Chapter 1

General introduction

1.1 Synaptic transmission

The human brain is a complex and plastic organ. It controls your actions and perception, memory storage/retrieval and executes higher cognitive functions like working memory and problem-solving. To provide the computational power required to manipulate and integrate the information needed to perform these functions, the brain consists of a huge network of nerve cells, called neurons. The 100 billion \((10^{11})\) neurons that make up a single brain make 100 trillion \((10^{14})\) contacts, or synapses, with each other. These synapses consist of a presynaptic site at the sending neuron, a synaptic cleft which constitutes a physical space between two neurons, and a postsynaptic site at the receiving neuron. To bridge the synaptic cleft, the electrical signal used to rapidly send information across a neuron has to be converted to chemical signal consisting of neurotransmitter stored in synaptic vesicles. To obtain the high temporal resolution required to rapidly and faithfully transmit information, the release cycle of these vesicles is under tight control (for review on the synaptic vesicle cycle see [240]). A schematic view of the synaptic vesicle cycle is depicted in Fig. 1.1. Most synaptic vesicles are stored in a reserve pool, while only a subpopulation \((\approx 5\%)\) is docked at a specialized site on the cell membrane called the active zone [219]. Here, vesicles undergo a maturation process called priming, which converts them into fusion competent or primed vesicles. The arrival of an action potential will trigger the opening of voltage-gated calcium channels resulting in local and transient elevation of intracellular calcium. This will trigger the fusion of primed vesicles with the target membrane resulting in the release of their cargo in the synaptic cleft. The strength of a presynaptic connection is determined by the amount of neurotransmitter released upon stimulation. Presynaptic strength is a function of the release probability of primed vesicles (vesicular release probability (Pvr)), the total number of primed vesicles (the readily releasable pool (RRP)) and the amount
Figure 1.1: *The synaptic vesicle cycle.* Synaptic vesicles are recruited from a reserve pool, translocated to and docked at the active zone (AZ) and primed to a fusion-ready state. Calcium influx caused by the arrival of an action potential stimulates synaptic vesicle fusion. Released neurotransmitters then bind to receptors at the postsynaptic density (PSD), causing them to open. This will generate a postsynaptic current, converting the chemical signal back into an electric signal. Empty vesicles are recycled by endocytosis.

The aim of this thesis is to disclose different aspects of the Sec1/Munc18 (SM) protein Munc18-1, an essential component of the synaptic vesicle release machinery [95, 264, 276], in regulating presynaptic strength. In this introduction, a theoretical framework is provided. Synaptic contacts are plastic, meaning they can get stronger or weaker based on previous information that entered the neuron, enabling synapses to store information about previous activity. Use-dependent changes in presynaptic strength can occur within milliseconds lasting up to several minutes, a process referred to as presynaptic short-term plasticity (STP). Several forms of STP are described in the following section. Next, a mechanistic view on the synaptic vesicle release machinery is provided with the focus on Munc18-1 function. Finally, two ways in which Munc18-1 function might be regulated are described, namely alternative splicing and tyrosine phosphorylation.
1.2 Presynaptic short-term plasticity

Several forms of STP are known, including synaptic depression and synaptic enhancement, of which the latter can be further subdivided into facilitation, augmentation and post-tetanic potentiation (PTP) [70, 199, 301]. While facilitation occurs between subsequent pulses and lasts for milliseconds to seconds, PTP refers to the increase in synaptic strength lasting tens of seconds to minutes following high-frequency stimulation. Augmentation is closely related to PTP but has a shorter duration and is more easily evoked than PTP, although the two can be hard to distinguish [199]. Different forms of STP often coexist and presynaptic strength changes as a net result of their combined effect. Presynaptic mechanisms also contribute to long/term plasticity, lasting for minutes to hours, but these effects are outside the scope of this thesis (for review, see [39]).

1.2.1 Paired pulses

STP can be induced by two closely spaced stimuli with intervals ranging from milliseconds up to seconds. Paired-pulse plasticity can be facilitating or depressing depending on the initial release probability [199]. Neurons are more prone to depression when the initial release probability is high, which results in a large fraction of the RRP being released during the first stimulus, rendering fewer vesicles available during the second pulse. In contrast, low initial release probability usually leads to facilitation caused by the build-up of intracellular calcium, also termed ‘residual’ calcium, which raises the release probability during the second stimuli as originally proposed by [132].

1.2.2 Repetitive stimulation

Synaptic depression mainly occurs during periods of high activity, when vesicles from the RRP are used faster than they are replenished. This is referred to as the depletion model of depression [199, 301]. However, this model cannot explain the extent of synaptic depression at all synapses, suggesting the existence of other depression inducing mechanisms like inactivation of release sites and depletion of the reserve pool [70, 199]. Recovery from depression occurs typically within seconds and is accelerated by elevations of intracellular calcium [105].

Residual calcium levels increase during repetitive stimulation, which raises the release probability of remaining vesicles and accelerates vesicle replenishment (for review, see [172]). The time course of the calcium signal is dependent on many factors, including endogenous calcium buffer capacity, uptake and release from intracellular calcium stores and activity-dependent modulation of calcium influx [70, 268, 284]. Furthermore, activity dependent modulation of presynaptic potassium channels, which shapes the action potential, has been implicated in synaptic plasticity [268].
Presynaptic strength can also be modulated by intracellular signaling cascades activated via presynaptic receptors or by repetitive stimulation (for review, see [52]). For example, synaptic depression can be induced by retrograde endocannabinoid signaling triggered by postsynaptic calcium elevation (depolarization-induced suppression of inhibition/excitation (DSI/DSE)) [59]. Another powerful example is diacylglycerol (DAG) signaling, which temporarily potentiates synaptic transmission by acting on two essential components of the release machinery, either directly in the case of Munc13 or indirectly via DAG-activated PKC for Munc18 [10, 151, 278]. Furthermore, recent insights led to the recognition of membrane residing lipids and their metabolites as important regulators of synaptic transmission [208]. A role for sphingosine in regulating the interaction between Munc18-1 and Syntaxin has been suggested [34].

