Morphological docking of secretory vesicles

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Abstract Calcium-dependent secretion of neurotransmitters and hormones is essential for brain function and neuroendocrine-signaling. Prior to exocytosis, neurotransmitter-containing vesicles dock to the target membrane. In electron micrographs of neurons and neuroendocrine cells, like chromaffin cells many synaptic vesicles (SVs) and large dense-core vesicles (LDCVs) are docked. For many years the molecular identity of the morphologically docked state was unknown. Recently, we resolved the minimal docking machinery in adrenal medullary chromaffin cells using embryonic mouse model systems together with electron-microscopic analyses and also found that docking is controlled by the sub-membrane filamentous (F-)actin. Currently it is unclear if the same docking machinery operates in synapses. Here, I will review our docking assay that led to the identification of the LDCV docking machinery in chromaffin cells and also discuss whether identical docking proteins are required for SV docking in synapses.

Keywords Docking · Electron microscopy · F-actin · Munc18-1 · SNARE proteins · Synaptotagmin-1 · Secretory vesicles

Introduction

Calcium (Ca\textsuperscript{2+})-dependent exocytosis of synaptic vesicles (SVs) can be elicited within fractions of a millisecond upon calcium-influx into the releasing cell. In order to achieve such exquisite temporal precision, secretory vesicles undergo a number of maturation steps before calcium influx. Neuroendocrine cells like chromaffin cells are established models for neurotransmitter release (Morgan and Burgoine 1997). These cells secrete adrenalin and noradrenalin that are both accumulated in large dense-core vesicles (LDCVs), which are recognized in electron microscopic (EM) pictures by a vesicular membrane containing an electron dense-core (Voets et al. 2001). Docking is considered the first necessary intermediate maturation step before either LDCVs or SVs gain fusion-competence...
and release their neurotransmitters. Recently, it was shown that only a fraction of docked SVs fuse upon Ca\textsuperscript{2+}-influx (Wadel et al. 2007) and that their release-readiness seems determined by their proximity to Ca\textsuperscript{2+}-channels (Sakaba and Neher 2001). Fusion involves the evolutionarily conserved soluble N ethylmaleimide-sensitive-factor-attachment-protein-receptor (SNARE)-complex (Ferro-Novick and Jahn 1994; Jahn 2000). The neuronal SNARE-complex contains plasma membrane proteins syntaxin-1 and synaptosome-associated-protein of 25-kDa (SNAP-25) and vesicle-associated-membrane-protein-2 (VAMP-2)/synaptobrevin-2 (Jahn and Scheller 2006; Rizo and Rosenmund 2008). Association of the SNAREs underlies the priming reaction (Walter et al. 2010), and final C-terminal-SNARE-assembly coincides with fusion-triggering (Sørensen et al. 2006). Genetic deletion of neuronal SNARE genes are lethal and has profound effects on neurotransmission (Schoch et al. 2001, 2003). Sec1/Munc18-1-related proteins (Jahn 2000) bind to the neuronal SNARE-complex (Dulubova et al. 2007) and are together with syntaxin-1 essential for docking (de Wit et al. 2006; Verhage and Sørensen 2008). Munc18-deficient mice die shortly after birth (Verhage et al. 2000). Previously we showed that deficiency of Munc18 (Voets et al. 2001) or the protein syntaxin-1 (de Wit et al. 2006) not only abolished exocytosis, but also produced robust docking phenotypes in chromaffin cells (Toonen et al. 2006a) as well as neurons (Toonen et al. 2006b). We also observed that manipulating Munc18 expressing levels affected the sub-membrane filamentous (F-)actin density (Toonen et al. 2006a) which is known to determine access of LDCVs to fusion sites (Malacome et al. 2006). In contrast to an increasing fundamental understanding of exocytosis the molecular mechanism of docking is only slowly being unraveled (Verhage and Sørensen 2008). This review summarizes how we achieved to resolve the minimal molecular docking machinery for LDCV secretion employing a unique docking assay in which ultrastructural approaches are combined with rescue experiments in genetically modified embryonic mouse chromaffin cells. In addition, the possible mechanism by which docking proteins control access to fusion sites by modulation of sub-membrane F-actin will be discussed. Finally our results in chromaffin cells will also be compared to docking phenotypes in synapses in an attempt to see whether docking machineries are conserved in rapidly secreting cells.

