CHAPTER 1

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General introduction

On nerve cells and synapses
Over the course of evolution, the brain has developed from a small relay station into an intricate network capable of complex cognitive functions, including the formation of memories, problem solving, communication skills, including language, and emotional responses. This is made possible by a repertoire of highly specialized cell types, including many kinds of neuronal and glial cells. The proper execution of brain functions critically relies on the communication between these cells. It is therefore not surprising that cell-to-cell communication in the brain is a highly regulated process, and subject of intense scientific study.

It has been recognized for over a century that neurons are the primary cell types that carry out most brain functions. During his systematic study of histological preparations, Ramon y Cajal recognized that each brain region holds a range of characteristic neuronal cell types, each with an elaborate dendritic tree. He suggested that neurons use these dendrites to connect to other cells, to transfer information [1]. We now know that the wiring of these connections during embryonic and postnatal development follows tight rules, and results in a highly organized network of neuronal connections. These connections – synapses – are essential mediators of neuronal communication, and are believed to be an important site for processing and storage of neuronal information. Over half a century of studies has revealed a remarkable richness in the composition and function of these synapses. Of key importance is the regulated release of neurotransmitter-containing synaptic vesicles that underlie the quantal nature of synaptic transmission [2,3]. Given the central role of synapses in brain function, a full understanding of synaptic transmission will be crucial for understanding signal processing and storage capacities of the brain.

Structure and function of presynaptic contacts

Heterogeneity in presynaptic structure and function
Different neuronal cell types form distinct types of synapses, with characteristics fitted to their functional requirements. For many synapses that transmit sensory information, reliability is of key importance. Therefore, these synapses are highly specialized structures, capable of releasing large numbers of vesicles at high rates. Examples are ribbon synapses in the retina and cochlear hair cells [4], the Calyx of Held in the auditory brain stem [5], and mossy fiber synapses in the cerebellum [6,7]. Many other types of synapses, including those involved in higher brain functions, are focused on flexibility. In these synapses, vesicle release is a stochastic process [2], and the strength can be rapidly modulated upon changes in neuronal activity [8,9]. By doing so, these synapses can modify incoming signals, and thereby contribute to information processing and storage processes in the brain [8,10]. Neurons in the hippocampus and cortex are involved in many cognitive functions, and make use of this kind of synapses.

Structure of presynaptic terminals in the hippocampus

Hippocampal neurons, both in situ and in vitro, are widely used to study synaptic transmission. The synapses formed by these neurons display a remarkable heterogeneity in morphological and functional characteristics. Presynaptic boutons have an average
volume of \( \sim 0.1 \ \mu \text{m}^3 \) \cite{11,12}. Synaptic vesicle fusion takes place at the active zone (AZ), a protein-dense structure at the plasma membrane (see \cite{13} for a recent review). Over 90% of the hippocampal synapses contain only 1 AZ, but boutons with 2 or 3 AZs are occasionally detected \cite{11}. The size of the active zone can range from 0.01 – 0.20 \( \mu \text{m}^2 \) \cite{11}. The number of synaptic vesicles per AZ averages around 200, but can range between 40 to over 1000, a 25-fold difference \cite{11,12,14,15}.

This total vesicle population is often subdivided in several “pools”, based on morphological and functional studies \cite{16} (Figure 1.1.1). 0 to 30 vesicles are in physical contact (‘docked’) with the plasma membrane, and this number closely correlates with the size of the AZ \cite{11,14,15,17}. The number of docked vesicles appears to correlate with functional properties of hippocampal terminals, in particular with the presynaptic strength and readily releasable pool (RRP) size \cite{11,14,15,17}. However, this relationship does not hold in other types of synapses, for instance in the cerebellum \cite{18}, and several pharmacological or genetic treatments affect presynaptic strength or RRP size independent of the number of docked vesicles \cite{19-21}. Therefore, processes downstream of docking are most likely decisive to determine principal functional parameters of the terminal.

Of all synaptic vesicles, a relatively small portion takes part in the vesicle release cycle during mild to moderate stimulation, and is usually referred to as the recycling pool \cite{16}. The size of the recycling pool is around 20-50% from the total number of vesicles in ex vivo preparations \cite{16,22-25}. In vivo, however, the recycling pool is probably much smaller, averaging 1-5% of the total vesicle pool \cite{25,26}.
The remaining vesicles, forming the so-called reserve (or resting) pool, do not appear to take part in activity-dependent release under physiological conditions [25,26], and their function is highly debated. It has been suggested that the resting pool contains a distinct set of vesicles that exclusively mediate spontaneous release [24,27]. Recent data indicate that resting pool vesicles contain proteins not found in vesicles from the release pool and vice versa, which fits with the idea of specialized pools for different modes of fusion [28,29]. However, other laboratories could not find evidence for separate pools for spontaneous release, and instead conclude that all vesicles can be released by prolonged stimulation with action potentials [30-32]. Vesicles from the resting pool can be recruited to the releasable pool by changes in Cdk5 and calcineurin activity (see also below) [22,25,33]. This indicates that these two pools mix, and that the (biochemical) distinction between the two may not be as sharp as previously suggested.

Others have hypothesized that reserve pool vesicles act as buffers to prevent washout of release machinery proteins, and are held together by cross-linking proteins [34]. Removal of the most likely candidates for such cross-linking function, synapsins, indeed increases the mobility of vesicles, resulting in dispersal of vesicles into the axon and reduction of vesicles in the terminal itself [35,36]. However, synapsin knockout neurons have only a mild vesicle release phenotype, showing increased synaptic depression during intense and prolonged stimulation [35,37]. The proposed protein-buffering function of the reserve pool thus seems to have, at most, only marginal effect on presynaptic function. It is most likely that the resting pool contains reserve vesicles, that are mature for fusion during high synaptic use.

It remains unclear why a synapse contains so many resting vesicles, and under what circumstances these vesicles are called for action. Importantly, it is not understood what defines a vesicle as ‘resting’ or ‘releasable’. For vesicle docking and priming we have a reasonable clear picture of the molecules involved (see [38,39] for recent reviews), but it is not known what prevents resting pool vesicles from fusion. Synapsins alone cannot fully account for this, since removal of synapsins only reduces, but not ablates the resting pool [37].

Recent experimental evidence has indicated that vesicles are not confined to an individual synapse, but are shared between terminals [23,40]. Vesicles from both the recycling pool and the reserve pool are interchanged between boutons to take part in secretion in neighboring synapses [40-42]. According to this model, synapses draw their synaptic vesicles from a single extrasynaptic or ‘superpool’ of vesicles, and exchange their vesicles based on synapse use [42,43]. Interchange of synaptic vesicles is remarkably fast - considerable vesicle exchange was observed within a few minutes [40,42,44], and is likely to be mediated by active transport along microtubules [44,45]. It is suggested that vesicle sharing serves as a mechanism to fine tune the strength of presynaptic terminals (reviewed in [45]), but this has not been directly demonstrated. In addition, vesicle sharing also occurs at rest, when presynaptic strength is relatively stable, and it seems to occur evenly among synapses, without targeting specific targeting [40,42]. This implies that vesicle sharing is constitutive and undirected, which is not to be expected for a mechanism to tune individual synapses. Whether vesicle sharing is indeed a mechanism
for synaptic plasticity remains to be tested.