1.3 Properties of neurotransmitter release

In response to an action potential (AP), neurotransmitter release occurs in a fast synchronous manner triggered by the short-lived local calcium influx through voltage-gated calcium channels. In hippocampal neurons, this type of fast release requires Synaptotagmin1 [75]. An asynchronous component however still persists in these neurons. Under normal conditions, this asynchronous component can be observed as a smaller but longer-lived increase in release rate following synchronous release, also known as delayed or asynchronous release [5, 9]. During intense activity, synchronous release declines while asynchronous release increases (compare responses at the start to those at the end of the train in Fig. 1.2a) [87, 180]. Asynchronous release is attributed to the buildup of residual calcium during stimulation and its relative resistance to RRP depletion suggests that asynchronous release reflects the immediate release of newly recruited vesicles [5, 180]. While synchronous release is essential for rapid communication, asynchronous release can act on a longer timescale and might provide long-lasting
inhibition or prolong postsynaptic spike activity in response to intense stimulation [100, 112]. Neurotransmitter release also occurs in the absence of stimulation, which is referred to as spontaneous release (Fig. 1.2b [68]). Spontaneous release is classically viewed as a byproduct of highly tuned synchronous release, but recent findings that spontaneous and evoked release can be differentially regulated, challenge this view and suggest that spontaneous release might function in synaptic homeostasis or maintaining connectivity [133, 196].

The function and origin of the different types of release are heavily debated. The most simplistic and commonly accepted idea is that they all originate from the same pool of synaptic vesicles (but see [133, 195] for opposing views). Calcium dynamics and the presence of multiple calcium sensors with a specific calcium affinity controlling release likely contribute to the differences in calcium sensitivity between release modes [75, 83, 172, 243, 270, 294]. In addition, heterogeneity in release kinetics of vesicles has been attributed to their distance from calcium channels [221, 269], referred to as 'positional priming', their molecular composition and/or maturation stage [215, 280, 282], referred to as 'molecular priming'.

1.4 The synaptic vesicle release machinery

Synaptic vesicle release is regulated by a specific set of proteins designed to execute regulated exocytosis. Although specialized for neuronal signaling, the fusion machinery of synaptic vesicle release uses the same basic principles as that of other secretory cells like neuroendocrine cells, or of intracellular membrane fusion reactions, which organize intracellular communication between organelles in eukaryotic cells. The following sections discuss the core proteins of the mammalian synaptic vesicle release machinery.

1.4.1 SNARE theory

The first clue about the proteins involved in synaptic vesicle release came from the identification of the SNARE proteins Syntaxin1, SNAP-25 and Synaptobrevin2 as targets for several neurotoxins that enter the presynapse and block synaptic vesicle release by cleaving these proteins [18, 19, 149, 218]. Their identification was quickly followed by the discovery that these proteins form a complex, the so-called SNARE complex [235], which together with their localization (Syntaxin on the synaptic membrane and Synaptobrevin on the synaptic vesicle) led to the hypothesis that SNAREs may form a universal vesicle fusion apparatus [94]. This was the start of a vast body of research directed to understand SNARE-driven membrane fusion (for review see [118, 204]). According to the SNARE theory, the formation of a four-helix bundle composed of the SNARE domains of these proteins exerts a pulling force on opposing membranes, which fuels membrane fusion (Fig. 1.3a). Current estimates suggest that assembly of at least two to three SNARE com-
plexes can provide the energy required to induce calcium-evoked vesicle fusion, and that heterogeneity in SNARE complex number associated with a single vesicle may contribute to heterogeneity in Pvr [167, 233]. Originally, SNARE proteins were considered the minimal membrane fusion machinery and thought to ensure vesicle-to-target specificity. However, SNARE assembly appeared quite promiscuous due to the high degree of structural conservation, enabling assembly of SNAREs from different organelles [118]. Furthermore, mammalian synaptic vesicle fusion does not occur in the absence of other key proteins, including Munc13 and the SM-protein Munc18-1 [262, 264]. At present, SM-proteins have been included as central components of the release machinery acting together with SNAREs across all steps leading to fusion [37, 242, 254].

1.4.2 Munc18-1

Screens for binding partners of the neuronal SNARE proteins led to the discovery of a 67kD mammalian brain protein, which tightly binds Syntaxin1 [97]. Since this protein was encoded by a homologue of the C. elegans gene unc-18, a gene involved in neurotransmitter release, it was named Munc18 (Mammalian homologue of unc-18). The same protein was identified in parallel as a mammalian homologue of the yeast Sec1 protein involved in exocytosis, which explains why Munc18 is also referred to as neuronal (n)Sec1 or rat brain (rb)Sec1 [73, 188]. There are three mammalian Munc18 isoforms expressed, of which only Munc18-1 is highly expressed throughout the brain [73, 98, 188, 245]. Munc18-1 belongs to the Sec1/Munc18(SM) protein family, of which several additional members involved in intracellular trafficking are expressed in mammals [251].

