Characterization of synaptic protein complexes in *Drosophila melanogaster*

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I hereby declare, that thesis has been written independently and with no other sources and aids than quoted.

Göttingen, March 2006

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Manuela Schmidt

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1. Introduction

"Proteins generally don't work alone, but instead assemble into complexes until their job is done. This can mean a long-lasting association, but often it is just a fleeting alliance" (Abbott, 2002).

Classically, cellular signalling cascades were depicted in a linear pathway. However, recent improvements in protein purification procedures together with technological advancements in protein identification and microsequencing provided evidence that most proteins do their jobs in association with other proteins. Those protein complexes are believed to act as "molecular machines" (Alberts, 1998) consisting of a "core unit" which is associated with "auxiliary units" depending on the biological context.
Chemical synapses are highly specialized cell-cell junctions with the purpose of communication between a nerve cell and a target cell being another neuron, a muscle or a gland cell. Synaptic transmission is directional: the presynapse is defined as the compartment from which neurotransmitter is released. Consequently, the neurotransmitter is sensed by specific receptors in the postsynaptic membrane. In fact, the notion is evolving that synapse assembly, maturation, maintenance and plasticity depend on multifaceted protein-protein and protein-lipid interactions within dynamic macromolecular complexes at both, the presynaptic and postsynaptic side (Gundelfinger et al., 2003; Ziv and Garner, 2004; Kim and Sheng, 2004). Studies on synaptic proteins are often based their over-expression in cultured neurons. However, data obtained from loss-of-function studies in mice indicate that many genes contribute to synaptic transmission in a redundant way. For this reason single-gene approaches, which rely on the over-expression of the protein-under-study, may not always be a suitable tool to study the molecular complexity of the synapse (Grant, 2006). Despite laborious efforts, relatively little is known about the mechanisms of synapse assembly and maintenance and basic questions still remain unanswered: How are the necessary constituents trafficked to the synapse? How is the local protein-network dynamically organized at the synapse? What are the mechanisms underlying the plastic reorganization of synaptic protein networks to ensure appropriate vesicle release under different physiological conditions?
The objective of this work was to identify and functionally characterize protein complexes in *Drosophila melanogaster*. Therefore, this work

concentrated on two proteins exhibiting a strong loss-of-function phenotype, which is thoroughly investigated by our laboratory. The first protein studied is the presynaptic protein Bruchpilot (BRP), which appears to be a key organizer of active zone structure and function (Wagh et al., 2006; Kittel et al., in review). The other protein is the Glutamate-receptor-interacting protein (DGrip), which seems to coordinate the complex process of muscle guidance during early embryogenesis (Swan and Wichmann et al., 2004; Swan and Schmidt et al., in review). Using a combination of biochemistry and genetics BRP and DGrip protein complexes should be characterized. To this end the function of newly identified molecular components should be assessed *in vivo* at a well-established synaptic model system, the larval neuromuscular junction (NMJ). Due to the high evolutionary conservation of synaptic proteins results obtained by this approach should also be applicable on vertebrates and humans.

1.1 *Drosophila melanogaster* as a model organism

Drosophila melanogaster, commonly known as the fruit fly, has been used as a model organism in genetic research for more than a century. Advantages of working with *Drosophila* include the short generation time, namely 10 days for one lifecycle at 25°C. This greatly eases the establishment and application of different transgenic and knock-out strategies compared to genetic approaches in vertebrates. In addition, the *UAS/GAL4* system allows spatio-temporal control over the expression of the gene of interest (Brand and Perrimon, 1993). Morphologically, all developmental stages are relatively easily accessible, hence permitting the analysis of individual cells. This in combination with the mentioned “genetic tools” greatly facilitated the functional analysis of cellular process *in vivo*, e.g. of synaptic transmission. Furthermore, most *Drosophila* genes are evolutionary conserved, which renders insights won in *Drosophila* applicable to vertebrates.

1.2 The Neuromuscular Junction of *Drosophila* – a synaptic model system

To understand the function and formation of neuronal circuits, the development of synapses has been extensively studied in both vertebrates and invertebrates. Indeed, the well defined and precisely described synapses of *Drosophila* have been used as a platform to explore mechanisms and principles of synapse formation, which find many counterparts in other animals (Prokop and Meinertzhagen, 2005). The notion that basic features underlying synaptic function are shared among vertebrates and invertebrates is strongly supported by the evolutionary conservation of most molecules identified in the context of intracellular trafficking and neurotransmitter release – a specialized form of membrane trafficking (reviewed by Prokop, 1999). Furthermore, the general "design" of synapses is comparable between vertebrates and invertebrates. Both exhibit morphologically and functionally defined sub-domains which constitute the presynaptic and postsynaptic sites. These domains should be shortly introduced using the *Drosophila* neuromuscular junction (NMJ) as an example.

Analogous to the majority of mammalian CNS excitatory synapses the NMJ is glutamatergic (Jan and Jan, 1976) and exhibits similar features like many central mammalian synapses in terms of plasticity (Wu and Bellen 1997; Matthies and Broadie 2003; Prokop and Meinertzhagen, 2005). The NMJ is formed by an array of 70 identified motor neurons (Truman and Bate, 1988) which innervate a segmentally repeated set of muscles (Fig. 1A). During larval development, muscle size increases more than 100-fold. To effectively depolarize and contract the muscle, NMJs grow extensively, as well (Fig. 1B). This growth must be tightly regulated to assure that synapses conform to constant densities on neuromuscular surfaces (Meinertzhagen et al., 1998).

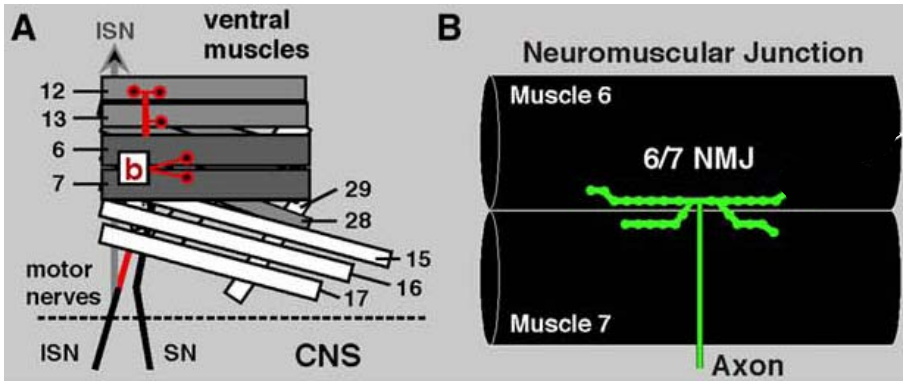


Fig. 1: Diagrams of the ventral body-wall muscles (numbered) and motor nerves from the dorsal aspect of the *Drosophila* larva (A) and the 6/7 neuromuscular junction (B). The intersegmental nerve b (red) projects to the ventral longitudinal muscle group (grey); muscles 6 and 7 (dark grey) are part of this group and are dually innervated by the most proximal branch (b). Modified from Johnson et al., 2006

The NMJ is composed of linked presynaptic specializations, so called boutons (Fig. 2A), which are added in an activity-dependent manner or in response to developmental cues (Schuster et al., 1996; Gramates and Budnik, 1999; Sigrist et al., 2002 and 2003). Usually, individual muscle cells are innervated by two different types of boutons. Type Is boutons are approximately 1.4 µm in size, whereas type Ib boutons can reach up to 8 µm (Johansen et al., 1989; Atwood et al., 1993). Each bouton harbours roughly 10-20 individual synapses. These are characterized by presynaptic active zones (AZ; Fig. 2B, C) - membrane thickenings decorated with synaptic vesicles. Hence active zones define the site of vesicle release (Fig. 2C; green line). Frequently an electron-dense matrix of different shape (T-shaped at the *Drosophila* NMJ, ribbons or pyramids in other organisms) extends from the active zone into the cytoplasm (Zhai and Bellen, 2004; Fig. 2C; marked by a green circle). The presynaptic region comprised by the presynaptic membrane apart from the active zone is usually referred to as the periactive zone (Fig. 2C; yellow line). It has been implicated in synaptic stabilization and growth. Directly apposed to the active zone on the postsynaptic site is an electron-dense membrane specialization, which is referred to as the postsynaptic density (PSD; Fig. 2B, C; marked by a red line). The PSD serves to cluster glutamate receptors (DGluRs), voltage-gated ion channels and various scaffolding molecules (Petersen et al., 1997; Sheng 2001, Garner et al., 2002, Prokop and Meinertzhagen, 2005). Underneath the PSD the muscle membrane is highly folded. This so called subsynaptic reticulum (SSR; Fig. 2C) harbours many adhesion and scaffolding molecules, which might be involved in dynamic

trafficking of DGluRs and ion channels (Budnik et al., 1996; Prokop and Meinertzhagen, 2005).

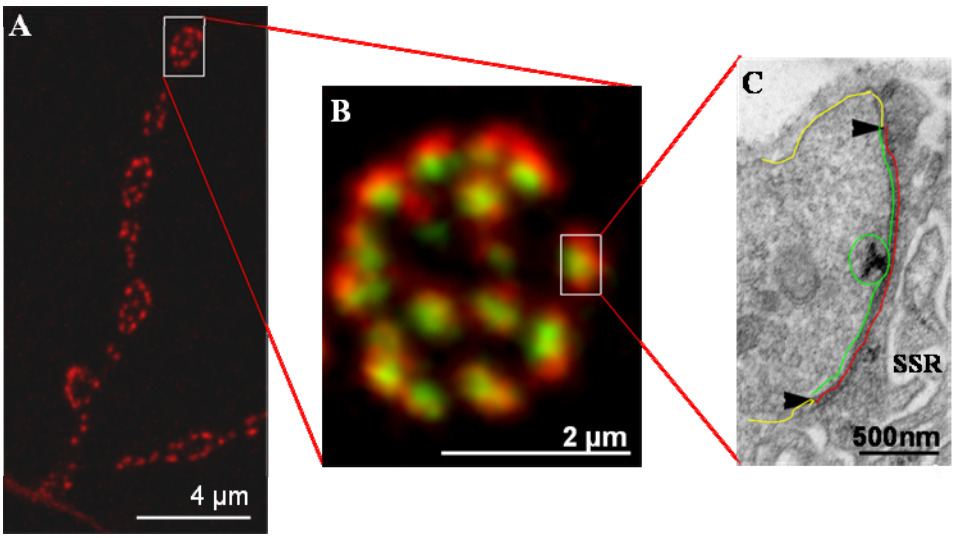


Fig. 2: The Larval NMJ. (A) Type Ib boutons are stained for the glutamate receptor subunit IIA (GluRIIA) identifying individual PSDs. (B) Closed-up view of a synaptic bouton showing the arrangement of presynaptic sites (visualized by BRP in green) and postsynaptic sites (visualized by GluRIIA in red). (C) Electron micrograph of a single active zone (in green) with the PSD just opposite of it (in red). The yellow line indicates the perisynaptic region. The subsynaptic reticulum is indicated as SSR.

1.3 Molecular assembly of synapses

During the development of the nervous system a functional network is built by neurons and their target cells. It has become obvious that synapse formation is a complex process relying on the coordinated interplay of multiple molecules. This protein network is believed to determine spatio-temporal aspects of synapse assembly and to ensure synaptic functionality. Despite recent advances in the identification of synaptic molecules, the elementary cell biological processes underlying synapse formation and maintenance are poorly understood. The following chapters aim to outline the current view on synapse assembly. For this purpose insights obtained from studies on neuronal cultures and from *in vivo* studies in different model

systems are integrated (Prokop, 1999; Gundelfinger et al., 2003; Ziv and Garner, 2004; Zhen and Jin, 2004).

1.3.1 Formation of presynaptic structures

A first requirement after target recognition of the axon is the site-specific recruitment of pre- and postsynaptic molecules. Proteins defining the presynaptic region seem to be pre-assembled prior to contact of potential synaptic partners. Ahmari and coworkers first reported clusters of 80 nm-dense core vesicles, later named Piccolo/Bassoon transport vesicles (PTVs) (Zhai et al., 2001), at newly forming synapses (Ahmari et al., 2000). Numerous scaffolding proteins of the active zone and components of the vesicle release machinery were identified on these vesicles. Among them the large coiled-coil proteins Piccolo and Bassoon (the only proteins among identified active zone components that have no obvious homolog in invertebrates), Rab3-interacting molecule (RIM; Zhai et al., 2001; Shapira et al., 2003) as well as Liprin-alpha (Kim et al., 2003), CAST/ERC/ELKS (CAZ-associated structural protein, synonyms: ERC/ELKS; Ohtsuka et al., 2002) and N-type Ca^{2+}-channels (Shapira et al., 2003). These studies promoted the notion that the presynapse is assembled by modular transport packets. In the presynaptic terminal these transport packages are believed to either contribute to the formation of the active zone or of the cytomatrix at the active zone (CAZ; Zhai and Bellen, 2004). The CAZ is believed to consist of a network of microfilaments and associated proteins, which regulate translocation of synaptic vesicles to the active zone and all consecutive steps necessary for vesicle exocytosis (Ziv and Garner 2004; Jahn and Sudhof, 1999). This might mainly be achieved by a tight spatio-temporal control of protein-interactions at the CAZ (Gundelfinger et al., 2003). A vast amount of *in vitro* studies investigated single proteins members of the CAZ (Fig. 3; Landis et al., 1988; Shapira et al., 2003; Zhai and Bellen, 2004; tom Dieck et al., 2005). However, the selective targeting and functional interactions among these proteins *in vivo* appear to be a “hen-and-egg-problem” (Rosenmund et al., 2003).

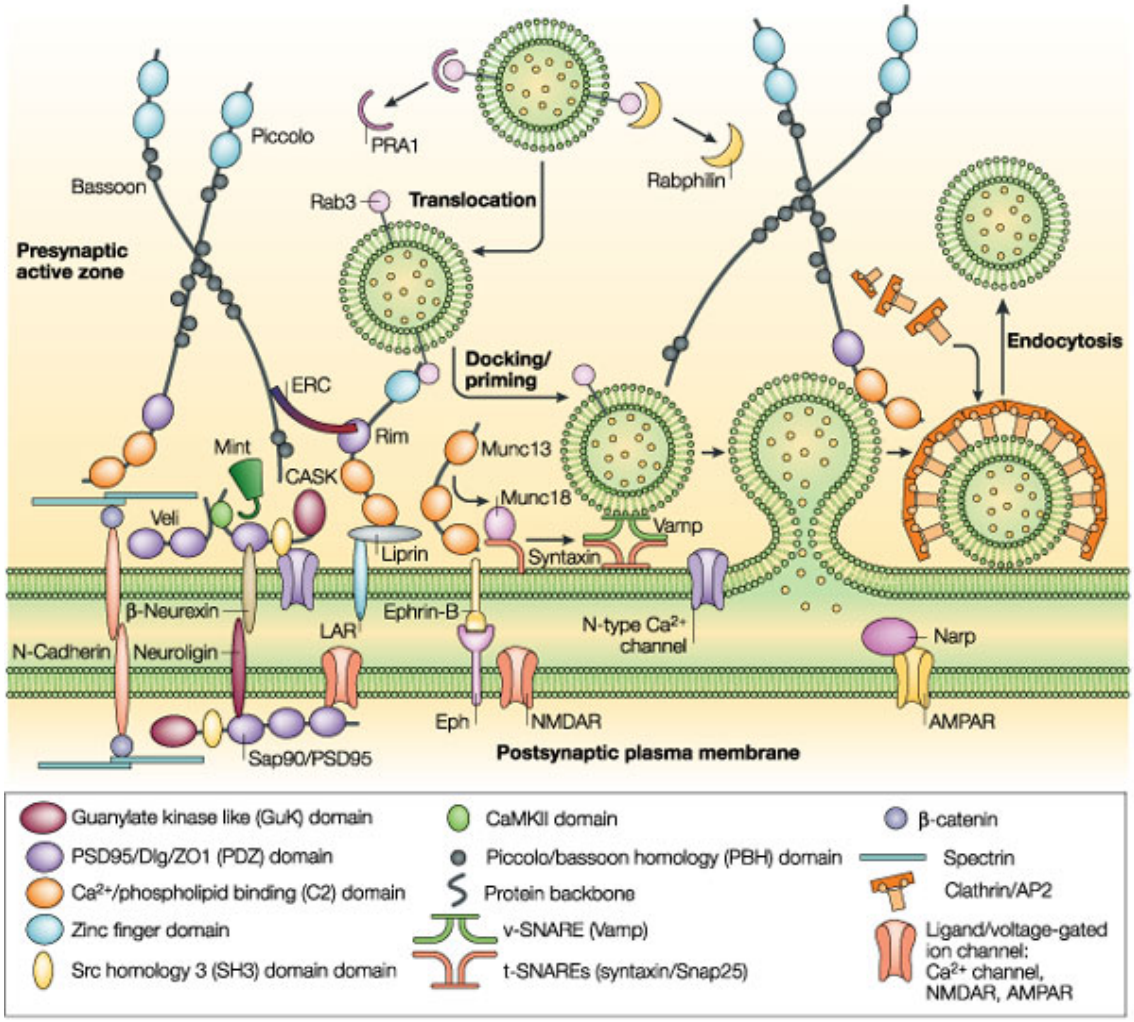


Fig. 3: Schematic overview of proteins identified at the CAZ and the active zone. Notably, this figure does not depict functionally proven interactions among single proteins. Modified from Ziv and Garner, 2004.

Several critical questions still remain to be elucidated: Where are the PTVs assembled? How are PTVs recruited to nascent presynaptic sites? What are the cues that link membrane trafficking processes to the immature presynaptic site? How is the functionality of a synapse – namely coordinated release of neurotransmitter- ensured?

The current view proposes that active zone precursors are pre-assembled at the Golgi apparatus (Garner et al., 2002). In fact, the prototypic active zone scaffold proteins Bassoon and Piccolo are localized to the trans-Golgi network in cultured neurons (Dresbach et al., 2006). Subsequently, these active zone precursors must be trafficked to the nascent presynaptic terminal by various motor proteins (Zhen and Jin, 2004). Target recognition between PTVs and the nascent presynaptic terminal might require appropriate

adhesion and docking proteins on both sides (Fig. 4). Several proteins of the cortical cytoskeleton, like Actin and Spectrin and cell adhesion molecules (CAMs), like Cadherins (Bamji et al., 2003; Salinas and Price, 2005), Neurexins (Dean et al., 2003; Nam and Chen, 2005) and Neuroligin (Scheiffele et al., 2000) are suggested to be involved. The complex architecture of cytoskeletal and membrane-bound scaffolding proteins is believed to finally build up the molecular machinery for vesicle release. Synaptic vesicle release by itself is a multifaceted and highly coordinated process triggered by Ca^{2+}-entry into the presynaptic terminal (Barrett and Stevens, 1972; Schneggenburger and Neher et al., 2000). Speed and fidelity of vesicle release is ensured by primary effector proteins which are shown to be functionally involved in different aspects of the exo-/endocytic cycle. Among them are Munc13s (Brose et al., 2000; Varoqueaux et al., 2002; Rosenmund et al., 2002) and Neurexins (Missler et al., 2003), voltage-dependent Ca^{2+}-channels (Jun et al., 1999; Ino et al., 2001), the Ca^{2+}-sensor Synaptotagmin (Geppert et al., 1994; Fernandez-Chacon et al., 2001; 2002) and the components of the SNARE complex (soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Sudhof, 2004). However, the dissection of molecular events at the active zone to coordinate the release of neurotransmitter is still awaited.

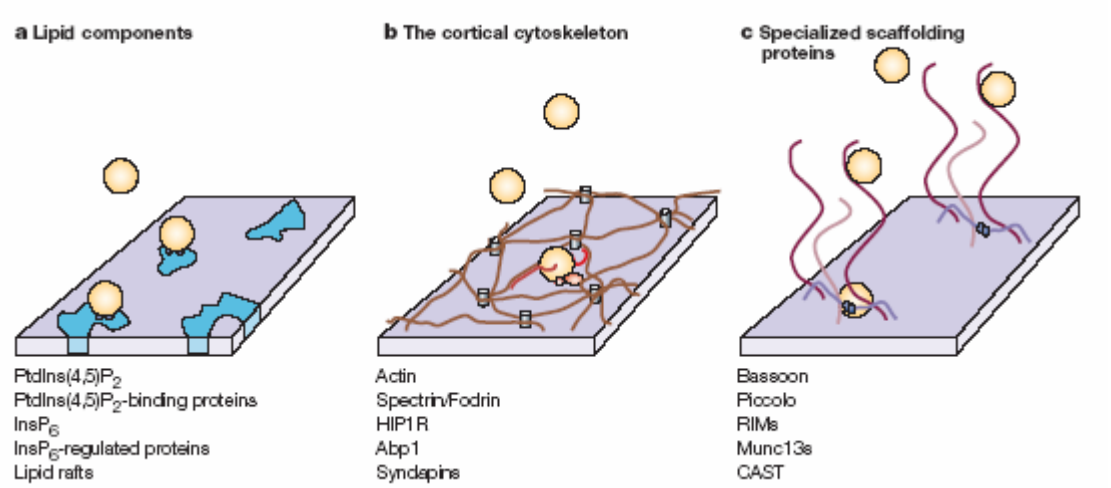


Fig. 4: Elements with an ability to compartmentalize the plasma membrane and thereby organize the machineries for membrane trafficking processes. Under each part of the figure, key examples of each type of organizing element are shown. Abp1, actin-binding protein 1; CAST, CAZ-associated structural protein; HIP1R, Huntingtin-interacting protein 1-related; InsP6, inositol hexakisphosphate; PtdIns(4,5)P2, phosphatidylinositol-4,5- bisphosphate; RIMs, Rab3-interacting molecules. Taken from Gundelfinger et al., 2003

1.3.2 Vertebrate CAST/ERC/ELKS proteins and Drosophila Bruchpilot

1.3.2.1 The vertebrate CAST/ERC/ELKS family

CAST/ERC/ELKS proteins were discovered upon purification from synaptic densities of rat brains (Ohtsuka et al., 2002) and on the basis of their RIM binding activity (Wang et al., 2002). Two genes encode for these proteins in vertebrates. Both produce several isoforms, among which are the two brain-specific ones CAST1/ERC2 and CAST2/ERC1b (Wang et al., 2002, Deguchi-Tawarada et al., 2004). CASTs are approximately 100-120kDa in size and contain several coiled-coil domains as well as a C-terminal PDZ-binding motif (IWA). Protein-interaction studies suggested that CASTs form large complexes with other prominent CAZ-members, like Munc-13, RIM1, Liprin-alpha, Piccolo and Bassoon. For this reason, CASTs are thought to be involved in the molecular organization and localization of members of the CAZ (Ko et al., 2003). They might anchor exocytosis events at the AZ membrane and mediate docking and priming of synaptic vesicles (Ohtsuka et al., 2002; Takao-Rikitsu at al., 2004). Even though the two amino-terminal coiled-coil domains of CAST have been identified as active zone targeting domains (Ohtsuka et al., 2002), the molecules responsible for active zone targeting of CAST remain to be identified. This is indicative for studies on presynaptic assembly: Even though single components have been identified and mapped in respect to their function in cultured neurons, their molecular interactions within the tight network of the CAZ is difficult to be addressed *in vivo*. Just recently, it was proposed that CAST/ERC/ELKS and RIM are redundantly anchored at the active zone by yet uncharacterized proteins in *C. elegans* (Deken et al., 2005). In contrast, previous investigations in cultured neurons suggested that RIM localization required an interaction with CAST/ERC/ELKS (Ohtsuka et al., 2002).

