GFP cell fill gained from the electroporation of a GFP encoding plasmid makes it possible to assess the localization and morphology of electroporated neurons. In addition, we used co-electroporation of synaptophysin-RFP (a synaptic vesicle protein) to analyze the formation of presynaptic release sites in layer V. We hypothesized, if mSYD-1A is required for presynaptic assembly in vivo, mice that lack msyd-1A expression in layer 2/3 neurons should show a reduction in the number of synaptophysin-RFP clusters in the axonal branches formed by layer 2/3 neurons in layer 5.

The effect of the loss of mSYD-1 on Presynaptic Assembly in vivo

miR-1A and synaptophysin-RFP or miR-CNT and synaptophysin-RFP were injected in utero into the lateral ventricle of E15.5 embryos of pregnant mice and electroporated with 5 pulses of 50V. Since we wanted to analyse the effect of a loss of msyd-1A, but suspected that mSYD-1A might function redundantly with mSYD-1B, we made use of the msyd-1B knock-out mice. Therefore, in addition to wild type (WT) embryos, we electroporated embryos that emanated from breedings of msyd-1B+/− females with msyd-1B−/− males. This yielded following knock-out / knock-down combinations: 1) wild type mice electroporated with miR-CNT (WT+miR-CNT), which continue to express msyd-1A and msyd-1B in layer 2/3 neurons, 2) wild type mice electroporated with miR-1A (WT+miR-1A), which presents a single loss of function for msyd-1A in layer 2/3 neurons and 3) msyd-1B−/− mice electroporated with miR-1A (1BKO+miR-1A), which presents a dual loss of expression of msyd-1B and msyd-1A in layer 2/3 neurons. The brains of electroporated animals
were analysed at P15 (a time when synapse formation is complete), after perfusion with paraformaldehyde and immunostaining for GFP and RFP. Almost all electroporated neurons (GFP positive) migrate to cortical layer 2/3 in brains of 1BHET+miR-CNT, WT+miR-1A and 1BKO+miR-1A, animals (Figure 3.15). The electroprated neurons in all conditions undergo polarization and grow extensive dendritic trees and axons that branch in layer V and then continue to project to the contralateral side of the brain (data not shown). From this initial analysis we conclude that the loss of msyd-1A expression alone or in combination with a loss of msyd-1B expression, has no major impact on the migration and morphology of layer 2/3 neurons. However, a detailed analysis of single cell morphology would require a decrease in the density of electroporated neurons, since the high density achieved in our experiments makes the identification of single cells difficult.

In order to analyze how the loss of msyd-1A or dual loss of msyd-1A and msyd-1B affects the formation of presynaptic release sites onto layer V neurons, high resolution (63x) confocal stacks of single GFP and synaptophysin-RFP expressing fibers within layer V, were acquired (Figure 3.15A, lower images). GFP is distributed along the entire axon, whereas synaptophysin-RFP is concentrated in clusters along axonal fibers, which are thought to represent presynaptic boutons. The goal of this analysis was to compare the density of synaptophysin clusters per 100µm of axon length between WT+miR-CNT, WT+miR-1A and 1BKO+miR-1A brains.

For the analysis the acquired confocal stacks of single axonal segments were projected in one plane and analysed with the image processing software “Metamorph”. The same intensity and size threshold for the synaptophysin-RFP channel (red) was applied to all images and only synaptophysin-RFP puncta above those thresholds were counted. After counting the synaptophysin puncta along an axonal segment, the length of the segment was measured and the puncta density per 100µm of segment calculated. A comparison of the puncta density of all axonal segments of three brains per conditions (WT+miR-CNT, WT+miR-1A, 1BKO+miR-1A) (Figure 3.15B and C) did not show a significant difference in synaptophysin puncta density after knock-down of msyd-1A (WT+miR-1A) or dual loss of expression of msyd-1A and msyd-1B (1BKO+miR-1A) (Anova, Tukey: p>0.05). Since the electroporation efficiency and therefore
synaptophysin-RFP expression levels can vary between different brains we also compared puncta densities of single brains that showed similar electroporation efficiencies, but also for this analysis we did not detect significant changes in synaptophysin puncta density (Figure 3.15D, Anova, Tukey: p>0.05).

In C. elegans DD motor neurons the loss of msyd-1A resulted in the mis-localization of synaptic vesicles and presynaptic proteins to the dendrite (Hallam et al., 2002). Therefore, it was suggested that SYD-1 could play a role in axon versus dendrite specification. We hoped to use the in utero electroporation assay described above to assess the possibility that synaptic vesicles mislocalize to dendrites after knock-down of msyd-1A. But this was not possible, since even under control conditions synaptophysin-RFP localized to dendrites.

In future, it should be confirmed that msyd-1A knock-down with miR-1A indeed results in a loss of mSYD-1A protein expression in layer 2/3 neurons. Since the anti-mSYD-1A antibody does not work in immunostaining, this could be assessed with Fluorescence activated cell sorting (FACS) for GFP expressing neurons, followed by a quantitative RT-PCR for msyd-1A RNA.

The lack of an effect of msyd-1A knock-down on the density of synaptophysin-RFP puncta, does not exclude that mSYD-1A could play a role in presynaptic assembly. It could affect the localization of other presynaptic proteins, as for example ELKS2 or Liprin-α2, which it interacts with. Therefore, going forward the localization of additional presynaptic proteins after loss of mSYD-1A expression should be analyzed.

The Effect of the loss of mSYD-1 on Synapse Formation in vitro

Due to the difficulties with the analysis of the in vivo loss of function experiments, we decided to address the effect of msyd-1A knock-down in primary cerebellar granule neuron cultures (GNC). Cerebellar granule neurons in culture have a very stereotyped morphology with small dendritic arbors, long axons and large presynaptic terminals, making them very useful for the analysis of presynaptic assembly. It should however be kept in mind that the synapses they form onto each other in culture do not exist in vivo.

To knock-down msyd-1A we used a mixture of siRNAs (Dharmafect) that are membrane
permeable, due to a chemical modification and are easily taken up by neurons. This has the advantage that in principle all neurons in the culture take up the siRNA, however this also makes it impossible to distinguish between a cell autonomous or non-autonomous effect of msyd-1A knock-down. The efficiency of the siRNA mediated msyd-1A knock-down, was assessed by comparing mSYD-1A protein levels in cultures treated with a mix of siRNAs targeting msyd-1A (si1A) and cultures treated with a mix of non-targeting control siRNAs (siCNT) for four days in vitro (DIV) (6 DIV until 10 DIV) (Figure 3.16A). To analyse an effect on synapse formation, granule neuron cultures from msyd-1B<sup>−/−</sup> and msyd-1B<sup>+/−</sup> mice were treated at 6DIV with si1A or siCNT. At 10 DIV, the cultures were fixed and immunostained. The immunostaining was performed with antibodies recognizing, VGlut1 (synaptic vesicle marker), Map2 (dendrite marker) and PSD95 (postsynaptic compartment marker). We determined the density of VGlut1 and PSD95 puncta along dendrites positive for the Map2 staining. For the analysis in Figure 3.16C, five neurons each on four independently treated coverslips were analysed for each condition. Under all conditions almost all PSD95 puncta were apposed to VGlut1. Whereas almost half of the VGlut1 puncta were not apposed by PSD95 puncta. This might indicate that synapse formation in granule neuron cultures (GNC) is not yet complete at 10 DIV. We did not observe a significant change in VGlut1 or PSD95 puncta density ( = number of VGlut1 or PSD95 puncta per 10µm) after si1A treatment of msyd1B<sup>−/−</sup> or msyd1B<sup>+/−</sup> neurons, as compared to cultures of the same genotype treated with siCNT. Neither single loss of msyd-1A or msyd-1B nor dual loss of both seems to have a strong impact on the number of synapses formed in granule neuron cultures. The observation that many VGlut1 puncta are not apposed by PSD95 puncta could hint at the cultures at 10 DIV being too young for an assessment of synapse density. Although we did not detect a significant reduction in VGlut1 or PSD95 puncta density, there seems to be a trend to a reduced density of VGlut1 and PSD95 puncta in msyd-1B<sup>−/−</sup> cultures treated with siCNT and si1A as compared to msyd-1B<sup>+/−</sup> cultures treated with siCNT or si1A. This would suggest that loss of msyd-1B expression can induce a small reduction in synapse number, but that loss of msyd-1A alone does not have such an effect. Another explanation might be that the siRNA mediated knock-down of msyd-1A is not efficient enough (Figure 3.16A) to uncover an effect of loss of
msyd-1A. Therefore these experiments should be repeated with optimized knock-down conditions.

**DISCUSSION AND FUTURE DIRECTIONS**

*Is mSYD-1A a True Orthologue of C. elegans SYD-1?*

By definition orthologues are genes in different species that evolved from a common ancestor by speciation, and retained the same function in the course of evolution. Therefore judging whether mSYD-1A indeed resembles a SYD-1 orthologue requires the consideration of information concerning its protein structure and function.

**Analysis of mSYD-1A Protein Structure**

The vertebrate candidate orthologues of SYD-1 were identified based on sequence similarities to the *C. elegans* protein. However, there are substantial differences in the primary amino acid sequence and domain structure between the identified mouse orthologues mSYD-1A / mSYD-1B and their invertebrate counterparts SYD-1 and DSYD-1. All proteins contain C2 and GAP domains but the invertebrate proteins both contain a PDZ domain, whereas neither mSYD-1A and mSYD-1B nor any other potential vertebrate orthologues are predicted to contain PDZ domains (Figure 3.1). The absence of a PDZ domain could mean that the vertebrate proteins differ in their function from the *C. elegans* protein. The high sequence conservation of the PDZ domain between SYD-1 and DSYD-1, with 76.6% similarity between the species, suggests that it is essential to the proteins function across species. However, other synaptic proteins with conserved functions between *C. elegans, Drosophila* and vertebrates do not necessarily show the exact same domain structure. For example Bruchpilot, the *Drosophila* ELKS homologue, has an extended N-terminus that is neither found in *C. elegans* ELKS, nor in the mammalian ELKS2. It has even been suggested that the N-terminus of Bruchpilot may substitute for functions that are carried out by Piccolo and Basson at vertebrate synapses (Stryker and Johnson, 2007). This example points towards the possibility that an mSYD-1A interacting protein with a PDZ domain could substitute for the lack of such a domain. Interestingly in our Y2H screen we found that the
C-terminal half of mSYD-1A (containing the GAP domain and C-terminus) can interact with the MAGUK family member MAGI-3. MAGI-3 is expressed in the brain and contains six PDZ domains (Adamsky et al., 2003). However, the direct interaction between mSYD-1A and MAGI-3 awaits further confirmation with biochemical methods. The absence of the PDZ domain from mSYD-1A does not exclude the possibility that mSYD-1A is a SYD-1 orthologue.

**Expression, Localization and Interaction Partners of mSYD-1A**

Our analysis of mSYD-1A expression indicates that it is expressed in the brain during the time of synapse formation (P0 – P7). Its presence in the insoluble PSD fraction after synaptosomal preparation suggests that it can localize to synapses.

We also found that mSYD-1A can biochemically interact with the CAZ proteins ELKS2 and Liprin-α2, which supports its possible synaptic function. Importantly, Liprin-α/SYD-2 is one of the proteins that have functionally been placed directly downstream of SYD-1 in *C. elegans* (Dai et al., 2006; Patel et al., 2006). Thus mSYD-1A expression, localization and biochemical interactions suggest that it is a SYD-1 orthologue.

**mSYD-1A GAP Activity**

The GAP domain of SYD-1 shows deviations from the consensus RhoGAP sequence in two residues that are key to efficient GTP hydrolysis in other GAPs. An arginine is replaced by a valine, in the position corresponding to residue R282 in human RhoGAP and an asparagine is replaced by an arginine, corresponding to N392 in human RhoGAP (Graham et al., 1999; Scheffzek and Ahmadian, 2005). This has led to the hypothesis that the SYD-1 GAP domain might be inactive (Hallam et al., 2002). Also DSYD-1 shows an amino acid exchange at the position corresponding to N392 in human RhoGAP. Neither mSYD-1A nor mSYD-1B contain those amino acid deviations (Figure 3.4). The GAP activities of SYD-1 and DSYD-1 have not been directly analysed so far (Hallam et al., 2002). We found mSYD-1A to have GAP activity towards RhoA (Figure 3.6). Introduction of the *C. elegans* and *Drosophila* GAP point mutations into mSYD-1A decreases its GAP activity significantly, suggesting that the invertebrate SYD-1s
might not be functional GAPs. However it is possible that the amino acid exchanges in their endogenous background, in *C. elegans* or *Drosophila*, respectively do not have an impact on GAP activity. We tried to test the GAP activites of full length *C. elegans* and *Drosophila* SYD-1 in the FRET assay, but were unsuccessful due to extensive degradation of the proteins in HEK293 cells. It will be necessary to gain a clear understanding of SYD-1 and DSYD-1 GAP activity. Going forward this will be better addressed in an *in vitro* environment with purified proteins.

