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**THE ROLE OF MULTIFUNCTIONAL SCAFFOLDING
PROTEINS IN THE SYNAPTIC VESICLE CYCLE**

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Cover Drosophila neuromuscular junctions stained with antibody against Fasciclin II (Fas II) and the presynaptic dense projection reconstructed in 3D using electron tomography.

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青春的美丽与珍贵，就在于它的无邪与无瑕，在于它的可遇而不可求，在于它的永不重回。

——席慕容

ABSTRACT

Fast synaptic transmission occurs at specialized junctions between neurons referred to as chemical synapses. Action potentials induce an influx of calcium ions into presynaptic terminals, which contain neurotransmitter-filled synaptic vesicles (SVs), triggering fusion of the vesicles with the plasma membrane and resulting in the release of neurotransmitters. After fusion SVs have to be recycled and refilled to maintain neurotransmission for a certain period of time.

Clathrin-mediated endocytosis serves as a major mechanism for synaptic vesicle recycling. It occurs at the periaxial zone and relies on a set of proteins such as clathrin and clathrin adaptors, which are essential for clathrin coat assembly, and the GTPase dynamin, which is required for budding of the newly formed vesicles from the plasma membrane. Multiple accessory and scaffolding proteins coordinate the assembly of the clathrin vesicles. In this thesis, the functional role of the scaffolding proteins Dap160 and Eps15 in the synaptic vesicle cycle was investigated. The genetically tractable *Drosophila* neuromuscular junction (NMJ) was used as an experimental model.

Several new methodological approaches, such as high pressure freezing, freeze substitution, and a correlative immunogold technique were developed or adapted in this work to study the *Drosophila* synapse. These approaches allowed for the characterization of the structure of the synapse and the organization of vesicles, and for the first time provided 3-dimensional reconstruction of the presynaptic specialization. The subcellular localization of Dap160 and Eps15 was determined. Biochemical experiments revealed that they form a molecular complex. Structural and functional analysis of *Drosophila* *dap160* and *eps15* mutants showed that these proteins have a dynamic localization in the nerve terminal: both molecules reside in distal pool of SVs at rest and relocate to the periaxial zone during synaptic activity. *dap160* and *eps15* single and double mutants display defects in synaptic vesicle recycling. Physiological experiments show that both proteins are required to maintain synaptic transmission at high activity rates. Genetic disruption of the interaction between the Dap160-Eps15 complex and the GTPase dynamin results in abnormal distribution of dynamin immunoreactivity at the periaxial zone during stimulation. We conclude that the Dap160-Eps15 molecular complex is essential to concentrate dynamin at the periaxial zone during synaptic activity.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text according to their Roman numerals:

- I. Koh, T. W., Korolchuk, V. I., Wairkar, Y. P., **Jiao, W.**, Evergren, E., Pan, H., Zhou, Y., Venken, K. J., Shupliakov, O., Robinson, I. M., O'Kane, C. J., and Bellen, H. J. (2007) Eps15 and Dap160 control synaptic vesicle membrane retrieval and synapse development, *J Cell Biol* 178, 309-322.
- II. **Jiao, W.**, Shupliakov, A., and Shupliakov, O. (2010) A semi-correlative technique for the subcellular localization of proteins in *Drosophila* synapses, *J Neurosci Methods* 185, 273-279.
- III. **Jiao, W.**, Masich, S., Franzen, O., and Shupliakov, O. (2010) Two pools of vesicles associated with the presynaptic cytosolic projection in *Drosophila* neuromuscular junctions, *J Struct Biol* 172, 389-394.
- IV. **Jiao, W.**, Winther. Å., Koh, T. W., Vorotsova, O., Bellen, H. J., Shupliakov, O. (2011) Dynamin targeting to the periactive zone in *Drosophila* synapses is mediated by Dap160, *Resubmitted to J Cell Biol*.

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LIST OF ABBREVIATIONS

AP2	Adaptor protein 2
AP-180	Adaptor protein 180kDa
AZ	Active zone
BAR	BIN-amphiphysin-RVS
BRP	Bruchpilot
CAZ	Cytomatrix of the active zone
CME	Clathrin-mediated endocytosis
CNS	Central nervous system
CSP	Cystein string protein
Dap160	Dynamin-associated protein 160kD
EH	Eps15 homology
EJP	Excitatory junctional potential
Eps15	Epidermal growth factor receptor pathway substrate clone 15
Fas II	Fasciclin II
FM1-43	N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide
GABA	γ -Aminobutyric acid
GED	GTPase effector domain
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
GTP	Guanosine-5'-triphosphate
HPF	High pressure freezing
LM	Light microscopy
NMJ	Neuromuscular junction
NT	Neurotransmitter
PDP	Presynaptic dense projection
PRD	Proline-rich domain
PSD	Postsynaptic density
RP	Reserve pool
RRP	Readily releasable pool
SH3	Scr homology 3
STED	Stimulated emission depletion

SV	Synaptic vesicle
TEM	Transmission electron microscopy
3D	3-dimensional

1 INTRODUCTION

Synapses are specialized sites for signal transduction between neurons and their targeted cells. They consist of presynaptic nerve terminals and postsynaptic compartments, separated by an extracellular space: the synaptic cleft. Fast neurotransmitters (NTs), such as glutamate, GABA, or glycine are stored in synaptic vesicles (SVs), which accumulate in presynaptic terminals in clusters. These vesicles are released into the synaptic cleft during synaptic activity, and the neurotransmitter receptors on the postsynaptic membrane become activated. Upon arrival of action potentials to the nerve terminal, Ca^{2+} ions enter through voltage-gated Ca^{2+} channels on the presynaptic membrane and trigger exocytosis. SVs dock and fuse, release NTs, and are rapidly recycled through endocytosis (Betz and Angleson 1998). The release is restricted to a specialized area of presynaptic membrane, called the active zone (AZ) (Couteaux and Pecot-Dechavassine 1970). The AZ is precisely aligned with the postsynaptic neurotransmitter reception apparatus, which referred to as the postsynaptic density (PSD) (Landis, Hall et al. 1988).

1.1 The synaptic active zone

Electron microscopy identifies the AZ as an electron dense structure, consisting of an electron dense membrane and an associated cytomatrix, often recognized as projections extending into the cytoplasm (Zhai and Bellen 2004). In different synapses, the projections may appear as different shapes. In the mammalian central nervous system (CNS), the cytomatrix of the active zone (CAZ) was shown as a web-like pattern, consisting of 50 nm pyramid shaped particles and interconnected by thin fibrils (Bloom and Aghajanian 1968; Landis, Hall et al. 1988; Phillips, Huang et al. 2001). However, a different structure has been revealed in the frog neuromuscular junction (NMJ) (Harlow, Ress et al. 2001). There, the CAZ is organized in an array-like structure. SVs are connected with ‘ribs,’ extending from the ‘beams,’ which run along the midline of the presynaptic ridge. ‘Pegs’ represent putative Ca^{2+} channels and connect directly to the ‘ribs.’ Functionally, the projections are thought to tether SVs and guide them to the presynaptic membrane (Brodin, Low et al. 1997; Muresan, Lyass et al. 1999; Harlow, Ress et al. 2001).

1.2 Synaptic Vesicle pools

In chemical synapses, all SVs look morphologically alike under the electron microscope, although some are closely apposed to the active zone, and others are further away. In 1961, Briks and MacIntosh, who investigated cat sympathetic ganglia, first suggested the existence of two distinct presynaptic vesicle pools: a 'readily releasable' fraction, which is depleted during low frequency stimulation, and a 'non-readily releasable' fraction. In the following years, a number of techniques including FM dye application, total internal reflection fluorescence microscopy, flash photolysis, and electrophysiological recordings, have been employed to determine functional properties of SV pools (Cochilla, Angleson et al. 1999; Schneggenburger, Meyer et al. 1999; von Gersdorff and Matthews 1999; Steyer and Almers 2001). These studies have confirmed the existence of the two SV pools in the *Drosophila* NMJ: the exo/endo cycling pool, which responds to mild stimulation (10Hz) or high K^+ , and the reserve pool (RP), which cycles only under intense stimulation (30Hz) (Kuromi and Kidokoro 2003). Research done in frog NMJs has led to a similar conclusion (Rizzoli, Richards et al. 2003). Recently a further distinction was made and a SV pool that releases more slowly than the readily releasable pool (RRP) but prior to the RP was identified (Rizzoli and Betz 2005). As a result, the concept of three SV pool has emerged. These three pools include: 1) the RRP, consisting of a few vesicles (~1%) docked and primed for release, 2) the recycling pool, containing about 5-20% of all vesicles and maintaining release during moderate stimulation, and 3) the RP, which includes the majority of vesicles (~80-90%) and becomes activated only upon intense stimulation.