In general, there is only limited understanding of the mechanisms that regulated the number of vesicles per terminal. As discussed in this section, the total pool size determines, at least to some extent, the size of the docked and recycling vesicle pool, and will probably also influence functional parameters. Total pool size is also strongly affected by certain forms of (homeostatic) plasticity [46]. In part, regulation of bouton- and AZ size could be an upstream factor that regulates vesicle content, since both correlate very well with the number of vesicles [11,14,46]. However, other mechanisms independent of synapse size contribute to the total pool size. These include synapsins [35], the cytomatrix proteins Piccolo and Bassoon [47] Brain-derived neurotrophic factor (BDNF) and its receptor TrkB [48]. Indeed, BDNF also affect vesicle sharing between synapses [42]. It is not known, however, which molecular events initiate and mediate changes in vesicle pool size.

Regulation of presynaptic strength

Release probability and its modulation by repetitive activity

The chance that a single action potential will trigger the release of a synaptic vesicle at a given hippocampal synapse is low. The value of this synaptic release probability (pr) is usually around 0.2, but as with total pool- and RRP size, a wide range of values is possible [49-53]. Correlative structural and functional studies showed that pr is for a large part dependent on the size of the RRP [15,17,46,52]. Another prominent source of heterogeneity in pr is the local intracellular Ca2+ concentration sensed by the vesicle release machinery. This is dependent on many factors, including the amplitude of the Ca2+ influx through voltage gated Ca2+ channels (VGCC), the release of Ca2+ from inositol-1,4,5-triphosphate (IP3) receptor (IP3R) and ryanodine receptor (RyR) gated intracellular stores, the Ca2+ buffering capacity of the terminal, and the distance between the VGCC and the primed vesicle [54-57]. Many of these variables differ from synapse to synapse, and have a direct effect on pr [58-62]. Furthermore, pr can adapt swiftly to changes in neuronal activity: repetitive stimulation of nerve terminals results in changes in the presynaptic input-output relation on the timescale of milliseconds to minutes (short term plasticity (STP), see Figure 1.2) [9]. Different synapses originating from a single presynaptic neuron can express highly variable forms for STP [50,60,63,64], see [65] for review. The pr at rest determines for a large part the sign of STP, since synapses with a low initial pr are more likely to facilitate, whereas high pr synapses tend to depress [50,51,66,67]. Several phases are being discriminated depending on their duration and effective stimuli [9]. The build-up of residual Ca2+ during repetitive stimulation and repetitive opening of Ca2+-channels is a central factor in each of these forms, together with the finite amount of vesicles available. The relation of residual Ca2+ to presynaptic plasticity is analyzed with exceptional precision in Calyx of Held synapses [5,68]. As a result of residual Ca2+, fusion is triggered more efficiently and the time course of presynaptic plasticity follows the time course residual Ca2+. In addition, residual Ca2+ is probably responsible for other aspects that contribute to presynaptic plasticity: an increase in the releasable pool of vesicles, probably via Ca2+-dependent acceleration of upstream steps in the secretory pathway (vesicle recruitment, docking and priming) and saturation of intracellular Ca2+-buffers and -stores and Ca2+-channel facilitation [5,9,68,69]. Underscoring their importance, VGCCs are one of the most highly regulated molecules in the presynaptic terminal, as
**BOX 1**

**How can presynaptic heterogeneity and release probability be measured?**

**Electrophysiological approaches**

*Paired pulse ratio* The $p_r$ can be determined by applying to consecutive action potentials at various intervals and measuring the evoked excitatory postsynaptic current (EPSC). The STP induced by this protocol is usually quantified by dividing the amplitude of the second response over the amplitude of the first response (the paired pulse ratio, or PPR). The PPR is a measure of $p_r$, since the synapses with a low initial $p_r$ are likely to facilitate and high $p_r$ synapses are likely to depress \cite{50,51,67}. Hippocampal synapses have an absolute refractory period of \~5 ms \cite{66}. By dividing the PPR at 5ms by the PPR at 30ms, one gets the average $p_r$ of the population of synapses that was recorded from \cite{209}.

*MK-801 rundown* Heterogeneity in $p_r$ of a population of synapses can be measured using the NMDAR blocker MK-801. This drug will irreversibly block the NMDAR channel once it is opened. By recoding the decline in NMDAR current amplitude during low frequency stimulation in the presence of MK-801, one gets a measure of the $p_r$ heterogeneity \cite{49,53}.

*Minimal stimulation* The only electrophysiological method to measure the $p_r$ of an individual synapse is minimal stimulation. By using a thin stimulation electrode, one can stimulate a single axon, which, because of the relatively low connectivity in the hippocampus, will usually result in the stimulation of a single synapse on a postsynaptic neuron. By counting the number of successes and failures at low frequency stimulation, one can directly calculate $p_r$ \cite{51,52}.

**Imaging approaches**

*FM dyes* Vesicle cycling can be visualized using fluorescent styryl dyes (FM dye, named after the researcher Fei Mao who first synthesized these molecules) \cite{210}. FM dyes bind lipid membranes with high affinity, and are thus taken up by vesicles during endocytosis. After washout of excess extracellular dye, the remaining fluorescence (FM staining) is proportional to the number of endocytosed vesicles \cite{211,212}. Upon subsequent stimulation, FM-dye containing vesicles can be exocytosed again (FM destaining), which is also used as readout of $p_r$ \cite{211,212}. Both FM-dye staining and destaining experiments report $p_r$ distributions similar to those found with electrophysiology \cite{50,191,213}. Using short FM loading periods, it is also possible to visualize individual vesicle release events \cite{214}. After FM dye staining, the dye can be fixed and visualized at an electron microscope, to see the faith of the endocytosed vesicles \cite{15}. This method was elegantly used to describe intersynaptic trafficking of releasable vesicles \cite{40,42}. Drawback of FM dyes is the harsh stimulation protocols required to load the releasable vesicle pool. Typical loading protocols are 1 minute 90 mM K+ \cite{191} or 600 action potentials at 20 Hz \cite{175}.
**pHluorin-based constructs** pH-dependent variants of GFP (pHluorin), which are reversibly quenched at a pH lower than 6.5, are now widely used to study vesicle release. Since synaptic vesicles are acidic (pH ≈ 5 [215]), intravesicular pHluorin is not fluorescent, but will light up when it is released to the extracellular space where pH ≈ 7.3. When fused to the intraluminar domain of a synaptic protein, it provides an excellent fluorescent marker for exocytosis [199]. Due to their low background fluorescence and limited bleaching, pHluorin constructs can be used to image vesicle release at high spatial and temporal resolution, including single vesicle release events [200,216]. Upon subsequent endocytosis, vesicles will be reacidified, and the fluorescence is lost again. In this way pHluorin constructs can also be used to measure the speed of both endocytosis and reacidification [200,216]. pHluorins can take part in many consecutive rounds of vesicle fusion, making it possible to image vesicle cycling for prolonged periods (up to several hours) without the need of reloading the sample with the fluorescent probe [217]. pHluorin based constructs are also widely used to study the reserve vesicle pool [23,24,218]. Many different pHluorin constructs have been created over the years, with pHluorin fused to VAMP2 (SynaptopHluorin, [199]), VAMP7 [28], synaptophysin (SypHy, [200]), vGlut [216], synaptotagmin [219] and SV2 [220]. Comparison of these constructs revealed that not every vesicular protein is released and endocytosed with the same kinetics, sparking hypotheses on multiple routes for synaptic vesicle release and reuptake [24,28,200]. Synaptic heterogeneity and $p_r$ reported by pHluorin constructs overlap with data obtained with other methods [192,200,221,222].