**mSYD-1A Function in Presynaptic Assembly**

A lack of mSYD-1A expression *in vivo* (Figure 3.15) or *in vitro* (Figure 3.16) did not affect the density of synaptic vesicle clusters. It is possible that the localiztion of other CAZ components is affected, but remained undetected so far. In *Dsyd-1* mutants, the major structural phenotype in the presynaptic bouton is an abnormal organization of T-bars (that mainly consist of Bruchpilot/ELKS) and the formation of ectopic Bruchpilot/ELKS clusters in axons. Therefore, to conclude if mSYD-1A is involved in presynaptic assembly we have to assess the localization of additional CAZ components, especially that of ELKS2 and Liprin-α2, since we found them to interact with mSYD-1A. Therefore, the results of our mSYD-1A knock-down studies do not exclude a role of mSYD-1A in presynaptic assembly. The question if mSYD-1A is a SYD-1 orthologue, regarding its role in presynaptic assembly, for now remains unanswered.

**Rescue Experiments in *C. elegans***

In collaboration with Yishi Jin and Hidenori Taru we performed rescue experiments with *msyd-1A* and *msyd-1B* in *C. elegans syd-1* mutants. Expression of *msyd-1A* or *msyd-1B* cDNA did not rescue the loss of function phenotype in GABAergic motor neurons (VD/DD MNs) in *C. elegans syd-1* mutants. Interestingly, expression of the *C. elegans* SYD-1-GAP domain alone also does not rescue the *syd-1* loss of function phenotype, nor does it result in a gain of function when overexpressed in a wild type background (personal communication Y.J.). This indicates that the N-terminal part of SYD-1 is essential for its function in presynaptic assembly. Whether it is sufficient is not known since results of rescue experiments with the N-terminal domain alone have
not been reported. However, it is known that overexpression of SYD-1 lacking the GAP domain in a wild type background causes neurite outgrowth arrest and defasciculation of the VD/DD motor neurons in *C. elegans*, suggesting that the GAP domain does have functional relevance. Expression of chimeric proteins containing the N-terminus of *C. elegans* SYD-1 and the C2 and GAP domains of mSYD-1A or mSYD-1B in *C. elegans syd-1* mutants, will help to investigate a conserved function of SYD-1 across species. If expression of the chimeric constructs results in a rescue of the *syd-1* mutant phenotype, it means that the N-terminal domain of SYD-1 is necessary for its function and that the mouse C2 and GAP domains can substitute for the corresponding *C. elegans* domains. If expression of the chimeric proteins does not result in a rescue, this would confirm that the N-terminal domain of SYD-1 in *C. elegans* is not sufficient for its function, however it would also mean that the C2 and GAP domains of the mouse SYD-1s can not substitute for their *C. elegans* counterparts. In this case, it would become more questionable if mSYD-1A and mSYD-1B are indeed SYD-1 orthologues, although in the end only further analysis of mSYD-1A / -1B function in vertebrate CAZ assembly will tell.

Our findings that mSYD-1A localizes to synapses and interacts with the CAZ components ELKS2 and Liprin-α2, strongly support our hypothesis of mSYD-1A being an orthologue of SYD-1. The observation that loss of mSYD-1A or mSYD-1A and -1B protein expression does not have a dramatic effect on presynaptic vesicle clustering, does not exclude that it is involved in presynaptic assembly by localizing other presynaptic proteins. To make a final conclusion about whether mSYD-1A is a true orthologue of *C. elegans* SYD-1, we need to analyze its function in presynaptic assembly more into detail.

*How is the GAP Activity of mSYD-1A Regulated?*

Our structure-function analysis revealed that mSYD-1A GAP activity is regulated through intramolecular interactions between the NTD and GAP domains. This finding provides a mechanism for mSYD-1A functional regulation by binding partners of the NTD.
Interestingly, a similar intramolecular mechanism has been described for the Rac GAP β2-chimaerins as well as the Rho GAP oligophrenin (Canagarajah et al., 2004; Fauchereau et al., 2003).

Our result that removal of the NTD from full-length mSYD-1A significantly increased its GAP activity, implies that a cellular mechanism or signal exists, which triggers a conformational change to remove the autoinhibition in the full length protein. In the case of β2-chimaerin, which contains an N-terminal SH2 domain, a C1 domain as well as a Rac GAP domain, the NTD is folded over the entire protein blocking access to the GAP domain as well as the C1 domain (Canagarajah et al., 2004). Binding of DAG to the C1 domain is suggested to induce a conformational change, allowing the SH2 domain to bind phospho-tyrosine residues thereby removing the N-terminus from the GAP domain (Canagarajah et al., 2004). Similarly for mSYD-1A, Ca^{2+} dependent phospholipid binding mediated by the C2 domain could induce such conformational changes. However, our attempts to detect membrane recruitment of GFP tagged mSYD-1A in HEK293 cells in response to an increase in intracellular Ca^{2+}, after treatment with the Ca^{2+} ionophore ionomycin, were unsuccessful. It is important to note that not all known C2 domains are regulated by Ca^{2+} and some play purely structural roles and again others have lost their Ca^{2+} binding ability completely (Nalefski and Falke, 1996). The C2 domain of mSYD-1A does not resemble any of the known C2 domains closely. The highest similarity is found with the synaptotagmin C2A domain, a Ca^{2+} dependent phospholipid binding domain. Based on our current data it is not possible to exclude Ca^{2+} dependent lipid binding and therefore membrane recruitment for mSYD-1A. Ca^{2+} dependent membrane recruitment can be very transient, since it depends on transient increases in intracellular Ca^{2+} concentration. Therefore, it may be necessary to use time lapse imaging to capture mSYD-1A membrane recruitment in response to cellular Ca^{2+} influx. Our finding that full-length myristoylated mSYD-1A, that comes into close proximity to membrane phospholipids, shows the same increased GAP activity as the isolated GAP domain, supports the idea that membrane binding could induce the conformational change necessary to open the protein. As mentioned above, in β2-chimaerins both DAG binding to the C1 domain and protein interactions via its SH2 domain, contribute to the activation of the GAP domain (Canagarajah et
al., 2004). This suggests that the mSYD-1A NTD might be required to engage in a protein interaction, that induces a conformational change removing the sterical hinderance caused by the NTD. We identified Liprin-α2 as interaction partner of the mSYD-1A NTD. Thus, it will be interesting to investigate an effect of this interaction on the GAP activity of mSYD-1A. The regulation of mSYD-1A GAP activity through phospholipid binding and protein interactions via its NTD, should be tested in in vitro GAP assays with purified proteins.

**mSYD-1A: GAP Signaling Downstream of Trans-Synaptic Adhesion?**

**A Role for mSYD-1A Downstream of the RPTP LAR?**

The identification of the interaction between mSYD-1A and Liprin-α, puts it into the context of synapse formation induced by the heterophilic trans-synaptic adhesion pair LAR/NGL-3. Liprin-α can directly interact with LAR (Serra-Pagès et al., 1998) and NGL-3 overexpressed in HEK293 cells can induce LAR dependent presynaptic assembly in co-cultured hippocampal neurons (Woo et al., 2009). A requirement of Liprin-α for LAR/NGL-3-induced synapse formation has not been demonstrated. However, the involvement of Liprin-α is possible considering that Liprin-α is required for the integrity of ultrastructural presynaptic morphology in *C. elegans* (Zhen and Jin, 1999) and *Drosophila* (Kaufmann et al., 2002). LAR RPTPs are involved in triggering cytoskeletal rearrangements during neurite growth and axon guidance in response to the LAR ligands, laminin/nidogen complex or heparan sulfate proteoglycans (Chagnon et al., 2004). The molecular pathways linking LAR RPTPs to cytoskeletal rearrangements have yet to be discovered, but the observation that LAR interacted with the GEF Trio in a Y2H screen (Debant et al., 1996), introduces the possibility that LAR triggers cytoskeletal remodeling through the regulation of Rho GTPases. Thus a role for mSYD-1A as link between LAR mediated trans-synaptic adhesion and the cytoskeleton is a hypothesis worth testing in the future. This could be performed by co-culturing HEK293 cells overexpressing NGL-3 with cultured neurons in combination with siRNA mediated knock-down of *msyd-1A*. If mSYD-1A is indeed necessary for presynaptic assembly downstream of LAR, knock-down of *msyd-1A* should result in a failure of NGL-3 induced
presynaptic assembly. Further it would be interesting to investigate a direct or indirect biochemical link between mSYD-1A and LAR. mSYD-1A may bind LAR indirectly via Liprin-α. Another potential link between mSYD-1A and RPTPs comes through MAGI-3, which we identified as interaction partner of mSYD-1A in our Y2H screen. MAGI-3 can interact with the RPTPβ, which differs from LAR in its extracellular domain but also contains the highly conserved intracellular Phosphatase domains of all RPTPs (Adamsky et al., 2003; den Hertog et al., 1999). Since the hypothesis of mSYD-1A acting downstream of RPTPs is based alone on our finding that it can interact with Liprin-α, it is important to consider other trans-synaptic adhesion molecules as potential upstream regulators of mSYD-1A.

A Model for mSYD-1A Function During Presynaptic Assembly

F-actin assembly is necessary for the clustering of synaptic vesicles and other AZ components in the presynaptic bouton (Zhang and Benson, 2001). Several trans-synaptic adhesion proteins as N-Cadherins and Nrx (Biederer and Sudhof, 2001; Hülsken et al., 1994) have been shown to regulate presynaptic actin assembly. In general active RhoA destabilizes F-actin whereas active Rac and Cdc42 stabilize F-actin. Thus one plausible hypothesis would be that downstream of trans-synaptic adhesion RacGEFs might activate Rac and Cdc42 whereas RhoGAPs inactivate RhoA to synergistically promote presynaptic assembly. For example In Drosophila the RacGEF ephexin couples synaptic Eph receptor signaling to the modulation of presynaptic Ca^{2+} channels via Cdc42 (Frank et al., 2009). mSYD-1A might inactivate RhoA downstream of trans-synaptic adhesion proteins to stabilize F-actin and thereby induce the clustering of synaptic vesicles and other AZ components (Figure 3.17). Another possibility might be that mSYD-1A recruits CAZ proteins through its interaction with Liprin-α. To verify these models the following points need to be addressed: 1) Is mSYD-1A and more specifically mSYD-1A GAP activity required for presynaptic assembly downstream of trans-synaptic adhesion proteins? 2) Is the interaction between mSYD-1A and Liprin-α necessary for adhesion induced synapse formation? 3) If mSYD-1A GAP activity is required what are mSYD-1As downstream effectors? The first questions could
be answered performing a screen using the co-culture assay of HEK cells and neurons. Different postsynaptic adhesion proteins can be expressed in HEK cells and their ability to induce synaptic vesicle clustering in neurons can be assessed in WT cultures and cultures treated with an siRNA targeting msyd-1A, Liprin-α or both. The second question could be addressed in a co-culture assay combined with the overexpression of truncated mSYD-1A or Liprin-α, lacking the domains required for their interaction. To identify which specific RhoGTPase is acting downstream of mSYD-1A, it will be necessary to perform an in vitro GAP assay with purified mSYD-1A and RhoGTPases.

**Does mSYD-1A have the Potential to be a Key Regulator of Presynaptic Assembly?**

In *C. elegans* loss of SYD-1 results in the mis-localization of synaptic vesicles and CAZ proteins to dendrites of DD motor neurons. In the *C. elegans* HSNL neuron loss of SYD-1 prevents clustering of synaptic vesicles and several CAZ proteins at presynaptic sites and leads to their dispersal in the axon. Overexpression of SYD-1 without a GAP domain induces neurite outgrowth arrest and axonal stalling, suggesting SYD-1 could be involved in the early stages of axon outgrowth. There are two possible interpretations of SYD-1 function to explain these phenotypes. 1) SYD-1 is necessary to induce the clustering of synaptic vesicles and CAZ proteins at presynaptic sites, 2) SYD-1 is required to determine axon-dendrite polarity (Hallam et al., 2002; Patel et al., 2006). To test these possibilities for mSYD-1A we co-electroprated an miRNA targeting *msyd-1A* (miR-1A) and a plasmid encoding synaptophysin-RFP into precursors of cortical layer 2/3 pyramidal neurons in E15.5 mouse embryos in utero. The electroporated mice were analysed at P15, an age at which neural migration, polarization and synapse formation is completed. This gave us the opportunity to assess an effect of loss of mSYD-1A on axon-dendrite polarity as well as synapse formation.

We found that neurons that lacked mSYD-1A expression grew normal dendritic arbors and axons, as did pyramidal neurons lacking both mSYD-1A and mSYD-1B expression. The density of presynaptic synaptophysin-RFP clusters in the axons of electroporated neurons did not change significantly after knock-down of mSYD-1A alone or in combination with knock-out of
mSYD-1B. Those findings suggest that mSYD-1A is not involved in the establishment of axondendrite polarity. Neither does mSYD-1A seem to be essential for the clustering of synaptic vesicles.