1.3 Synaptic vesicle recycling

SVs fuse with the presynaptic membrane during neurotransmitter release. In order to sustain synaptic transmission, the incorporated membrane needs to be retrieved rapidly to replenish the vesicle pools and restore the functionality of the presynapse. Several different mechanisms of membrane retrieval have been proposed: clathrin-mediated endocytosis (CME), kiss-and-run, and bulk endocytosis (Royle and Lagnado 2003). Among these processes, CME is the best characterized. It was first described by Heuser and Reese in the frog NMJ in 1973 (Heuser and Reese 1973). They proposed that SVs are retrieved from the presynaptic membrane as clathrin-coated pits, which become uncoated and fuse with an additional cellular recycling compartment, the endosome. An additional budding event leads to formation of a new SV. This set of events occurs in a minute scale at the lateral area of the active zone, which is referred to as the periaction zone (Heuser and Reese 1973; Sudhof 1995; Richards, Guatimosim et al. 2000). An

alternative hypothesis has been proposed by Ceccarelli and coworkers (Ceccarelli, Hurlbut et al. 1973). According to their model, SVs release neurotransmitters through an open fusion pore that closes before fully collapsing into the presynaptic membrane. This process is presumably fast and has time constant ranging from a few hundred milliseconds to several seconds (Klingauf, Kavalali et al. 1998; Pyle, Kavalali et al. 2000; Sara, Mozhayeva et al. 2002). Existence of this recycling pathway in synapses is still a matter of debate. Bulk endocytosis may also occur in synapses. Contrary to CME, this involves budding of large membrane invaginations or cisternae. It is believed that newly formed SVs pinch from these endosomal structures. The recycling of SVs through this pathway is presumably a slow process (Richards, Guatimosim et al. 2000).

1.31 Clathrin-mediated endocytosis

CME occurs both in neuronal and non-neuronal cells. It is important for many cell functions, including the uptake of nutrients, the internalization of receptors from the plasma membrane, as a defence against microorganisms, or synaptic vesicle recycling in nerve terminals (Slepnev and De Camilli 2000). Clathrin-mediated endocytosis is highly regulated and can be separated into several morphologically distinct steps: docking of clathrin molecules to the plasma membrane, invagination, formation of a deeply invaginated pit with a neck, fission, release of a free clathrin-coated vesicle and uncoating. These steps requires coordination of a number of proteins that sort the cargo, assemble clathrin, bend the presynaptic membrane, and constrict and cleave the invaginated pit (Robinson 1994; Schmid 1997; Slepnev and De Camilli 2000).

1.32 Clathrin and clathrin adaptors

Clathrin is the key building block of the clathrin coat. It consists of a heavy chain and a light chain, and three of each can form the triskelion, which can be assembled into polyhedral lattices or cages (Smith and Pearse 1999). The recruitment of clathrin requires the adaptor protein, AP2, which links clathrin to the presynaptic membrane (Hirst and Robinson 1998). AP2 is a heterotetrameric complex composed of four subunits: α , β 2, μ 2 and σ 2 adaptin. The C terminals of α and β 2 adaptin form two ear domains, and bind various accessory proteins, which coordinate endocytosis (Traub, Downs et al. 1999). AP2 also interacts with the integral membrane protein synaptotagmin and membrane lipids, and such interactions are required for recruiting AP2 to the fused SV membrane (Zhang, Davletov et al. 1994; Gaidarov and Keen

1999). AP-180 binds to both clathrin and AP2, and is proposed to regulate the size of newly formed vesicles (Ye and Lafer 1995; Hao, Luo et al. 1999).

1.33 Dynamin and scaffolding proteins

Fission of clathrin-coated pits releases vesicles from the membrane into the terminal, and dynamin has been shown to play a key role at this step (Schmid, McNiven et al. 1998; McNiven, Cao et al. 2000). The GTPase dynamin, together with BIN-amphiphysin-RVS (BAR) -domain proteins and possibly actin, form a helical structure around the neck of the invaginated pit. It hydrolyzes GTP and then undergoes a conformational change leading to fission of constricted coated pits from the presynaptic membrane (Hinshaw and Schmid 1995; Takei, McPherson et al. 1995; Sweitzer and Hinshaw 1998; Sundborger, Soderblom et al. 2011). Apart from the N-terminal GTPase domain, which binds and hydrolyzes GTP (Sweitzer and Hinshaw 1998), dynamin contains several other domains: the middle domain together with the GTPase effector domain (GED) forms a mechanochemical core and mediates self-assembly of dynamin (Okamoto, Tripet et al. 1999; Smirnova, Shurland et al. 1999; Mears, Ray et al. 2007); the PH domain interacts directly with the membrane bilayer (Salim, Bottomley et al. 1996); and the C-terminal proline-rich domain (PRD) binds to the Src homology 3 (SH3) domain of various accessory/scaffolding proteins including amphiphysin, endophilin, and intersectin (Wigge, Vallis et al. 1997; Simpson, Hussain et al. 1999). Amphiphysin and endophilin both contain BAR domains, which dimerize into banana-shaped modules and mediate membrane bending (Peter, Kent et al. 2004; Masuda, Takeda et al. 2006). Amphiphysin is proposed to target dynamin to coated pits through the SH3 domain (Shupliakov, Low et al. 1997) in vertebrate synapses. It also binds clathrin heavy chain and α -adaptin through its central region, promoting clathrin polymerization (Farsad, Slepnev et al. 2003; Miele, Watson et al. 2004). Endophilin is believed to regulate membrane curvature at the early steps of vesicle budding (Schmidt, Wolde et al. 1999; Sundborger, Soderblom et al. 2011). A recent study has shown that endophilin localizes to the rim of the clathrin coat and a restricted part of the neck of the pits, and aids in recruiting dynamin into a tight complex, thus promoting fission (Sundborger, Soderblom et al. 2011). Intersectin (Dap160 in *Drosophila*) is a large multidomain protein, which is composed of two Eps15 homology (EH) domains, a central coiled-coil domain, and five SH3 domains (four in *Drosophila*), through which it interacts with several endocytic proteins, including dynamin, Eps15, epsin, and Stoned B (Roos and Kelly 1998; Yamabhai, Hoffman et al. 1998; Martina,

Bonangelino et al. 2001). Studies performed in *Drosophila* mutants proposed that intersectin/Dap160 might function as a molecular scaffold for the endocytic machinery at the periactive zone (Roos and Kelly 1998; Koh, Verstreken et al. 2004). A model has been proposed, which suggested that intersectin/Dap160 is required for positioning and coordination of dynamin and for the actin matrix formation at the endocytic zone (Roos and Kelly, 1998; Broadie 2004). Another endocytic scaffold, epidermal growth factor receptor substrate 15 (Eps15), has also been shown to bind multiple endocytic proteins, such as intersectin/Dap160, AP2 and Stoned B (Benmerah, Gagnon et al. 1995; Marie, Sweeney et al. 2004; Majumdar, Ramagiri et al. 2006). Mutant studies in *Drosophila* and mammalian cells indicate its role in neurotransmission and CME (Benmerah, Bayrou et al. 1999; Majumdar, Ramagiri et al. 2006).

1.4 Model system-*Drosophila* neuromuscular junction

The *Drosophila* NMJ serves as one of the key model systems utilized to study synaptic transmission, and mostly the larval NMJ is used. Body wall muscles in *Drosophila* larvae can be easily identified. The majority of the muscle cells lining the body wall are innervated by type-I motoneuron nerve terminals, which can be subdivided into type-Ib (big) and type-Is (small) terminals (Johansen, Halpern et al. 1989; Gramates and Budnik 1999). Type-I terminals are glutamatergic, embedded in the muscle and surrounded by muscle membrane. These membranes, also referred to as the subsynaptic reticulum, are extensively folded. This organization is suggested to be favourable for glutamate diffusion (Johansen, Halpern et al. 1989; Atwood, Govind et al. 1993). In addition, another type of nerve endings containing proctolin or octopamine modulates the glutamatergic response (Anderson, Halpern et al. 1988; Monastirioti, Gorczyca et al. 1995). A variety of experiments, such as electrophysiology, FM dye uptake, and behaviour assays can be easily performed in *Drosophila* NMJs (Zhang 2003).