1.3.2.2 Bruchpilot - the *Drosophila* homolog of vertebrate CAST/ERC/ELKS

The newly identified *Drosophila* active zone protein Bruchpilot (BRP) exhibits significant homology to vertebrate CAST/ERC/ELKS in its N-terminal domain (Wagh et al., 2006; Fig. 5). Additionally, the large C-terminus consists of coiled-coil domains similar to cytoskeletal proteins, but does not show any homology to vertebrate proteins. Yet the entire protein is highly conserved among dipteran insects (Holt et al., 2002). Notably, the monoclonal antibody Nc82 (MAB nc82), derived from a monoclonal library produced against *Drosophila* head extracts, recognises BRP (Wagh et al., 2006). MAB nc82 was

found to label the active zones of NMJ synapses opposite of postsynaptic receptor fields (Wucherpfennig et al., 2003; Wagh et al., 2006). Consequently, BRP is localized to the active zone (Wagh et al., 2006) where it was found to form ring-like structures (Kittel et al., in review). Mutants of *brp* exhibit defective active zone membranes, a complete loss of presynaptic dense bodies and depressed evoked but sustained spontaneous vesicle release (Kittel et al., in review). Moreover, Ca^{2+}-channels were found to be inappropriately clustered at *brp* mutant synapses (Kittel et al., in review). The defects observed in *brp* mutants comprise one of the most severe phenotypes known in the "active zone field" so far. This should provide a platform to investigate the molecular mechanisms underlying BRP function employing the efficient genetics of *Drosophila*.

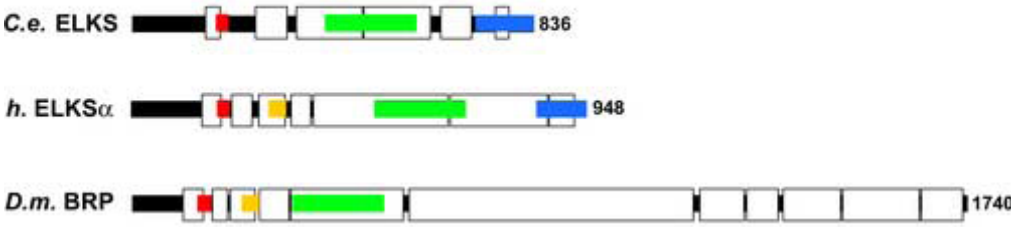


Fig. 5: Schematic domain structure of *Drosophila* BRP. Comparison of predicted coiled-coil domains (white boxes) and conserved regions (colour) for *C. elegans*, human, and *Drosophila* homologs. Modified from Wagh et al., 2006.

1.3.3 The postsynaptic compartment

As initially mentioned the NMJ of *Drosophila* is glutamatergic. Released glutamate is sensed at the postsynaptic membrane by glutamate receptors (GluRs) of two different kinds: (1) N-methyl D-aspartate (NMDA) receptors and (2) non-NMDA receptors (including AMPA [α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid] and kainate receptors). Ionotropic GluRs at the NMJ of *Drosophila* are of the non-NMDA-type. So far five subunits have been identified in larval muscles (termed GluRIIA to GluRIIE; Schuster et al., 1991; Petersen et al., 1997; Qin et al., 2005).

In contrast to presynaptic assembly, the PSD seems to be primarily assembled by gradual accumulation of molecules (Bresler et al., 2004; Ziv and Garner, 2004). Recent data point towards a requirement of a huge protein-network to establish and maintain the postsynaptic compartment during different states of activity (Fig. 6; Kim and Sheng, 2004). The

recruitment of synaptic non-NMDA receptors into PSDs is subject of intensive investigations. Currently, two pathways are discussed: Either lateral migration of GluRs into PSDs from a diffuse plasma membrane pool or an incorporation via subunit-specific constitutive or activity-dependent pathways (Bredt and Nicoll, 2003; Sigrist et al., 2002, 2003). Postsynaptic DGluR levels are regulated by various scaffolding molecules (Liebl and Featherstone, 2005), by adaptor proteins and kinases (Parnas et al., 2001; Albin et al., 2004; Chen et al, 2005) and via translational control (Sigrist et al., 2000). One prominent adaptor protein of vertebrate GluRs is the Glutamate-Receptor-Interacting Protein (GRIP), which seems to control trafficking of GluRs in cultured neurons (Dong et al., 1997, 1999; Wyszynski et al., 1999, 2002; Liu and Cull-Candy, 2005; Maher et al., 2005). In addition, there is an emerging consensus that GRIP is primarily involved in stabilising intracellular pools of receptors (Osten et al., 2000; Braithwaite et al., 2002).

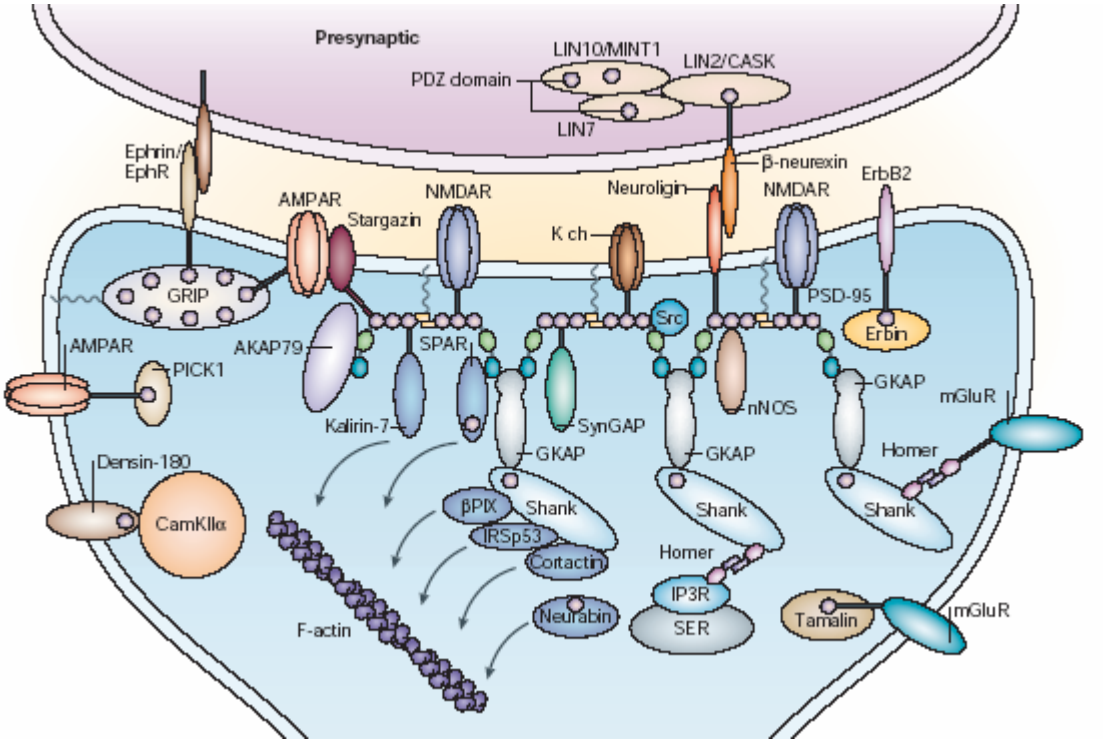


Fig. 6: Schematic diagram of the organization of the PSD at a mammalian excitatory synapse. The main PDZ-containing proteins of a glutamatergic synapse are shown, focusing on the postsynaptic density. PDZ domains are indicated by purple circles. The C-terminal cytoplasmic tails of membrane proteins are indicated by black lines. Specific protein-protein interactions are indicated by the overlap of proteins. Only a subset of known protein interactions is illustrated. Green and blue ellipses in PSD-95 represent SH3 and GK domains, respectively. Crooked lines indicate palmitoylation of PSD-95 and GRIP. Grey arrows indicate

binding and/or regulatory actions of proteins on the actin cytoskeleton. AKAP79, A-kinase anchor protein 79; AMPAR, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor; βPIX, PAAK-interactive exchange factor; CaMKIIα, α-subunit of $Ca2^{+}$/calmodulin-dependent protein kinase II; GK, guanylate kinase-like domain; Eph, ephin receptor; ErbB2, EGF-related peptide receptor; GKAP, guanylate kinase-associated protein; GRIP, glutamate-receptor-interacting protein; IP3R, IP3 receptor; IRSp53, insulin-receptor substrate p53; K ch, potassium channel; LIN7, lin7 homolog; LIN10, lin10 homolog; mGluR, metabotropic glutamate receptor; NMDAR, NMDA (N-methyl-D-aspartate) receptor; nNOS, neuronal nitric oxide synthase; PICK1, protein interacting with C kinase 1; PSD-95, postsynaptic density protein 95; SER, smooth endoplasmic reticulum; SH3, Src homology 3 domain; Shank, SH3 and ankyrin repeat-containing protein; SPAR, spine-associated RapGAP; SynGAP, synaptic Ras GTPase-activating protein. Modified from Kim and Sheng, 2004.

1.3.4 The Glutamate-receptor-interacting protein in vertebrates and Drosophila

Mammalian GRIP and its close relative AMPA Receptor Binding Protein (ABP/GRIP2) have been identified upon their physical interaction with AMPARs (Dong et al., 1997; Wyszynski et al., 1998). Initially they were considered to purely serve as "adaptors" in the construction of a postsynaptic scaffold thereby stably anchoring glutamate receptor complexes. Meanwhile GRIPs are thought to participate in multiple protein complexes with a role in membrane targeting of proteins or segregations of these proteins in cellular subdomains. GRIP1 mutant mice show kidney agenesis, polydactyly, syndactyly (Bladt et al., 2002) and gross morphological brain defects. This phenotype is comparable to the human Fraser syndrome potentially mediated by an interaction of GRIP with the cell adhesion molecule Fras1 (Takamiya et al., 2004). GRIP has also been shown to interact with members of several signalling pathways, e.g. Ephrins (Lin et al., 1999; Contractor et al., 2002; Hoogenraad et al., 2005) and Liprins (Wyszynski et al., 2002; Ko et al., 2003; Shin et al., 2003; Dunah et al., 2005). Recently, GRIP was found to mediate membrane localization of Membrane Type 5 Matrix Metalloproteinase (MT5-MMP) and might thereby regulate axon pathfinding or synapse remodelling (Monea et al., 2006).

Drosophila Grip (DGrip) was identified in our lab on the basis of its interaction with the C-terminal PDZ-binding motif of GluRIIC in a Y2H screen (Christine Quentin and Stephan Sigrist unpublished results). Surprisingly, further analysis revealed that DGrip is a key regulator of muscle guidance in the *Drosophila* embryo (Swan and Wichmann et al., 2004). Mutations in *dgrip* impair patterning of ventral longitudinal muscles (VLMs), whereas lateral

transverse muscles (LTMs), which attach to intrasegmental attachment sites, develop normally. DGrip protein concentrates at the ends of VLMs while these muscles guide toward segment border attachment sites. Therefore, DGrip might sense an attractive signal expressed at segment borders in order to specify the direction of muscle motility (Swan and Wichmann et al., 2004). Like its mammalian homolog DGrip consists of seven PSD-95/Discs-large/ZO-1 domains (PDZDs) and no other known protein-protein interaction motifs (Fig.). Insights obtained from the analysis of *dgrip* mutants and the prominent domain structure render DGrip a potential candidate for being a scaffolding molecule regulating many yet unidentified processes in *Drosophila*.

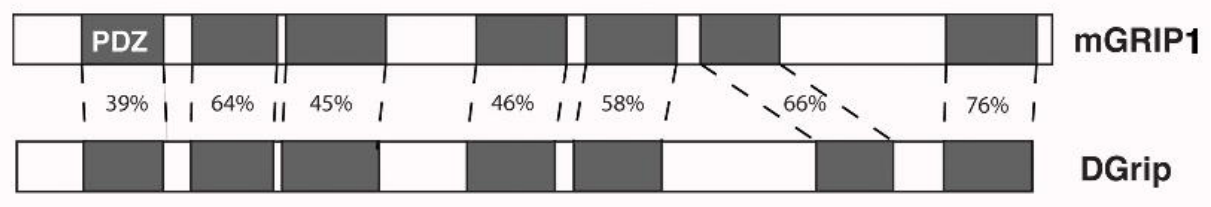


Fig. 7: Comparison between mouse GRIP1 and Drosophila protein CG5980 (DGrip), which both encode seven individually conserved PDZ domains. Sequence similarity between corresponding PDZ domains is indicated in percentages. Modified from Swan and Wichmann et al., 2004.

1.4 Proteomics-based assessment of gene function

Cellular processes and signalling pathways involve proteins to assemble in complexes where a few subunits to more than 100 components can be tethered by binary and ternary interactions. It is generally believed that proteins of similar function are found in these clusters and act together as "molecular machines" (Alberts, 1998). This can be utilized to predict protein function and get more insight into the molecular organization of signalling pathways – a principle that is referred to as the "guilt-by-association" concept (Choudhary and Grant, 2004).

The set of all expressed proteins encoded by the genome, but also the subset of proteins in a cell, tissue or organism is defined as the proteome. However, the analysis of the proteome, named proteomics, is significantly

more challenging than that of genomes which can be accounted for three basic problems:

1. The dynamic expression range of proteins (Corthals et al., 2000) influences the relative protein abundance. This is a limiting factor for proteome analysis, as protein amplification methods are lacking.
2. The diversity of differentially expressed isoforms and post-translationally modified proteins, which demands for very sensitive methods to detect small differences or alterations in protein expression.
3. Functionally relevant protein-protein interactions tend to be transient and are therefore difficult to be preserved during experimental handling.

These problems are challenging the characterization of protein-protein interactions in multiprotein complexes – an approach usually referred to as functional proteomics or interactomics.

1.4.1 Tools to study multiprotein complexes

Numerous approaches have been developed to study protein-protein interactions on different levels and in different expression systems. In the following the most common methods used to map protein interactions will be introduced.

A strategy widely used for detecting binary interactions is yeast two-hybrid (Y2H) screening. By means of Y2H large-scale interaction maps were produced for several organisms, among them *Drosophila* (Giot et al., 2003). Y2H can be quite sensitive to detect transient interactions and also allows domain-mapping for particular interactions of interest. However, the detected interactions may not be valid in a cellular environment, which is reflected by the high rate of false positives in Y2H studies (von Mering et al., 2002). Moreover, ternary protein-protein interactions or such, which are based on cooperativity, are out of the scope of Y2H studies.

Methods that aim to isolate protein complexes from cells or tissues usually employ generic affinity-capture strategies, where the protein of interest is genetically fused to an affinity tag (Shevchenko at al., 2002, Forler et al., 2003, Knuesel et al., 2003). This should enable the isolation of the tagged protein and its binding partners by standard methods and their identification by immunoblotting. Although these strategies have proven to be very useful tools in transgenic animals, their substantial problem remains the adjustment of protein expression close to natural levels. Finally, protein complexes can be captured using antibodies against endogenous proteins in

co-immunoprecipitations (IP) and subsequent detection on immunoblots. So-called “candidate approaches” enable sensitive and quantitative detection of proteins. However, they are limited by prior knowledge of likely components and by antibody availability. These limitations can be largely overcome using mass spectrometry to identify protein-protein interactions.

1.4.2 Mass spectrometry and its application in functional proteomics

Mass spectrometry (MS) is by now considered to be the fastest and most sensitive method for sequence analysis of proteins and peptides (Aebersold and Mann et al., 2003). By definition, a mass spectrometer consists of three major components: (1) the ion source, (2) a mass analyzer to measure the mass-to-charge ratio (m/z) of the ionized analytes, and (3) a detector for registration of the number of ions at each m/z value. Matrix-assisted laser desorption/ionization (MALDI; Karas et al., 1988) and electrospray ionisation (ESI; Fenn at al., 1989) are the two main methods to volatize and ionise proteins. MALDI is a pulsed ionisation technique in which the proteins or peptides are sublimated and ionized out of a dry, crystalline matrix by a laser pulse which mainly results in singly charged molecules. In contrast ESI is a continuous ionisation method capable of producing multiply charged molecules from a capillary electrode placed at high voltage. MALDI MS is usually easier to handle and identifies proteins by so-called peptide-mass fingerprinting (PMF). This method relies on matching a list of experimental peptide masses with the calculated list of all peptide masses in a database. On the other hand, sequence information obtained from ESI MS combined with collision-induced decay (CID; which generates even further fragmented ion spectra) is more reliable in identifying peptides. This method is also commonly known as MSMS or tandem MS. In addition high-efficiency capillary separation, like reversed-phase liquid chromatography, can be directly interfaced to tandem mass spectrometry (LC-MSMS) which generally results in automated, highly sensitive MS. This is why LC-MSMS is nowadays the technology base for a comprehensive analysis of complex protein mixtures (Peng and Gygi, 2001; Elias et al., 2005). Hence, it serves as a valuable tool for functional proteomics and promoted the efforts to construct a map of the synapse proteome and interactome (Collins et al., 2005; Grant, 2006).

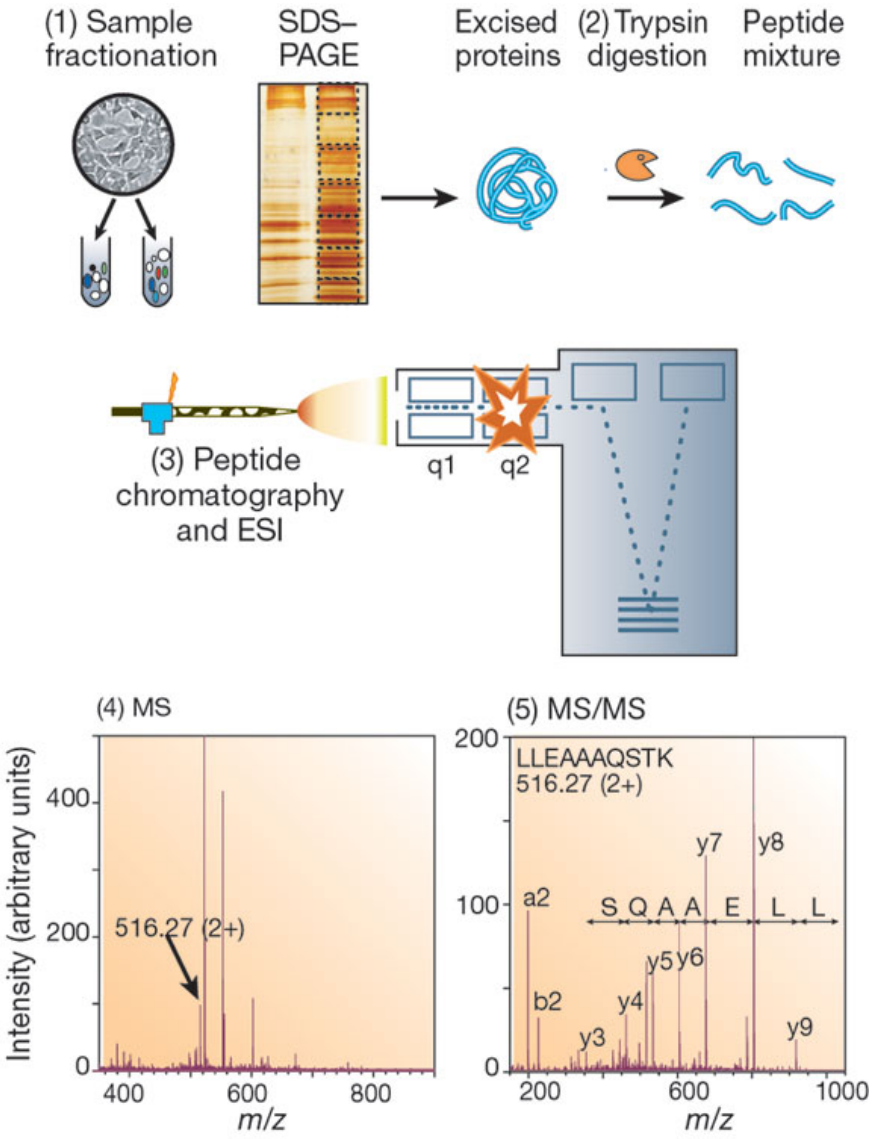


Fig. 8: Generic MS-based proteomics experiment as applied in this work. Firstly, proteins have to be extracted from cells or tissues and are separated by SDS-PAGE (1). Subsequent digestion with trypsin results in a complex peptide mixture (2) which is separated further by peptide chromatography which is online coupled to a mass spectrometer (3). First a mass spectrum of peptides eluting at one time point is taken (4) and then prioritized peptides are selected for fragmentation and a series of tandem mass spectrometric or MSMS experiment ensues (5). Modified from Aebersold and Mann, 2003.

2. Materials and Methods

2.1 Fly genetics and fly strains

Fly strains were reared under standard laboratory conditions (Sigrist et al., 2003). *Drosophila* germline transformation was performed as previously described (Horn et al. 2000). Transgenic animals were established in w^- flies (Castiglioni, 1951). *Dgrip* and *brp* mutants were produced in our lab (Swan and Wichmann et al., 2004; Kittel et al., in review). *Sif*-alleles sif^{ES11} *and* $sif^{98.1}$ were kindly provided by Chihiro Hama, NIN, Tokyo.

2.2 Molecular biology

All experiments were performed according to standard procedures (Sambrook, 1989). Enzymes, including T4 Ligase and Restriction Enzymes, were purchased from Roche (Mannheim, Germany) if not otherwise stated. All Polymerase Chain Reactions (PCRs) made for obtaining transgenic constructs were performed with Vent Polymerase (New England Biolabs).

2.2.1 Cloning of Y2H-construct for sif

All constructs were cloned into pGADT7 (Clontech). For all PCRs *sif1* cDNA (kind gift of C. Hama; Sone et al., 1997) was used as a template.

pGAD-sif1:
Forward primer: 5'CCGCTGATGCGCAAGGCCTAT3'
Reverse primer: 5'AACGCCAGCCATCCGAGTGA3'
Digest: *NdeI/EcoRI*

pGAD-sif2:
Forward primer: 5'AACTCGCGATGCGCCTCTG3'
Reverse primer: 5'CTCGTACGCGACGTTGGCTT3'
Digest: *NdeI/XhoI*

pGAD-sif3:
Forward primer: 5'GGTTGGAATGGCACTAGCA3'
Reverse primer: 5'TTCATCAGCACCGGCTGGTA3'
Digest: *NdeI/XhoI*

2.2.2 Cloning of Y2H-baits for brp (made by Sara Mertel)

The *brp* cDNA (Wagh et al., 2006) was used as template for the PCR. The PCR-fragment was cloned into pGBKT7 (Clontech).
Forward primer: 5'ATGGGCAGTCCATACTACGC3'
Reverse primer: 5'TATGTGCCGCTGGTAGTCCTG3'
Digest: *SpeI/KpnI*

2.2.3 Generation of pUAST-DGrip-myc

A C-terminal fragment of RE14068 cDNA was amplified using the following primers: 5'GACTAGTGAGCTCAATCGCTATGCCAGTGTC3' and 5'GACTAGTCTCAGAGCGCTGCATGATCATCTCGA3'.
The PCR-product was *SpeI*-digested and directionally inserted into *pSL1180* harbouring a 10xmyc-tag via *SpeI*. This insert was excised via the internal *SacI*-site and *NheI* and ligated to a *BglII-SacI*-product of RE14068 cDNA. This construct was directionally inserted into pUAST via *BglII* and *XbaI*.

2.3 Immunostaining

Embryos and larvae were prepared as previously described (Swan and Wichmann et al, 2004). The following antibodies were used: mouse monoclonal anti-Nc82 (MAB nc82; generous gift of Erich Buchner, Würzburg), 1:100; mouse monoclonal anti-Myc (9E10, Santa Cruz, USA), 1:500; rabbit polyclonal anti-GluRIIC (Qin et al., 2005), 1:500; rabbit polyclonal anti-GluRIID (Qin et al., 2005), 1:500. Secondary antibodies were goat anti-mouse and goat anti-rabbit coupled to Cy3 and FITC (Promega), 1:250; goat anti-HP coupled to Cy5 (Promega), 1:250. Imaging on larval body wall preparations was essentially done as described previously (Qin et al., 2005).

2.4 Quantitative real-time PCR

To assess expression levels of BRP and SIF in wild type compared to sif^{ES11} *and* $sif^{98.1}$ alleles 15 larvae were collected from each genotype and total RNA was extracted (Qiagen, RNeasy mini kit). Oligo-dT primed cDNA was synthesized (Omniscript), and relative amounts of *sif* and *brp* cDNA were quantified in duplicate using QuantiTect SYBR Green PCR kit (Qiagen) and the following specific primers.