**Other methods**
Quantitative immunocytochemistry is a powerful method to determine the synaptic expression levels in individual synapses, and provides a measure for synaptic protein content and vesicle pool size in fixed samples (see for instance [133,223,224]). More recently, fluorescently tagged antibodies against the intraluminar domain of vesicular proteins have been used to measure $p_r$ and releasable pool size with live imaging [28,133,218]. Ca2+ dyes like Fura-2, Fluo-2 and Calcium Green-1, which change their fluorescent properties upon calcium binding, can be used to measure localized changes in [Ca2+]. Presynaptically, they are routinely used to measure the (variation in the) amplitude of the presynaptic Ca2+ concentration in individual boutons [60,62,94]. When infused in the postsynaptic cell, it can serve as a reporter of $p_r$ [60].
they can be phosphorylated by PKA [70], PKC [71,72], MAPK/Erk [73], CaMK [72] and Cdk5 [74,75] (see [69] and [76] for review). On top of that, channel gating is modulated by G-protein βγ subunits [76,77], and several Ca²⁺ binding proteins, including calmodulin (CaM, [74]), and EF-hand proteins, such as frequenin/NCS, CaBP1 and VILIP-2 (reviewed in [69]).

At some types of synapses, intense stimulation induces long lasting increase in pr, known as presynaptic long-term potentiation (LTP). Presynaptic LTP can be induced in the mossy fibers of the hippocampus [78] and cerebellum [79], cortico-amygdal [80], corticothalamic [81] and corticostriatal synapses [82]. Although the role of cAMP and protein kinase A (PKA) in presynaptic LTP is undisputed [79,81-83], and several presynaptic proteins were found to be essential for LTP expression [83-85], the exact mechanism of presynaptic LTP remain to be identified to date ([86,87] and see below).

**Figure 1.2.** Relationship between firing pattern and release probability. A. Representative action potential firing pattern of a presynaptic hippocampal neuron. B Changes in average synaptic release probability as a result of the firing pattern in A. C. Types of presynaptic plasticity involved in release probability fluctuations in B. Note that several types of plasticity can act simultaneously. Abbreviations: PPF: paired pulse facilitation, Augm.: augmentation, PTP: post-tetanic potentiation, Depr.: depression.
Secretion modulation by presynaptic receptor activation

Presynaptic terminals express a range of surface receptors, that upon activation can modulate $p_r$ via a network intracellular signal transduction pathways. Over 70 different receptors have been described so far, many of them being sensitive to neurotransmitters, neurotrophins or opioids [88,89]. Many of the agonists of these receptors originate from spillover of neurotransmitter from neighboring synapses, glial cells, volume transmission or retrograde signaling from the postsynaptic cell [77,90,91]. Not every synapse expresses the same repertoire of receptors, which further broadens the variety within the synapse population (see for instance [92-94]). Furthermore, the exact location of the receptor (near release sites, peri-synaptic or extrasynaptic) naturally influences its effectiveness to modulate release [92,93,95]. Activation of presynaptic receptors triggers a complex cascade of signalling events (Figure 1.3). These cascades are not unique for the terminal and operate very similarly in other compartments in neurons and other cells, such as the adenyl cyclase (AC) and cAMP-pathway or the phospholipase C (PLC) and diacylglycerol (DAG) pathway. However, a specific set of effector proteins determines the specific effects of these canonical pathways in the terminal. Application of exogenous (synthetic) agonists and antagonists suggests that the presynaptic PLC/DAG pathway is the most powerful pathway to modulate secretion in many synapses, with >50% increase in synaptic transmission and up to 6 fold increase in spontaneous release events [19,96-98]. DAG potentiates transmitter release by increasing the apparent Ca$^{2+}$-sensitivity of vesicle fusion. The more than linear effect on spontaneous release can also be explained by such an increased Ca$^{2+}$-sensitivity [98]. The cAMP pathway may produce a similar extent of modulation as the PLC/DAG-pathway, but this is probably largely through cross-activation of the PLC/DAG-pathway ([99], see below). These pathways have been mapped to some extent (see below) but are often inferred from studies in postsynaptic or non-neuronal cells.

Secretion modulation by lipids

The synaptic proteome has been described extensively over the last decades, yielding a detailed understanding of the function and regulation of the vesicular release machinery [38,39]. But on top of that, the (phospho-) lipid composition of the vesicular- and plasma membrane strongly affects vesicle release, and modulation of individual lipids is a powerful mechanism to control synaptic output [100].

Phosphatidylinositol 4,5-biphosphase (PIP2) and its metabolites DAG and IP3 are important regulators of the synaptic vesicle cycle [100,101]. DAG and IP3 serve as major second messengers in intracellular signalling (discussed below), and PIP2 binds to an array of critical release machinery components. Ca$^{2+}$ sensors that trigger synaptic vesicle release, in particular synaptotagmins and doc2s, bear C2-domains, with which they bind to membranes in a Ca$^{2+}$ dependent manner [102]. The Ca$^{2+}$ affinity of these C2 domains is dependent on the presence of phosphatidylserine (PS) [103,104] and PIP2 [105-109]. Synaptotagmin-1 is reported have a 40 times higher Ca$^{2+}$ affinity in the presence of PIP2 [109]. Syntaxins form PIP2 dependent clusters on the target membrane, which might be of importance to target syntaxin to release sites [110]. PIP2 furthermore modulates the gating of several types of VGCCs [111] and is critical for the function of K+ channels [112,113]. It also binds to Munc18-interacting protein (Mint) and the vesicle
priming factor CAPS, but how this effects the synaptic vesicle cycle remains unknown [114,115]. Finally, the C2B domain of RIMs, essential presynaptic scaffolds [116,117], contains a polybasic sequence highly homologous to synaptotagmin-1 [118], which in synaptotagmins mediates PIP2 binding [109]. RIMs are therefore likely to bind PIP2. The in vivo relevance of PIP2 in vesicle fusion has been studied in bovine chromaffin cells to some extent, were it the size of the RRP [119]. The PIP2 content of membranes is also essential for the regulation of endocytosis of vesicles [100,120].