The most attractive and powerful feature of the *Drosophila* NMJ as a biological model system is the possibility for broad genetic manipulations. Techniques for deleting, mutating, or overexpressing genes of interest are available. The UAS-GAL4 system makes it possible to conditionally express a transgene or a modified transgene in specific tissues (Brand and Perrimon 1993; Parks, Cook et al. 2004). A variety of mutant flies with defects in synaptic function are currently available, including the *shibire^{ts1}* mutant, in which dynamin is not able to hydrolyze GTP at non-permissive temperatures, resulting in block of SV recycling (Ikeda, Ozawa et al. 1976; Koenig and Ikeda 1989; van der Bliek and Meyerowitz 1991; Ramaswami, Krishnan et al. 1994;

Grant, Unadkat et al. 1998); the α -adaptin mutant *d-ada*, which is deficient of the α -subunit of AP2 and completely devoid of SVs (Gonzalez-Gaitan and Jackle 1997); and the endophilin mutant *endo*, in which a severe loss of SVs is observed, which is correlated with depletion of excitatory junctional potentials (EJPs) during repetitive stimulation (Guichet, Wucherpfennig et al. 2002; Verstreken, Kjaerulff et al. 2002).

2 AIMS

The major goal of this thesis is to elucidate the functional role of the scaffolding proteins Dap160 and Eps15 in the synaptic vesicle cycle in *Drosophila* NMJs.

Specific goals:

- To develop approaches to localize synaptic proteins at subcellular resolution in *Drosophila* NMJs.
- To investigate the subcellular organization of *Drosophila* NMJs and determine the ultrastructural localization of the scaffolding proteins Dap160 and Eps15 at the synapse.
- To explore possible interactions between Dap160 and Eps15, and determine the role of these proteins in sustaining neurotransmitter release at NMJs.
- To elucidate the role of the scaffolding proteins Dap160 and Eps15 in dynamin targeting to the sites of endocytosis during synaptic vesicle recycling.

3 METHODS

3.1 Generation of transgenic flies

Drosophila melanogaster is a useful and attractive model organism for genetic studies, because it has a short life cycle and because it can be genetically manipulated much more easily than other organisms (Venken and Bellen 2005). The following strains were used in our experiments: w^{1118} control line (referred to as *wt*), *shibire^{ts1}* (*shi^{ts1}*), a temperature sensitive dynamin mutant, and *dap160* null mutant, *dap160^{Δ1}/Df(2L)bur-K1* (obtained from the cross: $w; dap160^{\Delta1}/CyO, KrGFP$ x $w; Df(2L)bur-K1/CyO, KrGFP$, as described earlier (Koh, Verstreken et al. 2004)).

In order to determine the function of Eps15, *eps15* null mutants were generated (Paper I). Transposable element insertion line EP2513(Rorth 1996) was used for P element excisions (Paper I, Figure 1B) and an imprecise deletion allele *eps15^{e75}* was achieved by crossing it with y^1w^* ; $CyO, H\{w^{+mC}=P\Delta2-3\}HoP2.1/Bc^1Egfr^{E1}$ (Gerlitz, Nellen et al. 2002). For FRT-mediated site-specific deletion, $P\{XP=FRT\}d00445$ and $PBac\{WH=FRT\}f02085$ were used and two FRT-bearing transposon insertions were placed in *trans* in the presence of heat shock-driven FLP recombinase (*hs-FLP*) (Paper I, Figure 1B). Allele *eps15^{Δ29}* was then achieved (Parks, Cook et al. 2004). *eps15^{e75}/eps15^{Δ29}*, *eps15^{Δ29}/eps15^{Δ29}*, *eps15^{e75}/Df(2R)Dll-MP*, and *eps15^{Δ29}/Df(2R)Dll-MP* were tested and double mutants *dap160^{Δ1} eps15^{e75}/Df(2L)bur-K1 eps15^{Δ29}* were generated to elucidate the functional relationship between Eps15 and Dap160.

In order to study the function of different SH3 domains of Dap160, transgenic flies with a specific SH3 domain deletion were required (paper IV). Genomic DNA of *dap160* without SH3B or SH3AB domain was amplified by PCR and cloned into P-element plasmids, downstream of the UAS sequence (which responds to GAL4 transcriptional activation). Both constructs were introduced into w^{1118} by P-element mediated transformation (Rubin and Spradling 1982). The pan-neural driver line, $elav^{C155-Gal4}; Df(2L)bur-K1/CyO, twi-GFP$ was used here to activate the expression of Dap160 with SH3B or SH3AB deletion in neurons. Thus, the following genotypes were used in our studies: $elav^{C155-Gal4}/UAS-dap160^{\Delta SH3B}$; *dap160^{Δ1}/Df(2L)bur-K1* (from cross: $elav^{C155-Gal4}; Df(2L)bur-K1/CyO, twi-GFP$ x $UAS-dap160^{\Delta SH3B}; dap160^{\Delta1}/CyO, twi-GFP$) and $elav^{C155-Gal4}/w; dap160^{\Delta1}/Df(2L)bur-K1; UAS-dap160^{\Delta SH3AB}/+$ (from cross: $elav^{C155-Gal4}; Df(2L)bur-K1/CyO, twi-GFP$ x $w; dap160^{\Delta1}/CyO, twi-GFP; UAS-dap160^{\Delta SH3AB}$).

Flies were maintained at room temperature on normal food. *dap160* mutant larvae were separated from the larvae bearing balancer chromosomes by the absence of GFP fluorescence and cultured on grape juice agar with yeast paste at 25°C to prevent overcrowding and competition from the larvae without mutations (Loewen, Mackler et al. 2001).

3.2 Dissection procedures

Third instar *Drosophila* larvae were used in our experiments. The larvae were briefly washed with HL3 buffer, and transferred onto Sylgard lined plates. The head and the tail were pinned to Sylgard with the dorsal side up. To access NMJs in larvae, the whole larvae were opened up. The gut, fat bodies and salivary glands were removed, and tissue debris was washed away with HL3 buffer. The body wall, brain, and connecting nerves were kept as a fillet.

3.3 Immunohistochemistry

Using immunohistochemistry we were able to visualize the morphology of NMJs with synaptic markers and detect the localization of proteins of interest. Fillets from third instar larvae were fixed in 4% paraformaldehyde, washed with PBS and permeabilized with 0.4% Triton X-100, and then incubated with primary antibodies and secondary antibodies conjugated to Alexa 488 or 555 (Invitrogen), sequentially. Fillets were mounted in Vectashield mounting medium (Vector Laboratories) and studied under a Zeiss 510 confocal microscope.

The following antibodies were used to determine the localization of synaptic proteins in NMJs: NC82, anti-Bruchpilot mouse monoclonal antibody served as the T-bar marker; anti-CSP mouse monoclonal antibody and anti-synaptotagmin rabbit antibodies were used to label SVs, anti-Fas II mouse antibody labeled boundaries of the active zone at rest, and anti-Dlg rabbit antibodies stained the postsynaptic compartment in NMJs.

The resolution of the conventional light microscopy is limited to 200 nm. In order to improve the resolution in our immunofluorescence studies of single synaptic boutons, we utilized a super-resolution optical approach, stimulated emission depletion (STED) microscopy. In the experiments described in Paper IV, the resolution about 90-100 nm was achieved. Z-stack serial images were also collected to obtain the 3D-information on the spatial organization of release sites (Papers II and IV).

3.4 Pre-embedding immunocytochemistry

Even though the resolution of the STED microscope is improved to the subdiffractional level, the electron microscope still provides a higher resolution. Since we aimed at relating the distribution of proteins of interest to subcellular structures, an immunogold technique was employed. We designed a semi-correlative technique that allowed us to localize proteins at the ultrastructural level in *Drosophila* synapses (Papers I, II and IV). Prior to immunogold staining, the muscle containing NMJs was cut on a Vibratome. The presynaptic boutons were cut opened, making them better accessible to antibodies. Fluoronanogold conjugated secondary antibodies (Nanoprobe) were used. Serial confocal images and/or 3-D confocal reconstructions were collected and analyzed to select best-stained areas for further EM analysis. Selected samples were post-fixed and silver enhanced. The samples were then dehydrated and embedded in Durcupan ACM resin (Fluka) for EM analysis.

3.5 TEM and 3D reconstruction of TEM images

In order to examine samples with transmission electron microscopy (TEM), ultrathin sections were prepared. Embedded samples were trimmed and sectioned with a diamond knife (Diatome). Ultrathin sections (50 nm) were collected onto Formvar-coated copper grids. Uranyl acetate and lead citrate were used for counterstaining to increase contrast. The sections were examined and photographed in a Tecnai 12 electron microscope (FEI).

After collecting images from serial ultrathin sections, we reconstructed positions of gold particles in 3D (Papers II and IV). Serial ultrathin sections were photographed using a bottom mounted 2k x 2k TemCam F224 CCD Camera (TVIPS) and images were aligned with Adobe Photoshop Software (CS4). Membrane contours were traced using a digitizer and transferred into Maya 8.0® 3D-reconstruction program and surface rendered. Spheres in the 3D-reconstructions represent weights of the silver enhanced particles.