For *brp*:
Forward primer 1: 5'TACGCCAGCCAAAAGCTGATC3'
Reverse primer 1: 5'GCTCTATCCGCTTCTGCCGTAT3'
Forward primer 2: 5'CGGCAAATCGCAATACGAGT3'
Reverse primer 1: 5'CTGCACCTCCATCTCGATCTTC3'
For *sif:*
Forward primer 1: 5'GGCCTATCGCTATGAGGATT3'
Reverse primer 1: 5'GCTCGCTGATACATGGAAGA3'
Forward primer 2: 5'ACCATCACAGCTCGGACATC3'
Reverse primer 2: 5'GATGTGGTGCTCAACGTCAG3'
Quantitative real-time PCR was performed using the GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). Obtained values were calibrated against total cDNA levels measured by RT-PCR primers 5'AAGCCCGTGCCCGTATTATG3' and 5'AAGTCATCCGTGGATCGGGAC3' for *tbp-1* (TaT binding protein-1), a housekeeping gene. Transcript levels are normalized to the level of wild type transcript detected.

2.5 *In situ* hybridization

Whole mount embryonic *in situ* hybridizations were performed essentially following the BDGP standard protocol (www.fruitfly.org). For preparing sense RNA probes LD28013 plasmid was cut with *XhoI* and in vitro transcribed using T7 RNA polymerase and for antisense probes LD28013 was cut with *EcoRI* and in vitro transcribed using SP6 RNA polymerase.

2.6 Yeast two-hybrid

2.6.1 Yeast two-hybrid screening

(in collaboration with Tobias Böckers, University of Ulm)
Bait constructs were cloned into pGBKT7 (made by Laura Swan) and transformed into the yeast strain AH109 (*MAT*a). Yeast mating was carried out between the bait-containing AH109 strain and the yeast strain Y187 (*MAT*α) pretransformed with the *Drosophila* embryo MATCHMAKER cDNA library (Clontech). Successfully mated diploids were identified on SD/-Leu/-Trp plates and subsequently replica-plated to SD/-Ade/-His/-Leu/-Trp/X-α-gal to select for true positives. Yeast DNA was isolated, amplified in bacteria and sequenced from the 5' end to identify candidate genes.

2.6.2 Yeast two-hybrid-based domain mapping

In principle all experiments were made according to the Yeast two-hybrid protocols of Clontech using the strain AH109. In brief, AH109 was co-transformed with the corresponding bait and prey constructs, grown on SD/-Leu/-Trp plates and 3 clones each were analyzed on SD/-Ade/-His/-Leu/-Trp/X-α-gal plates to select for positive interaction.

2.7 Immunoblots

Proteins were fractionated by standard SDS-PAGE and then transferred to a nitrocellulose membrane in prechilled western blot transfer buffer (25mM Tris, pH 8.0, 150mM glycine, 20% methanol,) for 90min at 90V at 4°C. The membrane was blocked in 5% milk-powder in 1xPBS and blots were probed with the respective primary antibodies. Primary antibodies were used at the following concentration: mouse monoclonal anti-Myc (9E10, Santa Cruz, USA), 1:500; mouse monoclonal anti-nc82 (MAB nc82; generous gift of Erich Buchner, Würzburg), 1:100; mouse monoclonal anti-SIF (MabH24.8.2 kind gift of C. Hama, NIN, Tokyo) 1:300; rabbit polyclonal anti-DGrip (Swan and Wichmann et al, 2004), 1:500; mouse monoclonal anti-ß-tubulin (E7, DSHB, University of Iowa); mouse monoclonal anti-HA (Santa Cruz), 1:500. Finally secondary antibodies (anti-mouse-IgG (Dianova) and anti-rabbit-IgG (Dianova), respectively) conjugated with horseradish peroxidase were used to visualize the signal after ECL detection (Amersham) using the LAS-3000 digital imaging system (Fujifilm).

2.8 Pulldown assay with immobilized C-terminal peptides

The general procedure is described in M. Soltau et al., 2004. Synthetic peptides representing the C-terminus of Echinoid (sequence: NRRVIREIIV) and the respective scrambled controls (for Echinoid: RIVRIRIEVN) were generated by peptides&elephants GmbH (Nuthetal, Germany). These peptides were coupled to NHS-activated Sepharose at a concentration of 3mg/mL matrix.
SF9 cell extracts expressing myc-tagged DGrip were solubilized in NTEP-buffer (50mM Tris-HCl, pH 7.9, 150mM NaCl, 5mM EDTA, 0.5% NP-40, 10mM iodacetamide, 1mM PMSF) and "precleared" with 400µl NHS-Sepharose-slurry for 3 hours to prevent unspecific binding to the NHS-Sepharose. Subsequently the "precleared" supernatant was applied to the peptide/NHS-matrix for 1hour at 4°C, the matrix was washed five times with

the respective buffers and was eluted by boiling in SDS sample buffer (50mM Tris, pH 6.8, 2% SDS, 10% (v/v) glycerol, 2% ß-mercaptoethanol, 0.1% bromphenol-blue).

2.9 Immunoprecipitation from SF9 cells

SF9 cells expressing single and double combinations of myc-tagged DGrip, HA-tagged GluRIIC, HA-tagged GluRIIA or HA-tagged 5HT1A were solubilized in NTEP-buffer (50mM Tris-HCl, pH 7.9, 150mM NaCl, 5mM EDTA, 0.5% NP-40, 10mM iodacetamide, 1mM PMSF), incubated for 15min on ice and centrifuged for 15min at 16,000g. The supernatant was used in IPs with anti-Myc, anti-HA and mouse IgG heavy chain coupled to Protein-A-Sepharose (Pharmacia) by rotation in PBS for 1h. After incubation at 4°C for 2h with slow rotation, the beads were washed three times (10min each) in NTEP-buffer and proteins were eluted by boiling in SDS sample buffer.

2.10 Solubilization of BRP

Adult wild type fly heads were homogenized under different conditions and equal amounts of the pellet fraction and soluble fraction.
Condition A: 20mM Tris, pH 7.5, 100mM NaCl, 1mM EDTA, 1% deoxycholate; 30min incubation on ice.
Condition B: 20mM Tris, pH 8.5, 150mM NaCl, 0,5mM EDTA, 1mM dithiotreitol, 0.1% SDS, 1% Triton X-100); 30min incubation on ice.
Condition C: 500mM Tris, pH 9.0 1% sodium-deoxycholate; 30min incubation at 36°C; addition of 1/10 volume of Triton-buffer (50mM Tris, pH 9.0, 1% Triton X-100); incubation at 4°C for 30min.
Condition D: 2% SDS; 5min at 95°C; 30min incubation on ice.

2.11 Crosslinking of antibodies to Protein-A-Sepharose

The protocol was adapted from the standard procedure used by the AG Knoblich (IMP, Vienna). For experiments concerning DGrip per IP 10µg of anti-Myc (kind gift of Jürgen Knoblich, IMP, Vienna) were bound to 50µl of Protein-A-Sepharose (beads) slurry for one h. For experiments concerning BRP 900µl of MAB nc82 and 44µg of mouse IgG heavy chain (Dianova; serving as control) were bound to 100µl Protein-A-Sepharose for 1h for each IP. The beads were washed three times with PBS, then with 50mM Borax, pH 9.0 and

subsequently the antibodies were crosslinked to the beads by incubation in 50mM Borax, pH 9.0 containing 40mM dimethyl pimelinediimidate dihydrochloride (Fluka) for 1h. Beads were washed three times with 1M Tris, pH 8.0 (2min each) and pre-eluted with 100mM glycine, pH 2.0 for 1min. After three consecutive washes with PBS the beads were ready-to-use for the IP.

2.12 Immunoprecipitations from adult fly heads

Adult fly heads were obtained by vortexing anaesthetized flies on liquid nitrogen and sieving.

Experiments concerning DGrip were essentially done as described by Betschinger and colleagues (Betschinger et al., 2003). In short: Adult fly heads of the genotype *elav-GAL4::UAS-dgrip-myc* and of wild type (for controls) were mechanically homogenized in extraction buffer (25mM Tris, pH 8.0, 27,5mM NaCl, 20mM KCl, 25mM sucrose, 10mM EDTA, 10mM EGTA, 1mM dithiotreitol, 10% (v/v) glycerol, 0.5% NP-40 containing protease inhibitor cocktail [Roche Applied Science, Germany]), incubated for 15min on ice and centrifuged for 15min at 16,000g. The supernatant was used in IPs with anti-Myc/mouse IgG heavy chain (for controls) crosslinked to Protein-A-Sepharose (Pharmacia). After incubation at 4°C for 2h with slow rotation, the beads were washed three times (10min each) in extraction buffer and proteins were eluted first under acidic conditions (100mM glycine, pH 2.0) and subsequently by boiling in SDS sample buffer.

For all experiments concerning BRP the protein extraction procedure was modified from Luo and colleagues (Luo et al., 1997). Wild type adult fly heads were mechanically homogenized in deoxycholate buffer (500mM Tris, pH 9.0 1% sodium-deoxycholate containing protease inhibitor cocktail [Roche Applied Science, Germany]) followed by incubation at 36°C for 30min. A 1/10 volume of Triton-buffer (50mM Tris, pH 9.0, 1% Triton X-100) was then added and the lysate was incubated at 4°C for 30min. After centrifugation for 15min 16,000g the supernatant was used in IPs with MAB nc82/mouse IgG heavy chain (for controls) crosslinked to Protein-A-Sepharose. After incubation at 4°C for 2h with slow rotation, the beads were washed five times (10min each) in deoxycholate/Triton X-100 buffer and proteins were eluted first under acidic conditions (100mM glycine, pH 2.0) and subsequently by boiling in SDS sample buffer.

2.13 Liquid chromatography and mass spectrometry

Two protocols at two different facilities were used to identify co-precipitating proteins in the IP-samples.

Protocol (a); all practical work was done in the lab of Dr. Henning Urlaub, MPIBPC, Göttingen

After removal of proteins from the MAB nc82-ProteinA-beads with SDS sample buffer, the samples were separated by one-dimensional (1D) SDS-PAGE (NuPAGE 4-12% gradient gel, Invitrogen) and protein bands were visualized using SYPRO Red (Molecular Probes). The elution and control lanes (controls i.e. immunoprecipitation with mouse IgG, see 2.12) were each cut in 2mm thick stripes, so that the regions of both lanes aligned to each other. Each individual stripe was in-gel digested with trypsin (from bovine, E.C.3.4.21.4, sequencing grade, Roche) and peptides were extracted according to Shevchenko et al., 1996. Dried samples from in-gel digests were dissolved in 10% (v/v) acetonitrile (CH_3CN, LiChrosolve grade, Merck), 0.15% formic acid (FA, Fluka). The sample volumes were adjusted to the sample amount. The dissolved samples were subjected to a nano-liquid chromatography (LC) coupled electrospray ionization (ESI) tandem MS using an orthogonal quadruple time-of-flight mass spectrometer (Q-Tof, Ultima, Waters). The nano-LC system was equipped with a C18 pepMap100 column (75µm ID, 3µm, 100, LC Packings) running with a flow rate of 180nl/min. The buffer used were as follows: Buffer A (H_20, 0.1% (v/v) FA) and buffer B (80% (v/v) acetonitrile, 0.1% (v/v) FA). The gradient applied was 90% (v/v) buffer A to 55% (v/v) buffer A in 60min, 55% (v/v) buffer A to 10% (v/v) buffer A in 5min and 5min with 10% (v/v) buffer A. Prior to separation of the peptides by nano-LC, samples were desalted with online coupled pre-columns (3mm) consisting of the same chromatrography material. The electrospray was generated with fused-silica 10-µm PicoTip needles (New Objectives, MA) and was operated at approximately 1.8-2.3kV. Fragment spectra of seqnecned peptides were searched against all entries of the Non-redundant Database from the National Center for Biotechnology Information (NCBI) using the software search algorithms MASCOT (Matrix Science Ltd, London, UK). For the database search no constraints on molecular weight or biological species were applied.

Protocol (b); in collaboration with Karl Mechtler, Proteomics Facility, IMP, Vienna

Proteins were *en masse* removed from the MAB nc82-ProteinA-beads with 100mM glycine, pH 2.0, reduced with dithiotreitol, carboxy-methylated using iodoacetamide and digested with trypsin (Betschinger et al., 2003). Peptides were extracted with formic acid and separated by nano-high-performance liquid chromatography on a PepMap C18 reversed-phase column. Eluting peptides were transferred online to an ion trap mass spectrometer (LTQ, Thermo, Finnigan). MS data were searched against all entries of the Non-redundant Database from the National Center for Biotechnology Information (NCBI) using the software search algorithms MASCOT (Matrix Science Ltd, London, UK). For the database search no constraints on molecular weight or species were applied.

Experiments on BRP protein structure; in collaboration with Hartmut Kratzin, Proteomics facility, MPIEM, Göttingen

After removal of proteins from the MAB nc82-ProteinA-beads with SDS sample buffer, the samples were separated by 1D SDS-PAGE (NuPAGE 4-12% gradient gel, Invitrogen) and protein bands were visualized by colloidal Coomassie staining (Neuhoff et al., 1985). The double bands corresponding to BRP and the respective region of the control sample were cut out and subjected to MALDI MS (Bruker Ultraflex I) according to the protocols of the proteomics facility, MPIEM (for details see www.em.mpg.de/proteomics). Some samples were additionally subjected to microsequencing using MALDI MS.

MS data were searched against all entries of the Non-redundant Database from the National Center for Biotechnology Information (NCBI) using the software search algorithms MASCOT (Matrix Science Ltd, London, UK). For the database search no constraints on molecular weight or biological species were applied.

3. Results

3.1 Mass spectrometry-based approaches to study protein complexes of DGrip and BRP

Numerous studies are nowadays dealing with the characterization of protein complexes which are localized at synapses and might be relevant for synaptic function. However, functional *in vivo* assays are laborious and difficult. Instead, *in vitro* studies are often performed in cultured neurons. In general, these rely on over-expression or modification (dominant active/negative variants) of the protein of interest and do not necessarily reflect physiological conditions. A recent survey compared synaptic phenotypes either obtained from single-gene manipulations *in vitro* to phenotypes resulting from knock-out of the particular gene (Grant, 2006). Grant concluded that upon *in vitro* modification of the gene of interest the effects were grossly overestimated (Grant, 2006). This is not surprising considering the vast molecular complexity of the synapse which is build up and maintained by a highly connected molecular network (Grant, 2003). For this reason functional assays are urgently required. These must be sensitive enough to examine single synaptic proteins *in vivo* and to determine their molecular dynamics at different developmental stages or under various environmental conditions.
This work aimed to combine a functional proteomics approach with *in vivo* characterization of the identified proteins in *Drosophila*. The larval NMJ serves as a valuable model system for functional genetic studies of the physiology and development of glutamatergic synapses (Koh et al., 2000). This thesis concentrated on two proteins, whose loss-of-function phenotypes are investigated by our group:

1. The *Drosophila* Glutamate-receptor-Interacting Protein (DGrip) which acts as a key regulator in embryonic muscle guidance (Swan and Wichmann et al., 2004).
2. Bruchpilot (BRP), the *Drosophila* homolog of vertebrate CAST/ERC/ELKS, which is required for presynaptic active zone assembly and Ca^{2+}-channel clustering to ensure efficient release at the NMJ.

To get a mechanistic understanding of the processes either of these proteins is involved in, this study aimed to unravel which protein-complexes DGrip and BRP are associated with, respectively. For this reason a mass-spectrometry (MS)-based approach should be established to characterize *in vivo*-derived protein-complexes. Immunoprecipitations (IPs) from adult fly heads or embryos should be performed and co-precipitating proteins should be identified by means of MS. Recent advances in MS made it possible to identify components of multiprotein complexes from tissue lysates with high sensitivity and accuracy, thereby facilitating analysis of protein interactions. The identified proteins should be investigated for their functional relevance applying biochemical and genetic techniques at the larval NMJ and heads of adult flies, respectively.

3.1.1 Analysis of in vivo-derived DGrip-complexes

The initial motivation to look at DGrip-protein-complexes in more detail was based on two *a priori* findings of our laboratory:

1. *Dgrip* loss-of-function exhibits strong muscle guidance defects in *Drosophila* embryos (Swan and Wichmann et al., 2004).
2. DGrip was identified as a potential binding partner of GluRIIC in a Y2H screen (Christine Quentin and Sigrist, unpublished results).
3. Electrophysiological characterization of *dgrip* mutants and *dgrip*-targeted RNAi (see chapter 3.5.2) pointed towards a requirement of DGrip for transmitter release at the larval NMJ (in collaboration with Robert Kittel).

These findings suggested that DGrip might act as a scaffolding and/or transport molecule in various cellular contexts. The search for binding partners of DGrip in *Drosophila* should give more mechanistic insight into these processes and provide a tool to study basic principles of DGrip function.

Firstly, the solubilization properties of DGrip were tested to optimize buffer conditions. A Tris-based buffer containing 0.5% of the non-ionic detergent NP-40 was used to solubilize DGrip in the following experiments. IPs from either embryos expressing myc-tagged DGrip (input ~1ml of wet, packed embryos) in the mesoderm (under the control of the *24B-GAL4* driver line) or adult fly heads expressing myc-tagged DGrip (input ~0.6g of heads) in all neurons (under the control of the *elav-GAL4* driver line) were performed. Figure 9 depicts representative examples of IPs submitted to 1D SDS-PAGE and western blotting. The blots were probed with anti-Myc antibody and

showed a strong signal corresponding to full-length myc-tagged DGrip (at a molecular weight of 150kD). However, several additional bands of lower apparent molecular weight were detected, as well. These bands stemmed most likely from endogenous processing of DGrip, as they could also be detected when the tissues were immediately denatured by boiling in 2% SDS. It was not possible to identify proteins which specifically co-precipitated with myc-tagged DGrip, neither by MALDI MS (in collaboration with Hartmut Kratzin, Proteomics facility, MPIEM, Göttingen) nor by a "gel-free approach" employing LC-MSMS (in collaboration with Karl Mechtler, Proteomics facility, IMP, Vienna). One major drawback of this approach was based on the over-expression of myc-tagged DGrip. This was necessary, as the weak affinity of anti-DGrip antibodies limited their use for IPs. The functionality of myc-tagged DGrip was assessed by rescue of the *dgrip* loss-of-function muscle guidance phenotype. However, myc-tagged DGrip was most likely produced in non-physiological amounts, which might have aggregated in non-functional pools interfering with transient interactions.

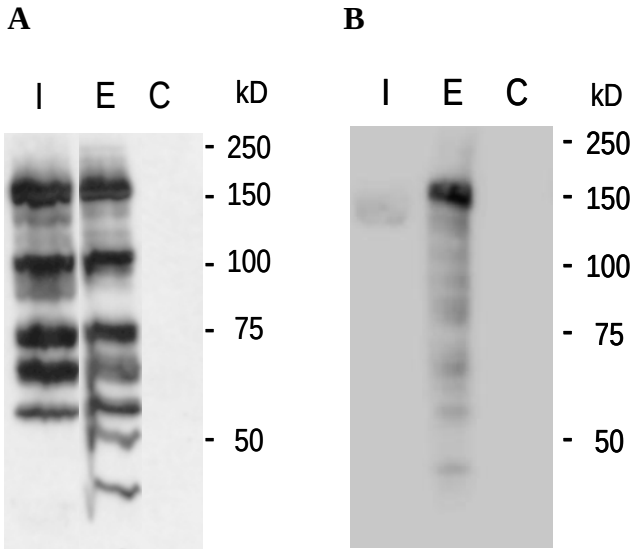


Fig. 9: Immunoblots of myc-tagged DGrip. Myc-tagged DGrip expressed in the embryonic mesoderm (A) or in heads of adult flies (B) was immunoprecipitated with anti-Myc antibody and detected by anti-Myc probing on western blots. The eluted fraction (eluate, E) is ten times more concentrated than the input fraction (input, I). For controls nonimmunogenic mouse IgG was used in the IP (control, C).

3.1.2 Characterization of BRP by mass spectrometry

The MAB nc82 selectively labels discrete spots surrounded by Dynamin at the larval NMJ. This implied that the target of MAB nc82 is localized to the active zone of synapses. Due to the specific phenotype of the first RNAi knock-down flies for the gene encoding the nc82 antigen (these flies could not fly) the name *bruchpilot* gene (*brp*) was coined (Wagh et al., 2006).

Pan-neural reduction of BRP expression by RNAi constructs pointed towards a role of BRP for intact active zone structure and normal evoked neurotransmitter release at chemical synapses (Wagh et al., 2006). In addition, *brp* mutants exhibited severe problems in vesicle release. This phenotype could be attributed to defects in active zone assembly and to inappropriate Ca^{2+}-channel clustering apart from vesicle release sites (Kittel et al., in review). However, many questions about the mechanistic role of BRP at the active zone still remain open.

To get a comprehensive view about the function of BRP at *Drosophila* synapses, BRP should be characterized by means of biochemistry and mass spectrometry. Firstly, the protein structure should be inferred by microsequencing peptides generated from BRP protein. Subsequently, BRP should be mapped systematically at the level of protein-protein interactions in *Drosophila* adult fly head lysates to resolve the composition of BRP protein complexes.

3.1.2.1 Solubilization of BRP from adult fly heads

As a prerequisite for further biochemical investigation of BRP, its the solubilization properties were tested in adult fly head extracts. BRP was resistant to combinations of any non-ionic and ionic detergents when moderate NaCl concentrations (up to 100mM) were used. Only very harsh conditions could effectively solubilize BRP (Fig. 10):

(a) A combination of the ionic detergent sodium-deoxycholate (Na-DOC) and the nonionic detergent TritonX-100 in a highly concentrated Tris-buffer (500mM).

(b) Boiling the sample in 2% SDS buffer (for detailed description of buffers see chapter 2.10).

The first condition is referred to as deoxycholate buffer in the following chapters and was used for IPs with MAB nc82 in further experiments (Fig. 12). The solubilization properties of BRP are reminiscent of vertebrate CAST1. Ohtsuka and colleagues used 1% SDS or 1% deoxycholate to

solubilize CAST1 from rat brains (Ohtsuka et al., 2002). Therefore, they inferred that CAST1 might be tightly associated to the cytoskeletal structure (Ohtsuka et al., 2002). The same might also apply to BRP.

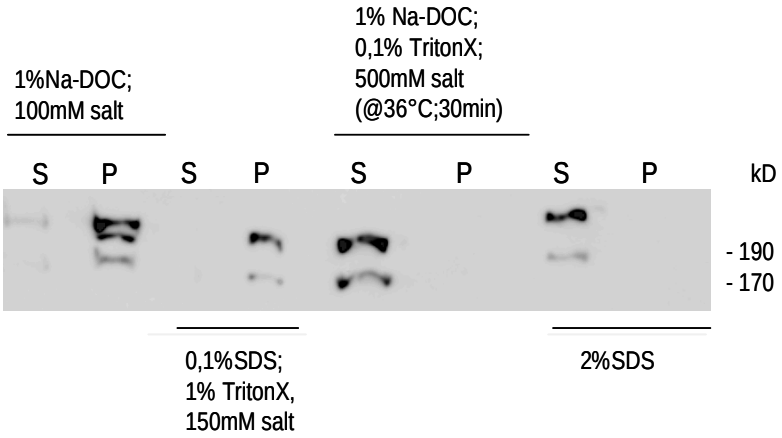


Fig. 10: Solubility of BRP extracted from wild type adult fly heads under the indicated conditions. The blots are probed with MAB nc82. BRP is only soluble using either high concentrations of ionic detergents (2 % SDS) or ionic detergents in combination with high salt concentrations. The slight differences in BRP reactivity are most likely caused by high detergent concentrations and high ionic strength of the sample. Samples representing equal amounts of protein extract in the pellet fraction (P) and the soluble fraction (S) were loaded on the gel.