Lipid rafts – cholesterol- and/or sphingolipid-rich patches in the membrane – are thought to be important for the proper targeting of synaptic proteins, as well as the proper function of some of these proteins [100,121]. Syntaxin and SNAP25 accumulate at cholesterol-rich patches at the plasma membrane, thereby facilitating vesicle docking at these sites [122]. Sphingosin is thought to affect the size of the RRP and spontaneous release frequency, by promoting SNARE complex assembly or altering Syntaxin - Munc18 interactions [123-125]. Confusingly, these studies disagree on sphingosin's effect on secretion, since some observe an increase in RRP size, evoked and spontaneous release [123,124], while others reported a smaller RRP and decrease $p_r$ [125]. Knockout of acid-sphingomylinase, which converts sphingomyelin into ceramide – the precursor of sphingosine, leads to a smaller presynaptic terminal and less docked vesicles [125]. Ceramide itself might affect presynaptic efficacy by its interaction with voltage gated ion channels [126].

The fact that many of the essential fusion proteins depend on lipid interaction suggest that lipids are important to target these proteins to specific locations on the plasma membrane. Vesicle fusion is likely to be restricted to specialized ‘release sites’ at the plasma membrane (reviewed in [127]). These sites would contain one or more VGCCs and a high density of release machinery components. Lipids, by sequestering and activating many of these components, form excellent candidates to define such sites. Unfortunately, we have only limited understanding how the (local) concentration of lipids is regulated at the synapse. Currently available tools are often too aspecific to pinpoint an observed phenotype to a specific lipid. In addition, complex and rapid metabolism networks of lipids make experimentation difficult, and often leave room for alternative interpretation.

**Secretion modulation by change in protein levels**

Adaptation of synaptic protein levels is a powerful mechanism to change synaptic strength. Experimental manipulation of protein levels showed that the abundance of an individual protein directly affects vesicle release [128-131]. Homeostatic plasticity – lasting changes in synaptic strength upon prolonged increased or reduced neuronal activity – has been shown to at least partly operate via changes in synaptic protein content. Chronic adaptations of network activity results in a several-fold change of many proteins involved in synaptic function [132-134]. Activity-induced removal of synaptic proteins is ubiquitin- and proteasome dependent, and can be prevented by blockade of the proteasome [135,136]. Inhibition of the proteasome also increases the size of the recycling vesicle pool [137] and affects synaptic vesicle release on a minute timescale [134,138]. The molecular mechanisms that select proteins for degradation are not fully understood. Ubiquitination – molecular ‘tagging’ of proteins for degradation – is in many cases dependent on the phosphorylation of the target protein [139]. Recent
### Functional Group

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<td>Vesicular and plasma membrane transporters</td>
<td>vGLUT, vATPase</td>
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work has shown that the phosphorylation of Munc18 by Erk dramatically increases its ubiquitination and subsequent degradation, and consequently lowers synaptic vesicle release (Schmitz et al., unpublished). Increased synaptic activity also seems to selectively tag presynaptic scaffold proteins for proteasomal degradation, while leaving other proteins unaffected [133,135]. Activity-induced protein degradation possibly depends on a yet unidentified GPCR [134]. How the synaptic levels of other specific proteins is increased during homeostatic plasticity remains to be elucidated, but likely involves both elevated protein expression and increased recruitment of proteins to the synapse [133].

**Mapping Ca\(^{2+}\) dependent pathways in the terminal**

Opening of voltage-gated Ca\(^{2+}\)-channels, Ca\(^{2+}\)-influx and elevation of intracellular Ca\(^{2+}\) primarily triggers the fusion of synaptic- and other secretory vesicles. The C2-domain proteins synaptotagmin-1 and -2 are the principal effectors for this primary effect in most neurons, with synaptotagmin-9 acting in limbic and striatal neurons [140]. Ca\(^{2+}\)-dependent spontaneous (i.e. non-action potential evoked) release can be initiated by both synaptotagmins [141] and doc2s ([108], but see [142]). During and shortly after repetitive stimulation, build up of residual Ca\(^{2+}\) accelerates synaptotagmin-dependent fusion and several upstream steps in the secretory pathway. Multiple C2-domain proteins have been implicated in the regulation of such upstream steps (see Figure 1.3), especially protein kinase C (PKC), which is involved in presynaptic modulation in many systems and contexts (see below). Other C2-domain proteins like other synaptotagmins, Munc13, rabphilin, ferlins or doc2 may contribute to the effects of residual Ca\(^{2+}\) on secretion modulation. For Munc13-2 it was recently shown that its binding to Ca\(^{2+}\) facilitates release during and after intense stimulation [143], while otoferlins are essential for synchronous release at ribbon synapses [144, 145]. The C2 domains of presynaptic scaffold protein RIM does not seem to require Ca\(^{2+}\) binding [118, 146], and deletion of the C2 domain of Piccolo does not affect synaptic vesicle release [47].

Ca\(^{2+}\) modulates the activity of several presynaptic proteins in a complex with EF-hand Ca\(^{2+}\)-binding proteins, primarily calmodulin (CaM, see Figure 1.2). Ca\(^{2+}\) -bound CaM (Ca\(^{2+}\)/CaM) has several targets, including Ca\(^{2+}\)/CaM-dependent protein kinase (CaMK), AC, myosin light chain kinase (MLCK), calcineurin, Ca\(^{2+}\-)channels and several proteins of the secretion machinery. Ca\(^{2+}\)/CaM modulates secretion by enhancing the refilling of the releasable vesicle pool, by binding to Munc13 [147], and activation of CaMK, which phosphorylates (among others) synapsin I [148]. Synaptotagmin has a shared CaMK/PKC