Munc18-1 is predominantly localized in the axon and is expressed at various degrees in different brain areas [74, 254]. Deletion of Munc18-1 in mice leads to a complete loss of neurotransmitter release [264], and similar results have been obtained in worm and fly model organisms [95, 276]. Since then, a large number of studies combined to the current view of Munc18-1 as a central component of the vesicle release machinery acting from the early steps of vesicle docking up through the final release event [37, 242, 254]. In addition, the recent discovery of several mutations in the munc18-1 gene in patients with severe epilepsy and intellectual disability together with the implication of Munc18-1 dysregulation in Alzheimer’s disease and schizophrenia highlight the importance of Munc18-1 for normal brain function [12, 38, 62, 78, 88, 89, 104, 114, 185, 213, 214]. Furthermore, Munc18-1 over-expressing transgenic mice show anxiety-like and schizophrenia-like behavior, including aversion to unprotected spaces, decreased social interactions and decreased prepulse inhibition of the acoustic startle reflex [260]. However, exactly how Munc18-1 executes all aspects of its function and how those aspects can be regulated is still under debate. The next section provides an overview of the current knowledge on Munc18-1 function followed by the proposed underlying molecular mechanism.
A

Syntaxin1

SNAP-25

Synaptobrevin2

B

C1

C2

C3

D1

D2

D3

Figure 1.3: SNAREs and Munc18 (A) The zippering of the four SNARE motifs from Syntaxin1, SNAP-25 and Synaptobrevin2 into a tight SNARE complex pulls the vesicle and target membranes together, initiating membrane fusion. (B) Crystal structure of Munc18-1 represented as a ribbon with domains 1-3 in blue-green-yellow respectively (adapted from (Misura, Scheller et al. 2000)). The flexible helix in domain 3 is shown in orange. (C) Different modes of Munc18-1/SNARE interactions. Munc18-1 interacts with monomer Syntaxin (closed or open, C1), with Syx1a/SNAP-25 acceptor complexes (C2) and to the fully assembled SNARE complex (via the N-peptide or directly to the SNARE bundle, C3).
**Vesicle docking**

It is undisputed that Munc18-1 plays a role in the initial docking step, where vesicles attach to the active zone, in concert with Syntaxin, SNAP-25 and Synaptotagmin [55]. Varying Munc18-1 expression levels results in parallel changes in the amount of docked vesicles in mice chromaffin cells and neurons [252, 255, 267, 276]. Dynamic analysis of the docking phenotype using total internal reflection fluorescence (TIRF) microscopy revealed that Munc18-1 is required for efficient vesicle delivery to and long-term retention at the target membrane [252]. Munc18-1 mediated regulation of the actin cytoskeleton, which forms a physical barrier preventing vesicles from accessing release sites, has been suggested as underlying mechanism for the effect of Munc18-1 on vesicle delivery [140]. Docking can be reinstated in the absence of Munc18-1 by stabilizing Syntaxin1-SNAP-25 acceptor complexes or by manipulating actin [55, 252], but these docked vesicles are unable to fuse. This provides strong evidence that, at least in chromaffin cells, Munc18-1 has an additional role after docking and is required to produce fusion competent vesicles. In both chromaffin cells and neurons, expression of the closely related isoform Munc18-2 on a munc18-1 null background rescued docking in excess of priming ([54, 86], Wierda et al., unpublished data), again hinting at a postdocking function of Munc18-1.

**Guiding SNARE assembly**

The ability of Muc18-1 to produce fusion competent vesicles has been supported by in vitro studies assessing the function of Munc18-1 using liposome fusion assays. A number of recent studies reported an accelerating effect of Munc18-1 in SNARE-driven liposome fusion [60, 207, 223, 226, 227], suggesting that Munc18-1 might guide SNARE complex formation. Conversely, SNARE assembly assays show that Munc18-1 strongly inhibits SNARE complex formation by sequestering Syntaxin [31, 154, 188, 203]. This discrepancy was resolved when Munc18-1 was shown to be capable of both inhibiting and stimulating SNARE-dependent fusion of liposomes depending on the presence of VAMP2 containing liposomes during incubation [223]. Furthermore, a recent study shows that Munc18-1-Syntaxin dimers can form an efficient and NSF-α-SNAP resistant starting point for SNARE assembly [155]. The authors convincingly argue that Syntaxin-SNAP-25 heterodimers are a poor starting point for SNARE complex formation due to their tendency to form a heterogeneous mixture of complexes and the ability of NSF and α-SNAP, proteins that recycle SNAREs for subsequent rounds of fusion, to disable these complexes in an ATP-dependent manner. In this view, Munc18-1 sequesters Syntaxin from SNAP-25, thereby seemingly inhibiting SNARE assembly, after which the fusion machinery can enter a more efficient pathway towards SNARE assembly.
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1.4.2 Short-term plasticity

Varying Munc18-1 levels not only lead to concomitant changes in the number of docked vesicles and the size of the RRP, but also influence the rate of depression during stimulation and the speed at which synapses recover [255]. RRP size and short/term plasticity are also regulated by phosphorylation of Munc18-1 by ERK or Cdk5 (Schmitz et al., unpublished results). Furthermore, the phosphorylation of Munc18-1 by PKC is essential in DAG-induced augmentation of release rates, and expression of a PKC-insensitive Munc18-1 mutant leads to faster synaptic depression [278].

1.4.3 Fusion pore dynamics

Expression of several Munc18-1 mutants in neuroendocrine cells influences the release kinetics of single vesicles, suggesting a role for Munc18-1 in controlling the dynamics of the fusion pore through which the vesicle content is released [45, 71]. Further research in lactotrophs (prolactin releasing cells in the anterior pituitary) shows that several Munc18-1 mutants increase the fusion pore dwell time [125]. This aspect of Munc18-1 function takes place after fusion pore opening and might involve direct or indirect regulation of membrane curvature, which stabilizes the fusion pore. Whether this aspect of Munc18-1 function also occurs in neurons is unknown.

