Chapter 6

General discussion

Synaptic transmission depends critically on the SM-protein Munc18-1 across species [95, 264, 276]. The origin of this defect was traced back to a defect in docking and release of synaptic vesicles, indicating that Munc18-1 is an essential part of the synaptic vesicle fusion machinery. Further studies pointed towards a role of Munc18-1 throughout the entire process from docking to the final fusion event [90, 115, 242, 254]. The individual aspects of Munc18-1 function at various steps within the vesicle release cycle are however still under debate. These likely involve various posttranslational modifications and protein-protein interactions. Synaptic transmission has to be precisely timed and highly adaptable to changing circumstances, and the synaptic vesicle release machinery is under tight control to accomplish this. The aim of this thesis was to gain more insight in the various aspects of Munc18-1 function by investigating the consequence of specific Munc18-1 modifications on synaptic transmission. We made use of genetic mutagenesis to create small changes in the amino acid sequence of Munc18-1 designed to alter or mimic specific events. All Munc18-1 constructs were expressed in neurons from munc18-1 null mutant mice to assess their capability to support synaptic transmission.

In chapter 2, we studied the effect of alternative splicing of Munc18-1 premRNA. This generates two closely related protein splice variants, Munc18-1a and Munc18-1b, which differ at their far C-terminus. We concluded that while alternative splicing does not change basal synaptic transmission, it does influence the capacity of Munc18-1 to sustain synaptic transmission during repetitive stimulation. Our results also suggest that a potential CaMKII phosphorylation site unique to Munc18-1a might be partially responsible for this effect.

Next, we identified Munc18-1 as a novel target for the neuronal tyrosine kinase n-Src. Mutating the main phosphorylation site of this kinase in Munc18-1, Y473, led to a severe reduction in presynaptic strength, which could be temporarily restored by high frequency stimulation. Hence, tyrosine phosphorylation of
Munc18-1 might constitute a potent regulatory mechanism of synaptic strength (see Chapter 3).

Chapter 4 describes the function of a specific mode of interaction between Munc18-1 and Syntaxin1, the target membrane SNARE protein whose assembly into a SNARE complex with SNAP-25 and the vesicular Synaptobrevin is thought to drive vesicle fusion. We showed that point mutations in Munc18-1 which interfere with binding to the N-terminus of Syntaxin1 and assembled SNARE complexes did not hinder docking, priming and fusion of synaptic vesicles. Based on these results, we argue that the interaction between Munc18-1 and the N-terminus of Syntaxin1 is not essential for synaptic transmission.

Finally, we tested the ability of a Munc18-1 mutant containing a transposon insert in domain 3a to support synaptic transmission (Chapter 5). Synaptic Munc18-1 levels were reduced in this mutant, which could explain the increased rate of depression during stimulation trains. Whether reduced Munc18-1 levels also explain the defect in asynchronous release remains to be seen. Furthermore, spontaneous release was more strongly affected than evoked release, and recovery after stimulation was delayed. Taken together, these results implicate the involvement of domain 3a in several aspects of synaptic vesicle release.

Modifications of Munc18-1 thus generate diverse effects on synaptic transmission, indicating that Munc18-1 is involved in various steps of synaptic vesicle release. Each aspect of Munc18-1 function seems to be regulated by different protein-protein interactions and pre- or post-translational modifications. In the next paragraphs, the role of Munc18-1 in several features of synaptic transmission will be discussed. One paragraph is dedicated to protein phosphorylation as a potent and diverse way to regulate Munc18-1 function. Afterwards, a molecular model of synaptic vesicle release containing recent insights in Munc18-1 function will be given. Finally, future research directions will be discussed.