**Table 1.1 (opposite page).** 562 known presynaptic proteins were divided into 17 groups according to their function in the presynaptic terminal, and groups were sorted on the percentage of phosphorylated proteins. The two rightmost columns show the number of phosphorylated proteins and phosphorylation sites per group. Data are derived from phospho.ELM database v8.1 [241], which includes data obtained from non-neuronal preparations. The numbers in brackets represent proteins and sites reported to be phosphorylated in synapses, and are derived from [242]. Currently, available data do not prove that the listed substrates are indeed phosphorylated in the presynaptic terminal or, alternatively, in the postsynaptic element of the synaptic preparation.
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Table 1.2. Phosphorylation and second messenger binding can modulate synaptic vesicle release in many ways. The effects of interactions that have been studied in detail in vivo (color-coded in Figure 1.3) are summarized here. Preparations: Ac. slices: acute slices, Aplysia: buccal ganglia of Aplysia Californica, Calyx: Calyx of Held, m. chromaffin: mouse chromaffin cells, b. Chromaffin: bovine chromaffin cells cult. neurons: cultured neurons. Helix sp. Buccal ganglia of Helix aspersa or Helix pomatia. All preparations are obtained from mouse unless otherwise stated. Mutant expression: BoNT/E+OE: SNAP25 removal with Botulinum toxin E with mutant overexpression, KI: knock in, KO+trans: KO background with transgenic overexpression, KO+OE: knockout background with mutant overexpression, WT+OE: wild type background with mutant overexpression. Parameters: Augm.: Augmentation, LTP: long-term potentiation, RRP: ready releasable pool, STD: short-term depression.
site, but its phosphorylation apparently does not affect vesicle trafficking or secretion in mouse chromaffin cells [128] (see Box 2). While the contributions of CaMK on synaptic plasticity on the postsynaptic side are prominent and well documented (see for instance [149,150]), its contribution to presynaptic plasticity may be less prominent. Ca\(^{2+}\)/CaM might also influence RRP size by activating MLCK. Data from different labs are, however, conflicting at this point, which may be related to the different experimental design and whether or not Ca\(^{2+}\) was present during refilling [151-154]. Binding of Ca\(^{2+}\) to other EF-hand proteins, such as frequenin/NCS, CaBP1 and VILIP-2 appears to specifically modulate the properties of Ca\(^{2+}\)-channels (see review [69]).

In addition to the two major groups of Ca\(^{2+}\)-effector proteins (C2-domain and EF-hand proteins), Ca\(^{2+}\) also stimulates the activity of several other components of presynaptic signaling cascades, for instance AC, ras-GEF, NO-synthase and PLC (see Figure 1.3). In addition to the influx via voltage-gated Ca\(^{2+}\)-channels on the plasma membrane, Ca\(^{2+}\) can also originate from intracellular Ca\(^{2+}\) stores gated by RyR or IP3R (reviewed in ref. [55]).

**Mapping GPCR-dependent pathways in the terminal**

Several presynaptic pathways are initiated by activation of GPCRs. Virtually all presynaptically expressed GPCRs couple to Gq, Gi/o or Gs [77]. Canonical pathways are well described and reviewed in detail elsewhere [155]; presynaptic pathways are summarized in Figure 1.3. Although crosstalk between pathways can occur at several stages ([156] and see below), Gq primarily acts via DAG and PKC, whereas protein kinase A (PKA) is the main effector of Gs activation. Gi/o signaling is more complex, as its G\(\alpha\) subunit inhibits PKA, while G\(\beta\gamma\) stimulates PLC, inhibits Ca\(^{2+}\)-channels and stimulates inwardly rectifying potassium channels. Not all canonical pathways operate in the presynaptic terminal. For example, Erk/MAPK pathway regulators like the Ca\(^{2+}\)- and DAG sensor RasGRP and Gi/o activated RapGAP, although expressed in neurons, ultrastructural analyses did not detect these molecules in presynaptic terminals [157,158].

To obtain a more systematic insight in the potential downstream targets of the kinases, we screened public phosphoprotein databases for proteins known to be present in the nerve terminal. Table 1.1 summarizes the functional groups of proteins present in the terminal. Among these, a remarkable high number of proteins was found to be phosphorylated in brain and synaptic preparations, especially cytomatrix proteins specific for the nerve terminal, such as Piccolo and Bassoon, and components of the presynaptic cytoskeleton. Transporters, small monomeric and heterotrimeric GTP-binding proteins appear to be scarcely phosphorylated and neurotransmitter synthesizing/metabolising enzymes appear not to be phosphorylated at all. Notably, the phosphorylation sites in these databases do not match very well with the phosphorylations found in studies on individual proteins (which are discussed below). Several phosphorylations which are known to affect secretion (for instance PKA-synapsin and PKC-SNAP25) are absent in the database. The reason for this discrepancy remains to be determined. We tested all presynaptic phosphorylation sites against 13 protein kinase consensus sequences to predict the kinase for each site (see ref [159], supplemental table 1). The dephosphorylation of candidate effectors by phosphatases, which may also be regulated,
has not been mapped systematically and remains an important open question. The best-described example in this respect is calcineurin, which, in conjunction with Cdk5, controls the recruitment of vesicles from the resting pool \([22,25]\).

Among the many potential effectors of GPCR-dependent pathways in the terminal, only a few are confirmed to modulate secretion upon activation/phosphorylation. The first and best-characterized effects are on presynaptic Ca\(^{2+}\)- and K+-channels. The four best-described kinases in presynaptic signaling (PKC, PKA, CaMK and Erk) all phosphorylate Ca\(^{2+}\)-channels, enhance Ca\(^{2+}\)-influx and thereby the release probability. Phosphorylation of K+-channels inhibits their rectifying role, broadens action potential duration in the terminal and thereby prolongs Ca\(^{2+}\)-channel open duration. Hence, the phosphorylation of K+- and Ca\(^{2+}\)-channels acts synergistically to potentiate secretion.

It has been postulated for decades that in addition to K+- and Ca\(^{2+}\)-channels, phosphorylation of components of the secretion machinery also contributes to secretion modulation. During the last decade, mutagenesis in vivo and in vitro using a knockout-and-rescue approach provides strong evidence that at least a few of such components indeed have prominent contributions to secretion modulation (Table 1.2). The largest modulatory effects are attributed to Munc13 and Munc18, which are both activated by DAG-pathways, although via different routes: Munc13 is activated by direct binding of DAG \([96,97,160]\), while Munc18 is phosphorylated by DAG-activated PKC (see Figure 1.3 and 4). Mutations that prevent DAG binding to Munc13 or PKC phosphorylation of Munc18 in synapses that do not express wildtype proteins show that both proteins are essential for DAG-dependent secretion modulation and that both DAG binding to Munc13 and PKC-dependent Munc18 phosphorylation are essential. An interdependent model for DAG induced potentiation has been proposed ([19], see below). In the Calyx of Held, PKC activation is not strictly required, but amplifies DAG induced potentiation \([98,154]\). Surprisingly, inhibition of PKC does not seem to have an effect in the neuromuscular junction, suggesting that this pathway operates differently in different synapses \([161]\).

SNAP-25 appears to be a third effector in the DAG/PKC pathway: in chromaffin cells PKC-dependent SNAP-25 phosphorylation increases the releasable vesicle pools \([162,163]\). Data from INS-1 \([164]\), PC12 cells \([165]\) and Aplysia neurons \([166]\) are consistent with this idea. However, proteolytic cleavage of SNAP-25 in mouse neurons followed by rescue with mutant SNAP-25 variants suggests that the protein is not involved in DAG-induced potentiation \([167]\). Phosphorylation also promotes the inhibition of Ca\(^{2+}\)-channels by SNAP-25, which reduces activity-induced calcium influx \([168]\).