However our results regarding presynaptic assembly have to be interpreted with caution. The in vivo analysis of the loss of msyd-1A expression (in utero electroporation assay) is based on the assumption that msyd-1A is expressed in layer 2/3 neurons in the cortex. Meaning if msyd-1A is not expressed in those neurons we would not be able to observe an effect on synaptic vesicle clustering, even if mSYD-1A plays a dominant role in presynaptic assembly elsewhere. A more detailed analysis of the mSYD-1A expression pattern will be necessary. In situ hybridizations in adult animals, accessible on the Allan Brain Atlas, do show msyd-1A and msyd-1B transcripts to be present in layer 2/3 of the cortex.

Another assumption this assay is based on is that overexpressed synaptophysin-RFP behaves like the endogenous protein. However a major problem during analysis of this experiment was the variability in synaptophysin-RFP expression, which in high expressing brains sometimes made it impossible to distinguish real synaptic vesicle clusters from background. We tried to address this problem by choosing fluorescence intensity and size thresholds for synaptophysin clusters. However, in future we should assess the juxtaposition of synaptophysin-RFP clusters with a postsynaptic marker as PSD95, which will help to distinguish real presynaptic vesicles clusters from artifacts.

The shortcomings of the in utero electroporation assay do not apply to the siRNA mediated knock-down assay in granule neuron cultures (GNC). We did show that granule neurons express mSYD-1A. We analysed the density and distribution of endogenous VGlut1 (vesicular glutamate transporter 1) clusters juxtaposed to PSD95. However no major changes were observed between control, msyd-1A knock-down and msyd-1A knock-down / msyd-1B knock-out conditions. Both assays have in common that we focused on synaptic vesicle clustering as readout for presynaptic assembly, but we did not analyse the localization of other presynaptic proteins, therefore it is still possible that a more subtle effect of presynaptic differentiation such as the localization of CAZ proteins is affected in our experiments.
Our experiments suggest that mSYD-1A might not be essential for presynaptic assembly, which could be explained with the synergy of many parallel protein interactions that ensure CAZ formation. Yet, our data on mSYD-1A’s synaptic localization and interaction with ELKS2 and Liprin-α indicate that it could play a role at the synapse. However, this role might be too subtle to be detected with the experiments we performed so far.

**New mSYD-1A Binding Partners**

We identified several interesting candidate interaction partners for mSYD-1A in our Y2H screen. Two proteins found to interact with mSYD-1A with high probability, were Sec5l and tankyrase2. Sec5l is a component of the exocyst complex mainly involved in regulating exocytosis. Interestingly depletion of components of the exocyst complex in neurons leads to axon loss (Lalli, 2009). Therefore the interaction with Sec5l does not only suggest a possible role for mSYD-1A in exocytosis but also in axonal growth.

We also identified an interaction between mSYD-1A and the poly-ADP-ribosylating (PARSylating) enzymes tankyrase1 and 2. Parsylation of target proteins results in their degradation through the ubiquitin-proteasome pathway. When we co-expressed mSYD-1A or -1B and tankyrase 1 in HEK293 cells we found mSYD-1A, as well as mSYD-1B protein levels to be reduced, which suggests that tankyrase can parsylate mSYD-1A and -1B and thereby trigger their degradation. Interestingly, tankyrase1 has been shown to be involved in the regulation of the canonical Wnt signaling pathway. Tankyrase PARSylates axin and thereby labels it for degradation. Axin is a component of the destruction complex for β-catenin, which means, Tankyrase indirectly stabilizes β-catenin and thus also β-catenin mediated transcription (Huang et al., 2009). How tankyrase is activated or if it is activated downstream of the Wnt receptor Frizzled, is not known. But its involvement in Wnt signaling is interesting, since Wnt signaling is involed in several stages of neural development: during cell type specification, axon guidance and later even synapse formation (Ahmad-Annuar et al., 2006; Ciani and Salinas, 2005). Therefore, it would be interesting to investigate if tankyrase could paly a role in the regulation of mSYD-1A / 1B protein levels downstream of Wnt signaling.
Conclusion

We identified mSYD-1A as a potential orthologue of C. elegans SYD-1. mSYD-1A protein is present in the brain during the time of synapse formation (P0 – P7) and associates with the PSD fraction after synaptosomal fractionation, suggesting that it could be part of the partially insoluble protein network at the CAZ. Furthermore, we discovered an intramolecular mechanism that regulates mSYD-1A GAP activity. mSYD-1A interacts with the CAZ components ELKS2 and Liprin-α2, which further supports its potential function in presynaptic assembly. And finally we identified several potential mSYD-1A interaction partners through a Y2H screen, which will be valuable to understand mSYD-1A’s synaptic function in the future.
FIGURES AND LEGENDS

FIGURE 3.1: Predicted SYD-1 mouse orthologues: mSYD-1A and mSYD-1B

(A) Domain predictions were made with the “SMART” server developed by the EMBL Heidelberg. None of the predicted mouse or human orthologues contain a PDZ domain, however for all the existence of a C2 and Rho-GAP domain is predicted. Values above domains indicate the similarity between the domain and the corresponding domain in C. elegans. Values on the left present the similarities between full length SYD-1 and its predicted orthologues. Global similarities between SYD-1 and its orthologues were determined using the Emboss pairwise alignment algorithm “needle”. (B) A phylogram representing the evolutionary relationship between the C. elegans, Drosophila and predicted mouse SYD-1 orthologues. The branch lengths are proportional to the amount of inferred evolutionary change. The phylogram is based on an alignment made with the ClustalW2 multiple alignment tool on the EMBL-EBI webpage.
FIGURE 3.2: Characterization of msyd-1A and msyd-1B RNA and Protein Expression

(A) msyd-1A and msyd-1B specific RT-PCR products were analyzed on agarose gels. Actin or β-tubulin RT-PCRs are input controls. msyd-1A and msyd-1B RNA was detected in adult mouse kidney, lung and brain. msyd-1B was also detected in liver, whereas low levels of msyd-1A were also detected in liver, spleen and muscle. msyd-1A and syd-1B transcripts are present in the mouse brain from E14 until adult. (B) Homemade anti-msyd-1A (mSYD-1A) antibody (Ab) recognizes mSYD-1A specifically as 100kDa band in lysate from HEK cells as well as cortical neurons, as shown by disappearance of the band after treatment of the cells with msyd-1A specific siRNAs. (C) Alignment of the N-terminal peptide recognized by the homemade anit-mSYD-1A antibody with the N-terminus of mSYD-1B. (D) The size and migration pattern of mSYD-1A in HEK cells overexpressing HA-mSYD-1A (HA-1A), HA-mSYD-1B, untransfected HEK cells (HEK) and P5 brain (P5) is the same. The mSYD-1A antibody does not bind to HA-mSYD-1B overexpressed in HEK cells. (E) mSYD-1A is detected in whole brain and cortical lysates between E14 and adult. (F) At P5 mSYD-1A is expressed at similar levels in whole brain (b), cortex (ctx) and cerebellum (ceb). Arrow head: mSYD-1A, star: unidentified cross reacting band.
FIGURE 3.3: Subcellular Localization of mSYD-1A

(A) mSYD-1A is detected together with the CAZ components Munc-18 and LIN-2/CASK in the insoluble membrane fraction P2, after subcellular fractionation of HEK 293 cell homogenate. ERGIC53: membrane marker, Tubulin and Actin: cytosolic markers, Histone: nuclear marker. (B) mSYD-1A is detected together with the CAZ components Munc-18, CASK and ELKS2 in the insoluble membrane fraction P2 after subcellular fractionation of P2 mouse brain homogenate. Neuroligin: membrane marker, Actin: cytosolic marker, Histone: nuclear marker. H: whole cell / brain homogenate, P1: nuclei, mitochondria, unbroken cells, PNS: post-nuclear supernatant containing membranes and cytosol, P2: membranes, S2: cytosol. (C) mSYD-1A is detected in the crude synaptosomal fraction P2, the lysed synaptosomal membrane fraction P3 and the insoluble PSD fraction, after synaptosomal fractionation of P10 mouse cortices. This suggests that mSYD-1A localizes to synapses and participates in insoluble protein complexes. H: whole brain homogenate, S1: brain cytosol and membranes, P1: nuclei, mitochondria, unbroken cells, S2: brain cytosol and plasma membrane, P2: crude synaptosomal pellet, S3: crude synaptic vesicles, P3: lysed synaptosomal membranes, SPM: synaptic plasma membrane, SPM-S1: Triton soluble SPM, PSD: Triton insoluble. Arrow head: mSYD-1A band, star: unidentified cross-reacting band.
The GAP domains of SYD-1, DSYD-1, mSYD-1A, mSYD-1B, α-chimaerin and human RhoGAP (hRhoGAP) were aligned using the ClustalW2 multiple alignment algorithm. Residues shown to be necessary for the catalytic activity of RhoGAP domains in general are marked in red (Scheffzek and Ahmadian, 2005). Arrows indicate amino acid deviations from the consensus sequence in SYD-1 and DSYD-1. In SYD-1 a conserved arginine is replaced by a valine (R723V) and a conserved arginine is replaced by an asparagine (R839N). In *Drosophila* the conserved arginine is replaced by an alanine. In mSYD-1A and mSYD-1B none of the residues shown to be essential for the catalytic activity of RhoGAPs deviate from the consensus RhoGAP sequence.
Measuring GAP activity with Rho sensors
(A) The GTPase cycle. The exchange of GDP for GTP is catalysed by guanosine nucleotide exchange factors (GEF). The GTP bound / active RhoGTPase binds to and activates downstream effectors, which can induce cytoskeletal rearrangements. The hydrolysis of GTP to GDP is catalysed by GTPase activating proteins (GAP). GEFs and GAPs can be activated by extracellular stimuli. (B) The Rho sensor is a fusion protein of the RhoGTPase, YFP, CFP and the Rho binding domain (RBD) of a Rho effector protein. In the GTP bound state Rho interacts with the RBD. Due to the close proximity between YFP and CFP in this conformation FRET occurs, and stimulation with light of 430nm (CFP stimulation) wavelength results in the emission of light at 530nm (YFP emission). If a GAP is co-expressed with the Rho sensor most sensor molecules are found in the GDP bound state. Rho therefore does not bind to the RBD and in this conformation no FRET occurs due to the distance between YFP and CFP. Therefore stimulation at 430nm results in an emission of 475nm light (CFP emission).
FIGURE 3.6: mSYD-1A is a functional GAP and membrane localization increases its GAP activity.

Results of FRET assays in HEK293 cells. A decrease in the Emission ratio of 530nm / 475nm presents an increase in GAP activity. y axis: emission ratio, x axis: transfected plasmids. Line in boxes = median. Upper part of the box = 75th percentile lower part = 25th percentile. The error bars indicate the highest and the lowest emission ratio out of n=12 experiments for each condition. *: p<0.05, **: p<0.001, ***: p<0.0001, Anova/Tukey. Western Blots show the protein expression levels in HEK293 cells for each condition. The RhoA sensor (α-GFP) is equally expressed in each sample. mSYD-1A full length and deletion fragments were detected with α-HA antibody. CASK and p50rhoGAP were detected with α-Myc antibody. α-actin serves as loading control. (A) Emission ratios after co-expression of the RhoA sensor with no protein (Mock), HA-mSYD-1A (HA-SYD-1A), Myc tagged p50RhoGAP as positive control (Myc-p50RhoGAP), Myc tagged CASK (Myc-CASK) and HA-mSYD-1A GAP dead (R436A) as negative controls. Co-expression of HA-mSYD-1A with the RhoA sensor significantly reduces the Emission ratio as compared to the Mock control. Which means HA-mSYD-1A is a functional GAP. (B) Emission ratios after co-expression of the RhoA sensor with no protein (Mock), full length HA-mSYD-1A (HA-SYD-1A), the isolated GAP domain of mSYD-1A (HA-GAP) and myristoylated and C-terminally HA tagged mSYD-1A (Myr-SYD-1A-HA). HA-GAP shows stronger GAP activity than HA-mSYD-1A. The myristoylated full length mSYD-1A shows the same GAP activity as the isolated GAP domain (HA-GAP).
FIGURE 3.7: The N-terminal domain (NTD) of mSYD-1A regulates its GAP activity

Results of FRET assays in HEK293 cells. A decrease in the Emission ratio of 530nm / 475nm presents an increase in GAP activity. y axis: emission ratio, x axis: transfected plasmids. Line in boxes = median. Upper part of the box = 75th percentile lower part = 25th percentile. The error bars indicate the highest and the lowest emission ratio out of n=12 experiments for each condition. *: p<0.05, **: p<0.001, ***: p<0.0001, Anova/Tukey. Western Blots show the protein expression levels in HEK293 cells for each condition. The RhoA sensor (α-GFP) is equally expressed in each sample. mSYD-1A full length and deletion fragments were detected with α-HA antibody. α-actin serves as loading control. (A) Co-expression of the RhoA sensor with HA-mSYD-1A deletion proteins. The NTD,C2 fragment of HA-mSYD-1A does not have GAP activity. The C2,GAP and GAP fragment reduce the emission ratio significantly (p<0.0001) as compared to full length HA-mSYD-1A and therefore show higher GAP activity than full length mSYD-1A. (B) Co-expression of the NTD,C2 fragment of mSYD-1A from a separate plasmid can reduce the GAP activity (increase in emission ratio) of the isolated GAP domain (HA-GAP) significantly.
FIGURE 3.8: *C. elegans* and *Drosophila* GAP domain mutations reduce mSYD-1A GAP activity.