1.4.4 Neuronal survival

Munc18-1 is essential for neuronal survival. In culture, neurons lacking Munc18-1 die within seven days [99]. This is most likely not a secondary effect of a lack of synaptic transmission, since Munc13-1/2 double knockout (DKO) neurons, that also show a complete lack of synaptic transmission, do survive in culture [262]. In addition, deletion of munc18-1 in a subset of neurons, both in vitro and in vivo, resulted in specific degeneration of these neurons, suggesting that these neurons die as a direct consequence of Munc18-1 deficiency, and not of a lack of synaptic input or trophic support [99]. Why lack of Munc18-1 causes neurodegeneration is unknown.

1.4.3 Molecular interactions of Munc18-1

Munc18-1 is a horseshoe-shaped hydrophilic molecule made up of three domains that have no recognizable functional domains or motifs (Fig. 1.3b) [164]. The molecular mechanism underlying Munc18-1 function has long been controversial because of the crystal structure showing closed Syntaxin1 embedded in the central cavity of Munc18 [164]. In this conformation, the Habc domain of Syntaxin is folded onto its SNARE domain making it inaccessible to other SNARE proteins [65, 188]. By binding closed Syntaxin, Munc18-1 would thus inhibit SNARE
complex formation, which is hard to reconcile with a requirement of Munc18-1 in neurotransmitter release. In addition, other Munc18-1 homologues showed a different way of interacting with their cognate Syntaxin [25, 36]. This controversy has recently been solved with the identification of other modes of interaction between Munc18-1 and SNARE proteins. While the overall structure of Munc18-1 is quite rigid, domain 1 can rotate with respect to domain 2 and 3, and domain 3a (residues 295-358) shows conformational flexibility (Fig. 1.3b) [24, 106]. These conformational changes probably aid the transition between multiple protein interactions. Furthermore, other proteins are likely to be involved in the switching between binding modes, including Munc13 [154] and phospholipids [34, 47, 50, 202, 266]. An overview of the different Munc18-1/SNARE protein interactions follows below.

Monomer Syntaxin1

The first identified mode of interaction involving the closed form of Syntaxin1 is with its nanomolar affinity also the strongest one (Fig. 1.3c1 left). Careful analysis of the crystal structure showed that this mode involves not only the Habc and H3 domain of Syntaxin1a, which bind to the central cavity of Munc18-1 formed by domain 1 and 3a [164], but also a small N-peptide at the far N-terminus of Syntaxin1 which binds to a pocket on the outside of domain 1 in Munc18-1 [31]. While the N-peptide of Syntaxin is not essential for this binding mode, it does contribute to the binding affinity of the complex and might be required for the inhibitory function of Munc18-1 on SNARE complex formation [31] (but see [203, 223]). Alternatively, Munc18-1/Syntaxin1 complexes might have a positive role in SNARE complex formation by providing an efficient and NSF-SNAP-resistant starting point for SNARE complex formation [155]. Once Syntaxin is in an open conformation, the Habc domain is released from the SNARE domain and Syntaxin is able to engage in SNARE complex formation. Munc18-1 can also bind Syntaxin in its open conformation (Fig. 1.3c1 right), albeit with lower affinity than to closed Syntaxin [31, 76, 203]. Since monomer Syntaxin assumes a closed conformation in solution [65], studies have used a Syntaxin variant with mutations in the linker (Syntaxin(LE)) which destabilizes the closed formation and renders it predominantly open. Expression of Syntaxin(LE) in hippocampal neurons increased the Pvr, suggesting that SNARE complex formation was facilitated [76]. Several mechanisms have been proposed to regulate this closed-to-open switch. Rotation of domain 1 of Munc18-1, potentially induced by N-peptide binding, has been implicated in the opening of closed Syntaxin by twisting the Habc domain away from H3[24, 106]. Furthermore, the transition of closed Syntaxin-Munc18-1 to assembled SNARE complexes is accelerated by the MUN domain of Munc13, another key protein in synaptic vesicle release [154]. The critical role of the MUN domain in priming suggests that this transition might be the molecular equivalent of priming [11].
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Syntaxin1/SNAP-25 dimers

Munc18-1 also binds the Syntaxin/SNAP-25 dimer (Fig. 1.3c2) [85, 207, 277], the so-called acceptor complex referring to their role as an acceptor for vesicles-associated Synaptobrevin. The structure of the acceptor complex is variable, existing in different states of dissociation. Munc18-1 is suggested to promote SNARE assembly by stabilizing a conformation of the acceptor complex, with both SNAP-25 SNARE domains engaged, which rapidly interacts with Synaptobrevin [277]. However, other proteins involved in synaptic vesicle release including Munc13, Complexin and Synaptotagmin had similar stabilizing effects on the acceptor complex, suggesting that this action might not be specific for Munc18-1.

Assembled SNARE complex

In the last five years, a vast body of evidence has formed showing Munc18-1 binding to fully assembled SNARE complexes (Fig. 1.3c3 left) [31, 63, 85, 135, 203, 227], which was originally thought not to occur [292]. This interaction has a much lower affinity than the interaction with monomer Syntaxin, and depends critically on the N-peptide of Syntaxin [31, 63, 227]. However, binding of Munc18-1 to Synaptobrevin and to the core SNARE bundle has also been reported (Fig. 1.3c3 right), although with even lower affinity [60, 85, 286]. Interfering with the SNARE binding capacity of Munc18-1 inhibits synaptic vesicle release, suggesting that SNARE complex binding is crucial aspect of Munc18-1 function [56, 135]. By being a bulky protein, Munc18-1 might provide leverage on the two membranes during SNARE zippering [206]. Conversely, other studies suggest that Munc18-1 is no longer required once the SNARE complex is formed [197, 300].