The cAMP/PKA pathway probably modulates secretion via direct activation of Epac/RasGGEF by cAMP and subsequent stimulation of the PKC-pathway \((99\), see Figure 1.3). In addition, PKA phosphorylates SNAP-25, which results in a larger RRP at least in chromaffin cells \([169]\), and RIM. While RIM is an essential factor in cAMP/PKA modulation in cultured cerebellar neurons \([83]\), endocannabinoid-induced PKA downregulation and long term depression \([170]\), PKA-phosphorylation of RIM itself is not required for cAMP/PKA modulation \([86,87]\). Overexpression studies suggest that snapin may also be a PKA-effector. Snapin phosphorylation increases the RRP size in chromaffin cells \([171]\), but decreases RRP size and enhances depression during repetitive stimulation in
Brain-derived neurotrophic factor (BDNF) has been shown to effect presynaptic efficacy [173]. Its receptor TrkB can activate Shc, and thereby lead to the activation of the MAPK/Erk pathway or Src (Figure 1.3). Src phosphorylates synapsin, thereby inducing an increase in RRP size [174].

Finally, overexpression studies provide hints for contributions of CaMK, and Erk pathways to presynaptic modulation. Phosphorylation of synapsin I by CaMK and Erk occurs in vivo in an activity-dependent manner and increases vesicle recruitment, which is enhanced as more sites become phosphorylated. Depending on stimulation frequency, an activity dependent switch between pathways occurs [175]. A CaMK site of synapsin can also be phosphorylated by PKA. This reduces synaptic depression during repeated stimulation and enhances recovery [176]. Erk-mediated phosphorylation of synapsin has a similar effect on short term plasticity, but increases LTP, spontaneous and evoked release as well [177].

A network of interconnected presynaptic signaling pathways
Presynaptic signaling cascades show a high degree of cross talk. At the level of effectors, a central aspect of signaling crosstalk exists between Munc13 activation by DAG and PKC-dependent phosphorylation (Figure 1.4). Both events are essential for presynaptic

**Figure 1.3 (opposite page).** Intracellular presynaptic signaling pathways under control of GPCRs and Ca2+. Activation of GPCRs leads to the dissociation of Ga and Gβγ subunits, which in turn change the activity of downstream pathways. Gβγ mainly originate from Gi/o and presumably also Gs, but not Gq coupled receptors. GPCR stimulation and Ca2+-influx ultimately leads to changes in second messenger levels and protein kinase activity, which modulate the activity of a range of proteins of the secretion machinery. The effect of ligand binding/phosphorylation on secretion is indicated by font-color, and is based on in vivo experiments in neurons or chromaffin cells where the endogenous protein is replaced by a mutated form with binding/phosphorylation abolished or mimicked. See table 1.2 for detailed description. Abbreviations: 5HT: 5-hydroxytryptamine, AC: adenylyl cyclase, α-SNAP: soluble NSF attachment factor, BDNF: brain-derived neurotrophic factor, Calmodulin, CaMK: Ca2+/calmodulin-dependent kinase, CaM KK: CaMK-kinase, CaMK kinase, cAMP: cyclic adenosine-monophosphate, CB1: endocannabinoid receptor 1, cdk5: cyclin-dependent kinase 5, CSP: cysteine ring protein, DAG: diacylglycerol, doc2: double C2 domain, Erk: extracellular signal-regulated kinase, GAP43: growth-associated protein 43, GIRK: G-protein coupled inwardly rectifying potassium channel, GPCR: G-protein coupled receptor, IP3: inositol 1,4,5-triphosphate, IP3R: IP3 receptor, mACHR: muscarinic acetylcholine receptor, MARCKS: myristoylated alanine-rich protein kinase C substrate, MEK: mitogen-activated or extracellular signal-regulated protein kinase, mGluR: metabotropic glutamate receptor, MLCK: myosin light-chain kinase, nCAs: neuronal Ca2+ sensing sensors NSF: N-ethylmaleimide sensitive fusion protein, P2Y: metabotropic purinergic receptor, PKA: protein kinase A, PKC: protein kinase C, PLC: phospholipase C, RasGEF: Ras guanylyl exchange factor, RyR: ryanodine receptor, SNAP-25: synaptosome-associated protein of 25 kDa, SNARE: SNAP and NSF attachment receptor, Syp: synaptophysin, Syt: synaptopagmin, TrkB: tyrosine receptor kinase B, VAMP: vesicle-associated membrane protein, VGCC: voltage-gated calcium channel.
plasticity [19,96] and sub-maximal stimulation of the DAG-pathway by presynaptic receptor activation and simultaneous sub maximal increases in intra-terminal Ca\(^{2+}\) following repetitive arrival of action potentials, is expected to produce additive effects, as outlined in Figure 1.4, to synergistically potentiate secretion. As such, Munc13 and PKC can be considered as presynaptic coincidence detectors, with Mun18 being a critical PKC substrate. It should be noted that PKC phosphorylates other release machinery proteins (discussed above, Figure 1.3). Of particular interest are synaptotagmin and NSF, which are both essential for proper synapse function. To what extent these substrate contribute to PKC-induced plasticity is an important open question, and should be clarified to understand the full scope of the effect of PKC.

Another level of cross talk could occur between the DAG/PKC and CaM/CaMK pathways, as they share two important effectors: Munc13 and synaptotagmin-1. The binding of Ca\(^{2+}\)/CaM to Munc13 increases its priming capabilities much like DAG does [147,178], and CaMK phosphorylates synaptotagmin-1 on the same site as PKC [179]. The contribution of CaMK in presynaptic plasticity is poorly understood (see above), but the sharing of effectors with the very potent DAG/PKC pathway suggests that it is of importance in the presynapse.

Besides sharing of effectors, intense cross talk occurs in the upstream signaling pathways. As shown in Figure 1.3, the levels of the second messengers Ca\(^{2+}\), DAG and to less extent cAMP are intensely controlled by multiple pathways. The MAPK/Erk pathway also appears as a major integrator of many separate signalling routes, and is heavily modulated by multiple kinases, cAMP and Ca\(^{2+}\). This illustrates that the classical idea of separate independent pathways that control synapse function does not hold. Instead, all these routes should be considered as one interconnected signaling network. The activation of an individual receptor or ion channel will not act on an individual pathway, but affects the activation balance of the entire network. It is therefore highly unlikely that the effect of receptor or kinase activation can be solely explained by an individual effector molecule. To understand the effect of such events, detailed understanding of the complete presynaptic signaling cascade is essential.