3.6 High pressure freezing and freeze substitution

TEM is a common tool to visualize the subcellular organization of tissues. Conventional methods of sample preparation often involve the application of chemical fixation and dehydration, which both introduce artifacts into the specimen. In order to characterize the near-native structure and subcellular architecture of the presynaptic

bouton in *Drosophila* NMJs, high pressure freezing (HPF) and freeze substitution protocols were adapted for our *Drosophila* studies (Paper III).

HPF is able to cryo-immobilize biological samples as thick as 200 μm . Beyond this, ice crystals form and the ultrastructure is destroyed. Since the thickness of the *Drosophila* body wall fillet is around 200 μm , it is advantageous to use HPF for this preparation. During freeze substitution the frozen water was replaced with acetone at -90°C , and the samples were then embedded into Durcupan ACM resin. Since samples were cryo-fixed at low temperature prior to the embedding, no evident distortion of the native organization of NMJs occurred.

3.7 Electron tomography

Electron tomography is a powerful method for obtaining 3-D structural information from 2-D images of cellular organelles. We applied electron tomography in our studies to elucidate the 3-D architecture of *Drosophila* PDPs (Paper III). After HPF and freeze substitution, samples were cut into 200 nm or 50 nm thick sections, and prepared for tomography. Sections were tilted in the electron microscope from -65° to $+65^{\circ}$ at 1° increments. In order to reduce reconstruction artifacts, another tilt series on the same region in the direction perpendicular to the first tilt were collected. After alignment of all tilt series, image intensity values were converted to electron density and tomographic reconstructions were produced by the weighted backprojection approach (Skoglund, Öfverstedt et al. 1998). Amira 4 software (Mercury Computer Systems Inc.) was used for rendering and segmentation. With electron tomography, we were able to slice the sample 100 times finer than the original section thickness and characterize structures that were previously impossible to resolve using conventional EM (McDonald and Auer 2006).

3.8 Biochemistry

Different *dap160* mutations and *eps15* null mutations were verified by absence of Dap160 or Eps15, or by the presence of different Dap160 deletions on Western blots of head extracts from different mutants (Papers I and IV). His-tagged and GST fusion proteins were expressed in BL21 cells and purified from soluble fraction using Ni-NTA and glutathione beads, respectively. To investigate protein-protein interactions, pull-down assays and immunoprecipitation experiments were performed (Paper I).

3.9 Electrophysiology

An essential objective in our studies was to examine the functional transmission in our mutants in order to define the role of proteins of interest in the SV cycle. Electrophysiological recordings were performed (Paper I). Third instar larvae were dissected and recordings were performed in HL3 saline with variable Ca^{2+} concentrations. Current clamp recordings were performed from muscle fibers with resting membrane potentials ranging from -65 mV to -75 mV. NMJs were activated with single action potentials and at 10-Hz. Motoneuron axons were stimulated extracellularly through a suction electrode.

3.10 Behavioural tests

A locomotion assay (Yang, Shaver et al. 2000) was used (Paper IV) to reveal defects in behaviour in different *dap 160* mutants. 24-27 third instar larvae for each genotype were picked up and tested. Distance travelled by each larva during a three minute period was measured by placing a Petri dish over a grid of 25 mm² squares and scoring the number of grid squares entered. Experiments were performed at 25°C and 34°C. At 34°C, a long protocol with a testing time of 13 minutes was also used.

4 ULTRASTRUCTURAL ORGANIZATION OF THE ACTIVE ZONE IN THE *DROSOPHILA* NEUROMUSCULAR JUNCTION

NMJs in *Drosophila* contain a characteristic presynaptic structure, also referred to as T-bar. Earlier electron microscopic studies have proposed that it is composed of two structural blocks, a “pedestal” attached to the presynaptic membrane and a wide “platform” residing on the pedestal (Feeney, Karunanithi et al. 1998; Zhai and Bellen 2004). SVs are present around this structure and are attached to it (Figure 1A). Our TEM experiments revealed a more complex structure of this presynaptic specialization (Figure 1B and Paper III, Supplemental Figure 1A and B), indicating that the T-shape does not represent the native structure of this specialization, and chemical fixation and dehydration applied during EM preparation procedures cause a distortion of its morphology.

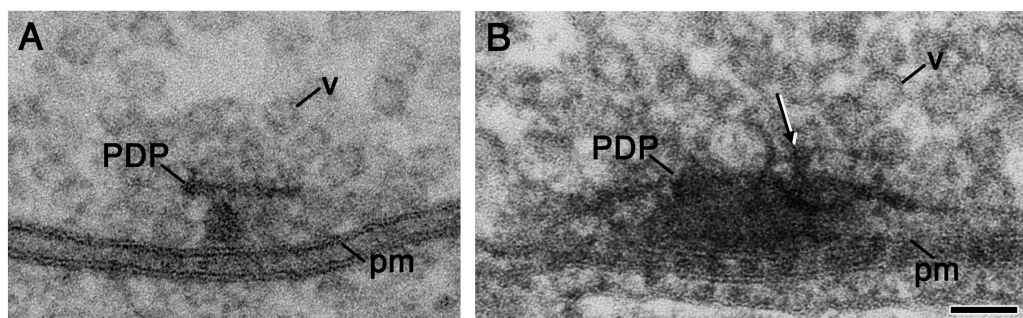


Figure 1. Electron micrographs of *Drosophila* presynaptic dense projections (PDPs) embedded for conventional EM. (A) A PDP has a characteristic “T-bar” shape, composed of a pedestal and a platform. (B) An image of a PDP, which displays complex extensions (arrow) emerging from the T-bar platform. v: synaptic vesicles; pm: plasma membrane. Scale bars: 50 nm.

To preserve the native structure of the NMJ, we froze *Drosophila* third instar larvae with a high pressure freezer EMPACT 2, and a freeze substitution protocol was used to substitute water in the tissue. Structures no longer resembling the T-bar were revealed. Our images display a dense central core with extensions of variable shape and length stretching into the vesicle pool (Figure 2 and Paper III, Supplemental Figure 1C-F).

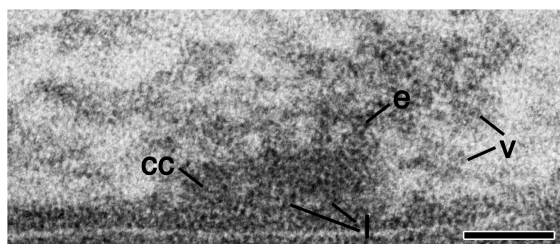


Figure 2. TEM image of a *Drosophila* PDP after HPF and freeze substitution. Note regular extensions (e) emerging from the central core (cc) of the presynaptic density, and legs (l) linking the central core to the plasma membrane. v: synaptic vesicles; Scale bars: 50 nm.

Since the presynaptic dense projection is a relatively large structure, 200 nm thick sections were cut to capture most of it in the middle. Tomographic reconstructions of dense projections revealed a number of new details: an irregular central dense mass-“central core,” a grid of connections linked the central core to the presynaptic membrane-“legs,” and dense clouds connecting to the upper lateral part of the central core-“cytoplasmic extensions” (Figure 3A and Paper III, Figure 1B-D). Double-tilt electron tomography of 50 nm-thick sections was performed to resolve the fine morphology of the extensions, which were found to be composed of filaments and to form bundles that are seen as electron-dense clouds in thick sections (Figure 3B and Paper III, Figure 2B-D).

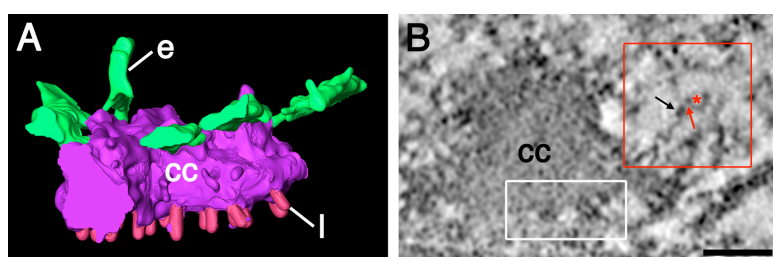


Figure 3. (A) 3D architecture of the PDP in *Drosophila* NMJs, illustrating three major blocks composing the dense projection: “central core” (cc; magenta), “extensions” (e; green) and “legs” (l; red). (B) Image of a digital section (7.8 Å) from the tomographic reconstruction of the 50 nm thick section. Black arrow points to a filament in the red rectangle. Legs are shown in white rectangles. Red star marks the vesicle connected to the filament by a small connector (red arrow). Scale bars: 50 nm.