Non-SNARE interactions

Munc18-1 directly interacts with other proteins implicated in synaptic transmission, amongst which are Doc2 [263], Rab3 [72], Mint [178] and possibly Munc13 [154]. Munc18-1 also binds to Granuphilin [257], a protein involved in regulated exocytosis in neuroendocrine cells. Furthermore, Munc18-1 binds and inhibits phospholipase D [144], an enzyme involved in phospholipid metabolism whose activity is required for normal synaptic vesicle release in Aplysia [109]. While phospholipids play a large role in regulated exocytosis [217], it is unknown whether an aspect of Munc18-1 function in mammalian synaptic transmission involves regulating lipid metabolism via phospholipase D. While these interactions have not been functionally studied in mammalian synaptic transmission, Munc18-1 mutants with altered affinity for some of these proteins have been developed and studied in other model systems. Munc18-1(E466K) shows an increased affinity for Rab3a and acts as a gain-of-function mutant in chromaffin cells, increasing the number of exocytotic events upon stimulation [45, 80]. Similar results were
found in transgenic *C. elegans* carrying an orthologous mutation in UNC-18 as demonstrated by hypersensitivity to the acetylcholinesterase inhibitor aldicarb, which is indicative of enhanced neurotransmitter release [81]. A transposon insert at site Glu379 in Munc18-1 reduces the affinity for Mint and decreases exocytosis in chromaffin cells and *C. elegans* [81]. However, expression of Munc18-1(P242S), which also lowers the affinity for Mint, has no effect on the amount of released vesicles but alters the kinetics of individual secretion events [45]. These results hint at the existence of SNARE-independent aspects of Munc18-1 function in synaptic transmission.

Taken together, these studies show that Munc18-1 is a central player in synaptic vesicle release, acting throughout different stages by regulating delivery, docking, priming and fusion of synaptic vesicle by guiding SNARE proteins towards efficient SNARE assembly. Although most research has focused on SNARE protein interactions, additional aspects of Munc18-1 function might involve interactions with other components of the release machinery and regulation of phospholipid metabolism and fusion pore dynamics. Because of its central role, regulation of Munc18-1 function or levels likely has a pronounced impact on synaptic strength and will contribute to short-term plasticity mechanisms.

### 1.4.4 Other major components of the synaptic vesicle release machinery

While SM and SNARE proteins are seen as the minimal fusion machinery in membrane fusion, several other proteins are also required for proper mammalian synaptic vesicle fusion (see [116, 206, 241] for recent reviews). For example, synaptic vesicle fusion is blocked in the absence of Munc13 [262]. Munc13s are required for priming via their MUN domain [11], which is probably involved in the transition of Munc18-1/closed Syntaxin dimers to SNARE assembly [154]. Furthermore, Munc13s contain many regulatory domains which determine their function in short-term plasticity, including domains involved in binding Calmodulin [128], diacylglycerol/phorbol esters [10, 201], Ca\(^{2+}\)-phospholipids [230] and RIMs [14, 58, 64]. These domains most likely regulate the activity of the MUN domain. Next to activating Munc13 [58], RIM is also involved in coupling calcium channels to the release machinery [93, 129].

Another MUN-domain containing protein involved in synaptic vesicle priming is CAPS (Ca\(^{2+}\)-dependent activator protein for secretion). Via its MUN domain, CAPS interacts with the Syx1a-SNAP-25 complex and the SNARE motifs of individual SNAREs [49, 134]. Furthermore, CAPS proteins contain a dynactin 1 binding domain (DBD), a C2 domain with unknown function and a pleckstrin homology (PH) domain which binds PI(4,5)P\(_2\) [239]. A large body of evidence supports a role for CAPS in dense core vesicle secretion [239]. Hippocampal neurons lacking CAPS-1/2 are defective in basal synaptic vesicle priming, but this defect can be restored by high-frequency stimulation [123]. CAPS can stimulate liposome
fusion in a PIP2 and SNARE dependent manner [119, 120], suggesting that CAPS functionally couples membrane and SNARE-protein interactions. CAPS might facilitate priming by facilitating SNARE-assembly via acting on t-SNAREs in a way similar to Munc13 or act downstream of Munc13 and stabilize the primed state of vesicles.

Synaptotagmin1 (Syt1) associates with synaptic vesicles via its transmembrane domain and is considered the main calcium sensor of the release machinery. Its two C2 domains bind calcium in a cooperative manner are responsible for Ca\(^{2+}\)-dependent phospholipid [51, 69] and Syntaxin/SNARE complex binding [40, 184]. Like Munc18-1, Syt1 functions at different steps of the release cycle. It is involved in docking synaptic vesicle to the active zone [55], but is also essential for fast calcium-triggered synchronous release of primed vesicles [75]. The view of Syt1 as the Ca\(^{2+}\)-sensor for synaptic vesicle release was however challenged by the observation that while evoked synchronous release is absent in neurons lacking Syt1, spontaneous release and asynchronous release following stimulation are massively increased [159, 283]. The current hypothesis attributes also a calcium-dependent clamping function to Syt1, preventing premature fusion at rest, and includes competition between multiple calcium sensors with different calcium-binding properties [270], for example with the high affinity Ca\(^{2+}\)-sensor Doc2 [83].

Knockout (KO) studies have revealed a positive role for Complexin in calcium-triggered release in mammalian neurons [200, 290]. Complexins tightly bind and stabilize SNARE complexes via a central helix [41], but their exact function has been hard to pinpoint due to mixed effects of Complexin manipulations on spontaneous and evoked release [287–289, 293]. These contradictory results were reconciled in the hypothesis that different accessory domains of Complexin have stimulatory or inhibitory functions and that there is both competition and cooperation between Complexin and Syt1 (but see ([170]).