**Morphological docking assay**

In electron micrographs of adrenal chromaffin cells, many LDCVs are found morphologically docked at the target membrane (Fig. 1). Morphologically docked vesicles are traditionally defined as those vesicles that have no measurable distance between vesicle and plasma membrane (Fig. 1), but some authors use less stringent criteria (for a review see Verhage and Sørensen 2008). We studied docking of LDCVs in mouse embryonic (embryonic day 18) chromaffin cells as a preferred docking model, because docking phenotypes are typically more evident than in other systems studied so far (for a review see Verhage and Sørensen 2008; Fig. 1). The reason why we use embryonic cells as a model system is because it seems that a genetic deletion of docking proteins results in a lethal phenotype.
causing death shortly after birth in all nulls studied so far (de Wit et al. 2009; Verhage and Sørensen 2008).

The first docking protein we identified was Munc18. After ultrastructural analysis of LDCV distribution we observed a tenfold reduction of ‘morphologically’ docked LDCVs in munc18-1 deficient chromaffin cells of intact adrenal glands (Voets et al. 2001), and which could have explained the severe secretion phenotype. In addition, we observed that gene-dose reductions in munc18-1 expression reduce docking, whereas gene-dose enhancements increase docking (Toonen et al. 2006a) indicating that Munc18-1 promotes docking. To reintroduce Munc18 we developed a primary-culture system for munc18-1 deficient chromaffin cells and readily infected those with the Munc18 gene using the Semliki-Forest-Virus (SFV) expression-system (Ashery et al. 2000; Toonen et al. 2006a). To obtain straightforward detection of infected cells based on their fluorescence we coupled the Munc18 cDNA via an internal ribosomal entry site to cDNA for green fluorescent protein (Toonen et al. 2006a) (Fig. 2).

Subsequently the distance of LDCVs to the plasma membrane is analyzed to determine the number of docked vesicles. As an example the same mutant cell (wild type cells expressing the light chain of Botulinum toxin C (BoNT/C) to delete syntaxin) as in Fig. 1 is used. Note that the SFV viral particles are clearly visible at the plasma membrane (arrowheads). See for details (de Wit et al. 2006, 2009)

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To easily find infected cells for EM analysis we grow our cells on coverslips that contain a grid (de Wit et al. 2006) which is kept after flat-embedding in the plastic resin, and can be used to select the region of interest for ultrathin sectioning and analysis of LDCV docking (Fig. 2). The analysis of LDCV distribution is usually performed manually, which is labor-intensive and subject to human bias and error. Recently we developed an algorithm to automatically analyze LDCV distribution and docking in electron micrographs (van Weering et al. 2008). In addition to neuronal Munc18-1 we also expressed two ubiquitous Munc18 isoforms, Munc18-2 and Munc18-3 in munc18-1 deficient chromaffin cells. Munc18-2 and -3 have relatively low expression levels in neuroendocrine cells (Hata and Südhof 1995; Tellam et al. 1995). Munc18-2 is involved in histamine secretion in mast cells (Martin-Verdeaux et al. 2003) and apical vesicle trafficking in epithelial cells (Riento et al. 2000), whereas Munc18-3 regulates cell surface expression of GLUT4 in adipocytes (Latham et al. 2006; Tellam et al. 1997). The amino acid sequence homology between Munc18-1 and -2 is greater than (60%) that of Munc18-3 (50%), and both Munc18-1 and -2 show strong affinity for syntaxin-1, -2, and -3, but not syntaxin-4 (Hata and Südhof 1995). Munc18-3 shows only high affinity for syntaxin-2 and -4 (Tellam et al. 1997). We found that Munc18-2 rescues the severe docking phenotype of munc18-1 null chromaffin cells indistinguishably from Munc18-1 while the more downstream vesicle priming steps are still impaired (Gulyás-Kovács et al. 2007). This suggests that Munc18-1 is additionally involved in priming vesicles for release. On the other hand docking is rescued only partially after Munc18-3 expression in munc18-1 null chromaffin cells (de Wit 2010).
The second docking protein we identified is the SNARE protein syntaxin-1. Like Munc18 we observed that deficiency of syntaxin-1 also produced robust docking phenotypes in chromaffin cells (de Wit et al. 2006). It is known that Munc18-1 interacts with syntaxin-1 and we therefore assumed that Munc18-1’s docking function depends on syntaxin-1-interaction (de Wit et al. 2007). Munc18-1 can interact with syntaxin in two distinct binding modes: either with isolated syntaxin-1 alone in a ‘closed’ conformation or with syntaxin-1 in an ‘open’ conformation in the assembled SNARE-complex (Toonen and Verhage 2007). However, it’s unclear which binding mode is essential in docking. Munc18-1 binding to open-syntaxin-1 involves the N-terminal-H(abc)-domain of syntaxin-1 (Dulubova et al. 2007; Dulubova et al. 1999; Khvotchev et al. 2007). We have previously shown that N-terminal-interaction is not sufficient for docking, since chromaffin cells from open-syntaxin-knock-in mice show a similar docking phenotype as syntaxin-1 and munc18-1 null (Gerber et al. 2008). In addition, Munc18-1 bearing a mutation that perturbs closed-syntaxin interaction [D34N/ M38V] (Naren et al. 1997; Schütz et al. 2005) cannot restore docking in munc18-1 null cells (Gulyás-Kovács et al. 2007). Other researchers have shown that Munc18-1 binding to open-syntaxin executes fusion (Barclay 2008; Burkhardt et al. 2008; Deak et al. 2009; Dulubova et al. 2007; Khvotchev et al. 2007; Shen et al. 2007). Together, our observations show that Munc18-1 and -2 promote docking by binding to closed-syntaxin-1 while a distinct interaction mode seems required to regulate the consecutive priming step.