SVs accumulate around the dense projection. 3D reconstructions of digital sections revealed that SVs positioned close to the projection are linked to the filaments/extensions by thin connections (Figure 3B and Paper III, Figure 2G-H, Supplemental Figure 1G-I). Most of these vesicles are connected to other vesicles in the vesicle pool (Figure 4A and Paper III, Figure 3), while a small group is linked to the filaments extending from the plasma membrane of the active zone, referred to as tethers assembled as triads (Figure 4B and Paper III, Figure 4A).

Previous studies suggested that Ca^{2+} channels cluster at the active zone between the presynaptic membrane and CAZ (Fouquet, Oswald et al. 2009), thus the legs revealed in our studies most probably correspond to the protein complexes that include the Ca^{2+} channel. Depolarization causes a Ca^{2+} influx through the voltage-gated Ca^{2+} channels, resulting in a local elevation of intracellular Ca^{2+} concentration in a limited range (~tens of nanometers) around the channels, which triggers exocytosis (Heidelberger, Heinemann et al. 1994; von Gersdorff and Matthews 1994). The tethered vesicles reside within 50 nm from the putative Ca^{2+} channels, indicating that

they may undergo fusion after single action potential (Paper III, Figure 4B and C). Other vesicles connected to the dense projection possibly lose their connections and translocate along the filaments to the plasma membrane during stimulation (Paper III, Figure 4D). It leads to the suggestion that the tethered vesicles are immediately available for release. The other groups of vesicles, which are not connected to the projection, presumably have slower release kinetics and might represent a different functional vesicle pool (Rizzoli and Betz 2005; Hallermann, Kittel et al. 2010).

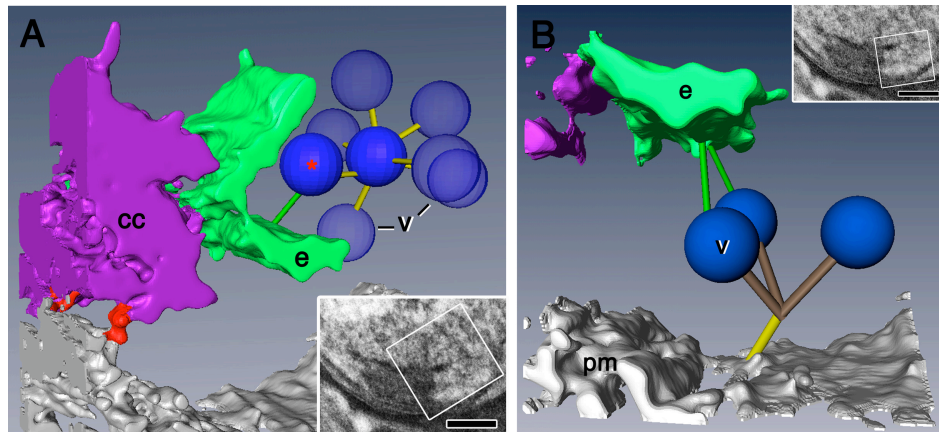


Figure 4. (A) 3D reconstruction of an area indicated by white rectangle in the EM image of a 200 nm thick section through a PDP (inset) from a sample embedded after high pressure freezing. Only one of the vesicles attached to the cytoplasmic extension and connected to other vesicles in the cluster is reconstructed. The reconstruction shows the relationship between the synaptic vesicle (v) associated with the cytosolic extension (e) and neighboring vesicles in the cluster (v). Connections between vesicles in the cluster are depicted by yellow bars. (B) 3D organization of vesicles (v) tethered to the presynaptic membrane, in relation to extensions (e). Reconstruction of an area marked with white rectangle in the inset, showing the TEM image of a 200 nm thick section through the whole PDP from a sample embedded after high pressure freezing. Connections (brown cylinders) link three vesicles to a density (yellow) extending from the plasma membrane.

The active zone is often defined by the electron dense membrane and the attached dense projection that extends into the cytoplasm (Zhai and Bellen 2004). Bruchpilot protein (BRP) is often used as a marker for active zones in *Drosophila* NMJs in immunocytochemical studies. *Drosophila* BRP has been shown to be involved in active zone assembly and is important for Ca^{2+} channel clustering. Genetic elimination of BRP results in the loss of presynaptic densities (Kittel, Wichmann et al. 2006; Wagh, Rasse et al. 2006). However, there is no correlation between the size of the dense projection and the size of the electron dense membranes in *Drosophila* NMJs. Some active zones only contain the dense membranes without the projections, which makes BRP hardly a precise component of the active zone. Fas II has been shown to accumulate at periaxial zone in adult NMJs (Marie, Sweeney et al. 2004; Ashley, Packard et al. 2005). In order to test if this protein outlines the active zones and can be

used as a reliable active zone marker, a semi-correlative immunogold technique was applied to visualize Fas II-immunolabeling at the EM level. We found that gold particles signalling for Fas II indeed associates with neuronal membranes at NMJs (Paper II). Gold particles were found both at the pre- and post- synaptic sides with equal distance to the membrane surface, corroborating with the suggestion that it links the pre- and post- elements in a symmetric fashion (Figure 5A-B and Paper II, Figure 2B and 3) (Kristiansen and Hortsch 2008; Mao and Freeman 2009). 3D reconstruction of Fas II labeling pattern shows that it indeed delineates the active zone area (Figure 5C and Paper II Figure 3E-G). Thus, Fas II can be used as a reliable marker for the active zone size under resting conditions.

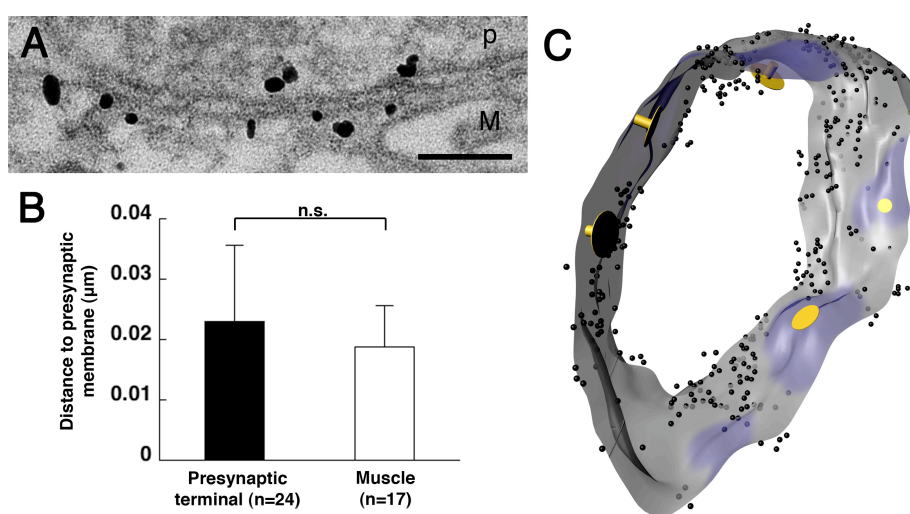


Figure 5. (A) High magnification EM image showing Fas II immunogold labeling from the area where pre- and post-synaptic membranes were cut transversally. Note that gold particles are symmetrically accumulated at the membranes in the cytoplasm of the presynaptic terminal (p) and the muscle (M). (B) Bar graph showing quantification of distances of gold particle from the membranes from seven areas. (C) 3D reconstruction of the Fas II labeling pattern in eight 50 nm serial sections of a neuromuscular junction. The presynaptic membrane is depicted in grey and is half transparent. Blue areas demark active zones. Yellow structures show positions of PDPs. Black spheres indicate position of geometrical centers of gold particles signaling for Fas II at the pre- and post- synaptic membrane. The muscle is not depicted. The spheres are grey when they are viewed through the membrane. Note that Fas II labeling delineates borders of active zones. Scale bar: 100nm.

5 RECRUITMENT OF SCAFFOLDING PROTEINS DAP160 AND EPS15 FROM THE SV CLUSTER TO THE PERIACTIVE ZONE DURING SYNAPTIC ACTIVITY

Previous experiments have shown that Dap160 is present at “hot spots” of endocytosis and co-localizes with dynamin. It has also been found that it directly binds dynamin and in addition to an array of other effector molecules, such as synaptojanin, and synapsin. This has led to the suggestion that Dap160 is a scaffolding protein that clusters effector molecules that are required for endocytosis at the periaxial zone (Roos and Kelly 1998; Brodie 2004). Interestingly, at the resolution of a light microscope Dap160 immunolabeling does not overlap with Fas II, which was found at the periaxial zone (Paper II, Figure 2A) (Marie, Sweeney et al. 2004). This strongly indicates that Dap160 is localized in a different compartment. To test this, immunogold labeling for Dap160 was performed and the protein was found in the vesicle pool and not at the periaxial zone at rest (Figure 6, Paper II, Figure 2C and Paper IV, Figure 1D and 2C). Its binding partners, Eps15 and dynamin, which co-localize with Dap160 at the LM level (Paper IV, Supplemental Figure 1C), were also found in the vesicle pool at rest (Paper I, Figure 2G and H; Paper IV, Figure 1G and H).