3.1.2.2 Determination of BRP protein structure by Mass Spectrometry

On immunoblots, MAB nc82 recognizes a double band of about 170kD and 190kD apparent weight, respectively (Fig. 10). To identify proteins harbouring the MAB nc82 epitope, *Drosophila* head homogenates were subjected to 2-D gel electrophoresis and western blotting. Finally, two spots were identified as isoforms of a protein encoded by the cDNA clone AT09405 of the predicted gene locus CG30337 (Wagh et al., 2006). Sequencing RT-PCR products of mRNA from third instar larvae and from adult flies revealed that CG12933, CG30336, and CG30337 actually belong to the same transcription unit (Wagh et al., 2006; Fig. 11C). From RT-PCR sequences and the cDNA AT09405 a cDNA, which contains the complete open reading frame (ORF) of *brp*, was constructed. A fourth predicted gene (CG12932) is located between CG12933 and CG30336, and thus could represent a large alternatively spliced exon of the *brp* gene (Fig. 11C). However, a linkage of CG12932 to the *brp* mRNA by RT-PCR consistently failed (Wagh et al., 2006). MAB nc82 recognizes an epitope, which maps to the C-terminal 1105 amino acids of the 1740 amino acid protein encoded by the composite cDNA (Wagh

et al., 2006). On immunoblots, over-expression of GFP-tagged BRP produced reactivity which corresponded to the upper band (Fig. 15) and *brp*-specific RNAi effectively suppressed both bands (experiment shown in Wagh et al., 2006). Furthermore, this cDNA was able to restore MAB nc82 label at active zones of *brp* mutant NMJs, partially rescued the physiological deficits, but could not rescue the observed lethality of *brp* mutants. For this reason it became necessary to clarify the exact composition of BRP protein structure and identify the disparity among the differentially migrating forms of BRP. To this end a MALDI-MS approach was chosen to firstly identify potentially different peptides among the two bands seen in immunoblots. BRP was enriched from wild type fly heads by immunoprecipitation with MAB nc82 and the elution fraction was subjected to 1D SDS-PAGE. The BRP double bands were visualized by colloidal coomassie staining (Fig. 11A), two spots of each band were cut out and subjected to MALDI-MS (in collaboration with Hartmut Kratzin, MPIEM, Göttingen). All peaks generated from the lower band were also obtained in the upper band and represented the open reading frames (ORFs) CG30336 and CG30337. Interestingly, four additional peptide peaks at a mass/charge ratio of 916.5, 1093, 1327 and 1771 were found in both samples of the upper band. Database mining with MASCOT (Matrix Science, London, UK) revealed that those peaks were generated by peptides which represent the protein encoded by CG12933. Additional microsequencing of peptides with a mass/charge ratio of 1327 and 1771 confirmed the results. These findings imply that BRP is represented by at least two isoforms recognized by MAB nc82 (Fig. 11B, C). The longer isoform additionally contains CG12933 (Fig. 11B, C). By now, it can not be determined if these isoforms exist only in adult flies or also throughout development, as several attempts to detect embryonic or larval BRP protein on western blots failed.

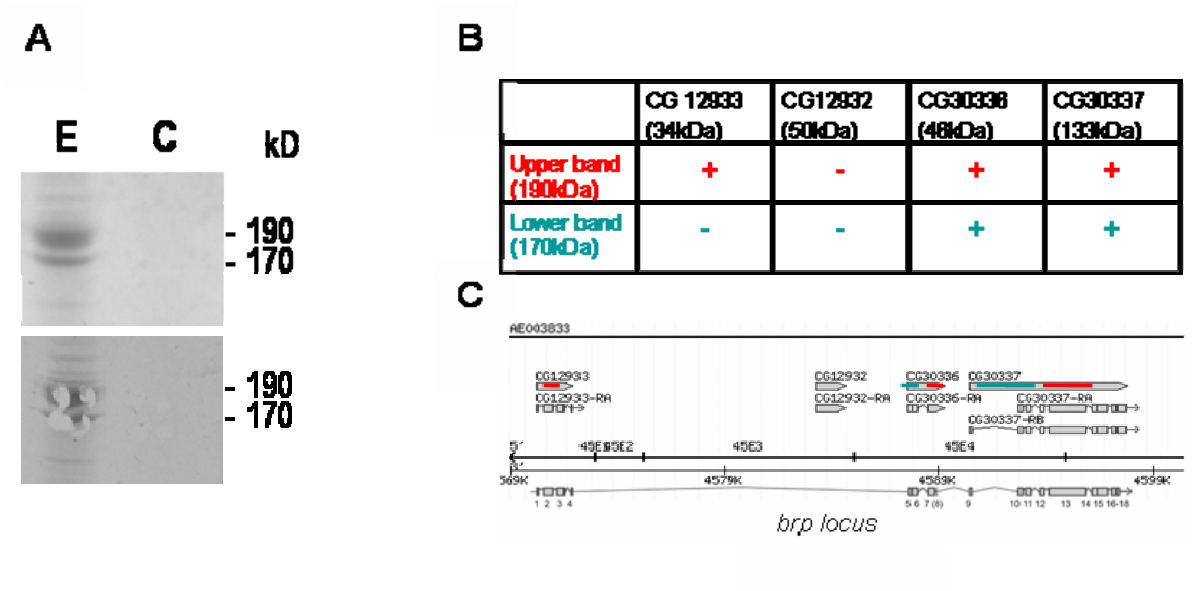


Fig. 11: Determination of BRP protein structure by mass spectrometry. (A) 1D SDS-PAGE stained with colloidal coomassie to visualize BRP, which was immunoprecipitated with MAB nc82 (eluate, E). The control IP (control, C) performed with mouse IgG heavy chain did not show BRP bands. The lower panel shows the position of the spots cut out for MS-analysis. (B) Scheme of ORFs identified by MS in the upper and lower band, respectively. (C) Schematic presentation of the *brp* gene locus with predicted ORFs corresponding to the upper (in red) and lower band (in yellow).

3.1.2.3 Functional proteomics to identify components of BRP protein complexes

The success of the proteomics-approach depended on the efficient immunoprecipitation of BRP from heads of adult wild type flies. For this reason native BRP and its associated proteins were extracted from wild type fly heads (input ~1.5g of heads) with deoxycholate buffer and were (co)-immunoprecipitated with MAB nc82 (Fig. 12A). Subsequently, the immunoprecipitates and corresponding controls of two independent experiments were subjected to microsequencing using two different protocols:

a) *In-gel digestion* (practical work performed in the lab of Henning Urlaub, MPIBPC, Göttingen):

Proteins were removed from the MAB nc82-ProteinA-beads with SDS sample buffer, separated by 1D SDS-PAGE (Fig. 12B) and in-gel digested with trypsin. Finally, the samples were individually submitted to online LC-MSMS using an LC-coupled orthogonal quadruple time-of-flight mass spectrometer (Q-Tof1, Micromass, Manchester, UK).

b) *Gel-free approach* (in collaboration with Karl Mechtler, Proteomics facility, IMP, Vienna):
Proteins were *en masse* removed (Fig. 12B) from MAB nc82-ProteinA-beads with 100mM glycine, pH 2.0, digested with trypsin and analysed by online LC-MSMS using an LC-coupled ion trap mass spectrometer (LTQ, Thermo Finnigan). By this means SDS-PAGE and extraction of proteins from the gel are circumvented which is supposed to minimize the potential loss of proteins during the procedure.

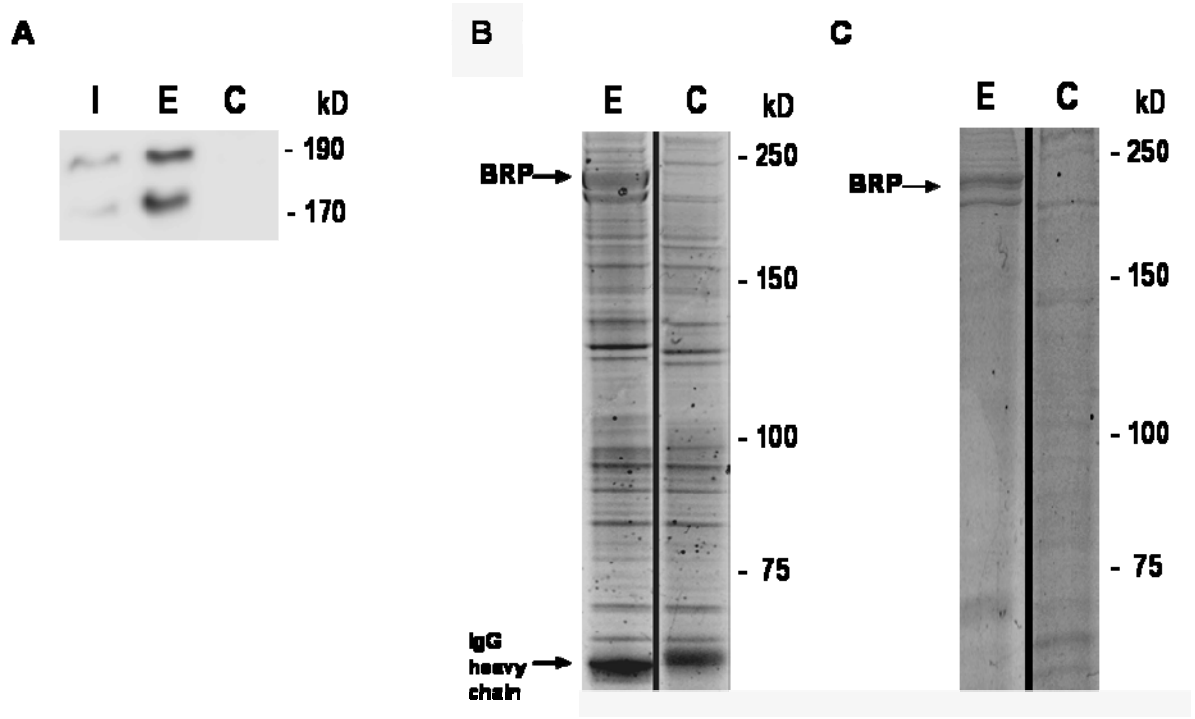


Fig. 12: Immunoprecipitation of BRP with MAB nc82. (A) Western blot of an IP of endogenous BRP from wild type adult fly heads with MAB nc82. The blot is probed with MAB nc82. An eluate of a control IP (Ctrl) was performed with mouse IgG heavy chain and did not show any signal when probed with MAB nc82. The input lane (I) represents 10 times less initial extract than the elution fraction (E). (B) SDS-PAGE of an anti-MAB nc82-IP and the corresponding control eluted with SDS sample buffer (protocol [a]). (C) SDS-PAGE of an anti-MAB nc82-IP and the corresponding control eluted with 100mM glycine, pH 2.0 (protocol [b]).
Proteins were visualized by SYPRO Red.

For both conditions, peptides were identified by searching against the Non-redundant Database from the National Center for Biotechnology Information (NCBI) using the software search algorithm MASCOT (Matrix Science Ltd, London, UK). For the database search no constraints on molecular weight or

biological species were applied. Most proteins were identified with several peptide matches. Only a few were assigned on the basis of a single peptide. In these cases the peptide sequence obtained was near-complete concerning either the b-type (ions that originate from the N-terminus) or y-type ions (ions that originate from the C-terminus) resulting in an ion score higher than the "identity" threshold of 46. Protein scores were derived from ion scores as a non-probabilistic basis for ranking protein hits according to the Probability Based Mowse Score used by MASCOT. Protein hits were only accepted as "positive" if the individual ion scores exceeded the "homology" threshold defined by MASCOT for each query and if the hit was absent in controls of both experiments (Table 1). The identification of many peptides matching to BRP itself (represented by the ORFs CG12933, CG30336 and CG30337 see above) with a total protein score >2000 in both approaches served as an internal control to distinguish MS-runs of the sample from the controls.

Protein	Mass (Da)	Score	Protocol
gi|6094287; CG5406 Still life	230344	113/787	ab
gi|7302107; CG1976-PA RhoGAP100F (DSYD-1)	195330	296/265	ab
gi|54641571; GG11661 Neural conserved at 73F	112442	162/341	ab
gi|6066229; CG1975 Drep2	51979	326/226	ab
gi|7303886; CG12932-PA	50122	90/106	ab
gi|7300865; CG7050-PA Neurexin I	199174	36	a
gi|157776; CG10693 Slowpoke	130216	54	a
gi|72151016; CG7254-PB Glycogen phosphorylase	96935	76	a
gi|23092777; CG1009-PC Puromycin sensitive aminopeptidase	122681	62	a
gi|6179938; CG1528; gamma-Coatomer protein	97243	41	a

gi|21626689; CG2987-PA alpha-Catenin related	88711	125	a
gi|21358039; CG5642	63182	47	a
gi|24585880; CG17337-PA	53130	51	a
gi|7291949 ; CG7008-PA Tudor staphylococcal nuclease	103037	96	a
gi|0733350; CG7762-PA Rpn1	102212	131	a
gi|1854503; CG5320 Glutamate dehydrogenase	62794	393	b
gi|17530879; CG2985 Yolk protein 1	48739	252	b
gi|10726425; CG3996	350737	342	b
gi|21645485; CG1516	131522	218	b
gi|7302508; CG11949-PA Coracle	184455	130	b
gi|433083; CG14994 Glutamic acid decarboxylase	58405	104	b

Table 1: Proteins specifically identified in BRP co-immunoprecipitates. Proteins were identified by microsequencing in two independent experiments using either in-gel digestion (indicated by "a") or a gel-free approach (indicated by "b") followed by online LC-MSMS. Proteins detected in both approaches are indicated as "ab". Note that the number of peptides sequenced from each protein does not reflect the quantity of protein. Furthermore, individual scores are not directly comparable between the two protocols, as these values are dependent on the properties of the type of mass spectrometer (score of protocol "a" is the first value and score of protocol "b" is the second value). Peptides were unique and about 7-20 residues in length. Proteins discussed in more detail are highlighted by bold font.

In the following chapters two of the identified proteins are discussed in more detail, namely Still life and RhoGAP100F (DSYD-1).

3.2 *Drosophila* Still life is a component of the BRP complex

Drosophila Still life (SIF) is a guanine nucleotide exchange factor (GEF) for the small GTPase Rac1 and was identified as part of the BRP protein complex by MS.

SIF is localized at the presynaptic site of the NMJ and is exclusively expressed in the CNS (Sone et al., 1997, 2000). The specific domain structure of SIF protein (Fig. 14B) is largely preserved in the mammalian homologs, Tiam-1 (invasion-inducing T-lymphoma and metastasis 1; Habets et al., 1994) and STEF (SIF and Tiam1-like exchange factor; Hoshino et al., 1999). As a hallmark of Dbl family GEFs, SIF exhibits a catalytic Dbl homologous (DH) domain flanked by a C-terminal pleckstrin homologous (PHc) domain. Furthermore it contains an N-terminal Ena/Vasp-homology domain 1 (EVH; Callebaut et al., 1998), an additional N-terminal PH domain followed by a highly conserved TSS domain (Hoshino et al., 1999) and a PDZ domain. PH domains are considered to be involved in protein-protein or protein-lipid interactions (Lemmon et al., 1997). Of note, also the mammalian homologs are expressed in the brain and seem to be required for Rac1-dependent neurite outgrowth (Leeuwen et al., 1997; Kunda et al., 2001; Matsuo et al., 2002).

In *Drosophila*, SIF is reported to regulate the outgrowth of the NMJ (Sone et al., 1997). Furthermore, SIF co-localizes with Fasciclin 2 (FAS2), an NCAM-like neural cell-adhesion molecule, at the periactive zone. The interplay of FAS2 and SIF might control growth and development of the NMJ (Sone et al., 2000).

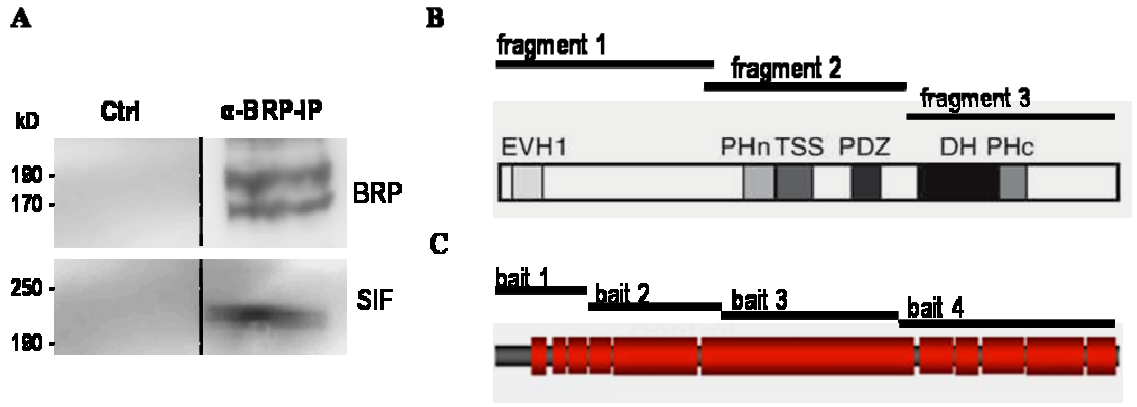
3.2.1 SIF binds BRP via the N-terminal domains

The finding that SIF is a member of the BRP complex is novel and should be verified by other means than MS.

Firstly, the binding of SIF and BRP was demonstrated by an independent co-precipitation of SIF with BRP. SIF could readily be detected in eluates of anti-BRP-IPs when western blots were probed with anti-SIF antibody (Fig. 13A).

In addition, it should be assessed if SIF binds BRP directly and the relevant interacting domains should be mapped by a Y2H assay. The domains of BRP and SIF which were used as baits and preys in the Y2H assay are shown in Fig. 13B and 13C, respectively. The N-terminus of BRP (encoded by

CG12933) strongly bound the N-terminal fragment of SIF and a little bit weaker a middle fragment of SIF. Therefore, it was concluded that SIF and BRP directly bind to each other. This interaction does not seem to exclusively depend on known protein-protein interaction motifs, as only the second SIF fragment contains such motifs like the N-terminal PH-domain and a PDZ-domain (Fig. 13B). Neither the C-terminus of SIF nor any other fragment of BRP was tested positively (Fig. 13D). However, this does not exclude a potential binding of the large coiled-coil domains of BRP to SIF as such domains are generally difficult to be handled in Y2H assays.



D

	SIF fragment 1	SIF fragment 2	SIF fragment 3
BRP bait 1	+++	++	-
BRP bait 2	-	-	-
BRP bait 3	-	-	-
BRP bait 4	-	-	-

Fig. 13: SIF binds BRP via direct interaction of their N-terminal domains. (A) Co-IP of BRP and SIF. BRP was immunopreciptated with MAB nc82 and visualized on blot with MAB nc82. SIF co-precipitated with BRP as proven by anti-SIF probing of the same sample. (B) Schematic representation of SIF protein structure and the domains used in the Y2H assay (modified from Sone et al., 2000). (C) Schematic representation of BRP protein structure and the domains tested in the Y2H assay. Modified from Kittel et al., in review. (D) Y2H-experiments revealed binding of the BRP N-terminus to the first and second fragment of SIF. "+++" indicates strong interaction; "++" indicates relatively strong interaction; "-" indicates no interaction.

3.2.2 Characterization of BRP in sif mutant alleles

After having established a physical interaction between SIF and BRP, the importance of this interaction should be examined functionally.

Two *sif* mutant alleles were previously described (Sone et al., 1997, 2000) and were used in this work: The hypomorphic $sif^{98.1}$ mutant (insertion of the P-element into the 12th intron of *sif*, Sone et al., 1997) and sif^{ES11} (an EMS-allele, which possibly produces a truncated protein as a result of a frameshift mutation; Fig 14; Sone et al., 2000). Both alleles are homozygous lethal (homozygous larvae die at 3rd instar larval stage) and show a reduction of *sif* mRNA to 30% of wild type level as tested by quantitative real-time PCR.

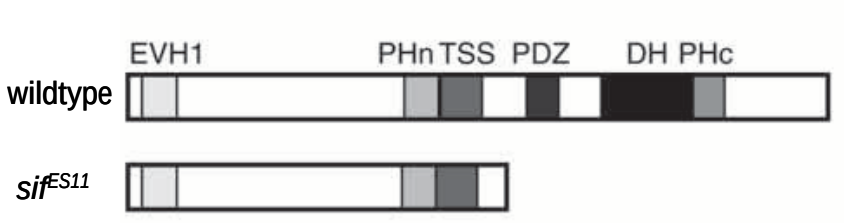


Fig. 14: Schematic presentation of the domain structure of wild type SIF and the truncated SIF protein possibly produced by sif^{ES11}. Modified from Sone et al., 2000.

When these *sif* alleles were crossed to the deficient chromosome *Df(3L)64DF*, which covers the genetic region of *sif*, adult flies could be obtained (genotype: sif^{ES11}/*Df(3L)64DF* and $sif^{98.1}$/*Df(3L)64DF*). However, eclosure rates of sif^{ES11}/*Df(3L)64DF* and $sif^{98.1}$/*Df(3L)64DF* were below mendelian ratio (33 % and 75% of mendelian ratio, respectively) and adult viability was severely compromised. In addition, adult flies exhibited reduced locomotor activity and were incapable to fly.

Adults of the genotypes sif^{ES11}/*Df(3L)64DF* and $sif^{98.1}$/*Df(3L)64DF* were used to examine the level of BRP protein head extracts. A strong reduction of BRP was observed on immunoblots when adult fly heads of sif^{ES11}/*Df(3L)64DF* and $sif^{98.1}$/*Df(3L)64DF* were compared to wild type (Fig. 15; assessed in two independent experiments).

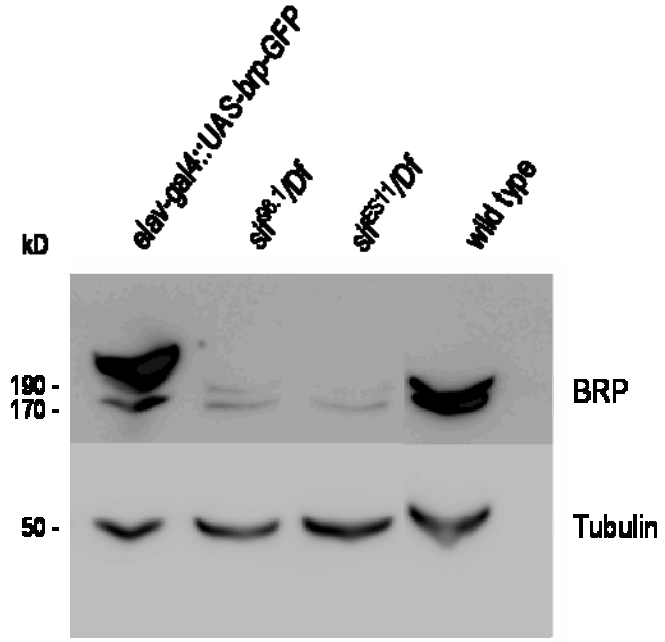


Fig. 15: BRP level were decreased in *sif* mutant alleles. Western blot of 5 heads of the genotype indicated probed with MAB nc82. BRP protein is reduced in adult fly heads of both sif mutant alleles (*sif*^{ES11}*/Df(3L)64DF,* indicated as *sif*^{ES11}*/Df,* and *sif*$^{98.1}$*/Df(3L)64DF*, indicated as *sif*$^{98.1}$*/Df*) compared to wild type. Anti-tubulin probing served as loading control.

To test if the reduction of BRP protein is a consequence of transcriptional regulation, mRNA levels of *brp* were examined by quantitative real-time PCR. Homozygous larvae of both *sif* alleles exhibited at least a 2-3 fold upregulation of *brp* mRNA (consistent in two independent experiments). This could be indicative of a compensatory upregulation of *brp* transcription due to the reduction of BRP protein levels.