6.1 Munc18-1 regulates RRP size

Munc18-1 levels regulate the number of docked, release ready synaptic vesicles [255]. The results in this thesis support a role for Munc18-1 in determining synaptic strength by regulating the size of this readily releasable pool (RRP) (Fig. 6.1a). In this thesis, RRP size is calculated using cumulative synchronous charge transferred during an RRP-depleting action potential train (100 pulses at 40Hz). A linear correction is applied to correct for RRP refilling during these trains, which assumes uniform and maximal refilling [168, 222, 238]. Since refilling is accelerated by calcium [237], this method tends to underestimate RRP size. Direct comparison revealed that RRP estimates generated this way are $\approx 70\%$ of those generated using hypertonic sucrose application [238], a calcium independent technique used to define the RRP [210]. Lack of release at the start of and strong facilitation during trains made Y473 mutants unfit for RRP assessment using action potential trains. Therefore, depletion of the RRP by hypertonic sucrose
was used for these mutants. Since we compare between groups using the same method, RRP defects will not depend on the method used. Only mutations that affect refilling, like M18$_{L307}$, should be interpreted with caution since RRP defects might be masked. M18$_{L307}$ expressing neurons do show a trend towards a $\approx 35\%$ reduction in RRP size. Since this is similar to the RRP defect seen in Munc18-1 heterozygous neurons, reduced Munc18-1 levels might be responsible for the smaller RRP size in M18$_{L307}$ expressing neurons.

The largest RRP defect was found upon mutating Y473, the main target of the tyrosine kinase n-Src. However, the RRP size increased immediately after HFS, suggesting that the priming deficit in these neurons can be overcome by activity. Mutating Y473 thus seems to increase the threshold for priming, but does not disrupt priming itself. Munc18-1 might facilitate priming by assisting in the initial SNARE assembly, generating release ready vesicles. Since CAPS-1/2 DKO neurons show a very similar phenotype to the Y473 mutants [123], this aspect of Munc18-1 function might also depend on CAPS. CAPS proteins are suggested to stabilize vesicles in their primed state [123]. The finding that Munc18-1 can enhance the binding of CAPS to Syntaxin1 suggests that Munc18-1 might be able to regulate the association of CAPS to the release machinery [49]. Based on the strong RRP size defect in the Y473 mutants, it is tempting to speculate that tyrosine phosphorylation of Munc18-1 controls this aspect of Munc18-1 function.

### 6.2 Munc18-1 mutants show diverse asynchronous release phenotypes

Action potential trains trigger slow asynchronous release in addition to fast synchronous release coupled to individual action potentials (Fig. 6.1b) [87, 181]. Asynchronous release is driven by the accumulation of calcium in the presynaptic terminal during sustained stimulation. During intense stimulation, asynchronous release increases while synchronous release rapidly depresses [87]. Asynchronous release can recover much faster than synchronous release [215, 216, 282] and might even outcompete synchronous release for newly recruited vesicles [181], making asynchronous release more resistant to depression. These findings, together with the relative resistance of asynchronous release to sucrose-induced RRP depletion [181], show that immediate release of newly recruited vesicles is responsible for a large portion of asynchronous release during sustained stimulation [181]. Several Munc18-1 mutants tested in this thesis altered asynchronous release. Remarkably, while sustained stimulation is able to overcome the defect in asynchronous release in Munc18-1 Y473 mutants, another Munc18-1 mutant, M18$_{L307}$, shows a progressive asynchronous release phenotype (Fig. 6.1c). In the next paragraph, I will try to provide an explanation for this difference.

Sustained stimulation is able to overcome the defect in synaptic transmission
Figure 6.1: Munc18-1 regulates RRP size and synaptic transmission during stimulation trains. Effects of manipulations described in this thesis normalized to wild-type Munc18-1b. (A) Effect on EPSC and RRP size. Several manipulations reduce synaptic strength by reducing RRP size. (B) Example of synaptic currents elicited by action potential stimulation at 10 or 40Hz. The red line in response to 10Hz stimulation depicts the separation between synchronous and asynchronous release created by custom-written Matlab routines. (C-D) Progression of the asynchronous phenotype during (C) 10Hz or (D) 40Hz stimulation trains. Shown is the charge released in the mutant neurons as a percentage of the charge released in WT neurons per pulse in a train.
in the Y473 mutants, including asynchronous release, generating delayed but relatively intact release as stimulation progresses (Fig. 3.3c-e). The aspect of Munc18-1 function affected by mutating Y473 therefore probably lies in facilitating or stabilizing upstream processes like priming. During stimulation, these processes are facilitated by the accumulation of presynaptic calcium [172, 237] and the defect is restored. Interestingly, continued stimulation seems to lead to a progressive increase in asynchronous release compared to M18<sub>WT</sub> (Fig. 6.1c-d). This might also be reflected in the cumulative asynchronous charge, were a trend to a steeper slope can be observed (Slope #60-100: M18<sub>WT</sub>, 626 ± 98 pC/s, n=17; M18<sub>Y473A</sub>, 1034 ± 211 pC/s, n=19, unpaired t-test with Welch correction, p = 0.0930). This suggests that a rate-limiting process in M18<sub>WT</sub> cells might not be present or delayed in Y473 mutants. For example, vesicles might be prevented from fusion in M18<sub>WT</sub> cells to ensure the build-up of an RRP. Alternatively, Y473 mutants might have a larger availability of vesicles for asynchronous release due to the competition between synchronous and asynchronous release [181].