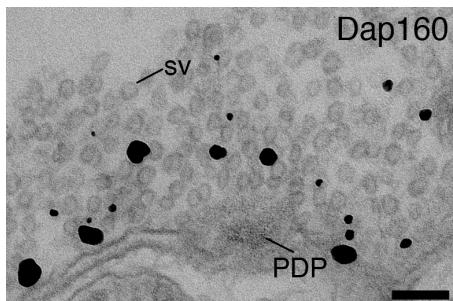


Figure 6. Dap160 localization in an NMJ of the third instar larva. Scale bar: 100nm

Careful analysis of labeling patterns for Dap160, Eps15 and dynamin revealed that a small group of vesicles close to the dense projection is not labeled, while antibody against the vesicle associated protein, cysteine string protein (CSP), stains the whole vesicle pool (Figure 7A, Paper II, Figure 1D and Paper IV, Supplemental Figure 2D-E). We also labeled the NMJs with NC82 antibody against the C-terminal of BRP, and found that the extensions coming from the dense projection are labeled (Figure 7B, Paper II, Figure 2D-E and Paper IV, Supplemental Figure 2B-C). The extensions penetrate into a few rows of SVs in the vesicle pool surrounding the dense projection, which appears to be not labeled with Dap160, Eps15 and dynamin antibodies. Thus, the differential distributions of immunoreactivities characterize different pools of vesicles.

Only the distal pool contains Dap160, Eps15 and dynamin, and the vesicles in the proximal pool do not.

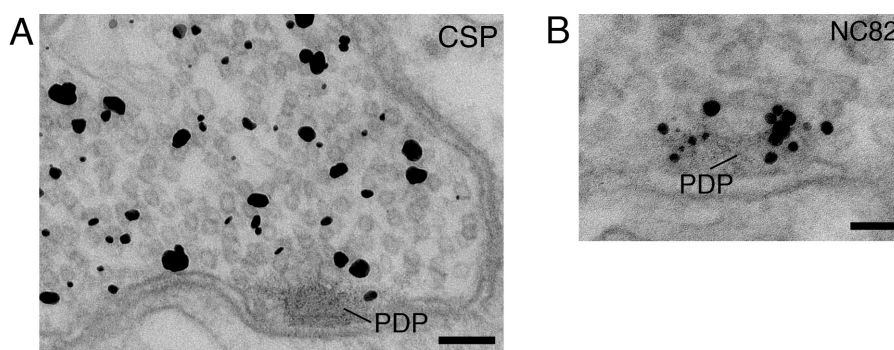
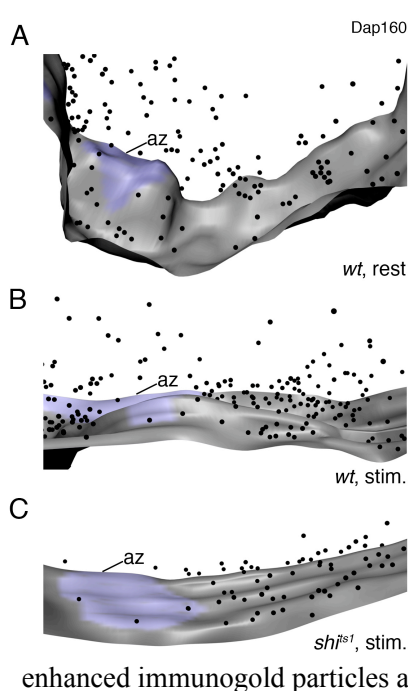


Figure 7. EM images of *wt* NMJs stained with CSP (A) and NC82 (B) antibodies. Scale bars in (A), 100nm; (B) 50nm.

SVs undergo the exo- and endocytosis cycle during synaptic activity. We then investigated what happens to the localization of the scaffolding molecules during synaptic activity. During stimulation with high K^+ , all three proteins relocate to the periaxial zone (Figure 8B, Paper I, Figure 2I and Paper IV, Figure 2A and F). The dynamin mutant, *shibire^{ts1}* was also tested in these experiments. *shibire^{ts1}* is a temperature sensitive dynamin mutant, in which one amino acid of dynamin is mutated, making it unable to hydrolyze GTP at temperatures above 29°C. This results in a block of endocytosis in NMJs. Prolonged stimulation of *shibire^{ts1}* NMJs at 29°C leads to the depletion of SVs at the active zone and an accumulation of endocytic intermediates (invaginated pits) at the presynaptic membrane around AZs (Koenig and Ikeda 1989). Immunogold labeling for Dap160, Eps15 and dynamin in stimulated *shibire^{ts1}* NMJs showed that gold particles are predominantly associated with the invaginated pits



(Figure 8C, Paper I Figure 3 and Paper IV, Figure 2B and G). The redistribution of these three proteins from the SV pool to the periaxial zone is shown in 3D in Figure 8 (Paper IV Figure 2C-E). All these observations suggested a mechanism that is triggered by synaptic activity, which controls the location of Dap160, Eps15 and dynamin. We proposed that this relocation is important to coordinate the efficiency of the endocytic machinery in NMJs.

Figure 8. 3D reconstruction of the subcellular localization of Dap160. The plasma membrane is gray. Active zones (az) are colored violet. Silver-enhanced immunogold particles are shown as black spheres.

6 DAP160 AND EPS15 FORM A MOLECULAR COMPLEX AND ARE ESSENTIAL IN THE MAINTENANCE OF SYNAPTIC TRANSMISSION

The relationship between two scaffolding proteins Dap160 and Eps15 in *Drosophila* synapses has not been characterized. To investigate if Eps15 is engaged in a molecular complex with Dap160, immunoprecipitation experiments were performed from fly head extracts using Eps15 antibodies. Our experiments show that Eps15 binds to Dap160, and that Dap160 is a major binding partner of Eps15 (Figure 9 and Paper I, Figure 1A). This is in a good agreement with co-localization of these proteins in NMJs (see last section). All these experiments lead to a suggestion that Dap160 and Eps15 work together to coordinate the synaptic vesicle cycle in NMJs. Direct interactions between mammalian orthologs have previously been reported (Sengar, Wang et al. 1999). All these findings indicate that the Dap160-Eps15 molecular complex is functionally important and evolutionary conserved.

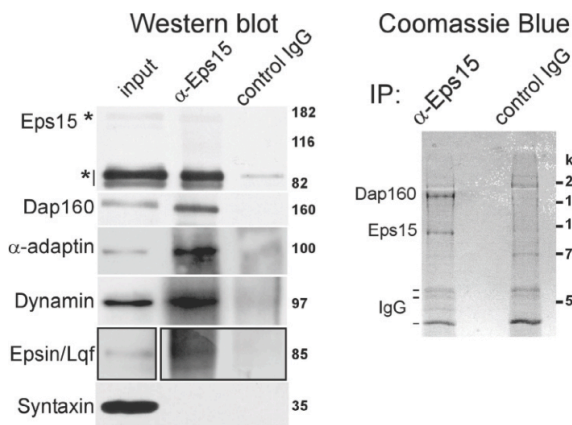


Figure 9. (Left) Anti-Eps15 antiserum coimmunoprecipitates Eps15 with Dap160, α -adaptin, dynamin, and epsin/Lqf, but not Syntaxin (input is 5%). Asterisks mark the positions of Eps15-specific bands. (Right) Coomassie staining shows that Dap160 is the major binding partner of Eps15 in the co-immunoprecipitate.

Earlier studies on *dap160* null mutants revealed that DAP160 protein has important functions in SV membrane endocytosis. Genetic deletion of Dap160 from *Drosophila* synapses results in reduced vesicle numbers at active zones, reduced styryl dye uptake during synaptic activity, and a decrease in the amplitude of excitatory junctional potentials (EJPs) during high-frequency stimulation (Koh, Verstreken et al. 2004; Marie, Sweeney et al. 2004). Endocytic intermediates and large cisternae were observed in *dap160* null mutant NMJs (Koh, Verstreken et al. 2004). *eps15* null mutants were generated to test whether the genetic removal of Eps15 will also lead to an endocytic defect (Paper I). Physiological experiments demonstrated that *eps15* null mutant synapses are unable to sustain neurotransmitter release during stimulation at 10 Hz (Figure 10 and Paper I, Figure 5). Additionally, FM1-43 dye uptake is impaired in these mutants (Paper I, Figure 6). EM analysis revealed an increased number of large

vesicles and cisternae under resting conditions. During stimulation a complete depletion of SVs and a profound accumulation of large cisternae and membrane invaginations occurred (Paper I, Figure 7). These data indicate that Eps15 is an important component of the Dap160-Eps15 molecular complex.