Results of FRET assays in HEK293 cells. n = 12 experiments. A decrease in the Emission ratio of 530nm / 475nm presents an increase in GAP activity and vice versa. y axis: emission ratio, x axis: transfected plasmids. Line in boxes = median. ★★★: p<0.0001, Anova/Tukey. Upper part of the box = 75th percentile lower part = 25th percentile. The error bars indicate the highest and the lowest emission ratio out of n=12 experiments for each condition. Western Blots show the protein expression levels in HEK293 cells for each condition. The RhoA sensor (α-GFP) is equally expressed in each sample. mSYD-1A full length and point mutants were detected with α-HA antibody. α-actin serves as loading control. *C. elegans* SYD-1 deviates from the conserved RhoGAP consensus sequence in two key residues. Introduction of the same amino acid exchanges into mSYD-1A (R436V, N552R) reduces the GAP activity of full length HA-mSYD-1A significantly (p<0.0001) as compared to WT HA-mSYD-1A. *Drosophila* DSYD-1 deviates from the conserved RhoGAP consensus sequence in one key amino acid. Introduction of the same amino acid change into mSYD-1A (R436A) reduces the GAP activity of full length HA-mSYD-1A significantly (p<0.0001) as compared to WT HA-mSYD-1A.
**FIGURE 3.9: mSYD-1A interacts with ELKS2**

(A) HA tagged mSYD-1A (HA-SYD-1A) and GFP tagged ELKS2 (GFP-ELKS2) were expressed together or individually in HEK293 cells as indicated. Proteins were immunoprecipitated (IP) with antibodies binding to GFP (GFP-IP) or HA (HA-IP) and Protein A sepharose beads. Precipitates were probed on Western Blot for the presence of HA-mSYD-1A (α-HA) and GFP-ELKS2 (α-GFP). HA-mSYD-1A and GFP-ELKS2 co-precipitate if co-expressed in HEK293 cells. (B) Two adult mouse brains were lysed and endogenous mSYD-1A precipitated with α-mSYD-1A (α-SYD-1A) antibody and Protein A sepharose beads. Lysates and precipitates were probed for the presence of mSYD-1A (α-SYD-1A), ELKS2 (α-ELKS2) and CASK (α-CASK). ELKS2 weakly precipitated with mSYD-1A whereas CASK did not co-precipitate.
FIGURE 3.10: mSYD-1A interacts through its NTD with the Liprin-α2 SAM domain
(A) GST Pull downs with GST or GST-Liprin-CC (Liprin-CC) from lysates of HEK293 cells expressing HA-mSYD-1A, Myc-ELKS2 or both proteins together. Input lysates and precipitates were analysed on Western Blots for HA-mSYD-1A (α-HA) or Myc-ELKS2 (α-Myc). HA-mSYD-1A does not bind to Liprin-CC neither in the presence or absence of Myc-ELKS2. Myc-ELKS2 does bind to Liprin-CC. (B) Pull down with His tagged Liprin-α SAM domains (Liprin-SAM) or a His-GST control protein from lysates of HEK293 cells expressing HA tagged mSYD-1A protein fragments. N-terminal domain (HA-NTD), N-terminal domain and C2 domain (HA-NTD, C2), C2 domain and GAP domain (HA-C2, GAP), GAP domain (HA-GAP). Input lysates and precipitates were probed on Western Blot for the mSYD-1A protein fragments (α-HA). mSYD-1A binds to Liprin-SAM through its NTD.
FIGURE 3.11: Interaction Partners of mSYD-1A identified with the Y2H screen.

(A) Potential interaction partners of mSYD-1A indentified in the Y2H screen. In red are proteins that were determined to interact with mSYD-1A with a high probability. The y axis indicates the cellular function that has been described for the identified interaction partners, the x axis indicates the number of identified interaction partners with a specific cellular function. The categories A, B, C, D indicate the probability that the interaction is biologically meaningful, with A being the highest probability and D being the lowest (see also text). The proteins in brackets are identified interaction partners with a specific cellular function with a high probability to interact with mSYD-1A (red) or proteins of specific interest because they could explain a potential function for mSYD-1A at the synapse (black) (B) Lysates of HEK293 cells expressing mSYD-1A / -1B (1A / 1B) or co-expressing tankyrase1 with mSYD-1A (1A+tnks1) or mSYD-1B (1B+tnks1) probed on Western Blot for HA-mSYD-1A / -1B (α-HA), tankyrase (α-tnks) and actin (loading control). Lysates from cells co-expressing 1A or 1B with tnks1 have reduced 1A / 1B protein levels as compared to expression of 1A / 1B alone. Inhibition of tnks1 through addition of the specific inhibitor XAV939 increases tnks1 expression levels and 1A / 1B levels in co-expressing cells. This indicates that the reduction in 1A / 1B protein levels is induced by tnks1 enzymatic activity.
FIGURE 3.12: Creation of Conditional Knock Out Mice for *msyd-1A* and *msyd-1B*  
(A) Creation of a conditional knock-out for *msyd-1A*. One loxP site was introduced before exon 1 the other loxP site in intron 4. A neomycin resistance gene (neo) flanked by FRT sites was introduced in intron 4 to allow for positive selection of targeted ES cell clones in neomycin containing selection medium. Due to the presence of the FRT sites the neo cassette can be excised by crossing mice with the targeted locus with mice transgenic for the FLIP recombinase. Further breeding with mice carrying the Cre recombinase transgene will result in excision of *msyd-1A* exons 1 – 4 in cells expressing Cre. (B) Creation of a conditional knock-out for *msyd-1B*. One loxP site was introduced in intron 1 the other loxP site in intron 3. The FRT-neo-FRT cassette was introduced in intron 3 and later excised by crossing mice with the targeted *msyd-1B* locus with mice transgenic for the FLIP recombinase. Crossing of mice with the knock in locus with mice carrying a Cre recombinase transgene resulted in excision of exons 2 and 3.
FIGURE 3.13: Characterization of msyd-1B knock-out mice

(A) msyd-1B transcripts in msyd-1B<sup>−/−</sup> (KO), heterozygous (HET) and wild type mice (WT). right: No transcripts containing exon 2 and 3 can be detected in KO mice. Transcripts containing exons 5 and 6 exist in the KO. Left: comparative qPCR analysis of transcript levels in msyd-1B KO mice compared to WT. KO: n=3, WT: n=3. WT transcript levels are set to 100% (=1), the transcript levels in the KO are presented as a fraction of the levels in the WT (= 2<sup>ΔΔCt</sup> values). Transcripts containing exon 5 and 6 are reduced to 56% of transcript levels in the KO as compared to the WT. Transcripts containing exon 2 and 3 can not be detected in the KO. (Anova, Tukey, p<0.001, error bars = SD) (B) Lysates of adult brain of msyd-1B KO and WT mice probed on Western blot for the expression of mSYD-1A. mSYD-1A (MW 100kDa) expression is unchanged in msyd-1B KO brain as compared to WT, tubulin = loading control. Arrow head: mSYD-1A, star: unidentified cross-reacting band. (C) Cytoarchitecture in WT and msyd-1B KO assessed with immunostaining for Calbindin (green, interneurons and cerebellar Purkinje neurons) and NeuN (red, general neuron marker). KO brains do not show any major changes in cytoarchitecture.
FIGURE 3.14: miRNA Mediated Knock Down and In Utero Electroporation

(A) The miR-vector allows co-cistronic expression of an engineered *msyd-1A* targeting miRNA in the 3’untranslated region of Emerald Green Fluorescent Protein (EmGFP) under control of a β-actin promoter. Lysates of HEK293 cells co-transfected with a plasmid encoding HA-mSYD-1A and increasing amounts (left to right) of the miR-vector encoding a control non-targeting miRNA (miR-CNT) or the miRNA targeting *msyd-1A* (miR-1A) were probed in Western blot for the expression of HA-mSYD-1A (α-HA). Actin = loading control. Co-transfection with the miR-1A vector results in a reduction of HA-mSYD-1A expression levels as compared to cells transfected with miR-CNT. (B) Mouse embryos were in utero electroporated with synaptophysin-RFP and miR-CNT or miR-1A at E15.5. Coronal sections of electroporated brains were prepared at P15. The neuronal precursors electroporated at E15.5 (green cells) differentiate into pyramidal neurons of cortical layers 2/3. After differentiation they grow a leading process and migrate along radial glia cells (black cells) until they reach layer 2/3. Following migration they grow axonal and dendritic arbors. Their axons branch in cortical layer 5 to form synapses onto dendrites of pyramidal neurons of layer 5 before they join the corpus callosum to project to the contralateral side of the cortex.
FIGURE 3.15: Effects of msyd-1A knock-down in WT and msyd-1BKO mice

(A) Upper panels: 10x confocal images of coronal sections of the sensorimotor cortex of representative electroporated brains of mice at P15, after electroporation with synaptophysin-RFP and a knock-down construct at E15.5. Genotypes and electroporated knock-down constructs as indicated. In all conditions the cell bodies of electroporated layer 2/3 neurons (green) are located in the upper third of the cortex (layer 2/3) and project axons towards layer 5, where they branch extensively. Scale bar = 1mm.

Lower panels: magnified axonal segments of electroporated layer 2/3 neurons in layer 5. GFP (green) fills the entire axon, whereas synaptophysin-RFP is localized in clusters (white) along the axon. The clusters presumably represent presynaptic boutons. Scale bar = 10µm. (B) Synaptophysin-RFP puncta were counted in 5-10 axonal segments for three brains per condition. Each dot in the scatter plot represents the number of synaptophysin-RFP puncta per 100µm (density) in one axonal segment. Axonal segments from the same brain are represented in the same color. In red are the puncta densities for the axonal segments from the three brains shown in (A). Line = median. No significant reduction in puncta density was detected after knock-down of msyd-1A in WT or msyd-1BKO (1BKO) animals (Anova, Tukey, p>0.05). (C) Mean synaptophysin-RFP puncta density was calculated for each analyzed brain (n=3 per condition), and from the mean density per brain, the mean density per condition was calculated. No significant reduction in synaptophysin-RFP puncta density was detected after knock-down of msyd-1A in WT or msyd1B-/-(1BKO) animals (Anova, Tukey p>0.05, error bars SEM).
FIGURE 3.16: Loss of *msyd-1A* and *msyd-1B* in Granule neuron cultures (GNC)

(A) Confocal microscope images of Granule neurons in GNC of the indicated genotype (*msyd1B<sup>+/−</sup> = 1BHET or *msyd1B<sup>−/−</sup> = 1BKO*) treated with control siRNA (siCNT) or a siRNA targeting *msyd-1A* (si1A) at 6 DIV and fixed and immunostained at 10 DIV. Map2 (dendritic marker, blue), VGlut1 (vesicular glutamate transporter 1, red), PSD95 (postsynaptic marker, green) scale bar = 10µm. Blow up: examples of dendritic segments along which VGlut1 and PSD95 puncta were counted to determine puncta density (# of puncta per 10µm). (B) Lysates of GNC probed in Western Blot for the expression of mSYD-1A (α-SYD-1A). GNCs from *msyd1B<sup>+/−</sup>* (1BHET) or *msyd1B<sup>−/−</sup>* (1BKO) mice were treated with siCNT or si1A at 6 DIV and lysed at 10 DIV. si1A efficiently knocked down *msyd-1A* in 1BHET GNC and less efficiently in 1BKO GNC. Actin = loading control. (C) Number of VGlut1 puncta (left) and PSD95 puncta co-localized with VGlut1 (right) per 10µm dendritic segment under different knock-out and knock-down conditions. No significant reduction in VGlut1 puncta or PSD95 colocalized with VGlut1 puncta density was detected (Anova, Tukey, p>0.05, error bars = SEM).
FIGURE 3.17: A Model for mSYD-1A Action During Presynaptic Assembly

Our experiments show that mSYD-1A interacts with Liprin-α2 via its N-terminal domain (NTD). Liprin-α2 can bind to the receptor protein tyrosine phosphatase (RPTP) LAR. LAR mediates presynaptic assembly after induction through NGL-3 (Woo et al., 2009). In this model mSYD-1A is recruited to LAR (or another trans-synaptic adhesion protein) through an interaction with Liprin-α2. The interaction with Liprin-α2 triggers the removal of the NTD, blocking the mSYD-1A GAP domain. After activation through Liprin-α2, mSYD-1A inactivates a GTPase, potentially RhoA, which promotes F-actin stabilization and thereby presynaptic assembly. Another possible way how mSYD-1A could promote presynaptic assembly, is by directly interacting with a trans-synaptic adhesion protein (e.g. LAR), which would lead to a recruitment of Liprin-α2. In such a scenario mSYD-1A would be acting upstream of Liprin-α2 in presynaptic assembly.
CHAPTER 4

GENERAL DISCUSSION AND FUTURE DIRECTIONS
GENERAL DISCUSSION

Neuronal circuit assembly encompasses a plethora of morphological transformations during neural migration, neurite outgrowth, axon guidance, axonal and dendritic arbor growth as well as synapse formation. Work over the past decades has led to the identification of extracellular signals that are detected by cell surface receptors and translated into cytoskeletal rearrangements. Cytoskeletal dynamics are mainly controlled by small RhoGTPases that function as switches and can be turned on (GTP bound) or off (GDP bound) in response to extracellular signals. The mediators between transmembrane receptors and the RhoGTPases are RhoGTPase regulators as GTPase exchange factors (GEFs) and GTPase activating proteins (GAPs). In this thesis I presented our findings on the role of the two GAPs α2-chimaerin and mSYD-1 in neural development. We have found α2-chimaerin to be an essential and specific mediator of repulsive growth cone behavior downstream of EphA4 receptor signaling in axon guidance in vivo. Our understanding of mSYD-1 is less complete than that for α2-chimaerin. However, the emerging picture hints at a role for mSYD-1A in presynaptic assembly downstream of trans-synaptic adhesion proteins.