In contrast, the asynchronous release phenotype in M18<sub>L307</sub> expressing neurons is not restored during HFS, but seems to reach a stable level. This phenotype is quite pronounced, reaching ≈ 70% reduction in asynchronous charge released, while the initial EPSC only shows a trend towards a ≈ 30% reduction in size. Since asynchronous release probably depends to a large extent on calcium-enhanced recruitment and priming, a reduction in the supply capacity of newly primed vesicles might have a modest effect under basal conditions but a more pronounced effect during more demanding conditions like high frequency stimulation. In line with a reduced supply of newly primed vesicles, M18<sub>L307</sub> mutant neurons show faster depression of synchronous release and less recovery after HFS.

What causes the reduced priming capacity in the M18<sub>L307</sub> mutant? The transposon insert in M18<sub>L307</sub> is located adjacent to a flexible helix in domain 3a, a domain suggested to be involved in SNARE complex interactions [106]. Restrictions in the flexibility of this domain caused by the transposon insert might render M18<sub>L307</sub> less efficient in priming. However, the fact that synaptic Munc18-1 levels are reduced in neurons expressing M18<sub>L307</sub> (to 50% of M18<sub>WT</sub>) provides a plausible alternative explanation. An effect of Munc18-1 levels on asynchronous release might have been previously overlooked [255]. A limited amount of Munc18-1 protein available for priming would explain a lower supply capacity of primed vesicles. Nonetheless, we cannot exclude that increased affinity for Mint, as reported for this mutant [81], might contribute to the vesicle supply defect, for example by inhibiting the clearing of release sites after use (see chapter 5).

### 6.3 Munc18-1 modulates Pvr

Mutating Y473 in Munc18-1 reduced the amount of vesicles released upon stimulation beyond what could be explained by a reduction in RRP size (chapter 3).
These results suggest that Munc18-1 not only regulates the amount of release ready vesicles, but also the probability that these vesicles will be released (vesicular release probability, Pvr). In line with a reduced Pvr, Y473 mutants display strong paired-pulse facilitation. Immediately following intense activity, synaptic responses in Y473 mutant neurons are strongly augmented. A large increase in Pvr is mostly responsible for this augmentation, indicating that high frequency stimulation can restore the defect in release probability.

CAPS-1/2 DKO neurons also display strong facilitation of EPSC size during repetitive stimulation and strong post-train augmentation [123]. However, Pvr is unaffected in these neurons. In addition, post-train augmentation in CAPS-1/2 DKO neurons is mainly due to an increase in RRP size, not in Pvr as is the case for Munc18-1 Y473 mutants. These differences suggest that the underlying mechanism causing the augmentation in these neurons might be different. On the other hand, Y473 mutants might disrupt several aspects of Munc18-1 function, one of which might be to stabilize primed vesicles via the recruitment of CAPS.

What could be the molecular mechanisms behind the role of Munc18-1 in regulating RRP and Pvr? One possibility is that a single aspect of Munc18-1 function regulates both. By facilitating SNARE complex assembly, Munc18-1 might not only facilitate priming but also, by increasing the number of transSNARE complexes associated with a single vesicle, increase Pvr [167]. A second possibility is that the reduced Pvr in Y473 mutants is the result of a compensatory mechanism triggered to prevent further RRP depletion. Such a mechanism has been observed previously in endocytosis defective dynamin mutant neurons [150]. Dynamin deficient synapses not only have a smaller RRP size due to impaired endocytosis, they also show a CaMKII-dependent decrease in Pvr. These results suggest the existence of a mechanism aimed at preventing synaptic transmission failure when vesicle supply is limited by reducing exocytosis. Finally, Munc18-1 might regulate the fusion barrier. In the next section, this concept and a possible role of Munc18-1 will be explained.