**Figure 1.4.** Crosstalk between Ca\(^{2+}\) and GPCR signaling in scaling release probability. Crosstalk between Ca\(^{2+}\)-dependent and GPCR-dependent pathways can occur at many stages, of which one example is shown here. DAG, generated by GPCR stimulation, potentiates release via Munc13 and PKC, which phosphorylates Munc18. Elevated residual Ca\(^{2+}\) caused by repetitive stimulation can have stimulatory effect on Munc13 (via CaM) and Munc18 (via PKC) as well. In this model, PKC-mediated phosphorylation of Munc18 and DAG binding to Munc13 are both essential for potentiation (shown in red), while the other interactions can be interchanged.
Functional relevance of synaptic heterogeneity and secretion modulation

Although counterintuitive at first, the heterogeneity and unreliability of hippocampal and cortical synapses is crucial for their information processing capabilities. Due to their wide variety in characteristics, every presynaptic terminal responds differently to a given action potential pattern. This renders each terminal as a unique signal processing unit, which largely expands the computational power of a neuronal network [8,10,180]. The in vivo relevance of presynaptic (short term) plasticity is poorly understood (in contrast to the in vivo role of postsynaptic plasticity for macroscopic brain functions, for instance learning and memory). The lack of suitable and specific mouse models is an important limitation, although a recent in vivo knockdown approach forms promising new direction [181]. Given the timescale at which it is most evident, it can be expected that presynaptic plasticity mainly contributes to the ‘real time’ processing capacities of the brain, for instance discrimination tasks, working memory, attention and decision making. Computer simulations show that build up of residual Ca2+ in the nerve terminal during repetitive stimulation is indeed a plausible mechanism for working memory [182]. Furthermore, presynaptic depression is thought to be involved processing of sensory information. With in vivo recordings it was shown that neurons in the somatosensory cortex respond to initial whisker stimulation, but stop responding if stimulation persists, which was caused by depression of the thalamocortical synaptic connections [183]. The result of this presynaptic depression is that novel whisker stimuli are transmitted to the somatosensory cortex, but persistent stimulation is ignored. A similar function of presynaptic depression was suggested for the localization of sound [184]. External modulation by activation of presynaptic receptors is important in a large variety of neuronal systems and phenomena, for instance in pain sensation and retrograde signaling. Central terminals of nociceptors express receptors for a range of signal molecules, like GABA, opioids, endocannabinoids and bradykinin, which modulate transmitter release and thus the level of pain perception [185]. Endocannabinoid-mediated regulation of neurotransmitter release is a widespread retrograde signal and regulator of synaptic strength in the CNS [170,186]. Several studies using knock-in mice with altered downstream signaling cascades show that these pathways are involved in (spatial) working memory [177,187]. In worms, phosphorylation of Munc18 is essential for proper processing of thermosensory input [188]. Recent statistical genome analysis suggested that presynaptic pathways might also be involved in higher cognitive processes in humans [189,190].

Rules that determine presynaptic strength of individual terminals

It is very clear that the properties of synapses in the CNS can be highly heterogeneous. However, we are only beginning to understand the rules that dictate the characteristics of an individual synapse. Regulation of presynaptic strength is naturally of the upmost importance brain function. Since hippocampal and cortical cells only form very few terminals on a target neuron, the strength of these few terminals is decisive for how the signal is transmitted to, and processed by the postsynaptic post cell Therefore, one would expect tight rules that regulate presynaptic efficacy. Much current research focuses on understanding these rules.
Experiments performed in various preparations suggest a tightly regulated interplay between the presynaptic terminal and its target dendrite. With hippocampal neurons grown in vitro, it was shown that neighboring synapses have a similar $p_r$ [50,191,192]. Interestingly, synapses formed on the same dendrite seem to have similar $p_r$, suggesting that rules for determining synaptic strength segregate by dendrite [191]. Furthermore, the expression of presynaptic GABAB receptors is also regulated by synapse location [193]. This is in line with data from hippocampal, cerebellar and cortical slices, where $p_r$, Ca$^{2+}$ influx and STP depend on the identity of the target neuron [60,63,64,194,195]. And like in culture, the expression of presynaptic receptors in controlled by the dendritic target [92-94,195].

Postsynaptic neurons have an arsenal of molecules capable of modulating presynaptic strength, which include lipids, gases, trans-synaptic adhesion molecules and neurotrophins [91,196,197]. The relative contribution of each of these mediators is yet unknown. It is most likely that most diffusible molecules, like endocannabinoids, nitric oxide and arachidonic acid are primarily involved in synaptic plasticity processes, like homeostatic plasticity and long-term depression [196,198], as continuous synthesis and release of these substances would make the process too energy-inefficient. For stable, activity independent regulation of presynaptic strength, trans-synaptic adhesion molecules are the most attractive candidates. Besides their essential role during the formation of synaptic connections, these molecules can have a lasting effect on the efficacy of presynaptic vesicle release (see [197] for a recent review). The extreme versatility of this group of proteins, and their differential expression along the dendritic tree make them perfect mediators to control the strength of presynaptic terminals.

**Box 2**

**Synaptotagmin: intensely studied, but what does it do?**

It is known for decades that the influx of Ca$^{2+}$ triggers the fusion of vesicles in presynaptic terminals [225], as well as in many other secretory cells [226]. Synaptotagmins were identified as the major Ca$^{2+}$ sensors that initiate fusion. Synaptotagmins are small, highly conserved proteins (synaptotagmin-1 contains 421 amino acids and is 97% conserved between rodents and humans), consisting of two Ca$^{2+}$-binding C2-domains and a transmembrane domain that anchors it on synaptic vesicles. So far 16 synaptotagmin isoforms have been described, [226]. Of these, synaptotagmin-1, synaptotagmin-2 and synaptotagmin-9 the synaptic Ca$^{2+}$ sensors for synchronous synaptic vesicle fusion, each operating in specific synapses [140]. In addition, synaptotagmin-1, 2 and 12 regulate a large share of spontaneous vesicle fusion [141, 226, 227]. Mutations that prevent Ca$^{2+}$ binding to synaptotagmin-1 lead to a ~50% reduction in spontaneous release [141]. Expression of synaptotagmin-12, which does not bind Ca$^{2+}$, increases spontaneous release, which is further enhanced by PKA-dependent phosphorylation [227]. Confusingly, removal of synaptotagmin-1 or 2 dramatically
Figure 1.5 Overview of residues studied with mutagenesis in rodent synaptotagmin-1, with their presumed function. Each line represents a residue, with their and position on synaptotagmin indicated. Symbols represent the effect of mutations in the residue and post-translational modifications.
increases spontaneous release, demonstrating that synaptotagmins also have an inhibitory function in vesicle fusion [141,228]. How synaptotagmins carry out these opposing functions is not understood.

Synaptotagmin is probably the most intensely studied protein in the presynapse, and how synaptotagmins exactly trigger vesicle fusion is hotly debated [229]. An important open question is whether or not synaptotagmins have additional, Ca2+ independent functions upstream of vesicle fusion, like vesicle docking [230,231], SNARE complex assembly [229] or “clamping” of vesicles prior to fusion [228,232,233]. Moreover, it is not understood how synaptotagmins interact with other Ca2+ sensors, in particular doc2s, in vesicle fusion [102], to which membrane (vesicle- or target membrane) it binds to trigger fusion [229], and whether the activity of synaptotagmins is regulated during plasticity. Mutagenesis studies revealed that mutations virtually every part of synaptotagmin-1 strongly affect the function of the protein, and that individual residues are important for multiple functions (Figure 1.5). A striking example is the polybasic sequence in the C2B-domain, between residues 321-327, that is critical for the proper function of the domain. These residues mediate binding to PIP2, which markedly increase the Ca2+ concentration of the C2 domain [106,109]. However, mutations in this region, especially of lysines 326 and 327, also interfere with binding to the SNARE complex [234] and to the endocytosis factors AP2 [235] and stonin [236]. Mutations throughout the protein affect binding to the SNARE complex and phospholipids (Figure 1.5). In most occasions, it is unknown whether these mutations are specific for the interaction, or act indirectly by interfering with protein structure or other properties of the protein. This naturally hampers the interpretation of existing data.