In addition to syntaxin-1 and Munc18-1, several other proteins have been implicated in docking in several types of secretory cells, such as rab3 and rab27, rabphilin3A, granuphilin, and exophilin4/Slp2a, and function mutations in several priming genes in C. elegans (RIM/unc-10, (M)unc-13, or CAPS/unc-31) produce a strong reduction of vesicles with a “contact patch” (for a review, see Verhage and Sørensen 2008). However, it seems that those proteins are not essential for LDCV docking in chromaffin cells (Ashery et al. 2000; van Weering et al. 2007), therefore we searched for other likely candidate docking proteins that together with Munc18 and syntaxin-1 form the minimal docking machinery (de Wit et al. 2009).

Minimal docking machinery

Previously we hypothesized that Munc18 promotes the formation of a docking acceptor at the plasma membrane (Voets et al. 2001) and we assumed that this docking acceptor consists of syntaxin (de Wit et al. 2006). Other researchers have shown that prior to binding of synaptobrevin-2 to syntaxin-1/SNAP-25 at the target membrane, syntaxin-1 is believed to form a 1:1-heterodimer with SNAP-25 as an intermediate step (Faschauer and Margittai 2004; Zilly et al. 2006). Recently we found that overexpression of either SNAP-25 or a C-terminal peptide of synaptobrevin-2 that was known to favor the formation of SNAP-25/syntaxin-heterodimers in a 1:1 stoichiometry (Faschauer and Margittai 2004; Pobbati et al. 2006) rescued docking in munc18-1 null chromaffin cells (de Wit et al. 2009) (Fig. 3). Therefore, we concluded that Munc18-1 promotes formation of docking acceptor complexes of syntaxin/SNAP-25 in chromaffin cells (Fig. 3) (de Wit et al. 2009). Since vesicle fusion is still impaired after overexpression of either SNAP-25 or a C-terminal peptide of synaptobrevin-2 we assumed that in addition to docking, Munc18-1 plays a critical function in downstream events leading to exocytosis (de Wit et al. 2009) (Fig. 3). The involvement of syntaxin-1/SNAP-25 acceptor complexes in docking suggests a direct requirement for SNAP-25 in docking, similar to syntaxin-1. However, previous observations argued against a docking function for SNAP-25 (Sørensen et al. 2003). Therefore, we decided to reanalyze docking in Snap-25 null cells. Indeed, Snap-25 deficient cells exhibited a strong docking phenotype, which was, however, slightly less severe than in Munc18-1 or syntaxin-1 deficient cells (de Wit et al. 2006; Voets et al. 2001) (Fig. 1). Different culture conditions and fixation methods could explain the observed differences to previous studies (Sørensen et al. 2003). Therefore, to circumvent possible culture or chemical fixation artifacts, we also analyzed docking in intact adrenal glands and in intact adrenal glands that were rapidly frozen under high pressure. We observed the same docking difference between Snap-25 null and wild type chromaffin cells after rapid freezing as compared to chemically fixed intact adrenal glands as well as cultured chromaffin cells (de Wit et al. 2009) (Fig. 1). In addition, we observed that overexpression of SNAP-25 on the Snap-25 null background rescued the phenotype (de Wit et al. 2009). Strikingly, in contrast to SNAP-25 overexpression in munc18-1 null cells, Munc18-1 overexpression in Snap-25 null cells did not rescue the docking phenotype (de Wit et al. 2009). Together, our observations demonstrate that SNAP-25 is the third docking protein essential for docking and that Munc18-1 cannot promote docking in its absence.