To allow rapid and synchronous fusion, vesicles need to be on the verge of fusion but actual fusion needs to be prevented until the appropriate trigger arrives. This led to the hypothesis of a fusion clamp that imposes an energy barrier and acts as a brake on fusion, for example by arresting partially zippered SNARE complexes [236]. Several proteins have been suggested to regulate or perform such a function, including Complexin, Munc13 and Synaptotagmin [10, 43, 146]. A fusion clamp could also be an intrinsic property of SNARE complex assembly. Based on the effects of several SNAP-25 mutants on synaptic transmission, tight N-terminal SNARE assembly was recently hypothesized to form an obstacle for further C-terminal assembly [274]. Munc18-1 might influence Pvr by regulating such a fusion barrier, potentially by influencing the tightness of N-terminal assembly or by regulating the association of a fusion clamp.
6.4 Munc18 mutants show a disproportionate effect on spontaneous release

One interesting observation is that the effect of several Munc18-1 mutations studied in this thesis is larger on spontaneous release than on action potential evoked release (Fig. 6.2a). A similar trend can be seen in other Munc18-1 manipulations previously tested in this lab (Fig. 6.2b). Meta-analysis of autaptic literature shows that the majority of studies report a proportional effect of perturbations on both types of release, often by regulation of RRP size [48]. Interestingly, a disproportionately large effect on spontaneous release has also been reported for proteins discussed above in the context of a fusion barrier (Fig. 6.2c) [10, 76, 274, 287, 288, 290]. In addition, a predominantly open form of Syntaxin, which is supposed to render vesicles more fusogenic, increases spontaneous release to a larger extent than evoked release.

How can such a disproportionate effect be explained? An allosteric model of calcium-activated fusion has been proposed to accommodate the fact that phorbol esters potentiate release rates to a larger extend in low calcium compared to high calcium concentrations [152]. This model introduces the term ‘fusion willingness’ to describe an intrinsic property of the molecular release machinery which drives fusion in the absence of calcium. Increasingly higher release rates can be obtained when more calcium binds to calcium-sensors within the release machinery, like Synaptotagmin. Vesicles will thus have a certain Pvr depending on their intrinsic fusion willingness and the actions of calcium-bound proteins. According to this model, increasing the fusion willingness of a vesicle will have a larger impact on Pvr in low calcium compared to high calcium concentrations (Fig. 6.2d). Since spontaneous release occurs in lower calcium concentrations than evoked release, regulating fusion willingness will have a larger impact on spontaneous release.

Further support for the hypothesis that a disproportional effect on spontaneous release might be the result of changed fusion willingness is provided by studies testing mutant variants of Munc13 and Syntaxin1. These studies show that M13-1(H567K) or Syx1(open) not only increase the rate at which vesicles are released spontaneously but also cause a faster response to hypertonic sucrose, which is a calcium-independent way to trigger vesicles release [10, 76]. The speed at which vesicles fuse in response to the osmotic challenge is thought to reflect their fusion willingness. One way to regulate fusion willingness is to impose an energy barrier for fusion. In the previous section, we suggested that Munc18-1 might influence this fusion barrier. The fact that some Munc18-1 mutants have a disproportionately large effect on spontaneous compared to evoked release further supports this hypothesis.

The observed disproportional effect on spontaneous release might also result from underestimation of effects on evoked release. Autaptic neurons generate a large current upon stimulation, which is susceptible to receptor saturation or loss of current due to simultaneous activation of proximal and distal synapses. This
could produce a ceiling effect masking the true difference between experimental groups on evoked release.

**Figure 6.2:** Manipulations in Munc18-1 effect spontaneous release to a larger extend than evoked release. (A-B) Effect of manipulations in Munc18-1 on spontaneous release (mEPSC frequency) and action potential triggered release (EPSC size) tested (A) in this thesis or (B) in previous studies using autaptic cultures. (C) Effect of manipulations in other components of the synaptic vesicle release machinery on spontaneous release (mEPSC frequency) and action potential triggered release (EPSC size) [10, 76, 274, 287, 288, 290]. (D) Effect of increasing release willingness (red line) in an allosteric model of calcium triggered vesicle fusion. Shown is the predicted vesicular release rate as a function of intracellular calcium concentration (adapted from [152]).