1.5 Regulation of the SV release machinery

To be able to adapt to changes and to be tuned for cell-type specific requirements, the synaptic vesicle release machinery is under the control of many levels of regulation. Individual components can be regulated at the level of their relative expression or by post-transcriptional and post-translational modifications, leading to changes in protein function, localization, stability or degradation. In the section below, two types of modifications known to affect Munc18-1 are described in more detail: alternative splicing of pre-mRNA leading to different protein products and protein phosphorylation which can temporarily modulate protein function by adding a negative phosphate group.
1.5.1 Alternative splicing

Alternative splicing of pre-mRNA increases the complexity of the genome by enabling cells to generate different mRNA strands from a single gene. It is a major regulatory mechanism of information processing in the nervous system and associated with many types of neurological diseases [143, 148]. It is a universal process estimated to occur in 95% of human multi-exon genes [183, 271]. During alternative splicing the spliceosome, a large macromolecular apparatus responsible for intron excision and exon ligation, chooses a different splice site on which to assemble. This decision is based on a combination of splicing factors, often the same ones that are used in constitutive splicing, which enable the spliceosome to recognize splice sites (for a review on the regulation of alternative splicing, see [147]). These splice factors are in turn recruited by regulatory elements in the pre-mRNA, including 5' and 3' splice signals, splicing enhancers and splicing silencers.

The decision for an alternative splice site can have several effects. It can lead to the inclusion/exclusion or lengthening/shortening of exons, to the failure to remove an intron (intron retention) or to the introduction of a frameshift, giving rise to protein variants that differ in their amino acid sequence and consequently in their function or localization. Alternatively, splicing might occur in an untranslated region impacting the stability or localization of the mature mRNA [17, 113]. However, not all cases of alternative splicing are functional. Long intron structures are susceptible to random mutations that will give rise to splice site-like motifs leading to non-functional and non-conserved alternative splicing. Mammals have especially weak splicing boundaries and disproportionately long introns, making them prone to non-functional alternative splicing compared to other species [113].

Splicing factors and their activity regulators show specific expression profiles at different stages, cell types and tissues, with the highest complexity found in testis and brain [84]. During development alternative splicing plays a role in cell-fate determination, axon guidance and synaptogenesis. In mature neurons, alternative splicing regulates and is regulated by neuronal firing. Presynaptic proteins that are affected by alternative splicing include the SNARE proteins Syntaxin and SNAP-25, Munc18-1 and several ion channels including the N-type voltage-gated calcium channel [74, 147]. In cultured hippocampal neurons expressing only one SNAP-25 splice variant, SNAP-25b was superior in vesicle priming which might underlie the developmental switch to a larger primed vesicle pool during synapse maturation [57]. These findings point at the importance of alternative splicing in synaptic transmission. Chapter 2 of this thesis will describe the role of alternative splicing of Munc18-1 in synaptic transmission.
1.5.2 Protein phosphorylation

Protein phosphorylation is the process in which enzymes, called protein kinases, transfer a negative phosphate group onto certain amino acids, most commonly serine and threonine. This can lead to local or conformational changes in the protein, which can modify protein location, function or for example target a protein for degradation. A second family of enzymes, the protein phosphatases, can in turn remove the phosphate group and reverse the modification. Many components of the synaptic vesicle release machinery undergo phosphorylation, and this has been shown to influence synaptic plasticity [52]. For example, serine phosphorylation of Munc18-1 by protein kinase C (PKC) is essential for certain forms of presynaptic plasticity [278]. Phosphoproteomic screens on murine brain also identified tyrosine phosphorylated residues on Munc18-1 [7, 46], suggesting that tyrosine kinases might regulate Munc18-1 function. There are two classes of tyrosine kinases, membrane attached receptor tyrosine kinases and cytosolic non-receptor kinases. Both classes are highly abundant in the central nervous system. While initially known for their role in development, they are currently also appreciated for their function in the adult brain, particularly in synaptic plasticity and memory [193]. Most focus has been on postsynaptic effects, but evidence is accumulating for an additional presynaptic role of tyrosine kinases. Presynaptic tyrosine kinase activity increases after depolarization within 30 seconds and lasts for 30 minutes [281]. The following section will describe tyrosine kinase families implicated in synaptic plasticity. In addition, table 1 provides examples of tyrosine kinases family members implicated in synaptic plasticity, with particular emphasis on presynaptic effects.

Receptor tyrosine kinases

Receptor tyrosine kinases constitute receptors for insulin and many growth factors and are grouped in 20 subfamilies. Some of these families have been implicated in synaptic plasticity, like the tropomyosin-related kinase (Trk) family. Trk kinases are activated by target-derived neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin (NT) 3 and 4/5. The synthesis, secretion and action of neurotrophins is regulated by synaptic activity, which in turn modulates synaptic efficiency [138, 192]. Interestingly, several of these signaling pathways are also implicated in adult axodendritic synaptogenesis and modulate morphological synapse/spine plasticity, which has been linked to LTP [228, 229]. For this reason, it is often difficult to distinguish whether modulation of synaptic strength results from direct effects on synaptic transmission or of indirect effects on synapse formation and synapse stability.
Non-receptor tyrosine kinases