Next, it was particularly interesting to investigate BRP protein levels at the NMJ of *sif* mutant alleles. Hence, larvae homozygous for either *sif* allele and additionally *sif*^{ES11}*/Df(3L)64DF* and *sif*$^{98.1}$*/Df(3L)64DF* larvae were stained with MAB nc82. Surprisingly, *sif*^{ES11} homozygous larvae (in the following named *sif*^{ES11} mutants) showed significantly reduced staining of MAB nc82 at the NMJ whereby the localization of BRP was unchanged (evaluation of the fraction of wild type mean grey value of MAB nc82 intensity: *sif*^{ES11} mutant: 0.8142 ± 0.1393, n=12; wild type: 0.9993 ± 0.1124, n=9; $P<0.0077$, Mann-Whitney Rank Sum test; Fig. 16A,B). Neither larvae of the genotypes *sif*^{ES11}*/Df(3L)64DF*, *sif*$^{98.1}$*/Df(3L)64DF* nor *sif*^{ES11}*/sif*$^{98.1}$ showed alterations of BRP signal at the NMJ. The overall morphology of the NMJ seemed to be unaffected in all tested genotypes. As reported before (Sone et al., 2000), a

slight reduction in bouton number was detected in *sif*^{ES11}/*Df(3L)64DF* larvae. For a more detailed view of synaptic structure, additional synaptic markers were examined immunohistochemically. No obvious defects in levels or localization of Cysteine-string-protein, (CSP), Syntaxin, the endocytic marker Dap-160 and FAS2 were observed in *sif*^{ES11} mutants (data not shown).
However, staining of *sif*^{ES11} mutants for GluRIID (Fig. 16A) and GluRIIC (data not shown) to visualize individual PSDs at the NMJ revealed an increased size of receptor fields compared to wild type. Enlarged receptor fields might be indicative of a compensatory upregulation of GluRs at the PSD due to a reduced release probability. This phenotype was also observed in *brp* mutants (Kittel et al., in review). To test for physiological consequences two-electrode voltage clamp (TEVC) recordings of postsynaptic currents were employed on *sif*^{ES11} mutants (in collaboration with Robert Kittel). At low stimulation frequencies (0.2 Hz) evoked excitatory junctional currents (eEJC) were unchanged at *sif*^{ES11} mutant NMJs when compared to wild type (*sif*^{ES11} mutants: -73 ± 5.7 nA; n=8; wild type: -75 ± 3.8 nA; n=10). To investigate possible changes in postsynaptic sensitivity miniature excitatory junctional currents (mEJCs; currents in response to single, spontaneous vesicle fusion events) were measured. Remarkably, mEJC amplitudes were slightly, but significantly reduced in *sif*^{ES11} mutants (*sif*^{ES11} mutant: -0.68 ± 0.04 nA; n=11, $P < 0.045$, Mann-Whitney Rank Sum test; wild type: -0.80 ± 0.03 nA; n=10), whereby mEJC frequencies were unaltered (*sif*^{ES11} mutant: 1.6 ± 0.3 Hz; n=11; wild type: 1.1 ± 0.2 Hz; n=10). Thus, it can be concluded that a reduction of BRP by approximately 20% does not influence evoked release at the NMJ upon low frequency stimulation. To date the observed decrease of postsynaptic sensitivity in *sif*^{ES11} mutants despite enlarged receptor fields can not be explained yet.

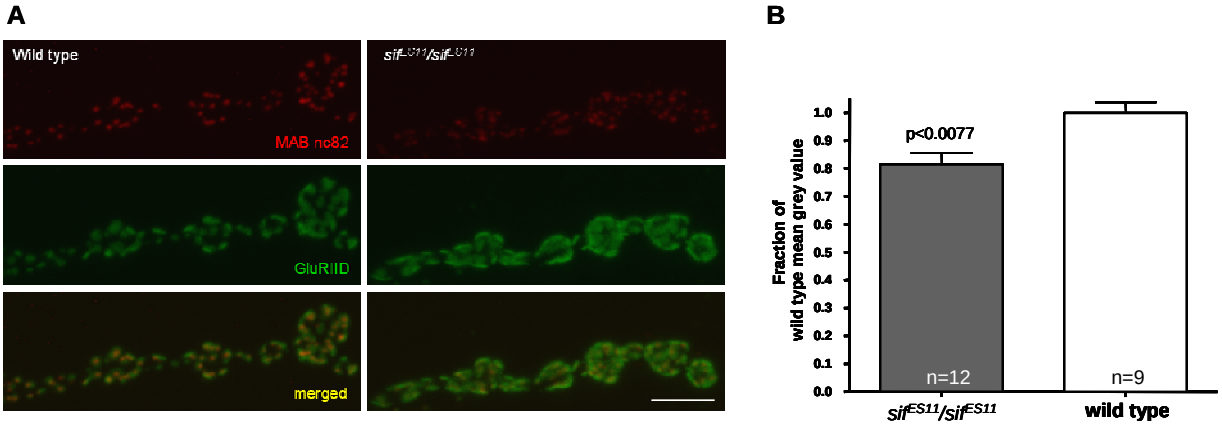


Fig. 16: BRP levels are significantly reduced at NMJs of *sif*^{ES11} mutants when compared to wild type. (A) Individual synapses of *sif*^{ES11} mutant larvae (genotype *sif*^{ES11}/*sif*^{ES11}) showed significantly weaker staining with MAB nc82 than those of wild type. Additionally, receptor fields, identified by the GluRIID label, were enlarged in *sif*^{ES11} mutants compared to wild type. (B) Quantification of the average intensity of MAB nc82 label revealed that active zones of *sif*^{ES11} mutants (12 NMJs of 7 animals) contain significantly less BRP (approximately 20% less; $P<0.0077$; Mann-Whitney test) than wild type active zones (9 NMJs of 6 animals). Scale bar in (A): 5µm;

3.3 *Drosophila* SYD-1 is a binding partner of BRP

Drosophila SYD-1(synapse-defective-1; synonyms: RhoGAP100F; DSYD-1) was identified as a member of BRP-protein complexes by MS, as well. SYD-1 was first described in *C. elegans* as a presynaptic protein important for the localization of presynaptic components at release sites of the nerve terminal (Hallam et al., 2002). For this reason, SYD-1 is assumed to play a role in specifying axon identity. DSYD-1 is closely related to SYD-1 of *C. elegans* and contains an amino-terminal PDZ domain, four putative SH3 binding sites, a C2 domain and a carboxy-terminal GTPase activating (GAP) domain (Fig. 17; Hallam et al., 2002). Closely related proteins can also be found in mouse and human by homology in the GAP domain (Fig. 17). The GAP domain of DSYD-1 is related to the RhoGAP family, but, like its relative in *C. elegans*, it exhibits a substitution of Asn by Val at position 194. This position was shown to be important for catalytic GAP activity (Rittinger et al., 1997). The deletion of the GAP domain in *C. elegans* caused strong dominant disruptions in neurite outgrowth and axonal transport (Hallam et al., 2002).

This phenotype could not be mimicked by expression of missense mutations in the GAP domain. These results indicate that SYD-1 may not be an active RhoGAP (Hallam et al., 2002). Instead, the GAP domain might sequester specific proteins and act as an interaction platform for its binding partners (Hallam et al., 2002) in *C. elegans*. Due to the lack of available full length *dsyd-1* cDNAs this issue could not be addressed in *Drosophila* yet.

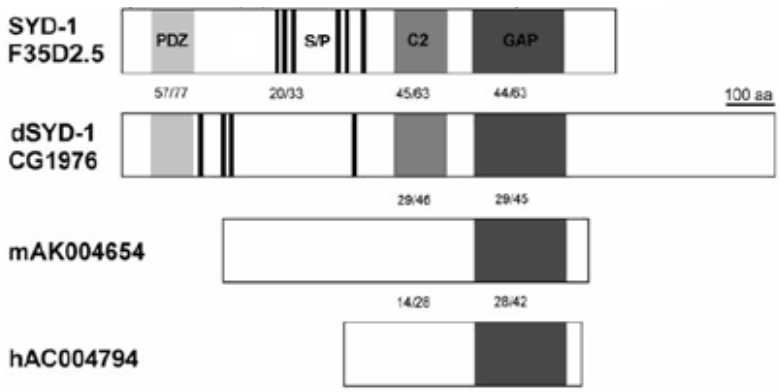


Fig. 17: Schematic overview of protein structures of SYD-1, DSYD-1 and closely related proteins in mouse and human (most likely represented by partial cDNA clones). DSYD-1 contains a PDZ-domain, C2-domain and a GAP-domain. Black bars indicate SH3-binding sites. Modified from Hallam et al., 2002.

Firstly, the spatio-temporal expression pattern of *dsyd-1* should be investigated. To this end *in-situ* hybridizations were performed throughout *Drosophila* embryogenesis. A strong specific label indicating selective expression of *dsyd-1* was detected in the CNS from stage 11 on (Fig. 18). The peripheral nervous system (PNS) and non-neuronal tissues were devoid of label. The onset of *dsyd-1* mRNA expression corresponds to the onset of neuronal differentiation and axon outgrowth (Broadie and Bate, 1993). Interestingly, the spatio-temporal expression profile of *dsyd-1* mRNA is highly similar to the one of *brp* (Wagh et al., 2006). As specific antibodies have not been available yet, further analysis of DSYD-1 on the protein level has to be awaited.

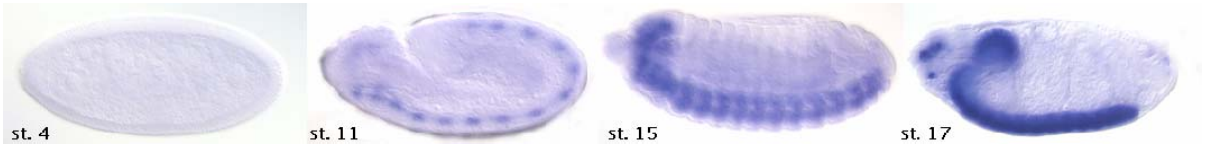


Fig. 18: *In situ* hybridization of Drosophila embryos to visualize the expression of *dsyd-1*. Specific staining was obtained when a 5' probe of *dsyd-1* cDNA was used. Sense probes did not produce any label in either experiment. *Dsyd-1* was specifically expressed in the CNS and ventral chord from stage 11 throughout embryogenesis.

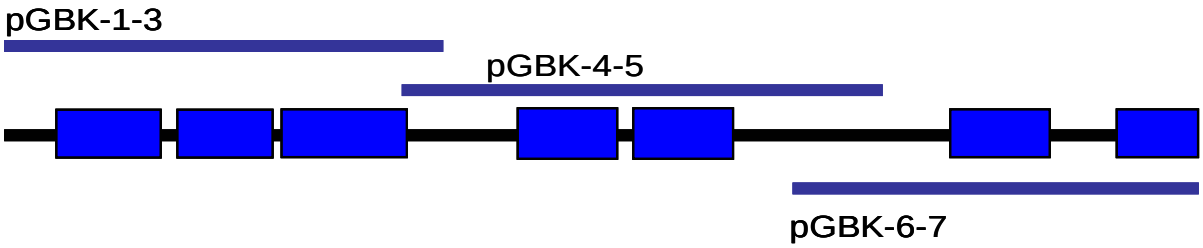
3.4 *In vitro* identification of DGrip binding partners

3.4.1 A yeast two-hybrid screen identified candidate proteins of the DGrip protein complex

The initial motivation of this thesis was to functionally study the composition of synaptic protein complexes at the NMJ. To this end this work concentrated on two proteins which are thoroughly investigated by our laboratory, BRP and DGrip. Several members of BRP-protein complexes were successfully identified by a MS-based proteomics approach. However, it was not possible to establish the same procedure for DGrip. For this reason DGrip binding partners should be alternatively identified using a Y2H approach.

A large-scale Y2H screen yeast two-hybrid screen was performed in collaboration with Tobias Böckers (University Ulm) using yeast mating to enable relatively easy screening of high numbers of transformants. To this end DGrip was subdivided in 3 fragments which were used as baits in the Y2H screen (Fig 19A; constructs were generated by L. Swan). Candidate proteins identified in the screen were subsequently tested for putative "auto-activation" and for interaction with all three baits at the Y2H level, respectively. Finally a set of eight putative binding partners of DGrip was obtained (Fig. 19B). Notably, candidate proteins have been reported as members of different signalling pathways and many of them exhibit PDZ-binding motifs.

A



B

Candidate description	Identified with bait	possible interaction with bait
CG7111; Rack1 (receptor for activated PKC)	PDZ 1-3	PDZ 1-3; PDZ 6-7
CG 30388; Magi (membrane assoc. protein with guanylate kinase activity; PDZ-motif)	PDZ 6-7	PDZ 1-3
CG3018; lesswright	PDZ 4-5	PDZ 1-3; PDZ 4-5
CG32677; Mint (Munc-18-interacting protein; PDZ-motif)	PDZ 1-3	PDZ 1-3; PDZ 6-7
CG4394; Traf6 (TNF-receptor associated factor)	PDZ 4-5	PDZ 4-5
CG1891; Saxophone	PDZ 1-3	PDZ 1-3; PDZ 6-7
CG10108; Phyllopod	PDZ 1-3	PDZ 1-3
CG16842; Echinoid (PDZ-motif)	PDZ 1-3	PDZ 1-3

Fig. 19: A Y2H screen identified putative binding partners of DGrip. (A) Schematic representation of the domain structure of DGrip and the baits used in the Y2H screen. PDZ domains are indicated as boxes. (B) List of putative binding partners of DGrip found in the Y2H screen. The middle column shows the DGrip bait used to isolate the respective candidate clone in the Y2H screen. The right column indicates the baits the respective candidate clones interacted with in a 2nd Y2H assay.

3.4.2 DGrip binds to GluRIIC in vitro

DGrip was originally identified in a Y2H screen for binding partners of the glutamate receptor subunit GluRIIC (Christine Quentin and Stephan Sigrist, unpuplished results). A fragment containing the region from the 5th PDZ domain downstream (Fig. 19) was found to bind to the C-terminal PDZ-binding motif of GluRIIC. Furthermore, the interaction of DGrip and GluRIIC was dependent on the PDZD-motif (EARV) at the C-terminus of GluRIIC.

Physical binding of DGrip and GluRIIC was tested by co-precipitation experiments *in vitro*. In SF9 cells recombinantly expressed HA-tagged GluRIIC coprecipitated with myc-tagged DGrip (Fig. 20). This interaction was specific, as neither HA-tagged DGluRIIA nor HA-tagged 5HT1A (a subunit of a serotonin receptor which contains a PDZ-motif at the C-terminus) were co-precipitated with DGrip in control experiments (data not shown). These data suggest that, at least *in vitro*, DGrip can bind GluRIIC, which suggested an involvement in postsynaptic GluR-trafficking as shown for its vertebrate homolog GRIP/ABP. Surprisingly, GluRIIC levels and localization at the PSD were unchanged in *dgrip*^{ex36} loss-of-function mutants. However, a P-element line (P{EP}1457) of *dgrip* shows increased clusters of GluRIIC at the PSD (V. Budnik, University of Massachusetts, personal communication).

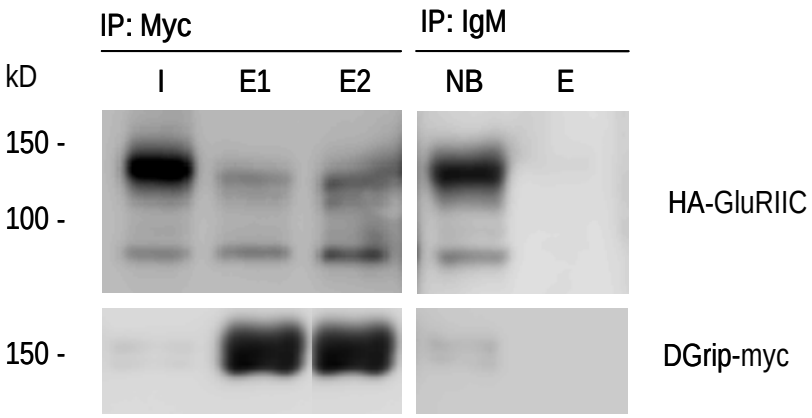


Fig. 20: Co-immunoprecipitation of HA-tagged GluRIIC with myc-tagged DGrip in SF9 cells. Shown is the input (I; 1/10 of elution) and elution fractions of two independent experiments (E1/2). HA-GluRIIC co-precipitates with DGrip-myc as revealed by detection of an HA-signal in the elutions of an anti-Myc-IP. In a control IP using IgM heavy chain HA-GluRIIC-signal could only be detected in the fraction not bound to the antibody-matrix (NB), but not in the elution (E). The lower panel shows anti-Myc probing of the same sample to visualize immunoprecipitated DGrip-myc.

3.5 A putative role of DGrip in transmitter release at the NMJ

The interaction of DGrip and GluRIIC *in vitro* implies a synapse-related function of DGrip. Indeed, several putative binding partners of DGrip identified in the Y2H screen would be consistent with this hypothesis. Among them are DMint1 (Munc18-1-interacting protein, also called X11-like proteins; Ho et al., 2002; Ashley et al., 2005), Saxophone (TGF-*beta* type I receptor; Rawson et al., 2003) and DMagi (membrane associated protein with guanylate kinase activity; Montgomery et al., 2004). All three molecules have been reported to influence NMJ outgrowth and/or transmitter release, respectively. However, despite laborious efforts those proteins could not yet be functionally associated to DGrip.
Nonetheless, it was of particular interest to unravel a potential synaptic function of DGrip.

3.5.1 DGrip is expressed in neuronal tissues

In situ hybridizations on embryos showed strong muscle expression of *dgrip* mRNA, whereby the CNS was devoid of *dgrip* signal (Swan and Wichmann et al., 2004). However, this does not necessarily reflect the absence of *dgrip* mRNA in neuronal tissue but could also be indicative for very low expression levels. For this reason the expression of *dgrip* mRNA was assessed by northern blotting at several developmental stages. To this end, mRNA was isolated from young embryos (0-2hours after egg laying), embryos aged for 12-18h after egg laying, larvae and adult fly heads. *Dgrip* message was readily detectable throughout development and showed particularly strong signals in adult fly heads (Fig. 21A). No differentially spliced isoforms of DGrip could be detected. The additional band at ca. 7 kb in adult fly heads presumably represents unprocessed mRNA. Next, western blots of extracts of larval fillets and larval brains were probed with anti-DGrip antibody. DGrip could readily be detected in the insoluble (pellet) and soluble (supernatant) fraction of larval fillets. A faint signal could also be identified in larval brain extracts (Fig. 21B).
The weak affinity of anti-DGrip antibody restricted further investigation of DGrip in the embryonic CNS and at the larval NMJ, respectively.

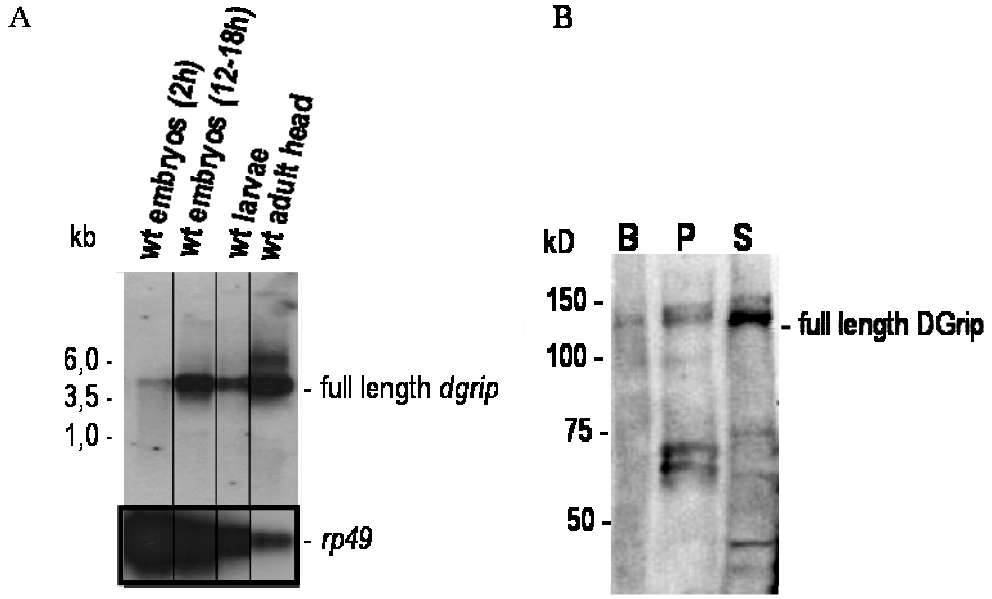


Fig. 21: Assessment of DGrip expression by northern and western blotting. (A) Distribution of *dgrip* mRNA throughout development. Northern blots were probed with radiolabeled full length *dgrip* cDNA. Radiolabeled *rp49* cDNA served as a loading control. (B) Western blot probed with anti-DGrip antibody. DGrip was readily detected in extracts from larval fillets (P=Pellet; S=Supernatant) and a faint band was also identified in larval brain extracts.

3.5.2 Presynaptic function of DGrip assessed by RNAi

By means of RNAi, a neuron-specific knockdown of *dgrip* mRNA to 40% of wild type level was achieved (using *elav-gal4* to target DGrip in all neurons; tested by quantitative real-time PCR). This proved the functionality of the RNAi-approach to induce motor neuron-specific knockdown of DGrip (genotype: *OK6-gal4/+::UASRNAi-dgrip/+*). Reduction of dgrip mRNA in motor neurons did not exhibit any morphological alterations at the NMJ or affect vitality in general. Furthermore, the overall architecture of the pre- and postsynaptic terminal seemed to be unaffected as revealed by immunohistochemistry against BRP, Syntaxin, Dlg (Disc-large, a postsynaptic scaffolding molecule) and GluRIIC. Nonetheless, vesicle release was strongly increased as demonstrated by TEVC at the larval NMJ (in collaboration with Robert Kittel). The amplitude of miniature excitatory junctional currents (minis) was unaltered (RNAi: -1.02 +/- 0.07 nA; control: -0.93+/- 0.03 nA; $P<0.27$; t-test), whereby the amplitude of evoked excitatory junctional currents (eEJC) was significantly augmented (RNAi: -95.6 +/- 6.97 nA; control: -64.25+/- 5.4 nA; $P<0.004$, t-test; ratio: 1.49; Fig. 22).

Consequently, this resulted in a significant increase of the quantal content (RNAi: 90.63 +/- 6.5; control: 68.36 +/- 6.95; P<0.036, t-test; ratio: 1.33Fig. 22). These physiological aberrations were reminiscent of the ones observed in *dgrip^{ex36}, twist-gal4/Y::UAS-dgrip/+* larvae, so called "*dgrip^{ex36}* by-passed larvae". These larvae are devoid of DGrip (*dgrip^{ex36}* is the loss-of-function allele of *dgrip*) except in mesodermal tissues, where DGrip was re-expressed to allow electrophysiological measurements (Robert Kittel, personal communication).
Altogether, these findings suggest a role of DGrip in regulating presynaptic neurotransmitter release at the larval NMJ.

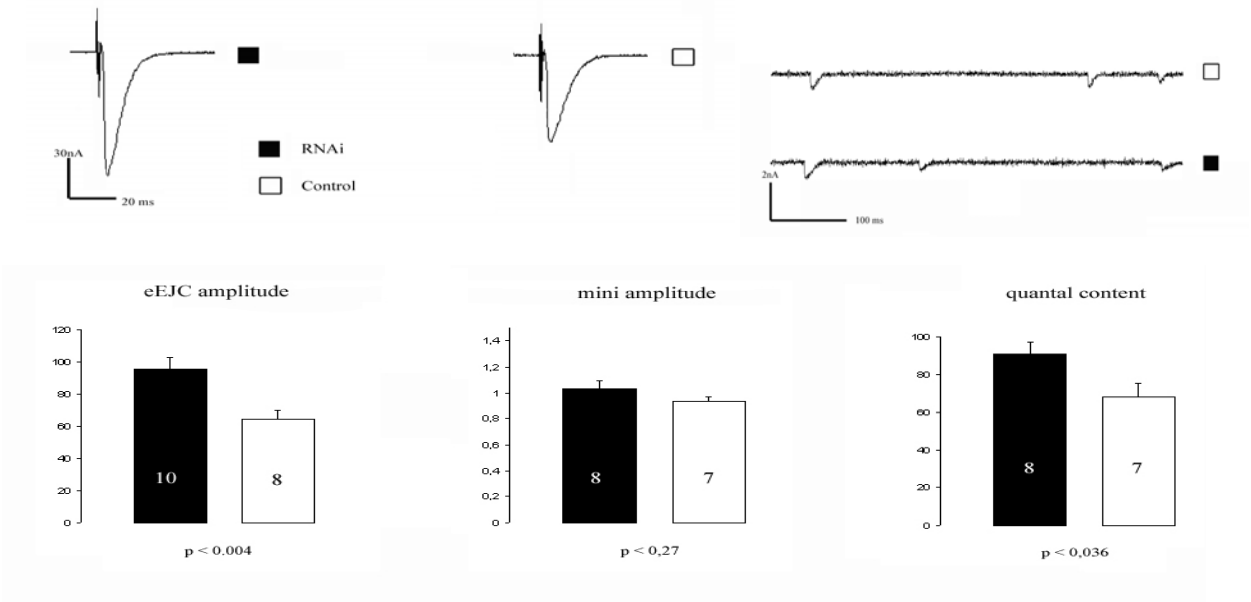


Fig. 22: Electrophysiological characterization of DGrip knockdown in motor neurons by RNAi. (A) RNAi (*OK6-gal4/+::UASRNAi-dgrip/+)* resulted in increased amplitudes of evoked EJCs when compared with the respective controls (*+/OK6-gal4*). (B) Miniature excitatory junctional currents (minis) were unchanged in size and kinetics. (C) A significantly higher quantal content is the consequence of increased evoked EJCs and unaltered minis. Traces are recorded from larval muscles 6 and 7 of segments A2/3. Figure and results are courtesy of Robert Kittel.