6.5 Regulation of presynaptic strength by Munc18-1 phosphorylation

Protein phosphorylation is a powerful regulatory tool due to its reversible nature and the wide variety and specificity of kinases and phosphatases. Indeed, many
components of the synaptic vesicle release machinery are regulated by phosphorylation [52]. Chapter 3 shows that the neuronal tyrosine kinase n-Src can phosphorylate Munc18-1 at site Y473 in vitro. Mutating Y473 causes severe priming and release probability defects. These results led to the hypothesis that tyrosine phosphorylation of Munc18-1 might be a powerful tool to regulate synaptic strength. Munc18-1 is a known target for several other kinases, including threonine/serine kinases PKC, ERK and Cdk5. In the following section, the circumstances leading to Munc18-1 phosphorylation by these kinases and the effects on synaptic transmission will be discussed.

![Figure 6.3: Posttranslational modifications on Munc18-1 can bi-directionally manipulate synaptic strength by regulating RRP size. (A) Effect of several non-phosphorylatable Munc18-1 mutants on EPSC and RRP size in autaptic cultures (normalized to Munc18-1 KO neurons expressing Munc18-1bWT). (B) High cross-talk between kinases.](image)

Each kinase targets Munc18-1 at a unique site, allowing kinase specific mutants to be created. Non-phosphorylatable versions of Munc18-1 show that phosphorylation of Munc18-1 by different kinases seems to regulate synaptic strength by increasing (tyrosine kinases and Cdk5) or decreasing (ERK) RRP size (Fig. 6.3a). Preventing phosphorylation of Munc18-1 also affects short-term plasticity, causing more depression in the PKC mutant, less depression in the Cdk5 mutant and strong facilitation instead of depression in the Y473A mutant.

Kinases are activated by different signalling cascades and with different timescales. Since most non-phosphorylatable mutants have an effect on the initial EPSC size, a subset of Munc18-1 is likely to be phosphorylated at rest. The proportion of phosphorylated Munc18-1 will increase once specific intracellular pathways are activated. For example, DAG-signalling and intense stimulation will enhance PKC phosphorylation of Munc18-1 which leads to larger EPSCs [278]. ERK-dependent phosphorylation of Munc18-1 occurs in response to increased activity and fear conditioning paradigms and will trigger the recruitment of the E3 ligase Fbxo41 and subsequent ubiquitination and proteasomal degradation of Munc18-1 (Schmitz et al., submitted). ERK phosphorylation of Munc18-1 is involved in the initial phase of cannabinoid induced synaptic depression, which acts on a timescale of minutes after application of a cannabinoid receptor agonist.
(Schmitz et al., submitted). These results show that increased activity can lead to phosphorylation of Munc18-1 by both PKC and ERK, but on different timescales and with different outcomes. PKC phosphorylation will acutely enhance synaptic transmission while phosphorylation by ERK is slower and probably is involved in homeostatic plasticity. Cdk5 in turn is activated upon presynaptic neuronal silencing [165], but its upstream signalling pathway is not identified.

To add to the complexity to this picture, many signalling pathways show a high degree of cross-talk. For example, the activation of ERK by NGF requires the Src tyrosine kinase family member c-Src [173], but is inhibited by Cdk5 [298]. Cdk5-dependent phosphorylation of Src leads to ubiquitination and subsequent degradation of Src [182]. PKC in turn activates Src upon DAG stimulation [27], stabilizes the Cdk5 activator p35, which is required for the activation of Cdk5 by BDNF [295] and activates the ERK pathway [258]. These examples illustrate the complexity of intracellular signalling (Fig. 6.3b). Taken together, a single signal might trigger a specific temporal pattern of activation of several kinases, which could converge on a single protein like Munc18-1 to regulate presynaptic strength.

### 6.6 Molecular mechanism of synaptic vesicle release: role of Munc18-1

Based on recently published findings and the results in this thesis, we propose a model in which Munc18-1 is an essential player of the synaptic vesicle release machinery guiding SNARE complex formation through most of its cycle. By acting on monomer Syntaxin, acceptor complexes and possibly Synaptobrevin, Munc18 steers the release machinery towards efficient N-terminal SNARE assembly, thereby regulating docking, priming and fusion willingness.