The non-receptor tyrosine kinases include members of the Abl, Ack, Csk, Fak, Fes, Jak, Src, Syk and Tec family. The largest of these is Src family kinase (SFK), of which five members are expressed in the mammalian central nervous system - three Src related kinases (Src, Fyn and Yes) and two Lyn related kinases (Lyn and Lck) [186]. The postsynaptic functions of SFKs are well known, such as modulating NMDA receptor function during LTP and AMPA receptor endocytosis during LTD [2, 130, 175]. However, SFKs have been demonstrated to positively and negatively modulate glutamate release from synaptosomes, isolated synaptic structures generated by sheering or homogenizing of brain tissue [6, 179, 232, 272]. In addition, SFKs inhibit glutamate release from cultured cerebellar granule cells and calcium induced insulin secretion from rat pancreatic islets cells [42, 176]. Due to their localization, targets and ability to regulating presynaptic strength, several SFK members including Lyn, Src and Fyn are promising candidates for a regulatory function in neurotransmitter release (Table 1.1). But which signals might lead to activation of presynaptic tyrosine kinases and potential downstream phosphorylation of Munc18-1? Since chapter 3 of this thesis concerns the potential role of Munc18-1 phosphorylation by n-Src, a neuronal splice variant of Src, an overview of signaling cascades leading to Src activation is given in Box 1.
### Table 1.1: Tyrosine kinases implicated in synaptic transmission

<table>
<thead>
<tr>
<th>Receptor tyrosine kinases</th>
<th>Function</th>
<th>refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trk</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TrkB</td>
<td>LTP, retrograde BDNF signalling enhances neurotransmitter release</td>
<td>[126, 192, 194, 226]</td>
</tr>
<tr>
<td>TrkC</td>
<td>LTP, spatial memory, inhibition of GABAergic transmission</td>
<td>[136, 229]</td>
</tr>
<tr>
<td>Ephrin</td>
<td>LTP induction, NMDA-receptor function, activity-dependent turnover of AMPA receptors, presynaptic actin dynamics</td>
<td>[67, 194]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-receptor tyrosine kinases</th>
<th>Function</th>
<th>refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFK</td>
<td>Spatial memory training, decrease RRP size, decreased recruitment from reserve pool</td>
<td>[162, 296]</td>
</tr>
<tr>
<td>Src</td>
<td>Threshold for LTP induction, NMDA receptor function and trafficking, decreased paired pulse facilitation</td>
<td>[82, 153, 256]</td>
</tr>
<tr>
<td>Fyn</td>
<td>Ethanol-induced up-regulation of dopamine release, ethanol reward</td>
<td>[77]</td>
</tr>
<tr>
<td>Lyn</td>
<td>Up-regulated upon neural activity, associated with clathrin-coated vesicles</td>
<td>[246, 259]</td>
</tr>
</tbody>
</table>
For receptor tyrosine kinases, most signaling cues have been identified, e.g. BDNF and NT3/4 for the TrkB receptor kinase. Non-receptor tyrosine kinases on the other hand are downstream of several signaling cascades that show a high level of cross-talk. Since SFKs have a high degree of functional redundancy and are often examined together, the regulation of the Src subfamily will be discussed when possible and otherwise SFKs will be discussed as a group. The activity and targeting of SFKs is related though intra-molecular interactions of the highly conserved SH3 and SH2 domains and by ancillary molecules such as the negative regulator C-terminal Src kinase (Csk) and Csk homologous kinase. The central method of regulation is by tyrosine phosphorylation or dephosphorylation of two sites, which regulate Src activity in opposing ways. Autophosphorylation of one site increases the kinase activity while phosphorylation of another site suppresses the kinase activity (an extensive review of the features of active and inactive SFKs and their intricate control by external factors can be found in [111, 175, 211, 212]). Here we will discuss pathways that regulate SFK kinases and are implicated in regulating presynaptic strength (summarized in Fig. 1.4).

**SFKs and Neurotrophins.** Neurotrophins are secreted proteins that belong to a class of neuron specific growth factors, and are ligands for receptor tyrosine kinases. There appears to be substantial interplay between SFKs and receptor tyrosine kinases. For example, TrkB activation by BDNF increases SFK activation in cultured neurons and, in turn, SFKs can promote TrkB activation [107]. Since BDNF can be released from axons and dendrites and TrkB activation by BDNF has been shown in synapse fractions from both sides [108], this mechanism could be similar for both sides of the synapse.

**SFKs and Zinc.** Zinc can also activate TrkB, but in a SFK dependent manner, and promotes the formation of a complex including TrkB and SFKs Src or Fyn [107]. This activation is dependent on neuronal activity, probably since Zinc is released upon stimulation together with glutamate in synaptic vesicles and can enter the cell through channels that are opened upon neuronal activity [108]. A critical role for Zinc in presynaptic mossy fiber LTP, which is caused by an increase of glutamate release, has recently been shown [182].

**SFKs and presynaptic serine/threonine kinases.** Src kinase is a substrate for protein kinases PKC, PKA and CDK1/cdc2 [212]. PKCδ has been shown to activate Src upon DAG stimulation via activating protein phosphatase PTPα [27]. Both cAMP and the neurotrophin NGF induce PKA-dependent activation of c-Src via phosphorylation at residue S17. The activation of ERK and Rap1 by NGF is dependent on PKA phosphorylation of c-Src and probably on the tyrosine kinase receptor TrkA in PC-12 cells [173]. The PLC/DAG-pathway and cAMP pathway are downstream of many presynaptic receptors and can strongly regulate...
synaptic transmission (reviewed in ([52]). A role for CDK1 in synaptic transmission is less clear, although it can phosphorylate the synaptic vesicle protein Synapsin1 [127].