Docking of LDCVs to the SNAP-25/syntaxin acceptor complexes at the plasma membrane must involve vesicular proteins. Therefore, we next attempted to identify the vesicular component to dock vesicles to syntaxin-1/ SNAP-25 acceptor complexes. While the proteins present on SVs have been systematically identified (Takamori et al. 2006), none of these have been assigned as a docking factor; to the best of our knowledge no docking
phenotypes have been observed in null mutants for any of these vesicular proteins. Biochemical evidence suggests that at least two vesicle proteins bind to established docking factors on the plasma membrane and can therefore be considered candidate docking factors: synaptobrevin-2 and synaptotagmin-1 (Chieregatti et al. 2002; Rickman et al. 2004; Schiaivo et al. 1997; Söllner et al. 1993). We recently confirmed that the synaptobrevin-2 null mutation does not produce docking phenotypes similar to munc18-1 or syntaxin-1 null (Gerber et al. 2008), consistent with earlier findings (Borisovska et al. 2005). Therefore, we realized that synaptotagmin-1 is the prime candidate for a vesicular docking protein. Indeed we observed a strong docking defect in synaptotagmin-1 null cells, similar to Snap-25 null cells (de Wit et al. 2009).

In addition we found that SNAP-25 no longer rescues docking in synaptotagmin-1/munc18-1 double null mutants. By using synaptotagmin-1 and SNAP-25 mutations that affect their interaction we confirmed that synaptotagmin-1 provides the direct link between vesicles and syntaxin-1/SNAP-25 acceptor complexes via interaction of its C2B domain with SNAP-25 (de Wit et al. 2009) (Fig. 3). Together, using EM analysis combined with null mutations and (cross-) rescue experiments we identified two novel proteins, SNAP-25 and synaptotagmin-1 that act in concert with our previously characterized

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**Fig. 3** Docking proteins and the minimal docking machinery. 

(a) Top panel electron micrographs from primary cultured munc18-1 null chromaffin cells expressing either Munc18-1 (left), SNAP-25 (middle) or the [49–96] C-terminal fragment of synaptobrevin-2 (right). Bottom panel electron micrographs from primary cultured synaptotagmin-1 null chromaffin cells expressing either wild type synaptotagmin-1 (middle) or the synaptotagmin-1 mutant with reduced SNAP-25 affinity [Y311N] (right) or the GFP negative control (left). These observations together with the strong docking defects for syntaxin and Snap-25 null mutants (as shown in Fig. 1) indicate that besides syntaxin and Munc18-1, also SNAP-25 and synaptotagmin are essential for LDCV docking, and that synaptotagmin-1 binds to SNAP-25 to anchor LDCVs. The scale bar represents 200 nm.

(b) Working model for subsequent steps in the exocytotic pathway. The model depicts the subsequent steps in the pathway, starting with several initial protein complexes consisting of syntaxin-1 (red), SNAP-25 (green) and/or Munc18-1 (light blue), until the final fusion, which also involves synaptobrevin/VAMP (dark blue) and synaptotagmin-1 (yellow). The bottom row indicates non-productive protein complexes, which do not lead to fusion, either by integrating two syntaxins and one SNAP-25 into a single complex (2:1 complex ‘off pathway’, bottom left) or by formation of a 1:1 syntaxin-1/SNAP-25 complex stabilized by the C-terminal fragment of synaptobrevin-2, with or without synaptotagmin-1 associated (bottom right). Note that complexin-1 is excluded in this model. See (de Wit et al. 2009) and main text for a detailed description of the proposed steps in the pathway. Adapted from (de Wit et al. 2009)
proteins Munc18-1 and syntaxin-1 in docking of LDCVs, and that Munc18-1 also plays a unique, orchestrating role.