As an initial step, Munc18-1 is targeted to the active zone by its strong affinity for closed Syntaxin, were it establishes the right conformation of Syntaxin to assemble into an acceptor complex together with SNAP-25. This may involve rotation in domain 1 of Munc18-1, which will twist the Habc domain of Syntaxin away from the H3 domain allowing this domain to enter SNARE complexes [106]. Synaptic vesicles are then able to dock to the active zone via the association of Synaptotagmin with the acceptor complex [55]. Munc18-1 is a limiting factor in this process, since the number of docked release-ready vesicles strongly dependents on Munc18-1 levels [255]. Y473A mutants had fewer docked synaptic vesicles while expression levels were unchanged. It is tempting to speculate that tyrosine phosphorylation of Munc18-1 at this residue facilitates the formation of acceptor complexes or their association with Synaptotagmin.

Munc18-1 also has an essential post-docking role. This is supported by the fact that the secretion defective Y473D mutant showed normal docking. Munc18-1 probably acts on the acceptor complex making it accessible to Synaptobrevin,
thereby facilitating the initial N-terminal SNARE assembly and creating partially zippered, trans-SNARE complexes. This likely involves conformational changes in Munc18-1, for example in domain 3a [106], and is regulated by Munc13 [154, 262]. The large reduction in RRP size in both Y473 mutants suggests a role for this site in priming. Located on the outer surface between domain 3a and 3b, Y473 might be involved in regulating domain 3a flexibility. Cdk5 and ERK phosphorylation also regulate this aspect of Munc18-1 function.

By facilitating SNARE assembly, Munc18-1 also regulates the release willingness of primed vesicles, potentially by regulating the number of SNARE complexes associated to a single vesicle or by regulating the tightness of N-terminal SNARE assembly. Tight N-terminal assembly might pose a barrier towards C-terminal SNARE assembly [274]. PKC phosphorylation of Munc18-1 within the flexible helix of domain 3a might promote this aspect of Munc18-1 function, thereby increasing spontaneous and evoked release [278]. After partial trans-SNARE complexes are formed and the fusion clamp is in place, vesicles are release-ready and the continued association of Munc18-1 to the fusion machinery is no longer required. Upon stimulation, calcium binding to Synaptotagmin will trigger the final fusion event. Synaptotagmin might release the fusion clamp or reduce the energy barrier for fusion in some other manner, e.g. by coupling the energy from calcium binding to the SNARE complex zippering or by membrane modulation [170].

Other aspects of Munc18-1 function, which do not directly involve the SNARE proteins, might also be envisioned. For example, Munc18-1 might regulate the association of CAPS, which stabilizes priming by preventing vesicles from returning to an un-primed state [123]. In addition, Munc18-1 might allow a fusion clamp, for example Complexin, to associate to the SNARE complex [110, 274], thereby regulating fusion willingness.

### 6.7 Future directions

One remaining issue within this thesis is whether the severe defects on synaptic transmission in the Y473 mutants are caused by a disrupting of the tyrosine phosphorylation cycle or not. Identification of upstream events leading to the alleged Munc18-1 phosphorylation would greatly aid in resolving this issue. Since chapter 3 shows that the neuronal SFK member n-Src is capable of targeting Munc18-1, at least in vitro, focus should be directed at SFK activation pathways linked to presynaptic transmission. This would include Trk receptor signalling via neurotrophins or Zinc, cAMP and DAG signalling, integrin signalling via extracellular matrix molecules like Reelin, and Insulin signalling (Fig. 1.4; SFK activation pathways are reviewed in detail in chapter 1). In order to better direct this search, other Src family members like Fyn and c-Src and other tyrosine kinase families expressed in the central nervous system should be screened for their ability to phosphorylate Munc18-1. Insulin receptor tyrosine kinases are of special interest since they are known to target Munc18-3, a ubiquitously expressed isoform of
Munc18-1 [4, 122]. Tyrosine phosphorylation of Munc18-3 is important in exocytosis in adipocytes and regulates Syntaxin binding [122]. A similar mechanism might underlie Munc18-1 driven exocytosis in neurons.

Once found, this upstream pathway could be activated in neurons expressing wild-type or non-phosphorylatable Munc18-1 to study effects of acute phosphorylation of Munc18-1 on synaptic transmission. A potential pitfall of this approach is the large defect in synaptic transmission caused by the non-phosphorylatable mutant, which renders this mutant unfit as negative control. Alternatively, tyrosine phosphorylation of Munc18-1 could be prevented by disrupting the kinase-binding site on Munc18-1. Src homology 3 (SH3) domains bind proline-rich sequences containing a PXXP motif close to arginine for specificity [1, 211]. Munc18-1 contains such a sequence (R235 . . . P239, S240, S241, P242), which is of particular interest since it comprises the main ERK phosphorylation site (S241). This would provide a link between ERK and Src phosphorylation of Munc18-1, although this is purely speculative.