**SFKs and the extracellular matrix.** The extracellular matrix molecule Reelin is mostly known for its role in neuronal migration, but has recently been shown to have presynaptic effects that influence paired pulse facilitation [101]. However, it is suggested that this role of Reelin does not require Reelin signaling via Dab1, which bi-directionally activates SFKs in neurons [20], but via Reelin binding to presynaptic integrins. At the postsynaptic site, Reelin signaling though its receptors and Dab1 or through integrin receptors both activates SFKs, leading to tyrosine phosphorylation of NMDARs. This increases NMDAR channel activity and induces LTP [61]. Whether SFKs are also downstream presynaptic modulation of neurotransmission by Reelin signaling via presynaptic β1-integrin remains to be clarified.

**SFKs and Insulin.** Insulin and insulin receptors have been demonstrated to modulate synaptic activities at both the pre- and postsynaptic site [297]. C-Src is activated by insulin and mediates insulin signaling to PI3K, which is essential for the postsynaptic effect of insulin on LTD via NMDA receptor activation [231, 261]. Munc18-3, an ubiquitously expressed isoform of the neuronal SM-protein Munc18-1, is a target for the insulin receptor tyrosine kinase [4, 122]. Its tyrosine phosphorylation disrupts binding to its cognate SNARE protein Syntaxin4 and is required for insulin stimulated GLUT4 vesicle exocytosis in adipocytes, which required the IR but not the PI3K/Akt pathway [122, 174]. Combined, these studies suggest a potential presynaptic modulatory effect of insulin via the phosphorylation of the SM-protein Munc18-1 by the insulin receptor directly. Alternatively, a mechanism similar to the insulin signaling in LTD might exist on the presynaptic site as well, involving SFK activation via presynaptic insulin receptors.

**SFKs and other signaling pathways.** Like the BDNF/TrkB example discussion above, growth factor activation of many receptor tyrosine kinases in canonical pathways leads to activation of SFKs, and SFKs in turn regulate the activity and signaling of receptor tyrosine kinases. SFK activation is downstream of platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF) and others, leading to activation of Myc and Ras signaling pathways and of PKCδ (extensively reviewed in [29]). Whether these pathways contribute to presynaptic plasticity is unclear. In addition, SFKs are also controlled by integrin receptors, G-protein coupled receptors, antigen- and Fc-coupled receptors, cytokine receptors, and steroid hormone receptors [186, 212, 247].

**SFKs and Protein tyrosine phosphatases.** Protein tyrosine phosphatases can influence SFK function either directly via dephosphorylation of activating or in-
hbiting residues on SFKs themselves, or via the dephosphorylation of SFK target proteins. There is still much to explore on this topic, although the function of a couple of protein tyrosine phosphatases targeting SFKs in the brain, like PTPα, STEP and Shp2, are reviewed elsewhere [111, 175, 248].

<table>
<thead>
<tr>
<th>Extracellular signals</th>
<th>Calcium</th>
<th>NGF</th>
<th>Zinc</th>
<th>BDNF</th>
<th>Reelin</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presynaptic receptors/channels</td>
<td>GPCR</td>
<td>VGCC</td>
<td>GPCR</td>
<td>TrkA</td>
<td>NMDAR/AMPAR/VGCC</td>
<td>TrkB</td>
</tr>
<tr>
<td>Intracellular signaling</td>
<td>DAG</td>
<td>AC</td>
<td>cAMP</td>
<td>PKC</td>
<td>PKA</td>
<td>PTPα</td>
</tr>
</tbody>
</table>

**Figure 1.4:** Presynaptic signaling cascades leading to SFK activation.

### 1.6 Aim and outline

Based on the studies reviewed in this introduction, Munc18-1 is considered to be a central component of the synaptic vesicle release machinery and probably exerts different functions at different stages of the synaptic vesicle release process. The aim of this thesis was to disentangle different aspects of Munc18-1 function and understand how these aspects can be regulated. For this purpose, we conducted a structure-function analysis of Munc18-1 in mammalian synapses. By using neuronal cultures of munc18-1 null mice, our protein of interest could be replaced with mutant variants. The ability of these Munc18-1 variants to support different forms of synaptic transmission was assessed using patch-clamp electrophysiology in a reduced model system, namely neurons grown in isolation on islands of glia cells, so-called autaptic neurons. These neurons make abundant connections with themselves, allowing the assessment of different physiological parameters from the same synapse population in which a sole Munc18-1 variant drives synaptic vesicle release.

**Chapter 2** describes the effect of alternative splicing of Munc18-1 pre-mRNA, which leads to two closely related variants of Munc18-1, on synaptic transmission. We found that alternative splicing of Munc18-1 regulates the rate of depression during repetitive stimulation, potentially via a CaMKII-dependent mechanism. In **chapter 3** we interfere with tyrosine phosphorylation of Munc18-1 in order to assess the functional consequence of this post-translational modification. This resulted in severe defects in basal synaptic transmission, which led us to postulate that tyrosine phosphorylation of Munc18 might be a novel and potent mechanism to regulate synaptic strength. In **chapter 4** we combine quantitative biochemistry with synapse physiology and electron microscopy to address the role of a specific interaction mode between Munc18-1 and the N-peptide of the SNARE protein Syntaxin1 in docking, priming and fusion. Since interfering with this binding
mode did not hinder synaptic transmission, we argue that the N-peptide interaction mode is not essential in synaptic vesicle release. Chapter 5 tests the effect of a transposon insert in domain 3a on synaptic transmission, thereby testing the involvement of this domain in synaptic vesicle release. Our results hint at a role for this domain in regulating spontaneous vesicle release and the priming capacity of Munc18-1. The implications of these results are discussed in chapter 6, and further research directions are suggested. An overview of methods used in this thesis is given in Chapter 7.