Currently, it is unknown whether the same docking machinery as we resolved in neuroendocrine cells (de Wit et al. 2009) also acts in synapses. In vertebrate-synapses, docking-phenotypes for munc-18-1/syntaxin-1/Snap-25/synaptotagmin-1 null are less evident (Verhage and Sørensen 2008) possibly arguing for distinct docking mechanisms. However, recently we observed that heterozygous expression of Munc18-1 reduced the number of docked SVs in cultured hippocampal neurons (Toonen et al. 2006b) (Fig. 4). Therefore, it seems more likely that docking principles are conserved among secretory systems. This idea is strongly supported by observations that invertebrate-synapses have docking-phenotypes upon munc18-1/syntaxin-1/synaptotagmin-1 mutations (Hammarlund et al. 2007; Jorgensen et al. 1995; Reist et al. 1998; Weimer and Richmond 2005). However, these phenotypes are generally subtle and sometimes require advanced methodology and new docking definitions to become evident (Hammarlund et al. 2007; Weimer and Richmond 2005). In case of synaptotagmin-1 null mutations, phenotypes have not been specifically interpreted in terms of docking due to additional phenotypes in these invertebrate synapses: large effects on undocked vesicle populations near the active zone (Reist et al. 1998), impaired recycling (Jorgensen et al. 1995) and/or impaired recruitment (Loewen et al. 2006). Interestingly, a mutation used in the latter study is in an area of the molecule that was later identified to interact with SNAP-25 (Rickman et al. 2006). Probably, docking phenotypes are less evident in vertebrate synapses either due to redundancy arising from the expression of multiple isoforms for some of the docking genes identified here or because structurally unrelated proteins, that are not expressed in chromaffin cells, restrict undocking of SVs even when essential docking factors are not expressed. Finally, it is plausible that undocking and docking phenotypes are simply not as evident in the densely packed nerve terminal.

**Sub-membrane F-actin controls access of vesicles to docking sites**

Bovine chromaffin cells possess a dense mesh of sub-membrane F-actin underneath the plasma membrane which is thought to provide a barrier for LDCVs to access exocytotic sites under resting conditions (Nakata and Hirokawa 1992). Consistent with this view perturbations of the sub-membrane F-actin modulate docking and secretion (for reviews see de Wit 2010; Trifaró et al. 2008). Upon a secretory stimulus it seems that two major sub-membrane F-actin severing pathways are activated. The first pathway is controlled by Ca$^{2+}$ entry that results in activation of scinderin (Zhang et al. 1996) whereas the second pathway depends on protein kinase C (PKC) activation and phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKs) (Vitale et al. 1992). Both scinderin and MARCKS are F-actin severing proteins, and their activation induces a local disruption of the sub-membrane

![Fig. 4 Morphological docking phenotypes in neurons. Left panel cultured hippocampal neuron grown in islands (making synapses on itself that are also known as autapses) stained for MAP2 (red) and synapsin (green) at DIV 14. The scale bar represents 20 μm. The bottom shows a higher magnification (scale bar represents 2 μm) of the part indicated by the rectangle (top) of synapsin-stained synapses making synaptic contact with MAP2-stained dendrites. This image was kindly provided by Dr. Toonen. For details see (Wierda et al. 2007). Middle panel schematic representation of synapses showing morphological docked (top) and undocked (bottom) SVs, respectively without any (0 nm) or with (>0 nm) measurable distance between vesicle and the active zone facing the postsynaptic density. Right panel electron micrograph of an autaptic synapse of a control (wild type) hippocampal neuron (top). Note the array of docked vesicles facing the postsynaptic density. In autapses of heterozygous Munc18 hippocampal neurons less SVs are found docked to the presynaptic membrane (bottom). See also (Toonen et al. 2006b) for details. The scale bar represents 100 nm](image-url)
F-actin architecture to facilitate recruitment of LDCVs towards release sites, however their involvement in docking remains unresolved. Recently we observed that the sub-membrane F-actin is severely altered in the absence of the docking protein Munc18-1 in embryonic mouse munc18-1 null chromaffin cells (de Wit 2010; Toonen et al. 2006a). Compared to embryonic wild type, munc18 null chromaffin cells contain a denser sub-membrane F-actin network, and only a few vesicles are docked (de Wit 2010; Toonen et al. 2006a) (Fig. 5). Strikingly, Latrunculin A (Lat A) reduced the sub-membrane F-actin, and completely restored docking (Toonen et al. 2006a). Munc18-1 is rapidly phosphorylated by PKC upon depolarization (Craig et al. 2003), and is an important downstream target in PKC-dependent potentiation of secretory vesicle recruitment and release (Wierda et al. 2007). Recently, we observed that phorbol esters can reduce sub-membrane F-actin, and partially restore docking (H. de Wit unpublished results) and secretion (Gulyás-Kovács et al. 2007) in the absence of Munc18-1. Our observation that phorbol esters only partially restore the docking/prosecretion defect can be explained by the fact that reduction of the sub-membrane F-actin network alone is not sufficient for functional docking/prosecretion, like shown before after Lat A application (Toonen et al. 2006a). Together, this makes Munc18-1 a likely candidate as a downstream target for PKC-dependent reorganization of sub-membrane F-actin to regulate docking. Further experiments are required to see whether Munc18-1 alone is sufficient to rearrange sub-membrane F-actin or whether additional factors are required. For example, previously it was found that Munc18-1 colocalizes with cytoskeleton proteins (Bhaskar et al. 2004) and is phosphorylated by cyclin-dependent kinase (Cdk5), which is found in connection with neurofilaments (Shetty et al. 1995), but it remains to be tested whether Munc18-1 directly acts as an actin-severing protein. Irrespective of