Our findings support the notion that RhoGTPase regulators play key roles in signal transduction during multiple steps in neural development. In the following I want to discuss some open questions about the role of GEFs and GAPs in neural development in the context of our findings and the general knowledge. First in “GAPs and GEFs as key regulators of neuronal signaling” I will discuss how our findings together with the knowledge about other GEFs and GAPs support the idea that they are designated to transduce the signals of specific upstream receptors. Second, I will consider the possibility that GEFs and GAPs can “multi-task” meaning that they can function at several steps during neuronal development downstream of the same receptors and last in “Future Directions” I will discuss experimental strategies that could help to answer some of the open questions concerning the specific functioning of GAPs and GEFs.
GAPs and GEFs as key regulators of neuronal signaling

The human genome is predicted to encode between 59 and 70 proteins containing a RhoGAP domain, about 22 mammalian genes encode RhoGTPases, which means that RhoGAPs outnumber RhoGTPases by a factor of 3:1 (Tcherkezian and Lamarche-Vane, 2007). This imbalance is also true with regards to RhoGEFs of which approximately 50 are predicted to be encoded in the human genome (Tcherkezian and Lamarche-Vane, 2007). An excess of GEFs and GAPs likely represents the complex regulation of RhoGTPases. RhoGTPases have to be regulated temporally and locally, to allow for changes of cytoskeletal dynamics at different time points, in different parts of a cell. The excess of GEFs and GAPs could bestow specificity to RhoGTPase signaling by linking them to specific upstream receptors. Thus, GEFs and GAPs would be recruited to specific activated transmembrane receptors allowing for the temporal and local regulation of RhoGTPases, thereby driving spatially restricted changes in growth and morphogenesis. Our findings about α2-chimaerin provide a remarkable example of a linear signaling pathway, relying on an obligate GAP, designated to specifically drive cytoskeletal rearrangements downstream of the EphA4 receptor in CST axons and spinal interneurons. These findings raise the question, if this is a general principle of RhoGTPase signaling?

Other RhoGTPase regulators have been found to be essential for the signaling downstream of Eph receptors during axon guidance decisions in other neurons. Ablation of ephexin-1 or Vav-2/Vav-3, that couple to activated EphA4 receptors, does not cause the axon guidance defects in locomotor circuits observed in EphA4 and α2-chimaerin mutant mice. Rather, these mutants show defects in retinal ganglion cell axon guidance (Cowan et al., 2005; Shamah et al., 2001). A GEF shown to function downstream of EphB receptors in vitro is kalirin-7 (Penzes et al., 2003). Knock-out of kalirin-7 does not result in locomotor or axon guidance defects in the visual system but in a decreased spine density in CA1 hippocampal pyramidal neurons, highlighting a potential postsynaptic function (Ma et al., 2008). Interestingly, the GEFs and GAPs mentioned here are co-expressed with Eph receptors in other neurons in addition to the neuronal
populations with detectable phenotypes. This suggests that GEFs and GAPs could link to specific receptors in a cell type specific manner.

How does a model of GEFs and GAPs as obligate effectors of specific receptors fit together with what we know from our studies about mSYD-1A?

We found that mSYD-1A can interact with Liprin-α2. Liprin-α2 interacts with the RPTP LAR, which has been demonstrated to induce presynaptic assembly in response to stimulation with NGL-3 (Woo et al., 2009). This suggests that mSYD-1A could be functioning downstream of the RPTP LAR to mediate presynaptic assembly. Implementing this hypothesis with the model introduced above, this could mean that mSYD-1A functions as an essential downstream effector of LAR in a cell type specific manner. Therefore, it is possible that mSYD-1A knock-out animals will not show a CNS-wide defect in presynaptic assembly, but rather only specific synapses will be affected. This could also explain why we did not observe a dramatic effect on presynaptic assembly after the knock-down of mSYD-1A in cortical pyramidal neurons of layer 2/3.

If this model is indeed true, an interesting question to investigate in the future is, what causes GAPs or GEFs to be essential effectors of specific receptors in some cellular contexts but not in others?

“Multi-Tasking”

Several signaling proteins play roles during multiple steps of neural development. A good example of “multi-tasking” proteins are the members of the BMP (bone morphogenetic protein), the Wnt (wingless and int) and the FGF (fibroblast growth factor) families and their receptors, which have evolutionary conserved functions as morphogens during early embryonic patterning (Godsave and Slack; Hemmati-Brivanlou and Melton, 1994; Sharma and Chopra, 1976), as positional cues during axon guidance (Butler and Dodd, 2003; Lyuksyutova, 2003; McFarlane et al., 1995) and retrograde signals during synapse formation (Hall et al., 2000; Lucas and Salinas, 1997; Marqués et al., 2002; McCabe et al., 2003; Umemori et al., 2004). Also ephrins and their
Eph receptors function during axon guidance as well as synapse formation (Dalva et al., 2000; Drescher et al., 1995). Therefore, several ligand and receptor pairs seem to have multiple functions during neural development, which introduces the question if their downstream effectors might "multi-task" as well.

GAPs and GEFs have been found to be necessary to mediate cell morphological changes downstream of Eph- and BMP-receptors in axon guidance as well as synapse formation (Ball et al.; Penzes et al., 2003; Shamah et al., 2001). Also the neuro morphological changes induced by binding of Wnt to its receptor Dishevelled (Dsh) during dendrite morphogenesis seem to be mediated by RhoGTPase signaling (Rosso et al., 2005), suggesting this could also be the case during axon guidance and synapse formation. Although specific GAPs and GEFs that regulate RhoGTPase signaling downstream of Dsh in neurons remain to be identified (Ciani and Salinas, 2005; Salinas and Zou, 2008). Morphological changes induced by FGF receptors have been linked to RhoGTPase signaling via the GEF ephexin in vitro in non-neuronal cells (Zhang et al., 2007). It will be interesting to investigate if GEFs and GAPs are involved in regulating RhoGTPase signaling downstream of FGF-receptors during neural development in vivo.

RhoGTPases mediate cytoskeletal rearrangements at all steps during neural development that involve morphological changes (Govek et al., 2005). In most cases the GTPases are regulated in their actions by GEFs and GAPs. The hypothesis I want to introduce here is that the same GEF or GAP could be functioning downstream of the same receptor during different steps in neural development.

We identified α2-chimaerin as essential downstream effector of ephrinB3 induced and EphA4-receptor mediated axonal repulsion during axon guidance at the spinal cord midline. The same ligand-receptor interaction is involved in regulating spine length on hippocampal CA1 neurons and EphA4 knock-out mice show a higher spine density along the dendrites of CA1 pyramidal neurons (Murai et al., 2003). In both cases ephrinB3-EphA4 signaling induces growth inhibition. Therefore, it is reasonable to hypothesize that the same downstream signaling
mechanism, namely Rac1 inactivation through α2-chimaerin, is active in the axon growth cone as well as the dendritic spine. If this hypothesis is valid than spine density on CA1 pyramidal neurons in α2-chimaerin knock-out mice should be increased, as it is in mice lacking EphA4 expression. Importantly, our laboratory and others have found that α1-chimaerin, the shorter α-chimaerin isoform, upon overexpression, induces the pruning of spines and dendritic branches in cerebellar Purkinje neurons in slice culture as well as cultured hippocampal neurons (Buttery et al., 2006; Van de Ven et al., 2005). However, α2-chimaerin overexpression in cultured hippocampal neurons suggested that it is not involved in the pruning of spines or dendrites. This would be an argument against the hypothesis that α2-chimaerin functions in the pruning of spines downstream of EphA4. However, it is important to keep in mind that α2-chimaerin is activated by the binding of its SH2 domain to activated phosphorylated Eph receptors. Thus, overexpressed α2-chimaerin might be inactive, and ephrin stimulation might be necessary to reveal its function. The hypothesis that α2-chimaerin could be a “multi-tasking” GAP is therefore still worth investigating.

The “multi-tasking” hypothesis becomes much more speculative regarding mSYD-1A, since we do not know yet, if it indeed functions in presynaptic assembly. However, the fact that we identified CAZ components to biochemically interact with mSYD-1A supports a potential presynaptic role. mSYD-1As interaction with Liprin-α2 links it indirectly to the RPTP LAR, since LAR and Liprin-α biochemically interact (Serra-Pagès et al., 1998). The hypothesis that mSYD-1A could play a role in presynaptic assembly as well as axon guidance is mainly based on the fact that Liprin-α as well as LAR are involved in both. In Drosophila, DLAR and DLiprin function together in the guidance of photoreceptor axons (Choe et al., 2006), as well as in presynaptic assembly at the NMJ (Kaufmann et al., 2002). In vertebrates, Liprin-α presynaptically interacts with several CAZ components and LAR mediates presynaptic assembly induced by postsynaptic NGL-3 (Ko et al., 2003a; Woo et al., 2009). Thus, mSYD-1As interaction with Liprin-α besides
supporting a potential function in presynaptic assembly, could also hint at a role in axon guidance.

That GAPs and GEFs function downstream of the same receptor at several steps during neural development, could be a common theme. One practical explanation for the “recycling” of specific RhoGTPase signaling pathways during different steps of neural development is that similar cytoskeletal rearrangements are required at different developmental stages. It will be interesting to investigate how much RhoGTPase signaling pathways downstream of neuronal transmembrane receptors, are conserved among different neurodevelopmental steps. Understanding the differences and commonalities in RhoGTPase signaling downstream of a given receptor at different developmental timepoints, could teach us about how signal transduction pathways are fine tuned to deliver a specific morphological output.

FUTURE DIRECTIONS
Morphological Changes in response to extracellular signals are key to the assembly of neuronal circuits. Neural migration, axonal and dendritic arbor growth, axon guidance and synapse formation are all dependent on changes in neuronal morphology. The extracellular signals inducing those changes are received by transmembrane receptors and are intracellularly translated into RhoGTPase regulated cytoskeletal rearrangements. From our and other studies it becomes apparent that GAPs and GEFs play essential roles to mediate the signaling between neuronal transmembrane receptors and RhoGTPases. Mutations in GEFs and GAPs have been found to underlie neural disorders and mental retardation in humans (Miyake et al., 2008; Ramakers, 2002). This emphasizes the fact that loss or misfunctioning of GEFs or GAPs can result in defects in neural circuit assembly that have drastic effects on behavior. Thus, it is important that we continue to investigate the functioning of GEFs and GAPs during neuronal development.
In the past GEFs and GAPs have mainly been studied in vitro by overexpression and knock-down in neuronal cultures. Under those conditions the manipulation of many GEFs and GAPs show dramatic and very similar effects on neuronal morphology, causing doubt about the physiological relevance of those findings. For example, the knock-down of the GEFs oligophrenin and Tiam1 or the overexpression of a dominant negative mutant of the GEF kalirin-7, in hippocampal neurons in vitro, results in all cases in a reduction in spine density (Govek et al., 2004; Penzes et al., 2001; Tolias et al., 2007). Also the observation that the knock-down of Tiam-1 as well as kalirin-7 inhibits ephrinB1/EphB2 induced spine growth suggests that their functions overlap (Penzes et al., 2003; Tolias et al., 2007). Another confusing finding coming from in vitro knock-down experiments is that kalirin-7 seems to be involved in the regulation of spine growth downstream of multiple receptors as, EphB2, N-Cadherin, AMPA and the 5 HT-2a receptors (Penzes et al., 2003; Xie et al., 2007). Although in vitro experiments have supplied us with a lot of useful information about the potential functioning of GEFs and GAPs, their true importance for neural development can only be assessed with in vivo loss of function studies. Surprisingly, knock-out studies have not been particularly informative as to the in vivo functions of most GEFs and GAPs. The phenotypes are often minor structural and small functional defects. Those shortcomings become especially apparent considering the knock-out of kalirin-7. Lack of kalirin-7 expression results in a rather subtle phenotype with a 30% decrease in spine density, which is surprising considering it was proposed to be a central signal integrator for spine growth, downstream of multiple surface receptors. Behaviorally kalirin-7 knock-out mice only show a subtle very specific impairment in anxiety and fear based learning (Ma et al., 2008). Conventional knock-out studies do not seem well suited to understand the specific in vivo functions of GEFs and GAPs for several reasons: 1) The knock-outs described for GEFs and GAPs so far cause detectable morphological phenotypes only in specific neurons as e.g. α2-chimaerin in the neurons of the motorcortex and spinal cord and Vav in retinal ganglion cells (Beg et al., 2007; Cowan et al., 2005; Iwasato et al., 2007; Wegmeyer et al., 2007). 2) Often knock-outs cause only
very subtle changes in neuronal morphology and are therefore difficult to detect. 3) Some GAPs and GEFs probably do function redundantly and therefore their knock-out would not result in any phenotype. 4) Knock out studies do not give us any information about what RhoGTPase a GEF or GAP has specificity for, when and where in a neuron it is active and what upstream receptors it links to. However there are no other experimental strategies available to uncover the in vivo functions of GAPs during nervous system development.