3.6 DGrip and Echinoid organize muscle guidance in *Drosophila* embryos

3.6.1 Echinoid binds DGrip in vitro

Echinoid (Ed) was identified via its interaction with the first three PDZDs of DGrip in the Y2H screen. Ed is a cell adhesion molecule consisting of 7 immunoglobulin domains (Ig), 2 Fibronectin Type III (FNIII) domains a transmembrane region (TM) and a type II PDZD interaction motif at the cytoplasmic tail (Fig. 23A). Four independent isolates encoding fragments of Ed were retrieved in the Y2H screen and all of them contained the C-terminal, intracellular end of the molecule including the PDZD interaction motif, EIIV (Fig. 23A). In fact, it could be shown that the interaction of Echinoid with DGrip in Y2H was dependent on the presence of this EIIV motif (Swan and Schmidt et al., in review). The binding of DGrip to Ed and the importance of the PDZD motif was further assessed by a pulldown assay in SF9 cells. Recombinant DGrip which was expressed in SF9 cells (Fig. 23B) efficiently interacted with a peptide representing the C-terminal 10 amino acids of Ed, but not with a scrambled control peptide.

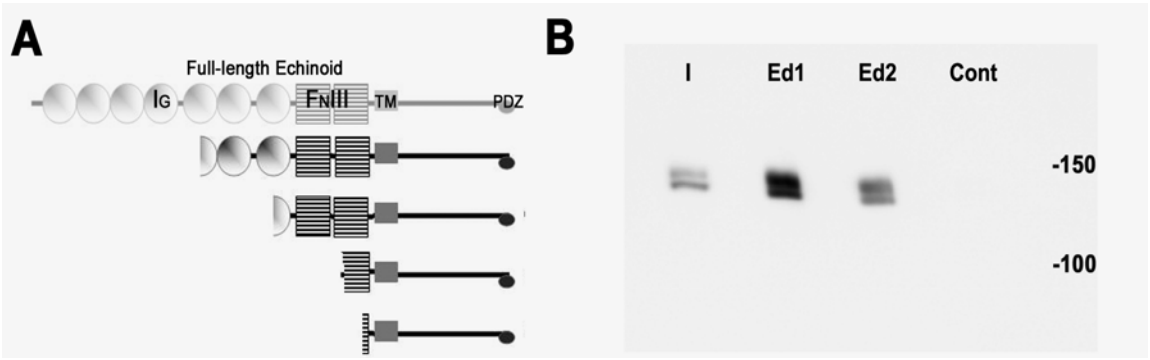


Fig. 23: Ed binds DGrip in vitro. (A) Schematic view of the domain structure of Ed and the four independent Ed-clones identified in the Y2H assay. (B) Full-length, C-terminally myc-tagged DGrip expressed in SF9 cells specifically bound to a 10aa peptide representing the C-terminus of Ed. Shown is the input to the experiment (I), two eluate fractions of independent replications showing binding of DGrip-myc to the Ed peptide (Ed1/2). The negative control with a 10aa-scrambled peptide did not show binding of DGrip-myc (cont). Figure modified from Swan and Schmidt et al., in review.

3.6.2 Loss of Echinoid provokes defects in embryonic muscle development

Ed is a L1-CAM-like molecule and is known as a regulator of both the EGF receptor (Bai et al., 2001; Escudero et al., 2003; Islam et al., 2003; Rawlins et al., 2003; Spencer and Cagan 2003) and Notch (Ahmed et al. 2003; Escudero et al. 2003) signalling pathways. Ed has not previously been reported to play a role in muscle development. After having proven physical interaction between Ed and DGrip, it was interesting to determine whether DGrip and Ed functionally interacted *in vivo.* Using the P-element insertion ed^{k01102} (insertion of P{lacW} into the first intron of *ed*, upstream of the coding region), muscle morphology in *dgrip*-negative background ($dgrip^{ex36}$) should be examined. Heterozygosity for ed^{k01102} strongly enhanced the ventral longitudinal muscle (VLM) defects in $dgrip^{ex36}$ hemizygous embryos, so that in the most severe cases individual muscles were hard to be identified (Fig. 24D). These findings could also be confirmed using another ed-allele, namely ed^{1x5} (a more severe allele, which causes high embryonic lethality; Swan and Schmidt et al., in review; Fig. 25).

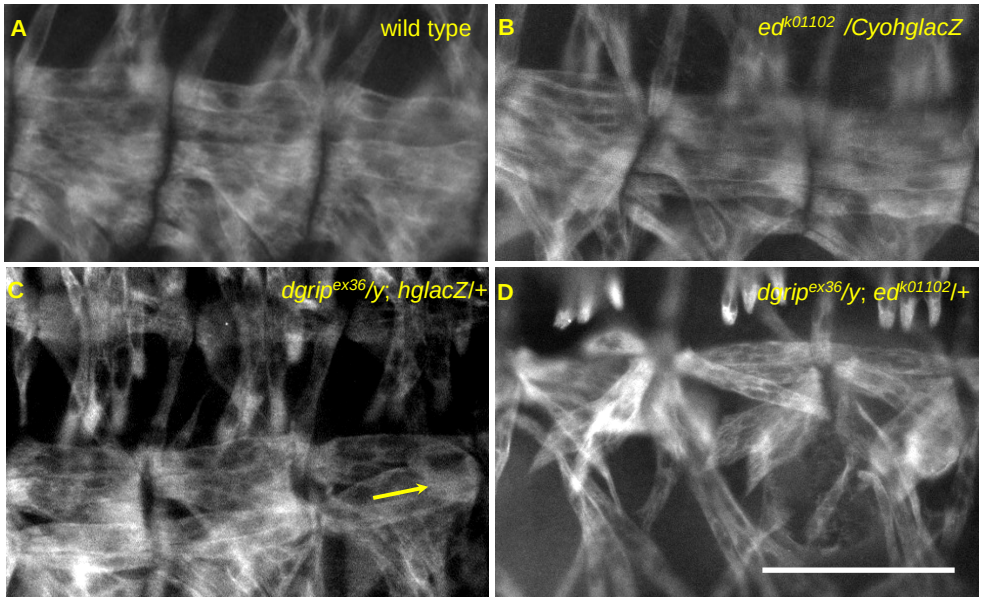


Fig. 24: Deficiency in *ed* enhanced defects in VLM morphogenesis. (A) Wild type; (B) $ed^{k01102}/+$ embryos did not exert defects in VLM morphogenesis. (C) $Dgrip^{ex36}$ mutants exhibit their characteristic aberrations in VLM morphogenesis (arrow), whereas VL muscles are completely misarranged $dgrip^{ex36}$; $ed^{k01102}/+$ embryos (D).
Images show lateral views on three hemisegments in muscle myosin staining of late stage 16 embryos. Scale bar in D: 50µm;

The analysis of several *ed-lof*-alleles for alterations in VLMs revealed phenotypes, which were reminiscent of partial loss of DGrip function (in collaboration with Laura Swan; Fig. 24D and 25D). This suggested that Ed could play a role in muscle guidance and that Ed and DGrip are functionally interacting to operate in VLM guidance (Swan and Schmidt et al., in review). Moreover, also the LTM group of muscles was affected in *dgrip^{ex36}; ed^{1x5}/+* embryos (Fig. 25D). In contrast, these muscles were unaltered in *dgrip^{ex36}* (Swan and Schmidt et al., in review; Fig. 25B).

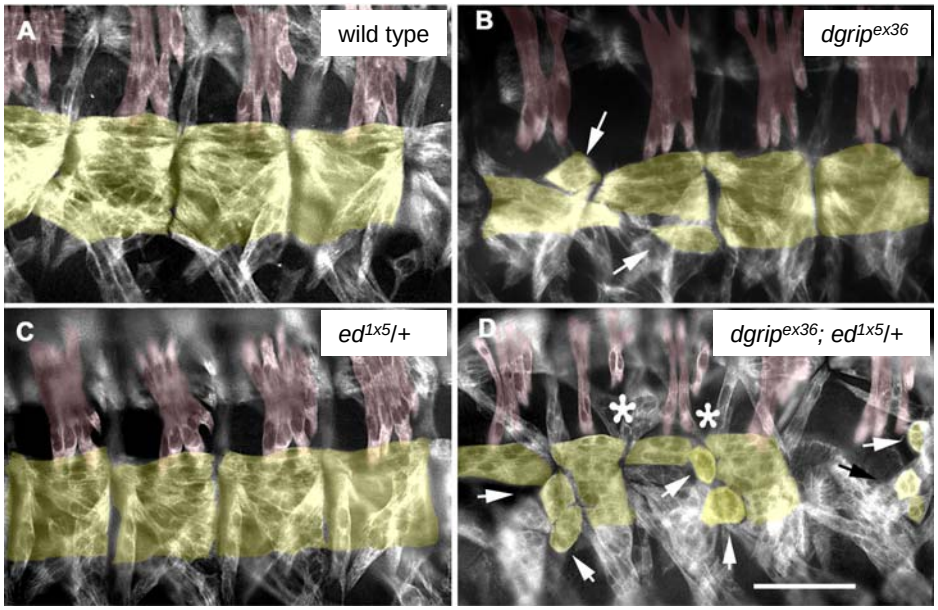


Fig. 25: Deficiency in *ed* also enhanced defects LTM morphogenesis. (A) Wild type embryo; (B) in *ed^{1x5}/+* embryos LTM and VLM morphogenesis are normal, respectively. (C) *Dgrip^{ex36}* mutants show their characteristic defects in VLM morphogenesis (arrows). (D) *Dgrip^{ex36}*; *ed^{1x5}/+* embryos have more frequent aberrations of VLM morphogenesis (arrows). Moreover, defects also emerge in the LTM field, where some LTMs appear to be missing (asterisks). More severe examples of *dgrip^{ex36}*; *ed^{1x5}/+* embryos (not shown) exhibit completely misarranged somatic musculature, where muscle identification is no longer possible.
Images show lateral views on four hemisegments in muscle myosin staining of late stage 16 embryos. Scale bar in D: 50µm. Modified from Swan and Schmidt at al., in review

4. Discussion

A challenging and pivotal task in cellular neuroscience has always been to resolve the composition of synaptic multiprotein complexes in order to understand synaptic physiology at the molecular level. However, main problems involve sample preparation and the generally limited amounts of synaptic proteins which still render proteomics on synaptic preparations a challenging task. For this reason most studies on synaptic protein networks predominantly used cultured neurons over-expressing the protein of interest. These approaches, however, can be prone to artefacts, as physiological expression levels are hard to mimic and protein networks are tightly regulated. Only recently, advancements in MS provided the basis for studies on *in vivo*-derived protein complexes, providing opportunities to determine their contents from native tissues. As one of the first, Neubauer and colleagues succeeded to identify proteins of the yeast U1 small nuclear ribonucleoprotein complex by MS (Neubauer et al., 1997). This study was followed by several others identifying components of the yeast nuclear pore complex (Rout et al., 2000) and of the human spliceosome (Zhou et al., 2002, Hartmuth et al., 2002). A pioneering MS-based study on the synaptic "interactome" characterized functional NMDA-receptor complexes from mouse brain (Husi et al., 2000). However, so far only few studies have used MS to establish neuronal interaction maps as reported for native metabotropic glutamate receptor 5 (Farr et al., 2004) from rat brain or for the presynaptic MALS/Veli-CASK-Mint1-complex from mouse brain (Olsen et al., 2005). The low number of studies performed is not surprising considering the requirements to establish a sensitively-working MS-platform, which is highly demanding in terms of knowledge, equipment and financial resources.

The main objective of this work was to establish a functional proteomics approach to characterize synapse-related protein complexes from *Drosophila* tissue. The physiological relevance of the obtained data should be further tested by a comprehensive genetic analysis at the NMJ. For this reason the thesis focused on two proteins our lab has been working on:

(1) Drosophila BRP

BRP exhibits homologies to mammalian CAST/ERC/ELKS proteins and contains large coiled-coil domains over its whole length. BRP is found at the active zone (Wagh et al., 2006), where it forms ring-like structures reaching approximately 150nm into the cytoplasm (Kittel et al., in review). Mutants of

brp exhibit defective active zone membranes, a complete loss of presynaptic dense bodies and depressed evoked but sustained spontaneous vesicle release (Kittel et al., in review). Moreover, Ca^{2+}-channels were not appropriately clustered at *brp* mutant synapses as observed by a decrease of Ca^{2+}-channel density and an apparent increase in the average distance of Ca^{2+}-channels and vesicle release sites (Kittel et al., in review). Taken together these data imply that BRP might establish a matrix, required for both T-bar assembly as well as the appropriate localization of active zone components including Ca^{2+}-channels. Thus, it was of particular interest to elucidate the mechanisms underlying BRP-mediated Ca^{2+}-channel clustering at the active zone. Furthermore, the interplay of BRP with other presynaptic proteins should be investigated. For this reason the composition of BRP protein complexes should be dissected and BRP binding partners should be functionally characterized at the larval NMJ. Based on the "guilt-by-association" concept the identification of binding partners should give more mechanistic insight into the function of BRP at the active zone.

(2) Drosophila Grip

DGrip is a seven PDZD-containing protein and was identified on the basis of its interaction with GluRIIC in a Y2H screen. In *Drosophila* embryos DGrip acts as a key regulator of muscle guidance as revealed by *grip* mutants (Swan and Wichmann et al., 2004). The mammalian homologs of DGrip, GRIP1 and GRIP2, are reported to serve as adapter-proteins of AMPARs and regulate their activity-dependent and subunit-specific trafficking (Dong et al., 1997, 1999; Wyszynski et al., 1998, 1999; Liu and Cull-Candy, 2005; Maher et al., 2005). GRIP also interacted with KIF5 (Setou et al., 2000) and with Ephrin- (Bruckner et al. 1999; Hoogenraad et al. 2005) and Liprin-mediated pathways (Wyszynski et al., 2002; Dunah et al., 2005). Moreover, GRIP is also involved in transport and localization of Membrane Type 5 Matrix Metalloproteinase (MT5-MMP) (Monea et al., 2006). Taken together, these data are indicative of GRIP being a scaffolding molecule with multifaceted functions. In *Drosophila*, the *dgrip* mutant phenotype implies that DGrip regulates the convergence of several signalling pathways during embryonic muscle guidance (Swan and Wichmann et al., 2004). However, so far no distinct signalling pathway could be related with DGrip function. For this purpose, protein complexes containing DGrip should be identified and their functional relevance should be genetically investigated in respect to muscle guidance and at the larval NMJ, respectively.

4.1 Mass spectrometry-based assays for functional proteomics

The prerequisite to examine protein complexes from native tissue by MS are optimized sample preparation and protein extraction considering the following issues:

(a) Efficient protein extraction from the tissue of interest;

(b) Preservation of protein-protein interactions during extract preparation;

(c) Compatibility with subsequent MS-approaches;

Noteworthy, BRP required very harsh conditions for solubilization (Fig. 10), which is consistent with the low solubility described for vertebrate CAST/ERC/ELKS and other members of the CAZ (Ohtsuka et al., 2002; Takao-Rikitsu et al., 2004; Olsen et al., 2005). However, the harsh conditions might have lead to the disruption of weak and transient protein-protein interactions within the BRP protein complex. Consequently, these might not be represented by this work. The following table presents an overview about the different experiments and MS-protocols performed for BRP and DGrip, respectively.

Protein	BRP		DGrip	
IP-condition	Proteins were extracted from heads of wild type flies (~1.5g) using a deoxycholate buffer. Protein extracts were subjected to IP with MAB nc82 (crosslinked to the matrix).		Proteins were extracted with 0.5% NP-40 from embryos (~1ml) and adult fly heads (~0.6g) expressing DGrip-myc (genotypes: *24B-gal4::UAS-dgrip-myc* and *elav-gal4::UAS-dgrip-myc* , respectively). IPs were performed with anti-Myc antibody (crosslinked to the matrix.	
Elution-condition	Elution under denaturing conditions (with SDS sample buffer; @95°C for 5min)	Elution under acidic conditions (with 100mM glycine pH 2.0; @4°C, for 5min)	Elution under denaturing conditions (with SDS sample buffer; @95°C for 5min)	Elution under acidic conditions (with 100mM glycine pH 2.0; @4°C, for 5min)
Peptide Extrac-tion	In-gel extraction and digestion of proteins	Gel-free approach	In-gel extraction and digestion of proteins	Gel-free approach
MS-method	LC-MSMS	LC-MSMS	MALDI MS; LC-MSMS	LC-MSMS
Result	15 proteins; among them 2 transmembrane proteins	11 proteins; no transmembrane proteins	No specific interactions could be retrieved	No specific interactions could be retrieved

Table 2: Summary of different approaches performed to identify protein interaction partners of BRP and DGrip, respectively. The experiments differ in the starting material, the conditions to obtain the immunoprecipitate and the extraction and digestion of proteins for MS. The result of each experiment is shortly summarized.

4.1.1 DGrip protein complexes could not be characterized by mass spectrometry-based techniques

The analysis of DGrip-protein complexes failed despite several diverse attempts to identify interacting partners from different tissues. The reasons can be manifold. The presence of multiple highly abundant protein bands derived from myc-tagged DGrip (Fig. 9) might have "covered" potentially low abundant binding partners during MS-acquisition. In the gel-free approach proteins were removed from the antibody-matrix *en masse* which might have increased the complexity of the sample to an extent which is problematic for MS analysis. Similar problems have been reported by other groups (Farr et al., 2004). Moreover, the relevant interactions DGrip undergoes might be too transient to be captured by the tested conditions.
For this reason Y2H-based techniques were alternatively employed to identify DGrip binding partners (chapter 3.4.1).

4.1.2 Components of BRP protein complexes

Upon immunoprecipitation of BRP with MAB nc82 the eluate containing putatively co-precipitating proteins were subjected to microsequencing using two different protocols. This lead to the identification of numerous putative interaction partners of BRP (Table 1), which are listed below according to their potential biological function:

(a) *Regulators of small GTPases*:

The Rac1-GEF Still life (SIF) (Sone et al., 1997, 2000); RhoGAP100F/Synapse defective 1(DSYD-1; Hallam et al., 2002); CG3996 (RabGAP-related protein, Bernards, 2003);

(b) *Ion Channels*:

Slowpoke (Slo), a Ca-activated K^+-channel (Atkinson et al., 1991);

(c) *Various enzymatic proteins*:

Tudor-SN (Caudy et al., 2003); Glycogen phosphorylase (Dombradi et al., 1984); Puromycin sensitive aminopeptidase (Schulz et al., 2001); Neural conserved at 73EF (Gruntenko et al., 1998); CG1516 (Gronke, 2003); Glutamic acid decarboxylase 1 (Jackson et al., 1990); Glutamate dehydrogenase (Papadopoulou and Louis, 2000);

(d) *Cell adhesion, cytoskeletal or transport proteins*:

Neurexin (Nrx) (Graf et al., 2004; Missler et al., 2005); alpha-Catenin-related protein (Goldstein and Gunawardena, 2000); gamma-Coatomer protein (COP) (Hahn et al., 2000); Coracle (Chen et al., 2005);

(e) *Proteins with other functions*:
Rpn1/p97 (part of the regulatory complex of 26S proteasomes; Holzl et al., 2000); Yolk Protein 1(Barnett et al., 1980);
(f) *Yet uncharacterized proteins*:
CG17337-PA; CG5642; CG1975 (DRep2); CG12932;

The composition of the BRP protein complex isolated from adult fly heads indicated that subsets of channels, intracellular signalling molecules, cell - adhesion and cytoskeletal proteins are organized together into a physical unit. The features of members of the BRP complex could provide insight into the specific function of BRP at the synapse. For this reason some of the identified binding partners will be discussed in more detail in the following chapters (chapter 4.6 and 4.7).

4.1.3 Comparison of mass spectrometry-protocols

Two protocols at two proteomic facilities were used to identify the proteins that co-immunoprecipitated with BRP. Whereas 15 proteins were identified when the immunoprecipitate was eluted using SDS-sample-buffer (protocol [a]), *en masse* elution identified 11 proteins (protocol [b]). Apparently, denaturing conditions for releasing bound proteins from the MAB nc82-matrix were very effective. Even a small fraction of MAB nc82 was released from the matrix, although it was crosslinked to the matrix before (Fig. 12). By this means, a high fraction of proteins bound to the BRP-matrix and additionally the heavy chains of MAB nc82 were collected in the eluate. However, subsequent 1D SDS-PAGE was required for "purification" of the immunoprecipitate from antibody constituents – disassembled heavy and light chains of IgGs, which could interfere with MS. In contrast, acidic elution is much "milder". Therefore, the co-elution of antibody constituents with the sample is prevented under acidic conditions and proteins are not effectively removed from the matrix.
Of note, transmembrane proteins were only identified using denaturing elution conditions (protocol (a)), which is again indicative for the differences in release efficacy among both protocols. However, only two transmembrane proteins, namely Neurexin and Slowpoke (Table 1), were identified in this study. Additionally, the scores obtained for Neurexin and Slowpoke were relatively low compared to cytoplasmic proteins. Transmembrane proteins are amphiphilic, which makes them difficult to be studied. This is most

evidently reflected by their very low representation in existing MS-analyses (Wu and Yates, 2003), despite their crucial roles in fundamental biological processes. A major problem for MS-based approaches on membrane proteins is the size and hydrophobicity of peptides generated by tryptic digest which is due to the limited accessibility of membrane-spanning regions for proteases. As a matter of fact this leads to the production of less peptides to be analysed and consequently explains the low sequence coverage usually obtained for membrane proteins (Wu and Yates, 2003). Great effort is undertaken to optimize the inclusion of membrane proteins in MS-based studies. These include on one hand the employment of organic solvents (Washburn et al., 2001) or strong detergents (Han et al., 2001) for more efficient solubilization. On the other different proteases are used to generate more overlapping peptides from transmembrane proteins (Van Montfort et al., 2002; Wu et al., 2003).
Intriguingly, there was a considerable overlap in proteins identified by either MS-protocol detectable. Five out of 15 (protocol [a]) and 11 (protocol [b]) proteins were found by both protocols, respectively. This is even more remarkable, as samples were prepared differently and two different MS-platforms were used (Q-Tof1 in protocol [a] and LTQ in protocol [b]). Just recently, Elias and co-workers demonstrated that under exactly the same conditions of sample preparation only 60% of the proteins were identified by two different types of mass spectrometers (Elias et al., 2005). This study demonstrated that different types of mass spectrometers might exhibit inherent ion preferences and dissimilar acquisition ranges, which strongly influence the amount and type of peptides sequenced (Elias et al., 2005). In summary, overlapping proteins from two independent trials can be considered to provide near complete assurance of correct identifications.

4.2 Identification of DGrip binding partners by means of yeast two-hybrid

Y2H is widely used to screen for protein-protein interactions *in vitro* and provides the possibility for fast high-throughput screens. Since our MS-based attempts to map DGrip at the level of protein-protein interactions *in vivo* failed, a Y2H screen was performed instead. To this end 3 fragments covering the sequence of *dgrip* were employed as baits in yeast mating. By this means, a number of candidate proteins were successfully identified. The

better performance of the Y2H system over the MS-approach to identify putative DGrip binding partners can not fully be explained. On one hand the problems encountered with anti-DGrip-myc immunoprecipitations and subsequent MS-approaches have to be taken into account and were discussed above (chapter 4.1.1). Moreover, interactions among proteins are dynamic and transient resulting in their fast formation and breakage. This represents one major obstacle of functional proteomics-approaches *in vivo*. Therefore, a Y2H approach might be advantageous to identify robust, but transient interactions of cytoplasmic proteins/regions *in vitro*. Partners of DGrip were likely to be transmembrane proteins. These might predominantly bind one of the seven PDZ-domains of DGrip via their cytoplasmic PDZ-binding motifs in a transient way.