To investigate if Dap 160 and Eps15 belong to the same functional pathway, a *dap160* and *eps15* double knockout was generated and defects in synaptic transmission in NMJs were investigated using the same paradigm as for *dap160* and *eps15* mutants. Interestingly, *dap160* and *eps15* double mutant displayed similar depression kinetics as observed in *dap160* and *eps15* null mutants (Figure 10 and Paper I Figure 10B). Moreover, the impairment of the FM dye uptake was also quite similar (Paper I Figure 10C). *dap160* and *eps15* single and double mutants all show normal evoked EJPs when stimulated at low frequency, indicating the Dap160-Eps15 complex has an essential role in maintaining synaptic transmission at high activity rates.

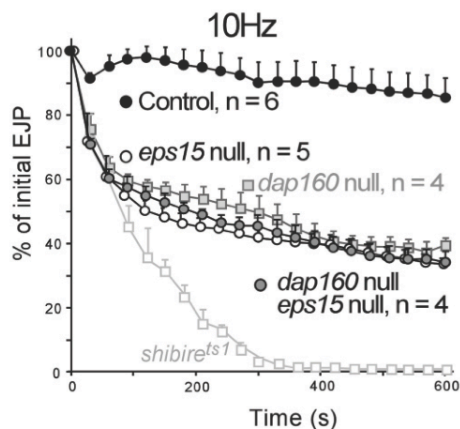


Figure 10. When stimulated at 10 Hz in 5 mM Ca²⁺, *dap160*- and *eps15*-null double mutants show synaptic depression kinetics that overlap with *eps15* null and *dap160* null single mutants.

7 ROLE OF THE DAP160-EPS15 MOLECULAR COMPLEX IN DYNAMIN TARGETING

Since the Dap160-Eps15 complex interacts with dynamin, we investigated if this molecular complex may control the function of this protein during the synaptic vesicle cycle. The GTPase dynamin has been shown to function at the fission step of the SV membrane endocytosis (Schmid, McNiven et al. 1998; McNiven, Cao et al. 2000). Dap160 co-precipitates with dynamin, and the GST pull-down experiments revealed that Dap160 binds to dynamin PRD domain through its SH3 A and B domains (Roos and Kelly 1998; Verstreken, Koh et al. 2003).

We first examined the level of dynamin in *dap160* and *eps15* mutant terminals and found that it is severely reduced (to 30% in *dap160* mutants and 10% in *eps15* mutants). Dap160 levels are reduced to 25% in *eps15* mutants (Koh, Verstreken et al. 2004). This leads to the conclusion that Dap160 and Eps15 are important for proper delivery of dynamin to nerve terminals during development.

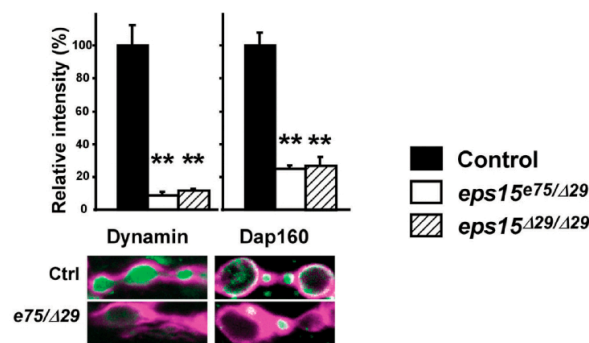


Figure 11. Dynamin and Dap160 are strongly reduced at *eps15* mutant NMJs. Anti-Dlg outlines the NMJ boutons (magenta). Dynamin and Dap160 stained in green are quantified. **, $P < 0.01$ (t test).

We then investigated how these proteins control dynamin function in synaptic vesicle recycling in third instar larvae. Our immunofluorescence experiments revealed that contrary to stimulated *wt* synapses, in *dap160* mutant NMJs Eps15 and dynamin are no longer co-localized. Eps15 accumulates in spots at the presynaptic membrane, while dynamin is diffused over the whole bouton (Paper IV, Figure 3E and F). This implies that breaking of the interaction between dynamin and the Dap160-Eps15 complex results in the abnormal localization of dynamin. To examine whether the recruitment of dynamin to the periaxial zone is impaired, immunogold experiments were performed in mutants. Immunogold labeling for dynamin in *dap160* mutants revealed that dynamin is associated with the SV pool at rest (as in *wt* NMJs). However,

upon high K^+ stimulation, it is no longer concentrated at the periaxial zone. Instead, it distributes evenly over the whole terminal membrane (Figure 12A₁₋₂ and Paper IV, Figure 3 A₁₋₃ and B₁₋₂). Constricted pits were found to accumulate at the presynaptic membrane in *dap160* mutants. Very few of them, however, appeared to be labeled by dynamin antibodies (a three-fold reduction compared to *shibire^{ts1}*), indicating that locating dynamin to the constricted pits is impaired (Figure 12B₁₋₂ and Paper IV, Figure 3B₃₋₄).

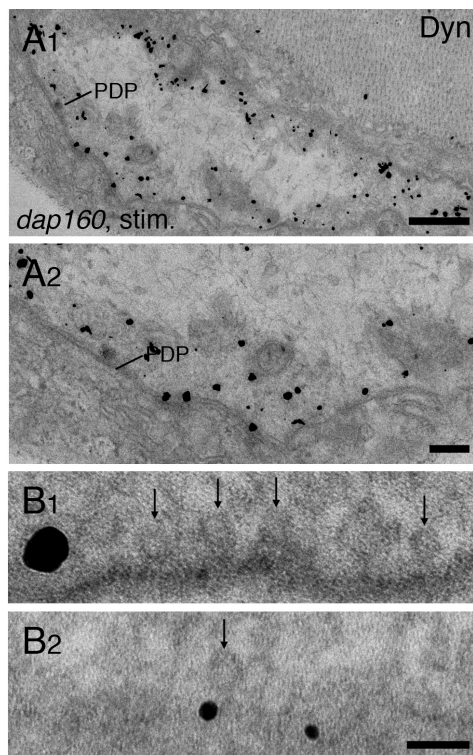


Figure 12. Mislocalization of dynamin from the periaxial zone in *dap160* mutants. (A₁₋₂) EM images showing mistargeting of dynamin to the plasma membrane in *dap160* mutant NMJs upon stimulation. (B₁₋₂) High magnification images showing constricted pits (arrows) accumulated at the periaxial zone upon stimulation. Only few pits are labeled for dynamin. The labeled constricted pit is indicated by arrow in B₂. Scale bars: (A₁), 500nm; (A₂) 200nm; (B₁₋₂), 50nm.

Immunogold labeling for Eps15 in *dap160* mutants was also examined. The pattern for Eps15 immunoreactivity is not changed and is similar to the pattern observed in *wt*. We also found that it associates with the SV pool at rest and is recruited to the periaxial zone during stimulation (Paper IV, Supplemental Figure 4D-G). AP2 also shows a similar spot-like pattern in stimulated *dap160* mutant NMJs, and co-localizes strongly with Eps15, indicating they are both accumulated at the sites of endocytosis during synaptic activity (Paper IV, Figure 3G and H).

Drosophila Dap160 possesses four SH3 domains, in which SH3A and SH3B bind to dynamin, and SH3B binds it preferentially (Roos and Kelly 1998). To test if this binding is important for targeting of dynamin to the periaxial zone, SH3B (ΔB) and SH3AB domain-deletion (ΔAB) rescue mutants were generated in the *dap160* null background.

Studies of these mutants using immunofluorescence revealed that ΔAB shows a strong mislocalization of dynamin upon high K^+ stimulation (Figure 13 and Paper IV, Figure 4B and C). Eps15 localization remains unchanged as compared to *wt*. Thus, interactions with these domains of Dap160 are essential to concentrate dynamin at the periaxial zone.