One possible strategy I would like to suggest is inspired by methods from the field of kinase signaling. The challenges in this field are in some ways very similar to the challenges in deciphering GAP and GEF signaling: 1) identification of specific substrates downstream of a kinase or of the specific RhoGTPase downstream of a GEF or GAP, 2) loss of function studies without the interference of redundancy. Different methods have been developed to overcome these challenges in the field of kinase research (Bishop and Shokat, 1999). One approach is a chemical-genetic approach for generation of monospecific inhibitors of any protein kinase. For this the target kinase is made specifically sensitive to the inhibitor N6-(benzyl)ATP through mutation of a conserved residue in its active site (Liu et al., 1998). Applied to GEFs and GAPs this would mean to create mutations in the PH or GAP domain that allow for the selective inhibition of the domain by a small synthetic molecule. To use this strategy in vivo the mutation would need to be directed at the genomic locus of a specific GEF or GAP by targeted knock-in. This strategy would enable rapid, reversible, local and graded (dose-dependent) inactivation of specific GEFs or GAPs and possibly could overcome the problems of redundancy, since the signaling system would have no time to adjust to the change. Treatment of knock-in animals with the specific inhibitor could be used to inactivate the targeted GEF or GAP at any time during development. This would allow for the dissection of a GEF’s or GAP’s function during different steps in neural development and due to the possibility of a local application of the inhibitor, it could even be used to assess the role of a GEF/GAP in different cell types. The FRET assay as described in chapter 3 can be used to resolve the temporal and spatial activity of RhoGTPases.
The assay makes use of a RhoGTPase sensor, which is a fusion protein between a GTPase of interest with YFP, CFP and a Rho binding domain (RBD) of a specific effector for the given GTPase (Figure 3.5, Pertz, 2006). If the RhoGTPase is bound to GTP it will interact with the RBD and FRET will occur. It could be interesting to combine a knock-in of a FRET sensor with a knock-in of an inhibitor sensitized GEF or GAP. Such animals would allow the study of changes in RhoGTPase signaling in response to the inhibition of a specific GEF or GAP in real time.

*Prospectus*

Due to their ability to link neuronal transmembrane receptors with intracellular cytoskeletal changes, RhoGTPase regulators are poised to play essential roles during neuronal circuit assembly. This notion is supported by the finding that mutations in several GEFs and GAPs lead to mental disorders that are thought to arise from disturbances of circuit structure (Govek et al., 2004; Pinto et al., 2010). In the future, the study of single GEFs and GAPs will help us to understand how the specific changes in neuronal morphology that underlie the assembly of neural circuits are achieved. In addition, it will further our understanding of the morphological changes that underlie disturbances in brain function.
CHAPTER 5

MATERIALS AND METHODS
CHAPTER 2: MATERIALS AND METHODS

Recombinant Proteins

The following recombinant proteins were purified from *E. coli* and coupled to glutathione sepharose or NHS-activated sepharose beads: Glutathione-S-transferase (GST); GST-SH2, containing amino acids 1-182 of murine alpha2-chimaerin; maltose-binding protein (MBP); MBP-A4, containing residues 572 – 978 (the intracellular domain) of murine EphA4; and MBP-A4/KD containing residues 572 – 978 (the intracellular domain) of murine EphA4 with a V635M mutation.

Biochemical Assays

Mouse brains from P14 animals were homogenized and separated into cytosol, membrane, and nuclear fractions. The membrane fraction was solubilized in 50mM Hepes pH 7.5, 1% Triton X-100, 0.5% sodium-deoxycholate, 10% glycerol, 100mM NaCl, 1.5mM MgCl2, 1mM EGTA, 10mM NaF, 1mM Na3VO4 and the solubilized membrane proteins were passed over a column with purified recombinant SH2 domain of α2-chimaerin fused to GST. Proteins were eluted with increasing sodium chloride concentrations (50mM – 2M NaCl) and eluted proteins were analyzed by silver staining and Western blotting. For the pull-down assays all cDNA expression vectors used for expression of proteins contained the CMV immediate early promoter and epitope tags for detection. HEK293T cells were transiently transfected using Fugene (Roche). Sixteen hours after transfection cells were lysed in 50mM Hepes pH 7.5, 0.5% Triton X-100, 10% glycerol, 1.5mM MgCl2, 1mM EGTA, 100mM NaF, and 1mM Na3VO4. Pull downs with GST-SH2 beads were performed with 150µl cell or tissue lysate diluted in 450µl 20mM Hepes pH 7.5, 50mM NaCl, 10% glycerol, 1mM EGTA, 1.5mM MgCl2, 1mM NaF, 1mM Na3VO4 for 3h at 4°C. MBP/MBP-A4/MBP-KD beads were pre-incubated in “Kinase Buffer” (20mM Hepes pH 7.5, 50mM NaCl, 6mM MgCl2, 4mM MnCl2, 0.1 mM Na3VO4) or λ - Phosphatase buffer (NEB) with 100µM ATP or λ - Phosphatase. Pull downs were performed with 150µl cell lysate diluted in 450µl “Kinase-Buffer”
for 3h at 4°C. After all pull downs the beads were washed 3x with 20mM Hepes, 200mM NaCl, 0.2% Triton X-100, 10% Glycerol, 1mM EGTA, 1.5, 6mM MgCl₂, 1mM NaF, 1mM Na₂VO₄. All pull downs were performed in the presence of complete protease inhibitor EDTA free (Roche), 1 tablet per 10ml. Proteins were resolved on SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with the indicated antibodies.

**Antibodies**

Rat monoclonal anti-HA (Roche, clone 3F10), mouse monoclonal anti-EphA4 receptor (Zymed, clone 4C8H5), rabbit anti-TrkA (Santa Cruz.), mouse anti phospho-tyrosine (Santa Cruz, PY99), anti α2-chimaerin (Buttery et al., 2006), rat anti-CTIP2 (Abcam), goat anti-b-galactosidase (Biogenesis), mouse anti-NeuN (Chemicon), goat anti-parvalbumin (Swant). Secondary antibodies were from Jackson Immuno Research, for IP-western analysis secondary antibodies specific to the antibody light chains were used to minimize detection of IgG used for immunoprecipitation.

**Retrograde Tracing**

Mice containing a gene-trap insertion in the α-chimaerin gene were obtained from Lexicon Genetics. Spinal cords of postnatal day 5 wild type and α2 gene-trap homozygotes were dissected by ventral laminectomy in cold L15 media (Mediatech). Spinal neuronal projections to the contralateral side of the cord were retrogradely labeled by applying crystals of Rhodamine Dextran (3000 MW, Molecular Probes) to a unilateral cut at the L3/L4 transition. The isolated spinal cords were incubated in oxygenated retrograde transport solution (in mM: 137 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 0.2% w/v glucose, 1 NaH₂PO₄, 24 NaHCO₃, pH = 7.4) for 8-12 hours before immersion fixation and sectioning. Labeled spinal cords were fixed in 4% PFA in 1X PBS for two hours and 50 mm serial vibratome sections were collected and mounted in Fluoromount-G.
(Southern Biotech). At least four consecutive sections were analyzed at the L2 level for retrograde labeling quantification.

**Corticospinal Tract Tracing**

Adult animals were anesthetized with a ketamine/xylazine mix (100/10 mg/kg body weight; i.p.) and the motor cortex was pressure injected with the anterograde tracer biotin dextran amine (10% BDA in 0.1 M PBS; Molecular Probes, 400 nl into four sites covering the forelimb to hindlimb representation in motor cortex). After 10-11 days, the animals received an overdose of ketamine/xylazine and were transcardially perfused with 1X PBS, followed by 4% PFA in 1X PBS. Spinal cords were isolated and postfixed for 2 hours at room temperature followed by overnight immersion in 30% sucrose. Frozen microtome sections of traced brain and spinal cord (50 mm) were collected and incubated overnight with and avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit, 1:100 in PBS) at room temperature. Sections were reacted with diaminobenzidine (DAB), washed, mounted, coverslipped on glass slides, and examined under darkfield optics using a Zeiss Axioimager microscope.

**Corticospinal Stimulation and EMG recording**

Cortical stimulation was performed by stereotaxic insertion of paraleen-insulated tungsten microelectrodes (Microprobe Inc.) into hindlimb motor cortex. Using a constant current stimulator, trains of biphasic initially cathodal stimuli (45 ms; 333 Hz; 0.2 ms pulse duration; trains every 2s) of increasing intensity were applied until a contralateral hindlimb movement was evoked; whereupon, we noted the current (threshold) and the presence or absence of an ipsilateral hindlimb response. Multiple stimuli at threshold intensities were applied to ensure reliable threshold measurements. In wild type, stimuli at multiples of threshold (up to 5 times) were applied to recruit and ipsilateral hindlimb response in addition to a contralateral hindlimb response. We recorded EMG activity using Ni-chrome wires inserted percutaneously into
muscle. The hindlimbs were shaved to facilitate muscle identification and to ensure accurate electrode placement. We used conventional amplification and filtration. For this study, we inspected the recorded EMG during the experiment for concordance between the kinematic effect of stimulation and the pattern of EMG activation.

**Growth Cone Collapse Assay**

Developing motor cortices from P1 mouse pups were dissected in cold L15 media (Cellgro). Cortical explants were plated in medium (Neurobasal, 1X B27 supplement, 1X Penicillin/Streptomycin/Glutamine mix, 1.2 mM MgCl₂, 35 mM Glucose) on MatTek glass bottom plates coated with 100 mg/ml poly D-lysine and 20 µg/ml Laminin (Invitrogen) and placed in a 37°C /5% CO₂ incubator. After 2 days in culture, explant plates were transferred to a 37°C/5% CO₂ environment microscope for time-lapse image analysis. Individual axonal growth cones from the explants were imaged for a total of 45 minutes with a 63X 1.4 NA Zeiss objective. Growth cones were imaged for 15 minutes before treatment to determine basal growth rates. After 15 minutes, 250 µl (1µg/ml of control Fc protein or ephrin-A1-Fc fusion protein) was bath applied to explants, and time-lapse imaging proceeded for an additional 30 minutes. Each growth cone was imaged at 30 second intervals before and after treatment. Growth cone collapse and distance traveled were scored blind towards genotype using Metamorph image analysis software.

**CHAPTER 3 : MATERIALS AND METHODS**

**Expression Constructs**

All eukaryotic msyd-1A and msyd-1B expression constructs were cloned into a pCAG (β-actin promoter) vector downstream and in frame with an HA tag, except of myr-SYD-1A which is cloned with a C-terminal HA tag. The cDNAs were PCR amplified and restriction sites necessary for in frame cloning with HA added with the PCR.
**HA tagged full length msyd-1A and msyd-1B**: cDNAs encoding the predicted full length cDNA for msyd-1A (Riken_1200008N06) and partial cDNA for msyd-1B (Riken_C430017H16) were obtained from the Riken Institute. The msyd-1A cDNA was PCR amplified adding a 5' SalI restriction site and a 3' BamHI restriction site and cloned into a SalI/BamHI opened pNICE-HA vector (containing a CMV promoter). This gave rise to a pNICE vector containing msyd-1A cDNA with a 5' HA tag. HA-msyd-1A was cut out of the pNICE vector using XhoI and HpaI and cloned into a pCAG vector (β-actin promoter) opened with XhoI/SphI, giving rise to pCAG-HA-mSYD-1A, which is the msyd-1A expression vector used for most experiments in this thesis.

Since only a partial msyd-1B clone (lacking 5' sequences) was available the full length cDNA was assembled according to the exon predictions of the UCSC Genome Browser and the translational start site prediction on NCBI. We had a 951bp fragment containing the predicted translational start and a 278bp overlap with the Riken clone synthesized by DNA2.0. Two silent point mutations (C709T and G925A) were introduced within the fragment to remove an XhoI and BamHI restriction site. The fragment was excised out of the delivery vector with PacI and EcoRV to be ligated 5' of the msyd-1B cDNA in the pFLC1 vector received from Riken. Full length msyd-1B was cut out of pFLC1 using XhoI/BamHI and ligated into a pNICE vector containing a 5' HA tag.