However, care should be taken with the interpretation of Y2H results. The major drawback of Y2H-based assays is the high percentage of "false positives" among identified proteins. Thus, Y2H results require independent confirmation by other assays. These problems were also encountered by this work. Most presumed interactions between the candidate proteins and DGrip could not be verified despite laborious efforts. Especially, none of the presynaptic proteins like DMint (Munc-18-interacting protein; Ashley et al., 2005), Saxophone (TGF-*beta* receptor type I; Rawson et al., 2003) and Magi (membrane associated protein with guanylate kinase activity; Montgomery et al., 2004) could be linked to DGrip function yet. Neither biochemical assays nor a functional relation of these proteins to muscle development could be successfully established. By now, it can not be determined if those candidate proteins were simply "false positives" or if more sensitive assays are needed. In the latter case, specific antibodies or careful examination of brain tissue from different developmental stages could provide more insight into the potential association of DGrip with DMint, DMagi and DSaxophone, respectively.

On the other hand, some candidate proteins originally identified by Y2H-based techniques were successfully confirmed by other methodological means. A Y2H screen performed earlier in our lab originally identified DGrip as a binding partner of GluRIIC. This interaction appeared to be dependent on the PDZ-binding motif of GluRIIC. Co-precipitation of recombinantly expressed DGrip and GluRIIC from SF9 cells (Fig. 20) confirmed the Y2H data. Additionally, the Y2H screen performed in this work identified the cell-adhesion molecule Echinoid as a novel DGrip interactor of functional relevance in muscle guidance (chapter 3.6).

4.3 DGrip and Echinoid organize muscle guidance in *Drosophila* embryos

Ed contains immunoglobulin (Ig) and Fibronectin type III (FNIIII) domains, however it differs from the L1-CAM family of cell adhesion molecules as it lacks ankyrin repeats in its cytoplasmic domain (Hortsch, 2003). Several lines of evidence indicate binding and a functional interaction between DGrip and Ed. Firstly, the interaction of Ed and DGrip was dependent on the C-terminal EIIV motif of Ed and was mediated via PDZDs 1, 2 or 7 on the Y2H-level (Fig. 23; Swan and Schmidt et al., in review). Secondly, recombinant DGrip-Myc expressed in SF9 cells specifically interacted with a peptide representing the last 10 amino acids of Ed, including the EIIV motif (Fig. 23). Thirdly, several experimental findings point towards a genetic interaction between Ed and DGrip in the *Drosophila* muscle system:

(1) The $dgrip^{ex36}$ muscle phenotype was strongly enhanced by heterozygosity of *ed*, as confirmed using two independent *ed*-alleles (Fig. 24 and Fig. 25).

(2) In addition, the LTM group of muscles exhibited severe alterations in $dgrip^{ex36}$; $ed^{1x5}/+$ embryos (Fig. 25).

(3) $Dgrip^{ex36}$ mutant muscles were sensible for Ed over-expression (Swan and Schmidt et al., in review).

(4) Zygotic mutants of *ed* show similar defects in embryonic muscle morphogenesis (Swan and Schmidt et al., in review) as reported for $dgrip^{ex36}$ (Swan and Wichmann et al., 2004).

These data reveal that both, Ed loss-of-function and over-expression, can produce similar phenotypes in muscles. Ed is reported to mediate homophilic cell adhesion (Islam et al., 2003, Spencer and Cagan, 2003). Ed clones produced in wing discs showed altered sorting behavior, causing aggregation and adhesion of only those cells expressing the same complement of cell adhesion molecules (Wei et al., 2005). Maternally, Ed is expressed in the epidermis, over which nascent muscles “crawl” during the muscle guidance process to reach their target apodeme. Hence, the modification of Ed-levels on the “muscular side” could lead to significant changes in the transient muscle-epidermal adhesion during muscle guidance. This would explain the phenotype of *ed*-alleles as well as the strong phenotype of $dgrip^{ex36}$; $ed^{1x5}/+$ embryos. In both cases muscles seem to be abnormally stabilized during the guidance process as indicated by ectopic adherence between muscles (Swan and Schmidt et al., in review).

4.4 DGrip and synaptic vesicle release at the NMJ

Besides the well-characterized *dgrip* mutant phenotype in embryonic muscle guidance, several lines of evidence implied that DGrip might have an additional synaptic function. *Dgrip* mRNA could be detected in high amounts in adult fly heads (Fig. 21A). Moreover, DGrip protein was present in isolated larval brains, though at very low levels (Fig. 21B). In addition, motor neuron-specific RNAi targeted against the N-terminus of *dgrip* (*OK6-gal4/+::UAS-dgrip-RNAi/+)* exhibited significantly increased evoked excitatory junctional current (EJC) amplitudes, whereby miniature excitatory currents were unaltered (Fig. 22; in collaboration with Robert Kittel). Thus, the quantal content was significantly increased. This phenotype was similar to the one observed in *dgrip^{ex36}, twist-GAL4/Y::UAS-dgrip/+* "by-passed larvae". Accordingly, electron microscopy (performed by Carolin Wichmann) revealed the existence of big vesicles (150-300nm) of putatively endocytic origin in larval boutons of *dgrip^{ex36}* and to a lesser extent in *OK6-gal4/+::UAS-dgrip-RNAi/+* larvae. Taken together, these findings point towards a presynaptic function of DGrip, possibly controlling synaptic vesicle release.
However, it is not yet clear, how DGrip exerts this effect on vesicle release. Given that vertebrate GRIPs have been proposed to be involved in trafficking (Braithwaite et al., 2002; DeSouza et al., 2002), endocytosis (Osten et al., 2000; Xia et al., 2000) and cytoskeletal binding (Seog, 2004), DGrip could possibly be involved at several steps of the exo/endocytotic pathways. Interestingly, also vertebrate GRIPs have been reported to be present in central nervous presynapses (Charych et al., 2004), but their roles there are unknown. Due to the apparently complex function of DGrip further experiments are required to dissect the functional role of DGrip in neurotransmitter release.

4.5 Determining BRP protein structure by mass spectrometry

Western Blots probed with MAB nc82 consistently showed a double band at 170 kD and 190 kD of apparent weight, respectively. The epitope of MAB nc82 resides in the C-terminal region of BRP encoded by CG30337 (Wagh et

al., 2006). Enrichment of BRP by immunoprecipitation with MAB nc82 and subsequent analysis of each individual band revealed the putative protein structure of BRP. The data indicated that BRP exists in two forms in adult fly heads: the longer isoform is encoded by the ORFs CG12933, CG30336 and CG30337, whereby the shorter one is translated from ORFs CG30336 and CG30337 (Fig. 11B, C).

The *brp* gene locus is quite complex (Wagh et al., 2006; Fig. 11C) and contains a yet uncharacterized ORF represented by CG12932 between CG12933 and CG30336. Therefore, it was speculated that CG12932 might be part of the BRP protein. However, this could not be confirmed by PCR (Wagh et al., 2006) and microsequencing (this study, Fig. 11B, C), respectively. Surprisingly, low amounts of CG12932 were detected to co-precipitate with BRP as revealed by both MS-protocols applied (Table 1). In addition, CG12932-representing peptides were identified from a gel-region corresponding to an apparent molecular weight of 50-60 kD (MS-protocol [a]). Thus, CG12932 might be part of another protein transcribed from the *brp* locus and associated to BRP complexes. Most likely this novel protein does not include the region encoded by CG30337, as it is not recognized by MAB nc82. Indeed, the isolation of a cDNA comprising CG12933 and CG12932 (Christine Quentin and Sara Mertel, personal communication) supported this idea. Accordingly, northern blots of adult heads probed with 12932 detected a band of approximately 4kb - the length of a putative transcript from CG12933 and CG12932 (Wagh et al., 2006).

4.6 Small GTPase signalling and BRP in the presynaptic terminal

Over the past several years, it has become clear that the Rho family of GTPases plays an important role in various aspects of neuronal development in both vertebrates and invertebrates (for comprehensive review see Govek et al., 2005). Among the various processes are neurite outgrowth (Luo et al., 1994; Yoshizawa et al., 2002), axon pathfinding (Ng et al., 2002), vesicle trafficking (Wang et al., 1997; Sudhof, 1995) and exo/endocytosis (Geppert et al., 1997; Wucherpfennig et al., 2003). *Drosophila* Rho family proteins are more than 85% identical in amino acid sequences to the corresponding mammalian proteins. The most prominent members of the Rho GTPase

family are Rho (RhoA-D and RhoT), Rac (Rac1-3) and Cdc42. These are low-molecular-weight guanine nucleotide-binding proteins which cycle between GDP-bound inactive and GTP-bound active states. Their activity is determined by the ratio of cellular GTP to GDP and is further controlled by several regulatory molecules, like guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Activated Rho GTPases interact with specific effector molecules and recent reports hint to the importance of GEFs in determining the specificity of the initiated signalling cascade. This could be achieved via association with scaffolding molecules that link them, and hence the GTPase, to specific GTPase effectors in a temporally and spatially controlled manner (Buchsbaum et al., 2002; Jaffe et al., 2004). Most of the Rho GTPase effectors were found to be involved in the organization of the actin cytoskeleton (Hall, 1998; Banerjee and Wedegaertner, 2004) and membrane trafficking (Ridley, 2001; Qualmann and Mellor, 2003) and therefore regulate the initially mentioned processes. Rho GTPase signalling in the presynapse is supposed to mediate synapse development and Ca^{2+}-dependent exocytosis. The actin meshwork underneath the presynaptic plasma membrane is assumed to act as a barrier to reject the access of vesicles to the active zone membrane (Vitale et al., 1995; Bernstein et al, 1998). For this reason, disassembly of the cortical actin network may be a key step to facilitate trafficking of vesicles to the active zone membrane and finally their release. These processes are most likely tightly regulated upon electrical activity and various extracellular cues. Apart from various actin-depolymerising proteins, such as Scinderin and Gelsolin (Vitale et al., 1991; Miyamoto 1995), several studies point towards a role of Rho GTPases in precisely regulating the reorganization of actin-based structures in the context of Ca^{2+}-dependent exocytosis in various secretory cells and neurons (Doussau et al, 1999; Harada et al., 2000, Pinxteren et al., 2000). Most of these reports employed co-localization assays or *in vitro* binding studies of RhoGEFs (Banerjee and Wedegaertner, 2004) and RhoGAPs (Harada et al., 2000) with the actin cytoskeleton, respectively. However, the molecular mechanisms how effector proteins associated to small GTPases and their regulating proteins exert their function in the presynaptic terminal remain largely unknown. This work identified several proteins involved in small GTPase signalling as part of BRP protein complexes, namely *Drosophila* Still life (SIF), *Drosophila synapse defective-1* (DSYD-1) and a yet uncharacterized

protein encoded by CG3996. The first two are described in more detail below.

4.6.1 The Rac1-GEF Still life and BRP

Drosophila sif encodes a Rac1-GEF (Rac1-guanine nucleotide exchange factors) and was found in a screen of mutants for abnormal motor activities (Sone et al., 1997). Its mammalian homologs Tiam-1 (invasion-inducing T-lymphoma and metastasis 1; Habets et al., 1994) and STEF (SIF and Tiam 1-like exchange factor; Matsuo et al., 2002) are both implicated in regulating Rac1 in the context of neurite growth during development of the mammalian nervous system (Kunda et al., 2001; Matsuo et al., 2002). Microsequencing identified SIF as a highly scored binding partner of BRP (Table 1). The physical interaction of both proteins could further be confirmed by Co-IPs and subsequent immunoblotting (Fig. 13A). Moreover, a Y2H assay proved that SIF directly binds to BRP. This binding was mediated by the N-terminus of BRP (encoded by ORF CG12933) and both, the very N-terminal part or the part encompassing the PHn, TSS and PDZ domain of SIF (Fig. 13B-D), respectively. PH and PDZ domains are prominent protein-interaction motifs. PDZ domains are reported to be important for the organization of membrane proteins particularly at cell-cell junctions (Kim and Sheng, 2004) and PH domains are considered to be involved in protein-protein or protein-lipid interactions (Lemmon et al., 1997). Interestingly, the region including the PHn and TSS domains of SIF is reported to exert dominant-negative effects on Tiam-1 and STEF (Bourguignon et al., 2000). This fragment was able to specifically block the GEF function of ectopically expressed STEF and Tiam-1 in KB cells and of endogenous STEF and Tiam-1 in N1E-115 cells (Matsuo et al., 2001). Remarkably, the binding motifs of BRP and SIF are highly conserved among species (Fig. 5 and Fig. 13) and the corresponding DNA regions are still present in the *brp* mutant (Kittel et al., in review) and sif^{ES11} mutants (Fig. 14). Due to the lack of appropriate antibodies against this region the potential translation of truncated SIF in sif^{ES11} mutants could not be investigated.

In addition to the physical binding of BRP and SIF, several lines of evidence suggested a functional genetic interaction of BRP and SIF in *Drosophila* neurons. In adult fly heads of $sif^{ES11}/Df(3L)64DF$ and $sif^{98.1}/Df(3L)64DF$ BRP protein is markedly reduced when compared to wild type levels on western blots (Fig. 15). Intriguingly, BRP levels were significantly decreased at active

zones of *sif*^{ES11} mutant NMJs, as well (Fig. 16). Quantitative real-time PCR ruled out that the decrease of BRP levels was due to changes in *brp* transcription. However, the reduction of BRP levels at the NMJ of *sif*^{ES11} mutants by roughly 20% did not cause alterations in evoked vesicle release upon low frequency stimulation (chapter 3.2.2). Previous studies demonstrated that *brp*-RNAi larvae, in which a majority of synapses had no discernable BRP label, exhibited diminished vesicle release by approximately 50%. These data imply that only a near complete loss of BRP might cause defects in vesicle release at the NMJ. Further work examining short-term plasticity and synaptic release under high frequency stimulation should clarify vesicle release properties in *sif*^{ES11} mutants.

Both, SIF and BRP are expressed in the embryonic CNS and ventral chord at the time when neuronal differentiation and axonal outgrowth set off (Sone et al., 1997 and this work, data not shown; Wagh et al., 2006). Due to the lack of antibodies the localization of SIF in respect to BRP at the NMJ could not be tested. But in contrast to BRP, which is localized to the active zone, immunohistochemistry and immunoelectron microscopy showed that SIF resides predominantly in the periactive zone at *Drosophila* larval NMJs (Sone et al., 1997; 2000). However, this does not necessarily exclude the existence of small amounts of SIF at the active zone.

In summary, there is growing body of evidence for a yet unknown role of SIF in trafficking BRP to the active zone. However, by now it can not be determined where the SIF-BRP interaction takes place, in the axon or in the periactive or active zone of the presynaptic terminal. This question should be addressed at the NMJ and in the adult brain, as synapses might differ in their demands for BRP function. To this end, also more sensitive assays like *in vivo*-imaging (Rasse et al., 2005) need to be applied to study the dynamics of the SIF-BRP interaction. Remarkably, FRAP studies (fluorescent recovery after photobleaching) indicates high turnover of BRP at the active zone of NMJs (Wernher Fouquet, unpublished results). SIF might well be involved in mediating the “shuffling” of BRP at the active zones (see also chapter 4.6.3).

4.6.2 DSYD-1, a Drosophila RhoGAP, is associated with BRP

DSYD-1 was unambiguously identified by both MS-protocols as a member of BRP protein complexes in adult fly heads. Alike SYD-1 of *C. elegans, Drosophila* SYD-1 (DSYD-1) contains PDZ and C2 domains, SH3 binding sites and a RhoGAP-like domain (Hallam et al., 2002). In *C. elegans* SYD-1 is

reported to reside at active zones. Moreover, Hallam and co-workers reported that SYD-1 might target presynaptic components to future release sites in the nerve terminal, thus SYD-1 may play a role in specifying axon identity (Hallam et al., 2002). However, the mechanisms underlying these processes remain to be elucidated. DSYD-1, as well as SYD-1, may not exhibit catalytic RhoGAP activity due to an exchange of critical amino acids. But, at least in *C. elegans,* the RhoGAP domain seems to be necessary for SYD-1 function and presumably serves as a platform for interactions with proteins that control cytoskeletal remodelling and hence the establishment of presynaptic sites. To our knowledge, no function has been assigned to DSYD-1 so far. *In situ* hybridization on embryos demonstrated a correlation of the spatio-temporal expression profile of *dsyd-1* and *brp*. Both are expressed from embryonic stage 11-12 on, which goes in line with the onset of neuronal differentiation. The data are indicative of DSYD-1 having a functional role in *Drosophila* neurons, as well. Due to the lack of antibodies against DSYD-1 no further studies in respect to co-localization of both proteins could be performed so far.

4.6.3 Is the synaptic presentation of BRP regulated by small GTPases?

Neurons extend multiple processes, among which only one becomes the axon and others develop into dendrites. It remains largely unclear, which signals are required for initially determining axonal identity. However, data obtained from cultured rat hippocampal neurons and different neuronal cell lines suggested a role for regulators of the actin cytoskeleton in specifying axon identity (Bradke and Dotti, 1999; 2000). The most prominent ones among them are members and regulators of the small GTPase family, including GEFs, GAPs and the respective small GTPases (Luo et al., 1994; Yoshizawa et al., 2002). Several studies in *Drosophila* have shown that some GEFs play important roles in neuritogenesis (Newsome et al., 2000; Bateman et al., 2000). Moreover, SIF is reported to regulate actin dynamics and therefore structural changes in neurons, like axonal extension and motor terminal arborisation (Sone et al., 1997). In fact, the vertebrate homologs of SIF, Tiam-1 and STEF, have both been implicated in axon formation and neurite outgrowth (Matsuo et al., 2001; Kunda et al., 2001). These studies suggest that GEFs activate the small GTPase Rac1 which leads to disassembly of the local actin cytoskeleton. In turn, microtubule invasion within selected growth cones is facilitated (Kunda et al., 2001). In addition, investigation of

SYD-1 in *C. elegans* revealed that GAPs might be involved in the specification of axonal identity by a yet undefined mechanism (Hallam et al., 2002). There is growing body of evidence that small GTPase-mediated signalling pathways might be crucial for the polarization of neurons. However, cytoskeleton-mediated effects are not the only inducers of axon identity. Extracellular signals have been shown to provide spatial cues for axon formation (Esch et al., 1999). A network of signalling interactions is required to finally mediate axonal outgrowth and, concomitantly, the transport of axonal and future presynaptic components. It has been suggested that active zone components are delivered to the synapse in modular transport packages, so called active zone precursor vesicles (Ahmari et al., 2000; Zhai et al., 2001). The first proteins identified on these vesicles were the large coiled-coil active zone proteins Piccolo and Bassoon, hence the alternative name Piccolo/Bassoon transport vesicles (PTVs) (Shapira et al., 2003). Several more molecules like Munc-13, Syntaxin, alpha-Liprin, RIM and CAST/ERC/ELKS were identified on PTVs and are implicated in linking together components of the CAZ (Zhai et al, 2001; Shapira et al., 2003). However, little is known about the molecular determinants of PTV assembly and trafficking.

Where does the assembly of *de novo* PTVs take place? Data from studies on the development of active zones indicate that PTVs are pre-assembled at the Golgi apparatus (Garner et al., 2002). In fact, a recent study revealed that Bassoon and Piccolo are trafficked to the synapse via a trans-Golgi compartment (Dresbach et al., 2006). A distinct Golgi-binding domain of Bassoon appeared to be crucial for Golgi transit, as its deletion resulted in reduction of synaptic Bassoon levels (Dresbach et al., 2006). Taken together, CAZ-proteins seem to be preassembled in the Golgi-apparatus from which they are trafficked to synapses in concrete packages.

What are the factors involved in recruiting PTV components to the site of assembly? The Golgi apparatus has evolved as central junction for membrane traffic. This requires a controlled recruitment of a distinct set of proteins to specific sites of the Golgi apparatus to ensure accurately trafficking to its destination, e.g. the plasma membrane. Members of Ras-related GTPases (including the Rho, Rab and ARF families) play a key role in membrane traffic, as they are believed to specify the target membrane at the Golgi apparatus (Short et al., 2004; Munro, 2005). Small GTPase-regulators are found exclusively on distinct sites of the Golgi apparatus, where they activate specific GTPases. Those activated GTPases can recruit several effector proteins, which bind the protein set to-be-transported via so-called

tethering factors and thereby promote target-specific trafficking (Collins, 2003; Jordens et al., 2005). Tethering factors are defined by their composition of extensive coiled-coil domains and large multi-subunit complexes (Sztul and Lupashin, 2006). Structurally, they appear to be long rod-like molecules (Sapperstein et al., 1995). Bassoon, Piccolo and also BRP exhibit exactly these structural features and might therefore be good candidates for acting as tethers. It still remains to be elucidated if presynaptic proteins are delivered in a pre-assembled state in *Drosophila*, as well. Nevertheless, the structural similarities of BRP to prototypical tethers imply that BRP might recruit other synaptic proteins to be transported together. This work identified three distinct regulators of small GTPases as part of a BRP protein complex. SIF and DSYD-1 seem to be specific for Rho family GTPases and CG3996 is a putative GAP of Rab-GTPases. These findings propose an interesting working hypothesis how long-distance traffic of BRP to the CAZ might be controlled by small GTPases.

On the other hand a different model could also implicate small GTPases in short-distance traffic of BRP, namely the “deposition” of BRP at the CAZ, once BRP has been transported to the synapse. SIF is reported to be localized at submembraneous compartments of the periactive zone (Sone et al., 1997). Interestingly, several proteins of the periactive zone were found to supply the active zone with necessary components. Studies in *C. elegans* demonstrated that SAD-1 (a serine/threonine kinase similar to Par-1) accumulates at the periactive zone and controls the localization of synaptic vesicles in a neuron-type-dependent manner (Crump et al., 2001). Moreover, Zhen and colleagues showed an involvement of the putative GEF RPM-1 (regulator of presynaptic morphology) in the structural organisation of the active zone (Zhen et al., 2000). RPM-1 resides in the periactive zone and elicits diverse neuron-type dependent abnormalities of synaptic morphology when mutated (Schaefer et al., 2000; Zhen et al., 2000). Highwire is the *Drosophila* homolog of RPM-1 and has been reported to restrict growth of the NMJ (Wan et al., 2000), but does not exhibit active zone defects. The necessity of regulated transport of synaptic material is nicely demonstrated in motor neurons of *Drosophila liprin-alpha* mutants (Miller et al., 2005). These mutants exhibit defects in synapse structure and physiology (Kaufmann et al., 2002), similar to the ones seen in *kinesin* mutants (Schnapp et al., 2003). Live-observation of axonal transport in *liprin-alpha* mutants implies that the observed synaptic defects might stem in part from a failure in the delivery of putative synaptic-vesicle precursors, as well as BRP

(Miller et al., 2005). Combined with data from *C. elegans* on SYD-1 and SYD-2, the homolog of Liprin-alpha (Zhen and Jin, 1999), these studies provide evidence that synaptic scaffolding molecules might exert their action in transport and recruitment of synaptic constituents as well as local organization of these factors at the active zone.
In summary, SIF, DSYD-1 and CG3996 might be involved in delivery of BRP to the active zone at two sides (Fig. 26): On one hand, they might regulate long-distance transport of BRP from the Golgi apparatus to the synapse. On the other hand, the short-distance delivery of BRP within the presynaptic terminal might depend on these factors. Due to the limited availability of antibodies and fluorescently-labelled variants of SIF, DSYD-1 and CG3996, their subcellular distribution could not be tested yet. However, future experiments involving *in vivo* imaging of protein dynamics (Rasse et al., 2005) in the axon and the synapse should shed light on the site of action of SIF, DSYD-1 and CG3996. In addition, the generation of loss-of-function mutants for either protein is a prerequisite for their functional characterization in the context of synapse assembly.