**Fig. 5** Comparison of docking and sub-membrane F-actin in chromaffin cells. a Top panel rhodamin–phallolidin staining of sub-membrane filamentous (F-)actin in a wild type (left) and munc18-1 null (right) chromaffin cell (bar represents 2 μm). In the absence of Munc18-1 the % of intact sub-membrane F-actin is significantly increased compared to wild type chromaffin cells (middle). Bottom panel in munc18-1 null chromaffin cells fewer LDCVs (right, middle) are docked compared to wild type controls (left, middle) as observed by electron microscopy and might be explained by the differences in intact sub-membrane F-actin (middle). This docking phenotype is the opposite for wild type embryonic chromaffin cells (left). The scale bar represents 100 nm. b Putative cartoon model summarizing the role of Munc18-1 in docking and modulation of sub-membrane F-actin. In embryonic chromaffin cells sub-membrane F-actin is less developed and more vesicles are docked at the plasma membrane (PM) compared to mature chromaffin cells (de Wit 2010; Nili et al. 2006). Upon depolymerization of sub-membrane F-actin stimulated by PKC, Munc18-1 or Ca²⁺ (e.g. scinderin) LDCVs have easier access to docking sites. We hypothesize that in the absence of Munc18-1 F-actin depolymerization and access to docking sites is severely reduced. In wild type embryonic cells most of these sites are already occupied which is indicated by the mild increase of docked LDCV after overexpression of Munc18-1 in these cells (Toonen et al. 2006a) and also by the strong increase in mature chromaffin cells (de Wit 2010; Nili et al. 2006). Adapted from (de Wit 2010)
the mechanism it seems that Munc18-1’s function here does not depend on binding to syntaxin-1 because syntaxin-1 deletion by Botulinum toxin C (BoNT/C) does not affect the sub-membrane F-actin (de Wit et al. 2006).

Similar to chromaffin cells, synapses seem to have a sub-membrane F-actin network (Dillon and Goda 2005; Doussau and Augustine 2000). F-actin is abundantly distributed throughout the presynaptic terminal and is often associated with the synapsin filaments (Landis et al. 1988) to link SVs (Pieribone et al. 2001). Biochemical (Phillips et al. 2001), ultrastructural (Hirokawa et al. 1989), and functional (Morales et al. 2000) studies indicate that actin also constitutes an important component of the active zone. In hippocampal synapses, Lat A treatment promotes neurotransmitter release, but the readily releasable pool size and its rate of refilling are not altered by Lat A (Morales et al. 2000). This suggests that Lat A exerts its effect on vesicles that are already docked at the active zone. Similarly, actin depolymerizing agents are ineffective in increasing neurotransmitter release in hippocampal slices of mouse harboring a homozygous deletion of the LIM kinase 1 gene (LIMK-1), a kinase that modifies actin dynamics by phosphorylating and thereby inactivating cofilin (Meng et al. 2002). Taken together, these observations suggest that the actin cytoskeleton negatively regulates neurotransmitter release by forming a structural barrier for exocytosis at or near release sites. However, it can be expected that the organization of the actin cytoskeletal network at release sites in chromaffin cell might be different from synapses, probably because their release sites do not contain a subcellular specialization like the active zones in synapses and expression of certain synaptic actin-associated proteins (e.g. synapsins, LIM kinases) is absent (Dillon and Goda 2005; Doussau and Augustine 2000).