A largely unexplored question is how the activity of synaptotagmins can be regulated by posttranslational modifications. One of the best examples is synaptotagmin-12, which upon phosphorylation by PKA increases spontaneous release [227]. Because of their central role in vesicle fusion, synaptotagmins are attractive candidate molecules to be regulated in plasticity. Changes in synaptotagmin activity are likely to have a dramatic effect on vesicle fusogeneity. Synaptotagmin-1 bears several confirmed phosphorylation sites, mostly with unknown function (Figure 1.5). Of special interest is phosphorylation of threonine 112, which is situated in an α-helix in the linker between the transmembrane and C2A domains [237]. This site is very well conserved across species and can be phosphorylated by both PKC and CaMK [179,238]. It is known to be phosphorylated in vivo [179,238,239], and potently increases binding to members of the SNARE complex [207]. But despite this strong molecular effect, phosphorylation of T112 did not affect RRP size or refilling in mouse chromaffin cells [240]. It is currently unknown if this site has any role on vesicle release or endocytosis in neurons.
Ultimately, one should be able to faithfully predict the $p_r$ of any given terminal in every point in time. Our current understanding of presynaptic heterogeneity is a far cry of what it should be to reach that goal. We need to have a very exact understanding of all the factors that affect presynaptic strength: the release machinery, the modulatory signaling network, expression of receptors, retrograde signaling pathways and location-specific rules. Only then we can understand how the terminal is tuned, to predict how information will flow through the neuronal network and is processed and stored on the way.

Outline of this thesis

The general aim of this thesis is to investigate how presynaptic strength is regulated, both on the molecular and cellular level. The first part, Chapter 2 and 3, deals with cellular process, and aims to identify rules that determine the strength of individual synapses. The second part of this thesis studies provides insight in two molecular aspects. Chapter 4 identifies a novel substrate of the DAG/PKC pathway, and chapter 5 deals with a component of the gene transcription program responsible for synapse formation.

As outlined in the previous sections, the strength and protein content of presynaptic terminals is highly variable, even among terminals formed by the same axon. The nature behind this diversity is largely unknown. It is not understood how the strength of an individual terminal is regulated, and which parameters are most important to regulate synapse output. The identity of the postsynaptic cell is known to be a critical factor, but this alone is insufficient to explain the wide heterogeneity among presynaptic terminals. Available experimental procedures are an important limitation, and most studies are limited to few terminals per experiment. This naturally hampers interpretation of the data, and potentially reduces the power to detect effects, due to variation between individual experiments. To overcome these technical differences, new methods need to be developed that measure the strength of large populations of synapses.

In Chapter 2, we describe and validate an optical method using SypHy, a genetically encoded reporter for vesicle release [199,200], to measure presynaptic strength in large sets of synapses. This method is utilized to address three questions on presynaptic functions. Firstly, we tested whether spontaneous release is mediated by vesicles from the resting pool, that is not released by action potential stimulation. The existence of such an ‘spontaneous release pool’ was postulated based on the observation that spontaneous release persists after depletion of the releasable vesicle pool [24,27]. We provide evidence that, after depletion of the releasable pool, unreleased vesicles mature and can be released by action potential stimulation. This challenges the hypothesis that spontaneous release is derived from the resting pool. Secondly, we tested if the strength of presynaptic terminals is dependent on synapse location, and observed a surprisingly organized spatial organization, with some areas containing only weak, and others containing predominantly strong synapses. Finally, we tested if this spatial organization can be modified by brief periods of network activity, since synaptic heterogeneity is activity dependent [173,191].
Chapter 3 provides a detailed description of the spatial organization of presynaptic strength. Based on the target-cell dependency of \( p \) [60,64] and the prominent location-dependency of postsynaptic signal integration [201-203], we tested whether presynaptic strength is dependent on dendritic location. Using the method described in chapter 2, together with quantitative immunofluorescence, we discovered that the strength and protein content of a synapse is highly dependent on its location on the dendritic tree, with proximal synapses being the strongest. In addition, we tested to what extent this distance dependency is regulated by network activity and the identity of the postsynaptic cell.

Chapter 4 focuses on one intracellular signal transduction pathway that modulates presynaptic strength. Activation of the DAG/PKC pathway is well-known to increase spontaneous and evoked vesicle release [204-206], which known to operate via Munc13 and Munc18 [19,96] (see Figure 1.4). Synaptotagmin-1 is also phosphorylated by PKC, which enhances its binding to syntaxin and SNAP25 [179,207]. However, mutations in synaptotagmin-1 preventing this phosphorylation have no effect on vesicle fusion in chromaffin cells [128]. However, since neurons and chromaffin cells are known to differ in the contribution of PKC in vesicle release, we hypothesized that phosphorylation of synaptotagmin-1 is of importance for vesicle release from synapses. Surprisingly, we found that phosphorylation of synaptotagmin-1 is essential for potentiation induced by phorbol ester, a pharmacological activator of the DAG/PKC pathway. Several other forms of PKC-dependent plasticity were unaffected. This chapter discusses how synaptotagmin-1 is involved in distinct steps of DAG/PKC-induced plasticity, and how it interacts with Munc13 and Munc18, the known effectors of this pathway.

Chapter 5 focuses on the genetic program that orchestrates synaptogenesis. The formation of functional synapses involves the production, transport and delivery of hundreds of proteins, and the exact regulation of this process is critical to form the required number of synapses between specific neurons, with a predefined strength. The execution of this process is precisely controlled by gene transcription factors, but many of these factors remain to be identified. Studies in invertebrates previously identified the tumor necrosis factor Menin as an essential transcription factor for synapse formation [208]. Here, we tested the role of Menin on synapse formation in cultured hippocampal neurons obtained from MEN1-lox mice. In contrast to expectations, we did not find an effect of MEN1 deletion on the formation of synapses, presynaptic strength or postsynaptic response.

Chapter 6 provides a general discussion of the new experimental findings of this thesis, in the context of existing literature. A detailed discussion is given on the relevance of distance-dependent scaling of presynaptic strength, and the possible mechanisms. In addition, it postulates an new working model for the molecular mechanisms of the DAG/PKC pathway, and discusses how this pathway operates in different types of release machineries. Finally, it provides open questions that arise from this thesis, that can form the foundations for future research.
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