**GAP domain mutants**: Quick Change Lightning Multi Site, Directed Mutagenesis kit, Agilent-Stratagene

**Myristoylated and palmitoylated full length msyd-1A**: myr-SYD-1A. The minimal MARCKS-3/4 Cys (MGCCFSKT) sequence (R. Tsien, UCSD) containing a myristoylation and palmitoylation sides was added to the full length msyd-1A with the forward PCR primer. A C-terminal HA tag was added with the reverse PCR primer.
**Liprin-α Constructs for Recombinant Protein Expression**

Constructs in the pGEX-4T vector expressing the GST fused mouse Liprin-α1, -α2, -α3, -α4 minimal ELKS binding domain (Liprin-α-CC, amino acids α1: 351-673, α2: 369-696, α3: 333-645, α4: 185-419) were a gift from Eunjoon Kim at KAIST, Daejeon, Korea. Constructs in the pET vector expressing the 6xHis fused human Liprin-α2 SAM domains (Liprin-α-SAM, amino acids: 866-1258) were a gift from Mingjie Zhang at HKUST, Hong Kong, China.

**Origin of other Expression Constructs**

The RhoA sensor construct was a gift from Olivier Pertz, University of Basel (Pertz et al., 2006). The ELKS2 construct with N-terminal GFP tag was a gift from Toshihisa Ohtsuka, University of Yamanashi (Ohtsuka et al., 2002). The tankyrase 1 and 2 constructs with N-terminal Flag tag were a gift from Nai Wen-Chi, UCSD (Chi and Lodish, 2000).

**Design and Production of mSYD-1A/B Antibodies**

All antibodies were raised in rabbit. The following peptides were used for immunization:

SYD1A-1: FLRLDHTFHELEAARLLRA (aa 312-331)
SYD1A-2: GREKLPRKKSEAKDRGHPA (aa 15-33)
SYD1A-3: MAEPLLRKTFSRLGREK (aa 1-18)
SYD1A-4a +b: DTRRPSDTPDGAVAPYC + APYLRPKRQPPLHLPC (aa 602-617 and 615-629)
SYD1A-5: ILDLERELSKIQINVC (aa 722-737)
SYD1B-1: EKATLKMDDHLKLVASY (aa 1071-1088)
SYD1B-2: RYHLDTTVSSRHSYRK (aa 712-728)
SYD1B-3: MPGASGGQKRASEPLTLESY (aa 1-18, for a predicted ORF)
SYD1B-4: EDLPLKPPAVTVKQLQ (aa 520-535)
The immunizations with peptides SYD1A-1, -2, -3 and SYD1B-1, -2, -3 were performed by Covance, USA the immunizations with peptides SYD1A-4a+4b and SYD1A-5 were performed by Eurogentec, CH. The fourth bleed anti-serum received from the immunization with peptide SYD1A-3 was affinity purified over a column containing the peptide. The resulting antibody is referred to as anti-mSYD-1A. For purification the peptide was coupled to a Sulfo-link coupling gel (Pierce). After peptide coupling unspecific binding sites were blocked with 50mM L-cystein-HCl. The affinity matrix was incubated with the anti-serum for 1h at room temperature. The anti-serum was drained once and reapplied to be incubated with the affinity matrix for another 1h. After draining of the anti-serum bound antibodies were eluted with 100mM glycine pH 2.5. Collected fractions were dialyzed against 1xPBS/50% Glycerol and stored at -20°C.

**HEK293 Cell Subcellular Fractionation**

HEK293 cells were mechanically (cell cracker) homogenized in 10 mM HEPES pH 7.4, 2 mM EDTA, 1 mM MgCl₂, 1 mM DTT, complete protease inhibitor (Roche) yielding the homogenate (H). A first spin at 2900 x g for 20min yielded the post-nuclear supernatant (PNS) and the pellet P1, containing nuclei, mitochondria and unbroken cells. P1 was resuspended in 10 mM HEPES pH 7.4, 2 mM EDTA, 1 mM MgCl₂, 1 mM DTT, complete protease inhibitor and underwent a second spin at 100,000 x g for 2h, which resulted in the separation of the membranes and insoluble associated proteins (pellet, P2) from the cytosol (supernatant S2). P2 was resuspended in 50 mM Tris-HCl pH 6.8, 2 % SDS, 100 mM DTT, complete protease inhibitor (Roche).

**Brain Subcellular Fractionation**

Brains of mice at postnatal day 2 (P2) were dissected on ice and weighed. Homogenization was performed using a hand homogenizer in 10 volumes 10mM HEPES pH 7.4, 2 mM EDTA, 1 mM MgCl₂, 1 mM DTT, complete protease inhibitor (Roche) yielding the homogenate. A first spin at 2900 x g for 20min was performed and the resulting pellet resuspended in 10mM HEPES pH 7.4,
2 mM EDTA, 1 mM MgCl₂, 1 mM DTT, complete protease inhibitor (Roche) and passed five times through a syringe with a 22G needle resulting in the homogenate (H). The homogenate was centrifuged again at 2900 x g for 20 min yielding the post-nuclear supernatant (PNS) and the pellet P1, containing nuclei, mitochondria and unbroken cells. P1 was resuspended in 10 mM HEPES pH 7.4, 2 mM EDTA, 1 mM MgCl₂, 1 mM DTT, complete protease inhibitor (Roche). The PNS was centrifuged at 100,000 x g for 2h, which resulted in the separation of membranes and insoluble associated proteins (pellet, P2) from the cytosol (supernatant S2). P2 was resuspended in 50 mM Tris-HCl pH 6.8, 2 % SDS, 100 mM DTT, complete protease inhibitor (Roche).

**Synaptosome Prep**

P10 mouse cortices were homogenized with a motor driven homogenizer in 4 mM HEPES pH7.4 containing 0.32M sucrose (10ml / 1g of tissue). The homogenate (H) was centrifuged at 1000 x g for 10 min resulting in the pellet P1, containing nuclei and unbroken cells and the supernatant S1, composed of brain cytosol and membranes. A second high speed spin of S1 at 10,000 x g for 15 min, resulted in the crude synaptosomal pellet (P2) and a cytosolic supernatant that was not further fractionated. P2 was resuspended in 4 mM HEPES pH7.4 containing 0.32M sucrose (ca. 10 volumes) and centrifuged again at 10,000 x g for 15 min. The pellet (washed crude synaptosomal fraction) P2', was lysed by hypoosmotic shock in 9 volumes of ice cold H₂O with 10 strokes in a glass homogenizer. The pH was adjusted to 4 mM HEPES using 1M HEPES, pH 7.4 (40ul for 10 ml). The lysed synaptosomes were incubated for 30 min at 4°C to ensure complete lysis. Following the incubation the lysate was centrifuged at 25,000 x g for 20 min, giving rise to a pellet (P3) containing lysed synaptosomal membranes and associated insoluble proteins and a supernatant (S3) containing soluble synaptic proteins and synaptic vesicles. To further purify synaptic plasma membranes P3 was resuspended in 4 mM HEPES, pH to 7.4 containing 0.32M sucrose and 50 mM NaCl (ca. 1/15 of the volume used to resuspend the cortices in the beginning) and centrifugated over a sucrose gradient of (0.8M / 1.0M / 1.2M) at 150,000 g for 2h. The
synaptic plasma membranes (SPM) collect at the interface between the 1.0M and 1.2M sucrose layers and were extracted from the ultracentrifuge tube with a syringe. The recovered SPM was diluted and spun again at 150,000 x g for 2h to yield the final SPM fraction. To purify postsynaptic densities (PSD) The SPM fraction was extracted for 15min at 4°C in 50 mM HEPES pH 7.4, 50mM NaCl, 2 mM EDTA, 0.5% Triton X-100 and centrifuged at 200,000 x g for 20min. This yielded SPM-S1, the supernatant containing presynaptic soluble proteins and the pellet (PSD) mainly containing postsynaptic proteins and insoluble CAZ components. All steps were performed on ice and in the presence of complete protein inhibitor (Roche).

**FRET Assay**

HEK293 cells were transfected with the RhoA sensor (Pertz et al., 2006) and either a msyd-1A expression construct or an empty control vector (mock). After 48h the cells were suspended in 1xPBS. The emission spectrum between 450-600nm after excitation with 430nm light was measured in a Fluorescence Spectrophotometer (F-4500, Hitachi). Following the measurement the cells were pelleted and lysed in a PBS based buffer containing 1% Triton and complete protease inhibitor (Roche). The cell lysates were analyzed on Western Blot to control for the equal expression of the RhoA sensor in all samples. The RhoA sensor was detected with a homemade antigen-GFP antibody (rabbit) at a 1:1000 dilutions. The expression of mSYD-1A and all its deletion or mutant constructs was detected with an anti-HA antibody (rat) from Boehringer Mannheim. All Western Blots were probed for actin (loading control) with an anti-actin antibody (mouse) from Sigma. Myc-tagged protein were detected with an anti-Myc antibody (mouse) from Sigma.

**Co-Immunoprecipitation**

HEK293 cells were transiently transfected (Fugene, Roche) with a plasmid encoding HA-mSYD-1A or GFP-ELKS2 or both plasmids together. After 48h the cells were washed with ice cold
1xPBS and lysed in 20mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1mM NaF, 1mM Na$_3$VO$_4$, 1mM DTT. All steps were performed on ice. The lysate was centrifuged at 13,000 rpm, 4°C in an Eppendorf table top centrifuge for 10min and the supernatant transferred into a new tube. 1 part lysate was mixed with 4 parts Lysis buffer without Triton (final triton concentration in mix 0.25%). 1µl of homemade anti-GFP antibody (rabbit) or no antibody was added and the mix incubated for 90min on ice. After the incubation the mix was centrifuged at 13,000rpm, 4°C for 5min and the supernatant transferred into a new tube. ProteinA sepharose (GE Healthcare) was added to the samples containing anti-GFP antibody, anti-HA conjugated Agarose (Sigma) to the samples without antibody. The mix was incubated on an overhead rotator over night in the cold room. The next morning the sepharose/agarose beads were precipitated at 1000 rpm in an Eppendorf tabletop centrifuge and washed three times in Lysis Buffer. All steps were performed in the presence of Complete Protease Inhibitor EDTA free from Roche. After the last wash step beads were resuspended in 2x SDS-sample buffer (200mM DTT, 100mM Tris-HCl pH 6.8, 4%SDS, 20% glycerol, 0.05% bromphenolblue). Proteins were resolved on SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with anti-GFP (rabbit, 1:1000, homemade) and anti-HA (rat, 1:1000, Boehringer Mannheim).

For Co-Immunoprecipitations (IP) from brain lysates the same buffers as for the HEK cell Co-IPs were used. Endogenous mSYD-1A was precipitated and detected (1:1000) with the homemade anti-mSYD-1A antibody. For Western Blot detection of endogenous ELKS2 the anti-ERC1b/2 antibody (rabbit,1:500) from Synaptic Systems was used.

**Pull Down**

*Recombinant Proteins*

The following proteins were purified from E. coli and coupled to glutathione sepharose or Ni$^+$ agarose beads: 6 x His-Glutathione-S-transferase (GST), GST-Liprin-α1-CC containing
aminoacids 351-673 of murine Liprin-α1, GST-Liprin-α2-CC containing aminoacids 369-696 of Liprin-α2, GST-Liprin-α4-CC containing aminoacids 158-419 of Liprin-α4, 6 x His-Liprin-α2-SAM containing aminoacids 866-1258 of human Liprin-α2.

Pull Downs

HEK293 cells were transiently transfected (Fugene, Roche) with HA-mSYD-1A, HA-NTD, HA-NTD+C2, HA-C2+GAP, HA-GAP, CASK or ELKS2. After 48h cells were lysed. The lysis buffer for pull down with Liprin-CC immobilized on GST beads was: 50mM HEPES pH7.4, 1% Triton X-100, 0.1% SDS, 10% glycerol, 1.5mM MgCl₂, 1mM EDTA, 100mM NaF, 1mM Na₃VO₄, complete protease inhibitor (Roche). The lysis buffer for the pull down with Liprin-SAM on Ni²⁺ beads was: 20mM Tris-HCl pH 8.0, 1% Triton X-100, 10% glycerol, 100mM NaCl and complete protease inhibitor (Roche). The lysates were centrifuged at 13,000 rpm for 10min in an Eppendorf tabletop centrifuge and the supernatants transferred into a new tube. Lysates were diluted 1:6 in Dilution Buffer: Dilution Buffer Liprin-CC: 20mM HEPES pH 7.4, 50mM NaCl, 10% glycerol, 1mM EDTA, 1.5mM MgCl₂, 1mM NaF, 1mM Na₃VO₄. Dilution Buffer Liprin-SAM: 20mM Tris-HCl pH 8.0, 10% glycerol, 100mM NaCl, 5mM Imidazole pH 8.0. For the pull downs10µl of beads coupled to the indicated recombinant protein were incubated with the diluted lysate for 3h at 4°C. The beads were precipitated at 1000rpm and washed three times in wash buffer. Wash Buffer Liprin-CC: 20mM HEPES pH 7.4, 200mM NaCl, 0.2% Triton X-100, 10% glycerol, 1mM EDTA, 1.5mM MgCl₂, 1mM NaF, 1mM Na₃VO₄. Wash Buffer Liprin-SAM: 20mM Tris-HCl pH 8.0, 0.2% Triton X-100, 200mM NaCl, 10mM Imidazole pH 8.0. After the last wash the beads were resuspended in 2 x SDS-sample buffer. All steps were performed in the presence of Complete Protease Inhibitor EDTA free (Roche). Proteins were resolved on SDS-PAGE, transferred to nitrocellulose and immunobotted with anti-HA (rat, 1:1000, Boehringer Mannheim) or anti-Myc (Sigma) antibodies.
**msyd-1B Conditional Knock-Out Mice**

We commissioned the University of Connecticut Gene Targeting and Transgenic Facility for cloning and creation of mice carrying two loxP insertions in the *msyd-1B* (*syde2*) gene on chromosome 3 (*msyd-1B* flox). The 5′ loxP site is inserted into intron 2 the 3′ loxP site into intron 4. *msyd-1B* flox mice were crossed with mice carrying a Cre-recombinase transgene expressed under CMV promoter control. This gave rise to the *msyd-1B* knock-out mice.