Several kinases converge on Munc18-1 in response to changes in activity, although probably on different timescales. Nonetheless, while we currently only have information on the consequence of phosphorylation at a single site, multiple sites within Munc18-1 might be phosphorylated simultaneously. To gain more insight in the temporal pattern of Munc18-1 phosphorylation, it would be very informative to monitor kinase activity in live neurons with high spatial and temporal resolution. Genetically encoded fluorescent reporters of kinase activity have been created for this purpose, including reporters for CaMKII, ERK, PKA, PKC and Src activity [139, 166, 249]. Promising in this respect is the recent generation of transgenic mice expressing FRET biosensors for PKA and ERK [131]. Live imaging of protein kinase activity will no doubt lead to exciting new insights, especially when combined with electrophysiological recordings to monitor synaptic output.

A limitation of the current approach of using non-phosphorylatable and phosphomimicking mutants is that only chronic effects of phosphorylation can be studied. It could be that not the phosphorylated state itself, but the dynamic force of switching between states is vital for Munc18-1 function. It would be a huge technical advantage if phosphorylation states could be dynamically modulated. Recent developments in optogenetics enable the dynamic control of protein interaction and intracellular signalling cascades by using light-controlled association and dissociation of plant phytochromes and phytochrome-interacting factors [137]. When applied to neurons, this technique might enable researchers to dynamically manipulate kinase activity. The same also holds for the dynamics of protein-protein interactions.

Furthermore, the molecular action behind the role of tyrosine phosphorylation of Munc18-1 should be probed. As suggested above, the current hypothesis assumes the involvement of tyrosine phosphorylation of Munc18-1 in vesicle priming by promoting SNARE assembly. The resemblance between Munc18-1 Y473 mutants and CAPS-1/2 DKO or Munc13-1 KO cells asks for cross-rescue experiments. In addition, it would be of interest to probe whether the Y473 mutants are
still capable of increasing the binding between CAPS and Syntaxin. Furthermore, if the SNARE assembly promoting function of Munc18-1 is compromised in neurons expressing M18Y473 mutants, promoting SNARE assembly in other ways, for example by expression of the Synaptobrevin C-terminal fragment [191], should restore synaptic transmission.

In chapter 4 we show that interfering with Syntaxin1 N-peptide binding to Munc18-1 has no discernible effect on synaptic transmission in mammalian neurons. However, a recent study using rescue experiments in syntaxin-deficient neurons came to the opposite conclusion, namely that the Munc18-1/N-peptide interaction is essential for synaptic transmission [299]. These results add to the controversy about the function of Munc18-1/Syntaxin interactions, which has already resulted in various hypotheses depending on the model organism studied or the experimental design used. There are several possible explanations for these apparently contradictory results. First of all, the N-peptide of Syntaxin might have an additional role in synaptic transmission, which does not involve binding Munc18. This might be resolved by introducing the N-peptide in neurons expressing Munc18-1 mutants that interfere with N-peptide binding (M18L130K or M18F115E). The peptide will compete with endogenous Syntaxin1, and potential effects could be attributed to binding partners other than Munc18-1, although it will be difficult to control for a-specific effects of the peptide. Second, other interactions might take over the function of the N-peptide interaction and hold Munc18-1 close to assembling SNARE complexes, like interactions with Synaptobrevin or the partly assembled SNARE bundle [106, 226, 286]. It would be insightful to combine Syntaxin1 N-peptide deletion mutants with M18L130K or M18F115E. Third, conserved proteins can behave very differently across organisms due to small changes in the relative strength/contribution of different domains, which has been observed for mouse and fly Complexin [288]. Such a different contribution of the Munc18-1/N-peptide interaction could account for the divergent effects of disrupting this interaction in mouse and worm Munc18-1 [31, 124, 161].

To summarize, this thesis has contributed to increase knowledge on several aspects of Munc18-1 function and has shown that Munc18-1 is an important part of the synaptic vesicle release machinery. Munc18-1 is involved in the initial vesicle docking step as well as in priming and regulating the release probability of vesicles. Further research will be needed to complete the picture of Munc18-1 function in exocytosis and should preferably include dynamic modulation of post-translational modifications and live imaging of these events.