In synapses, SV fusion occurs at specialized sites of plasma-membrane, referred to as the presynaptic active-zonal-cytometrix (CAZ) that accumulates the molecular machinery to orchestrate SV docking/fusion (Schoch and Gundelfinger 2006). Light-microscopic studies in reduced systems indicate that distribution of essential docking factors is complex, with accumulations of individual factors in partially overlapping microdomains (Lang et al. 2001; Lang and Jahn 2008). However, currently little ultrastructural information is available on distribution of these factors in neurosecretory cells, like chromaffin cells especially not in mutant systems with docking-phenotypes that we previously generated (de Wit et al. 2009). We are planning to study the distribution of docking proteins using immuno-(I)EM on ultrathin cryosections of chemically-fixed chromaffin cells and synapses (Oorschot et al. 2002). In secretory cells the number of morphological docked vesicles typically exceeds the number of release-ready vesicles (Gulyás-Kovács et al. 2007). Docked vesicles can be in different stages of release-readiness and seems determined by their positioning relative to Ca\textsuperscript{2+}-channels (Sakaba and Neher 2001); however, ultrastructural evidence for this is currently unavailable and which genes modulate this positioning is unknown. The 3D-reconstructions of frog neuromuscular junction and rat brain synaptosomes revealed readily-releasable-pool (RRP) organization and identified protein-linkages (Fernandez-Busnadiego et al. 2010; Harlow et al. 2001; Rizzoli and Betz 2004) that could presumably dock SVs in proximity to Ca\textsuperscript{2+}-channels. Pharmacological manipulations were show to affect these linkages, but the molecular identity of these tethers remains unclear and as a consequence the molecular mechanism how these protein-linkages function in nano-meter-distance positioning of SVs to Ca\textsuperscript{2+}-channels is unknown. Electron-tomography also revealed that SVs associate to sub-membranous cytoskeleton inside CAZ (Rostaing et al. 2006; Sikou et al. 2007) and seems to play a central role in LDCV docking in neuroendocrine cells (Toonen et al. 2006a) and synapses (Sikou et al. 2007). Taken together novel EM approaches are required to investigate whether the previously identified protein-linkages are composed of our four identified docking proteins. In the end, this will help to characterize the molecular mechanism of secretory vesicle docking in the proximity of Ca\textsuperscript{2+}-channels and relate this to release-readiness and how this positioning is controlled by the sub-membrane actin.

Concluding remarks

In conclusion, we show that docking is established between syntaxin-1/SNAP-25 acceptor complexes at the target membrane and synaptotagmin-1 on the vesicle membrane. Munc18-1 promotes the formation of a 1:1 syntaxin-1/SNAP-25 acceptor complexes and is capable of determining the number of docking sites by modulating sub-membrane F-actin. In addition to a role in docking, we observed that Munc18-1 plays a critical function in the downstream events that lead to membrane fusion. The importance of these docking proteins is illustrated by the fact that they are strongly linked to brain disorders, in particular neurodegenerative diseases (Ghiani et al. 2010; Greber et al. 1999) and mental illnesses like attention-deficits (McKee et al. 2010), and also associate with intelligence (Gosso et al. 2006). Currently, it is unclear how these docking proteins operate in synapses and regulate SV docking near Ca\textsuperscript{2+}-channels. In addition it is unknown how the unique composition of sub-membrane cytoskeleton may help to orchestrate this docking process. Advanced imaging experiments are required to unravel possible differences in docking phenotypes between chromaffin cells as well as neurons and determine whether the same docking
machinery is used during neurotransmitter release at neuronal active zones. This difference has probably evolved to keep docking sites available during certain stimulatory demands. Finally, these future studies will help to resolve how our four identified docking proteins orchestrate the docking process and will improve our understanding of neurological disorders linked to these docking genes in human brain.

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