**In Utero Electroporation**

*micro RNA Mediated knock-down*

To reduce mSYD-1A protein levels *in vivo* we used micro RNA (miRNA) mediated knock-down.

We obtained the pcDNA 6.2-GW/EmGFP-miR vector from Invitrogen, which allows co-cistronic expression of an engineered *msyd-1A* targeting miRNA in the 3′untranslated region of Emerald Green Fluorescent Protein (EmGFP) under control of a Pol II (CMV) promoter. Following transcription of the co-cistronic RNA the miRNA hairpin is cleaved off the EmGFP part of the transcript by Drosha in the nucleus, and further processed by Dicer in the cytoplasm to yield mature miRNA that can be incorporated into an RNA induced silencing complex (RISC), which induces cleavage of the target mRNA (Invitrogen, BLOCK-iT manual). The fluorescence of EmGFP allows tracking of the miRNA expression and provides strong correlation of EmGFP expression with the knock-down of the target gene by the miRNA. We transferred the EmGFP-miR cassette from pcDNA 6.2 through Gateway recombination into a pCAG destination vector, which allows for the expression of the EmGFP-miR transcript under control of the β-actin promoter (pCAG-miR). The miRNA targeting *msyd-1A* encodes a short RNA complementary to nucleotides 1051-1071 (gtgctattgccaaccatcttt) in exon 3 within the ORF of *msyd-1A*. The efficiency of the *msyd-1A* knock-down was assessed by co-transfecting HEK293 cells with HA-*msyd-1A* and pCAG-miR containing the miRNA targeting *msyd-1A* (pCAG-miR-1A) or a control
vector containing an miRNA targeting β-galactosidase (pCAG-miR-CNT). The cells were lysed and HA-mSYD-1A protein levels in the lysates analyzed on Western Blot with anti-HA (rat, Boehringer Mannheim) antibody.

*In Utero Electroporation*

For *in utero* electroporation timed-pregnant mice (E15) were anesthetized with an intraperitoneal injection of ketamine/xylazine (100/10 mg/kg body weight). The surgical procedure was performed as described in Saito, Nature Protocols, 2006 (Saito, 2006). The glass capillaries used for injection were "wiretrol II" capillaries (Drummond scientific), pulled on a micropipette puller (model: P87, settings: heat 80, pull 30, velocity 40, time 200, pressure 500, Sutter Instruments Co.). The pCAG-miR (0.75 µg/µl) construct was injected together with a synaptophysin-RFP expression construct (0.5 µg/µl) into the lateral ventricles of the E15 embryos in utero. The electrical pulses (5 x 50mV, 950ms interval) were delivered with 7mm diameter platin tweezer electrodes (BTX) connected to a square wave electroporator (model: CUY 21 Edit, Sonidel). The electrodes were positioned to achieve electroporation of precursor neurons in the cortical subventricular zone (LoTurco et al., 2009). After the surgery the electroporated mice were left to develop normally. The electroporated pups were perfused at P15 with 4% Paraformaldehyde in 0.1M Phosphate Buffer. The brain was dissected and coronal sections (50µm) were cut on a vibratome (Leica VT1000S). For immunostaining the sections were treated with pepsin (0.15mg/ml in 0.2N HCl) for 10min at 37°C and washed three times for 5min with 1 x PBS. For permeabilization the sections were incubated in 50mM Tris, 150mM NaCl, 0.1% Triton, 10% Donkey Serum for 30min at room temperature (RT). Incubation with primary antibodies (anti-GFP, rabbit, 1:1000, homemade; anti-GFP, chick, 1:1000, Upstate; anti-RFP, guinea pig, 1:1000, homemade; anti-ER81, mouse, 1:20,000, generously provided by the Jessel lab, Columbia University) was carried out for 36h at 4°C in staining buffer (50mM Tris, 150mM NaCl, 0.1%
Triton, 2% Donkey Serum). After two 30min washes in wash buffer (50mM Tris, 150mM NaCl, 0.05% Triton) secondary antibodies (donkey anti rabbit Cy2, 1:200; donkey anti chicken Cy2, 1:200; donkey anti guinea pig Cy3, 1:500; donkey anti rabbit Cy5, 1:500; all from Jackson Laboratory) were applied in staining buffer at 4°C overnight. Sections were washed three times for 30min and than mounted on glass object slides (Superfrost Plus, Fisherbrand, Fisher Scientific) with fluoromount-B (Southern Biotech). Mounted sections were stored at -20°C.

**Image Acquisition and Analysis**

Microscopy was carried out with LSM 5 Exciter confocal microscope from Zeiss. The following objectives were used: 10x 0.45 NA for overview images of electroporated brain sections, 63x 1.4 NA oil immersion for imaging of single axonal fibers in electroporated brain sections. All images were acquired, using the LSM 5 Exciter software by Zeiss. Confocal stacks were projected into one plane and analyzed in Metamorph. An intensity and size threshold for synaptophysin-RFP was chosen to exclude puncta too small and too faint to be presynaptic synaptic vesicle clusters.

**siRNA Mediated Knock-Down in Primary Granule Neuron Cultures**

P5 mouse cerebella (wild type, *msyd-1B +/−* or *msyd-1B −/−*) were dissected and dissociated using 0.44mg/ml trypsin (in the presence of DNase and followed by incubation with trypsin soybean inhibitor) and mechanical tituration using a fire polished glass pipette. Granule neurons were purified by size exclusion using centrifugation through a 40% Percoll cushion. The granule neuron pellet was resuspended and centrifuged over a 4% BSA (bovine serum albumine) cushion. The resulting cell pellet was resuspended and neurons were plated on glass coverslips (previously coated with Poly-D-ornithine and Laminin) at a density of 100,000 cells per well (24 well cell culture dish) in Neurobasal (Gibco) containing B27 (Invitrogen) and 1 x GlutaMax (Invitrogen) media supplements (no serum, no antibiotics).
To reduce *msyd-1A* expression in granule neurons the neurons were treated at 6 days *in vitro* (DIV) with an *msyd-1A* specific pool of siRNAs (SYDE1 Accell SMART pool, Dharmacon) or a control pool of non-targeting siRNAs (Accell Non-targeting-pool, Dharmacon) at a final concentration of 0.2µM in the cell culture medium. The Accell siRNAs from Dharmacon are modified to be membrane permeable and therefore can be added to the culture medium (serum free) without the need of any transfection reagents. The granule neurons were incubated for 4 days after addition of the siRNAs. At 10 DIV the cultures were fixed through addition of 4% PFA, 4% Sucrose in 0.1M Phosphate Buffer. After three washes with 1xPBS the neurons were permeabilized for 10min in 100% Methanol at -20°C. The cells were re-hydrated for 5min in 1xPBS and than incubated in blocking buffer 1xPBS, 10% Donkey Serum, 0.1% Triton X-100 for 30min at RT. Incubation with primary antibodies (anti-VGlut1, guinea pig, 1:5000, chemicon; anti-PSD95, mouse, 1:200, Affinity Bioreagents; Map2, rabbit, 1:500, Chemicon) was carried out in blocking buffer overnight at RT. After three 5min washes in 1xPBS coverslips were incubated with secondary antibodies (donkey anti guinea pig Cy3, 1:500; donkey anti mouse Cy2, 1:200; donkey anti rabbit Cy5, 1:500 all from Jackson Laboratory) in blocking buffer for 2h at RT in the dark. After three washes in 1xPBS the coverslips were mounted with fluoromount-G (Southern Biotech) on glass object slides.

*Image Acquisition and Analysis*

Microscopy was carried out with LSM 5 Exciter confocal microscope from Zeiss with the 63x 1.4 NA oil immersion objective (z step size 0.37µm). All images were acquired using the LSM 5 Exciter software by Zeiss. Images were analyzed using Metamorph. Intensity thresholds were chosen to exclude background puncta. The VGlut1 and PSD95 puncta selected that way were counted and their density per 50µm of dendritic segment calculated.
**RT-PCRs and qPCRs**

RNA was extracted using TRIZOL reagent (Invitrogen) from wild type or *msyd-1B* KO mouse brains and treated with Turbo DNase (Applied Biosystems). Reverse transcription (RT) was carried out using the ImProm II RT system from Promega. The resulting cDNA was used in PCR reactions (basic characterization of *msyd-1A* and *msyd-1B* expression) or qPCR (*msyd-1B* expression in wild type compared to KO). PCRs were run with the PCR Master Mix from Promega and analysed on 2% agarose gels containing Gel Red (Biotium). qPCRs were run with the Power SYBR green PCR Master Mix in the StepOne Cycler (both from Applied Biosystems). The StepOne software (Applied Biosystems) was used to determine C_T values of the samples. PCRs for β-tubulin were used as active reference control (normalizer). The Comparative C_T Method was used to calculate comparative expression levels of *msyd-1B* in RNA extracts from wild type and *msyd-1B* KO brains.

**PCR primers:**

<table>
<thead>
<tr>
<th>cDNA-orientation</th>
<th>sequence</th>
<th>location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>msyd-1A</em>-fwd</td>
<td>cccaacatccaaggtgcgc</td>
<td>border exon2/3</td>
</tr>
<tr>
<td><em>msyd1A</em>-rev</td>
<td>gcctggcaacctcgaaagatg</td>
<td>border exon3/4</td>
</tr>
<tr>
<td><em>msyd1B</em>-fwd</td>
<td>gttgagaaggcaaacctgaag</td>
<td>border exon5/6</td>
</tr>
<tr>
<td><em>msyd-1B</em>-rev</td>
<td>ttaagcttgccactggccag</td>
<td>border exon6/7</td>
</tr>
<tr>
<td><em>msyd-1B</em>-KO1-fwd</td>
<td>gaatcacactgacagacttatttg</td>
<td>border exon1/2</td>
</tr>
<tr>
<td><em>msyd-1B</em>-KO1-rev</td>
<td>tatgatccgggacctgttag</td>
<td>border exon2/3</td>
</tr>
<tr>
<td><em>msyd-1B</em>-KO2-fwd</td>
<td>gttgagaaggcaacctgaag</td>
<td>border exon5/6</td>
</tr>
<tr>
<td><em>msyd-1B</em>-KO2-rev</td>
<td>ttaagcttgccactggccag</td>
<td>border exon6/7</td>
</tr>
<tr>
<td>actin-fwd</td>
<td>gatgcagatacctgctgctgtg</td>
<td>exon2</td>
</tr>
<tr>
<td>actin-rev</td>
<td>ctgtatgcccctgtgtgctgaccagc</td>
<td>exon4</td>
</tr>
<tr>
<td>β-tubulin-fwd</td>
<td>ggcctcctctcaagatg</td>
<td>exon1/exon2 border</td>
</tr>
</tbody>
</table>
### β-tubulin-prec

<table>
<thead>
<tr>
<th>cDNA-orientation</th>
<th>sequence</th>
<th>location</th>
<th>use</th>
</tr>
</thead>
<tbody>
<tr>
<td>msyd-1B 2/3 fwd</td>
<td>ccatgcattcagaaattaQg</td>
<td>exon2</td>
<td>detection exon2+3</td>
</tr>
<tr>
<td>msyd-1B 2/3 rev</td>
<td>gactagatcccggacctg</td>
<td>exon3</td>
<td>detection exon2+3</td>
</tr>
<tr>
<td>msyd-1B 5/6 fwd</td>
<td>tgaagatgtgatcctctctg</td>
<td>exon5</td>
<td>detection exon5+6</td>
</tr>
<tr>
<td>msyd1B 5/6 rev</td>
<td>aagggctctcaagatgtt</td>
<td>exon6</td>
<td>detection exon5+6</td>
</tr>
<tr>
<td>β-tubulin-fwd</td>
<td>tgaggctctctctcaaagt</td>
<td>border exon1/2</td>
<td>detection of β-tubulin</td>
</tr>
<tr>
<td>β-tubulin-rev</td>
<td>cagcaccactctgaccaagat</td>
<td>border exon2/3</td>
<td>detection of β-tubulin</td>
</tr>
</tbody>
</table>

### Cell and Tissue Lysis

All lysates of cultured cells (HEK293 or neurons) or mouse brains were prepared on ice in 1% Triton, 0.1% SDS, 0.5 mM EDTA, 1x PBS, 2 mM DTT containing 1 Complete Protease Inhibitor tablet / 10 ml, unless indicated differently.
REFERENCES


Yang, L., and Bashaw, G.J. (2006). Son of sevenless directly links the Robo receptor to rac activation to control axon repulsion at the midline. Neuron 52, 595-607.