4.7 BRP and Ca^{2+}-dependent exocytosis

Fast and efficient coupling of synaptic excitation and subsequent secretion of neurotransmitter is pivotal for synaptic transmission. Since more than a century ago the concept of Ca^{2+}-dependent transmission of neuronal excitation has been established - even before the concept of chemical synaptic transmission was conjectured (Locke 1894). Further work led to the Ca^{2+}-hypothesis, which proposed that neurotransmitter release from synaptic vesicles is triggered by elevations of the Ca^{2+}-concentration in the presynaptic terminal (Katz & Miledi 1965). Depolarization of the presynaptic nerve terminal causes the influx of Ca^{2+} through voltage-activated Ca^{2+}-channels. Presynaptic voltage-activated Ca^{2+}-channels are usually of the P/Q-and N-type (Jun et al., 1999; Ino et al., 2001). Ca^{2+}-dependent release is believed to occur within 100-200 µs after the increase of intracellular Ca^{2+}. To ensure this fast effect, both, the distance between Ca^{2+}-channels and the distance between Ca^{2+}-channels and vesicles at release sites are decisive for the transmission characteristics of a synapse (Neher, 1998;

Schneggenburger and Neher, 2000). In this context, the precise organisation of the presynaptic active zone (Burns & Augustine 1995; Zhai & Bellen 2004) and the density, coupling and direct juxtaposition of Ca^{2+}-channels, Ca^{2+}-gated K^{+}-channels and synaptic vesicles are critical (Robitaille et al., 1993; Atwood & Karunanithi, 2002). In fact, the probability of a vesicle being released following the opening of a single Ca^{2+}-channel has been calculated to decrease threefold when this distance is doubled from 25 to 50 nm (Bennett et al., 2000).

Several studies have shown that the presynaptically expressed Ca^{2+}-channel subunit Cacophony (Cac) dominates release at *Drosophila* neuromuscular junctions (Kawasaki et al., 2004). The analysis of *brp* mutants pointed towards a pivotal role of BRP in Ca^{2+}-dependent vesicle release at the NMJ of *Drosophila*. Electrophysiological analysis suggested an increased distance between Ca^{2+}-channels and vesicle docking sites in *brp* mutants (Kittel et al., in review). In addition, *in vivo* imaging of a GFP-tagged variant of Cac (Cac^{GFP}) revealed a reduced density of Cac^{GFP} at *brp* mutant synapses (Kittel et al., in review).

In this context, it is particularly interesting that the neuronal adhesion molecule Neurexin (Nrx) and Slowpoke (Slo), a Ca^{2+}-activated K^{+}-channel, could be isolated as putative members of BRP complexes. Both proteins were found by microsequencing, though with relatively low scores (for discussion on scores of transmembrane proteins see chapter 4.1.3): Neurexin was identified by two peptides and a total score of 36 and Slowpoke by one peptide with a score of 54, respectively. One of the peptides representing Nrx does not exhibit the complete series of y-type ions. Consequently, it does not exceed the “homology” criterion defined by MASCOT, however, it has still been assigned to Nrx as a top-scoring match.

4.7.1 Neurexin and BRP – players in Ca^{2+}-channel clustering?

Neurexins are synaptic cell-adhesion molecules that are localized at presynaptic terminals (Ushkaryov et al., 1992). In vertebrates, Neurexins are encoded by three genes. From each of them a longer alpha-Neurexin and shorter beta-Neurexin are transcribed, whereby the latter is not evolutionary conserved (Tabuchi and Sudhof, 2002). The only *Drosophila* homolog, Neurexin 1, is similar to alpha-Neurexin of vertebrates (Tabuchi and Sudhof, 2002). Neurexins serve as receptors of postsynaptic Neuroligins in the context of synapse formation (Ichtchenko et al., 1995; Scheiffele et al., 2000;

Dean et al., 2003). Alpha-and beta-Neurexins exhibit a PDZ-binding motif at the C-terminus which – among others- interacts with the cytoplasmic scaffolding molecules CASK (Hata et al., 1996) and Mint (Biederer and Sudhof, 2000). Both molecules are implied in the modulation of presynaptic Ca^{2+}-channels (Atlas, 2001). Several lines of evidence suggest that Neurexins are involved in the assembly of the presynaptic protein scaffold and the secretory apparatus (Dean et al., 2003; Missler et al., 2003). The latter might be mediated by an alpha-Neurexin-dependent coupling of Ca^{2+}-channels to presynaptic release sites (Missler et al., 2003). Triple knockout mice lacking all members of the alpha-Neurexin family exhibited impaired evoked synaptic transmission; however, no changes in synapse formation could be detected.

Brp mutants failed to cluster Ca^{2+}-channels properly, which resulted in a longer average distance between Ca^{2+}-channels and vesicle release sites. This sufficiently explains the low vesicle release probability and impaired short-term plasticity of *brp* mutant synapses. Mechanistically, these data could point towards a requirement of a BRP-Neurexin interaction to functionally organize Ca^{2+}-channels at the active zone of the NMJ. Disrupting this interaction might result in the observed failure of Ca^{2+}-channel anchorage at the active zone, hence a longer average distance between Ca^{2+}-channels and vesicle release sites. Importantly, this might not be the only explanation for the defects in *brp* mutants and most likely BRP organizes the active zone in association with other factors within the synaptic protein scaffold. Noteworthy, a recent study in primary hippocampal cultures hints towards a syntenin-1-mediated interaction between CAST1/ERC2 and Neurexin, which might contribute to the molecular organization of the CAZ (Ko et al., 2006).

4.7.2 Slowpoke is a putative member of BRP protein complexes

Drosophila Slowpoke (Slo) is a subunit of a Ca^{2+}-activated K^{+}-channels (CAK channel; Atkinson et al., 1991) and exhibits structural and functional homology to vertebrate BK-type CAK channels (Butler et al., 1993). Slo is expressed in various tissues like muscles, tracheal cells and the CNS throughout fly development (Broadie and Bate, 1993; Becker et al., 1995). Furthermore, it is found in presynaptic endings of rat brain (Knaus et al., 1996) and frog neuromuscular junction (Robitaille et al., 1990), where it influences transmitter release. At the larval NMJ of *Drosophila* its activity

seems to be regulated by Slowpoke binding protein (Slob) and the 14-3-3 protein in a dynamic manner (Schopperle et al., 1998; Zhou et al., 1999): upon a rise in Ca^{2+} in the presynaptic terminal Ca^{2+}-Calmodulin-kinase II (Wang et al., 1994) might phosphorylate Slob, which could in turn promote binding of 14-3-3 protein to Slo causing Slo downregulation (Zhou et al., 1999). Apart from local Ca^{2+}-concentrations and the membrane potential in the presynaptic terminal, Slo-activity might therefore be additionally controlled by modulative protein-protein interactions. Generally, Slo contributes to membrane repolarization and therefore limits transmitter release in *Drosophila* nerve terminals (Gho and Ganetzky, 1992). Presynaptic Ca^{2+}-channels and Ca^{2+}-gated K^{+}-channels are in close vicinity at the active zone. This spatial and functional arrangement appears to be organized through interactions with active zone proteins, which may define "slots" - loci of interaction that ultimately govern synaptic efficacy (Harlow et al., 2001; Cao et al., 2004). *BRP* mutants exhibit desynchronised evoked vesicle release, which is attributable to an altered presynaptic current waveform. This could result from a disruption in the co-localisation of Ca^{2+}-channels with Ca^{2+}-activated K^{+}-channels, which in turn could slowdown K^{+}-channel-mediated presynaptic repolarisation. Consequently, Ca^{2+}-channels would remain open longer, and thus cause prolonged vesicle release.
However, a detailed molecular explanation can not yet be drawn from these results, as important features are still unknown:

(a) Ca^{2+}-channels are mislocalized at the CAZ. Is this related to alterations of Ca^{2+}-channel properties in terms of conductance and voltage sensitivity?

(b) Are the physiological properties of Slo altered in *brp* mutants?

(c) Is the distribution of Slo influenced by the absence of BRP? Is BRP also important for the localization of Slo at the active zone?

Only after answering these questions, it will be possible to functionally connect Slo with the observed phenotype in *brp* mutants.

4.7.3 BRP is a master organizer of active zone structure

In vertebrates, most of the known presynaptic proteins are described in the context of vesicle exocytosis and endocytosis (Sudhof, 2004; Gundelfinger et al., 2003). In contrast, much less is known about the molecules and protein complexes required for the functional assembly of the presynapse *in vivo*. However, a vast amount of studies investigated CAZ-proteins by over-expression in cultured neurons (Landis et al., 1988; Shapira et al., 2003; Zhai and Bellen, 2004; tom Dieck et al., 2005). These conditions might not be appropriate to examine the selective targeting and functional interactions among CAZ proteins. This becomes obvious by the disparity observed, when *in vivo* studies of loss-of-function phenotypes are compared to data obtained from *in vitro* approaches (Zhen and Jin, 2004; Grant, 2006).

This work discovered the Ca^{2+}-activated K^{+}-channel Slowpoke and the neuronal adhesion molecule Neurexin as putative members of a presynaptic BRP complex. In combination with data from *brp* mutants this provides a glimpse, how BRP might coordinate active zone assembly. Structurally *brp* mutants show ruffling of the presynaptic membrane and a lack of electron-dense projections. Therefore, BRP may be involved in organizing T-bars, a matrix of unknown origin. In turn, this matrix might be required for a functional organization of the active zone to ensure speed and fidelity of vesicle release. Indeed, activity-induced addition of presynaptic dense bodies has previously been suggested to elevate vesicle release probability (Wojtowicz et al., 1994). The observed mislocalization of Ca^{2+}-channels in *brp* mutants might be a consequence of the lack of T-bars and therefore explain the severe defects in vesicle release. In addition, Slowpoke might be localized by BRP in close vicinity to Ca^{2+}-channels. Antibodies and fluorescently labelled variants of Slowpoke will be generated to examine this issue in more detail.

STED-microscopy revealed that MAB nc82 detects BRP at a distance of approximately 150nm from the active zone. This distance would be in accordance with the length of T-bars and the putative length of the BRP protein. BRP might act as a prototypical tether that spans the region from the active zone membrane to the roof of the T-bar with its large coiled-coil domains (Fig. 26). Even though BRP appears to be the “master-organizer” of T-bars, it might not act alone in the structural and functional assembly of the active zone, but rather in concert with other CAZ proteins. Several proteins of different nature have been suggested to play an important role in organizing the active zone in *Drosophila*, among them Liprin-alpha

(Kaufmann et al., 2002), members of the Kinesin family (Schnapp et al., 2003), members of TGF-beta-signalling (Aberle et al., 2002), Neuroglian (Godenschwege et al., 2006) and Syndecan and Dallylike (Johnson et al., 2006). In this thesis Neurexin was identified as a putative component of BRP complexes. In vertebrates Neurexins have been implicated in Ca^{2+}-channel clustering at the active zone. It is not clear yet, if *Drosophila* Neurexins exert similar functions, but if so, it would provide a molecular explanation of the Ca^{2+}-channel clustering phenotype observed in *brp* mutants.

In summary, the *de novo* identification of putative members of a BRP complex by a MS-based proteomics approach nicely complements genetic and physiological studies on *brp* mutants. Taken together, these data led to the proposal of the following working-model of BRP function (Fig. 26). This thesis should provide a framework to assess BRP function at the CAZ at the molecular level.

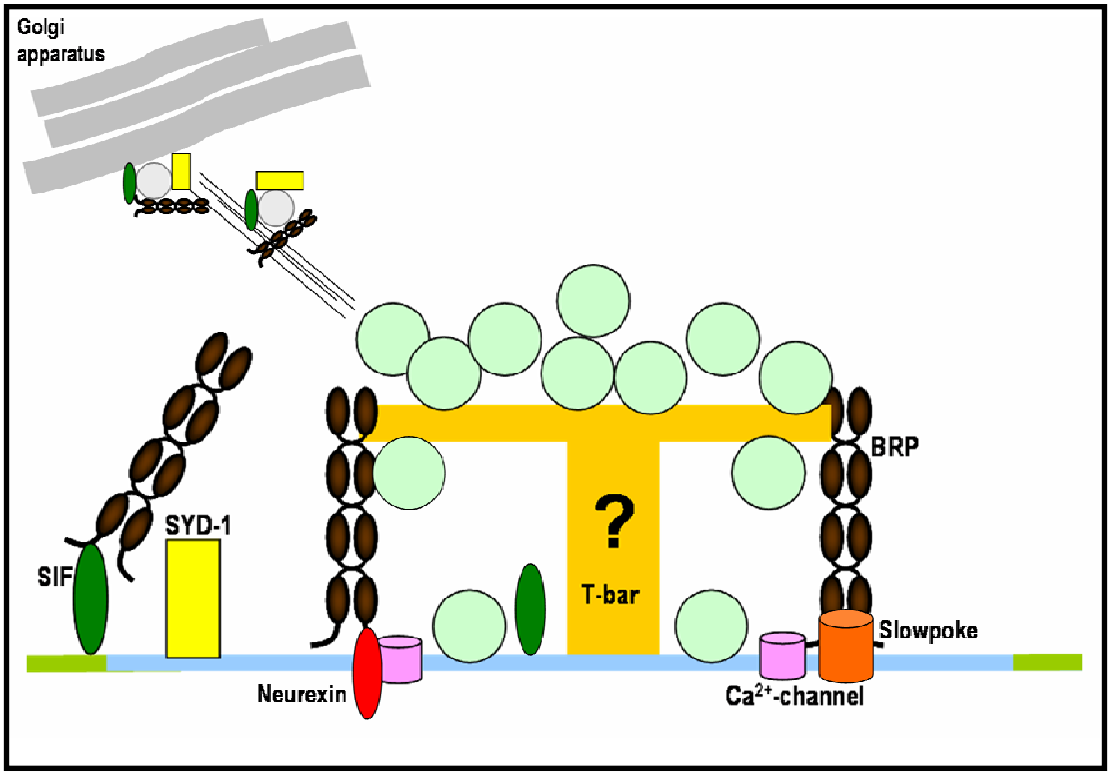


Fig. 26: Working-model of active zone assembly in *Drosophila*. Long-distance transport of BRP from the Golgi apparatus to the synapse might be mediated by small GTPase signalling. One of the regulators could be SIF and SYD-1. SIF and SYD-1 might recruit BRP and specify its traffic to the active zone membrane. In turn, BRP could act as a tether at the Golgi apparatus to assemble synaptic components for trafficking to the synapse. In a second step or in an alternative scenario, SIF and SYD-1 could be involved in the "deposition" of BRP at the active zone, thus mediate short-distance transport of BRP. Once at the active zone, BRP might act as a tether to functionally organize the presynaptic protein network including the

formation of T-bars of yet unknown origin. Consistent with its size, BRP could span the region from the active zone membrane to the roof of the T-bar (~150nm) and thereby coordinate the delivery of active zone components to the membrane.

5. Summary

Chemical synapses are highly specialized cell-cell junctions with the purpose of communication between a nerve cell and a target cell being another neuron, a muscle or a gland cell. Synaptic transmission is directional: the presynapse is defined as the compartment from which neurotransmitter is released. The postsynaptic site harbours the machinery for neurotransmitter reception and integration, which is located precisely opposite to presynaptic release sites. At both sites large protein complexes are assembled by multidomain scaffolding proteins. In fact, the notion is evolving that synapse assembly, maturation, maintenance and plasticity depend on multifaceted protein-protein and protein-lipid interactions within dynamic macromolecular complexes. In order to understand synaptic physiology at the molecular level, the determination of the composition of synaptic multiprotein complexes is of particular interest in cellular neuroscience. Most studies on synaptic protein networks were predominantly performed on cultured neurons cells over-expressing the protein of interest. Such *in vitro* approaches, however, might be difficult to be interpreted. Only recently, advancements in mass spectrometry (MS) provided the basis for studies on *in vivo*-derived protein complexes enabling the determination of their contents from native tissues.

The objective of this work was to identify and functionally characterize synapse-related protein complexes in *Drosophila melanogaster*. The physiological relevance of the obtained data should be further tested by a comprehensive genetic analysis at the larval NMJ, a well established synaptic model system.

To this end, the thesis focused on two proteins our lab has been working on:

1. The Glutamate-receptor-interacting protein (DGrip), a seven PDZ-domain containing protein. Previous studies in our lab revealed that DGrip coordinates the complex process of muscle guidance during early embryogenesis.

A yeast two hybrid (Y2H) screen identified the cell adhesion molecule Echinoid (Ed) as a binding partner of DGrip. Further analysis suggested that Ed and DGrip might act in concert to regulate embryonic muscle guidance possibly by regulating adhesion between muscle and epidermal cells.

In addition, a combination of Y2H and RNAi approaches provided hints towards a role of DGrip in regulating presynaptic neurotransmitter release at the larval NMJ.
The data support the notion that DGrip functions as a dynamic scaffolding molecule to mediate many transport-dependent processes.

2. *Drosophila* Bruchpilot (BRP), a novel coiled-coil protein with homologies to the CAST/ERC/ELKS family. BRP is a component of transmitter release sites (active zones) in presynaptic terminals. The analysis of *brp* mutants pointed towards a role of BRP in organizing active zones and synaptic vesicle release.

A MS-based functional proteomics approach identified proteins, which co-immunoprecipitated with BRP from adult fly head extracts. Among them were Still life (SIF) and SYD-1, both regulators of the Rho family of small GTPases. A combination of biochemical and genetic approaches suggested that SIF might influence the presentation of BRP at synapses. In addition, the identification of Neurexin and Slowpoke as putative components of BRP protein complexes strengthened the role of BRP as an organizer of active zones.
In summary, this work dissected *in-vivo*-derived BRP complexes on the molecular level. These data could provide considerable insight into transport of active zone-components and the assembly of the presynapse. As basic principles of synapse formation are often evolutionary conserved, the obtained data should be translatable into other animals.

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7. Abbreviations

ABP	AMPA receptor binding protein
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AZ	active zone
BRP	Bruchpilot
CAST	CAZ-associated structural protein
CAZ	cytomatrix at the active zone
CNS	central nervous system
Co-IP	Co-immunoprecipitation
ECM	extracellular matrix
Ed	Echinoid
EJC	evoked junctional current
ESI	Electrospray ionisation
FNIII	fibronectin type III
GFP	green fluorescent protein
GluR	Glutamate receptor
GRIP	Glutamate receptor interacting protein
Ig	immunoglobulin
IP	immunoprecipitation
KIF1A	Kinesin motor protein
LC	Liquid chromatography
LTM	lateral transverse muscle(s)
Maldi	Matrix-assisted laser desorption/ionization
Mini	miniature excitatory junctional current
Mint	Munc-18 interacting protein
MS	mass spectrometry
Munc	Mammalian homologue of unc
NMDA	N-methyl D-aspartate
NMJ	neuromuscular junction
PDZ	PSD-95/Discs-large/ZO-1 domain
PFF	peptide fragmentation fingerprint
PMF	peptide mass fingerprint
RIM	Rab3-interacting protein
RPM	Regulator of presynaptic morphology
SAD	Synapse of the amphid defective
SIF	Still life

SNARE	Soluble n-ethylmaleimide-sensitive component attachment protein receptor
SYD	Synapse defective
Syt	Synaptotagmin
TGF	transforming growth factor
UAS	upstream activating sequence
UNC	uncoordinated
VLM	ventral longitudinal muscle(s)
Y2H	Yeast two hybrid

8. Curriculum vitae

1. Personal Details

1.1	Surname:	Schmidt
1.2	Forename:	Manuela
1.3	Nationality:	German
1.4	Date and Place of birth:	01.02.1978 in Wasserburg/Inn
1.5	Marital status:	Single
1.6	Work Address in Göttingen:	ENI-G; AG Sigrist Grisebachstr. 5 37073 Göttingen Germany Tel: 0551/3912354 e-mail: mschmida@gwdg.de

2. Scientific Education

2.1 University Entrance Qualification:
June 1997 Ruperti-Gymnasium Mühldorf; grade: 1,2

2.2 University Studies:
1997-1999: Undergraduate studies in Biology; University of Würzburg
1999-2002: Graduate studies in Biology; University of Würzburg

Since Sept.01: Master/PhD Program Neurosciences; International Max Planck Research School; Göttingen

Internships:
2000: "Identification of QTLs for Ammonium Resistance in *Arabidopsis*"
Supervisor: Prof. Bennett; Plant Science Division; University of Nottingham, UK
Duration: 3 months
2001: "The role of the STAT6 Gene in the Development of Asthma bronchiale"
Supervisor: Dr. Wjst; Group of Molecular Epidemiology; GSF, München-Neuherberg, Germany
Duration: 2 months

2.3 Examinations:
20.06.2002: Diploma in Biology; University of Würzburg; grade: 1,0
16.08.2002: Master Examinations; International Max Planck Research School; Göttingen; Grade: A

2.4 Diploma Thesis (Sept.2001-June 2002):
"Molecular analysis of ABA- and stress-dependent gene expression of GORK in *Arabidopsis thaliana*"
Supervisor: Prof. Dr. R. Hedrich; Institute of Plant Physiology and Biophysics; University of Würzburg, Germany

2.5 Grants/Fellowships:

Oct. 1999-March 2002:	"Stipendium für Hochbegabte"; Bavarian Ministery for Cultural Affairs
June 2001:	Book-Grant; Oskar-Karl-Forster-Stiftung
July 2003:	Travel Allowance, Boehringer Ingelheim Fonds

3. PhD Thesis and PhD Curriculum

3.1	Thesis Title:	Characterization of synaptic protein complexes in *Drosophila melanogaster*
3.2	Location:	European Neuroscience Institute (ENI-G) Grisebachstr. 5 Göttingen
	PhD committee:	PD Dr. Sigrist (direct supervisor; ENI-G) Dr. Wouters (ENI-G) Prof. Dr. Jäckle (Department of Molecular Biology; MPI for Biophysical Chemistry; Göttingen)
	Commencement of Project:	Oct. 2002

3.3 Advanced method courses (outside the PhD program):

2003:	"Neurobiology of Drosophila" Cold Spring Harbor Laboratories, New York, USA Duration: 3 weeks
2004:	"Brain Proteomics" TU Kaiserslautern, Kaiserslautern, Germany Duration: 1 week

3.4 Attended International Conferences:

2003	18th European Drosophila Research Conference Göttingen, Germany
2004	4th Forum of European Neuroscience Lisbon, Portugal
2004	10th European Symposium on Drosophila Neurobiology Neuchatel, Switzerland
2005	ENI-Christmas Meeting (poster contribution) Alicante, Spain

9. List of Publications

Swan, L.E., Wichmann, C., Prange, U., Schmid, A., Schmidt, M., Schwarz, T., Ponimaskin, E., Madeo, F., Vorbruggen, G., and Sigrist, S.J. (2004). A glutamate receptor-interacting protein homolog organizes muscle guidance in Drosophila. Genes Dev *18*, 223-237.

Rasse, T.M., Fouquet, W., Schmid, A., Kittel, R.J., Mertel, S., Sigrist, C.B., Schmidt, M., Guzman, A., Merino, C., Qin, G., Quentin, C., Madeo, F.F., Heckmann, M., and Sigrist, S.J. (2005). Glutamate receptor dynamics organizing synapse formation in vivo. Nat Neurosci *8*, 898-905.

Dhananjay A. Wagh*, Tobias M. Rasse*, Esther Asan, Alois Hofbauer, Isabell Schwenkert; Heike Dürrbeck; Sigrid Buchner; Marie-Christine Dabauvalle, Manuela Schmidt, Qin Gang, Carolin Wichmann, Robert Kittel, Stephan J. Sigrist, Erich Buchner. Bruchpilot, a protein with homology to ELKS/CAST/ERC, is required for structural integrity and function of synaptic active zones in Drosophila.
Accepted in Neuron.

Laura Swan*, Manuela Schmidt*, Tobias Schwarz, Evgeni Ponimaskin, Ulrike Prange, Tobias Boeckers, Ulrich Thomas and Stephan J. Sigrist. Echinoid and *Drosophila* Glutamate receptor interacting protein organize *Drosophila* muscle guidance via a complex interaction. *In review.*

Robert J. Kittel*, Carolin Wichmann*, Tobias M. Rasse*, Wernher Fouquet, Manuela Schmidt, Andreas Schmid, Dhananjay A. Wagh, Christian Pawlu, Robert R. Kellner, Katrin I. Willig, Stefan W. Hell, Erich Buchner, Manfred Heckmann, and Stephan J. Sigrist. *Drosophila* Bruchpilot is required for presynaptic active zone assembly and Ca^{2+}-channel clustering to ensure efficient vesicle release. *In review*.