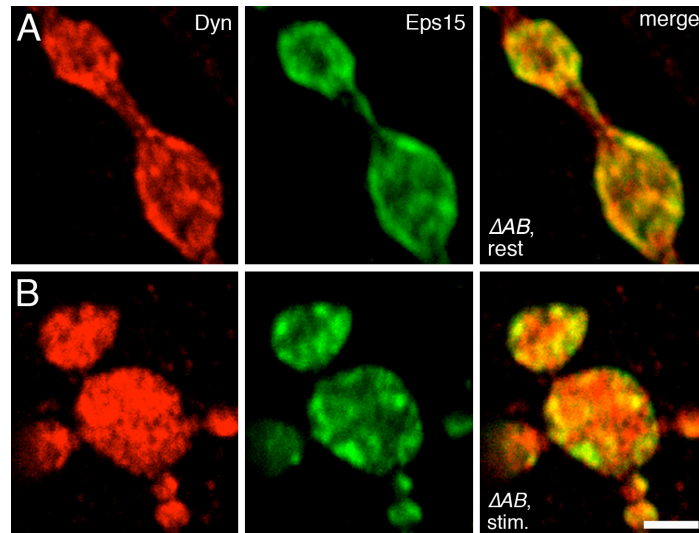


Figure 13. Confocal images of NMJs from ΔAB rescue line at rest and during stimulation. Scale bars: 2 μ m.

We further investigated functional defects in ΔB and ΔAB mutants in behavioural tests. A locomotion assay was employed. At 25°C and 34°C (3min), both ΔB and ΔAB show slightly decreased locomotion activities, but not significantly different compared to *wt* (Paper IV, Figure 4D). Since *dap160* mutants show significant depression of synaptic activity under intense stimulation (Koh, Verstreken et al. 2004), we extended the duration of the test to 13 minutes. Impaired locomotion was clearly seen in ΔAB at 34°C (Figure 14 and Paper IV, Figure 4E), indicating the importance of the interaction between dynamin and the Dap160-Eps15 complex in synaptic activity under high frequency stimulation.

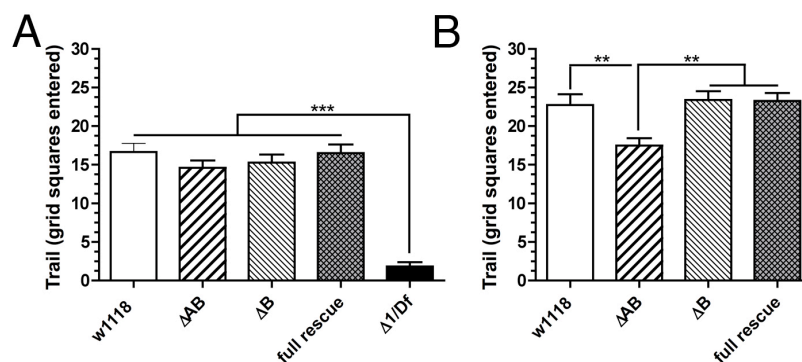


Figure 14. Bar graphs illustrating differences in locomotion behaviour in *dap160* mutants. (A) At 25°C only *dap160* null mutants ($\Delta 1Df$) are significantly different. (B)

At elevated temperature (34°C; 13mins), a significant reduction (~24%) in grids squares entered was detected in ΔAB . **, P<0.01; ***, P<0.001 (ANOVA).

Repetitive stimulation of *shibire^{ts}* NMJs at non-permissive temperature results in a complete block of SV recycling, while *dap160* and *eps15* single or double mutants only show moderate slow-down of endocytosis (Paper I, Figure 5D). This is due to both a reduced level of dynamin in the mutants and an inability to concentrate dynamin at the periaxial zone. Removing Dap160 or its dynamin interacting domains results in mislocalization of dynamin during stimulation. Dynamin is no longer accumulated at the periaxial zone and not efficiently recruited to constricted pits. SV endocytosis may occur even at reduced levels of dynamin, but this is not sufficient to support sustained activity. In the stimulated *dap160* mutant, Eps15 is still able to relocate to the periaxial zone, indicating other proteins such as AP2 or/and stonin, may target Eps15 to the endocytic site. In summary, our experiments show that the Dap160-Eps15 complex is essential to concentrate dynamin at the sites of endocytosis during synaptic vesicle recycling.

8 THE MODEL FOR DYNAMIN TARGETING

Our experiments allow us to propose a model, which explains the role of the Dap160-Eps15 molecular complex in endocytosis (Figure 15 and Paper IV, Figure 5). At rest, the Dap160-Eps15 complex and dynamin reside in the distal pool of SVs in the NMJ. Upon stimulation, the Dap160-Eps15 molecular complex recruits and concentrates dynamin at the periactive zone, the sites of endocytosis, to aid its fast assembly to promote the budding of newly formed vesicles from the presynaptic membrane. Removing Dap160 breaks the molecular link between dynamin and the Dap160-Eps15 complex, resulting in mistargeting of dynamin at the periactive zone.

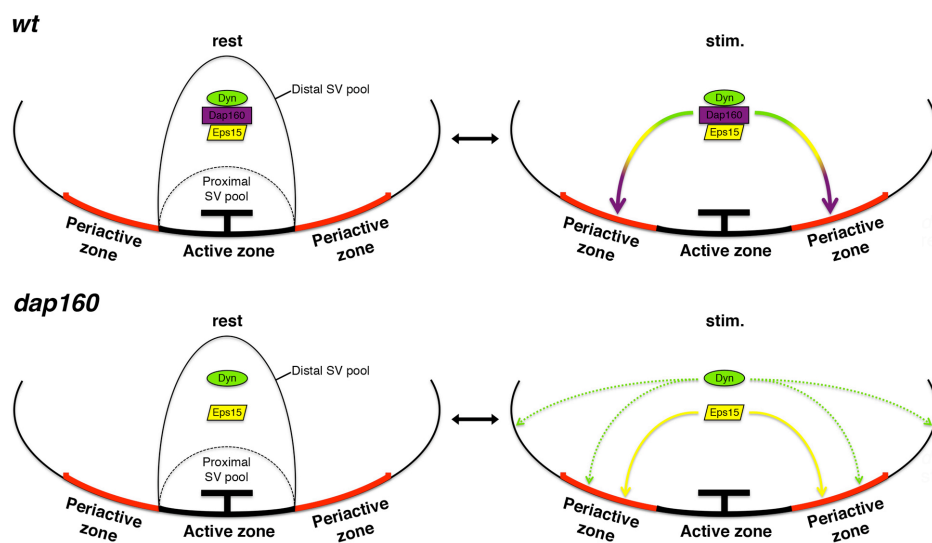


Figure 15. Schematic illustration of the mechanism for dynamin targeting to the periactive zone during the synaptic vesicle cycle.

Recent studies have shown that the Dap160-Eps15 complex is recruited early to the periactive zone (Henne, Boucrot et al. 2010). In agreement with this, we propose that this complex concentrates dynamin at hot spots of endocytosis via the interactions of Dap 160 SH3AB domain. Since the rate of dynamin oligomerization is concentration dependent (Roux, Koster et al. 2010), an increase in dynamin concentration accelerates dynamin spiral formation and the subsequent vesicle-budding reaction. The function to concentrate dynamin becomes critical during high-frequency stimulation, when SV membrane accumulates at the periactive zone and a proper dynamin concentration required for fission complex assembly at endocytic sites is more difficult to achieve.

9 CONCLUSIONS

- Immunogold and electron tomography protocols were developed, making it possible to localize synaptic proteins in *Drosophila* NMJs and to study the structural organization of this synapse at high resolution.
- Fas II delineates the active zone area in the *Drosophila* NMJ under resting conditions and immunolabeling for this protein can be reliably used to evaluate changes in the size of the active zone region.
- The presynaptic dense projection of the active zone consists of three distinct components: the central core, legs connecting the central core to the plasma membrane, and cytoplasmic extensions emerging from the central core.
- The SV pool in the *Drosophila* synapse is structurally composed of the proximal pool attached to the dense projection by thin filaments and distal pool constituted by interconnected vesicles. Vesicles tethered to the presynaptic membrane are able to release immediately in response to stimulation.
- The scaffolding molecules Dap160 and Eps15 and the GTPase dynamin reside in the distal pool of SV at rest and relocate to the periaction zone during synaptic activity.
- Dap160 and Eps15 directly interact and form a complex, which is required to maintain synaptic transmission in the *Drosophila* NMJ at high activity rates. This complex is essential to concentrate dynamin at the periaction zone during synaptic activity.

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所有在瑞典相识的朋友，这个寒冷的国度，半年的白雪，一季的阳光，因为有你们，才显得温暖。

戴琦，汪神球，谢谢你们对刚来瑞典的我的照顾，为了打升级而手制的纸牌依旧是我美好的回忆。

巧琳，谢谢你姐姐一样的关怀，血拼的时候也总是不忘记帮我注意好东西。金哲，你的好手艺让我很饱口服啊。

绍波，曾几何时，来去路上都有你做伴，现在住的远了，一个人等公车的时候还是会想到你。赵建，很想偷师你的川菜手艺啊。（好吧，我承认我是吃货。）

隶宁，还记得两人在Gotland东走走，西看看的悠闲，谢谢你时不时来自旧金山的问候。

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