Vesicle transport and neurotransmitter release in central nerve terminals
Leenders, A.G.M.

Citation for published version (APA):
Chapter 7

General discussion
The two major aims of the research described in this thesis were to characterize differential regulation of neurotransmitter release from SVs and LDCVs in more detail, and to gain more insight in the activity-dependent mobilization of SVs in central nerve terminals. In order to address these aims we introduced two new approaches. First, we developed a method to measure endogenous neurotransmitter release at a sub-second timescale. Second, we used ultrastructural analysis to determine depolarization-induced changes in intrasynaptic SV distribution. By combining these approaches we derived new insights on the following topics, which will be shortly discussed in the next sections of this chapter.

I) Difference in sub-second kinetics of release and mobilization between SVs and LDCVs (chapter 2, 3).

Already within 100 msec depolarization not only transmitter release from SVs was observed, but also recruitment and docking of SVs did occur. Release of the LDCV transmitter CCK could not be observed until depolarizations lasting over 100 ms were applied. In agreement with such a delayed release, a decrease in amount of LDCVs was not observed until 1 s depolarization. This implies that the release kinetics of LDCVs is, at least, two orders of magnitude slower than that of SVs.

II) Differential regulation of SV and LDCV release by different Ca\(^{2+}\)-channel subtypes (chapter 3, 6).

The same set of presynaptic Ca\(^{2+}\)-channels, e.g. P-, Q-, N-type Ca\(^{2+}\)-channels, is involved in triggering release from both vesicle types. We found, however, differences in the relative contributions of these Ca\(^{2+}\)-channel subtypes to the release of amino acids from SVs and CCK from LDCVs. Furthermore, adaptation of Ca\(^{2+}\)-channels in the regulation of presynaptic release became visible when studying fast transmitter release from central nerve terminals of tottering mice, which have a mutation in the pore-forming subunit of the P- and Q- Ca\(^{2+}\)-channels. A dramatic decrease in presynaptic expression of this Ca\(^{2+}\)-channel subtype and its contribution to transmitter release was accompanied by an enlarged contribution of N-type Ca\(^{2+}\)-channels, thereby compensating for changes in overall release capacity.
III) Rab3A is involved in SV mobilization (chapter 4).

Parallel experiments on fast SV recruitment, docking and amino acid release in central nerve terminals of rab3A null-mutant mice did unravel a facilitatory role of rab3A in depolarization-induced transport of SVs.

IV) Sequential changes in SV pools during depolarization (chapter 5).

The morphometrical analysis of SV distribution upon stimulation with increasing depolarization durations, showed dynamical changes in three functionally separate SV pools, a docked pool at the active zone, a recruited pool near the active zone (<150 nm) and a reserve pool at a more distant site (>150 nm) in the terminal. The sub-second depolarizations enabled us to discriminate exocytotic steps of the SV cycle, i.e. recruitment, docking and transmitter release, from the presumably slower process of endocytosis. Indeed, depolarizations lasting seconds to minutes were required in order to observe decreases in total SV numbers, which were accompanied by increases in endocytotic structures.

1. Sub-second stimulation of release and vesicle mobilization from purified nerve terminals

Synaptosomes are a widely used model system to investigate presynaptic regulation and modulation of neurotransmitter release. In this thesis synaptosome preparations of both rats (chapter 2, 3) and mice (chapter 4,5,6) have been used. The latter because of the availability of genetically altered mice, which enabled studies on regulation of release by distinct presynaptic proteins (rab3A, chapter 4 and mutated Ca$^{2+}$-channels, chapter 6). Although we purified the mice synaptosomes from whole forebrain, instead of cortex and made some minor adjustments in the stimulation protocol, the release and ultrastructural characteristics in mice and rat synaptosomes were very similar.

Since the small nerve terminals in this preparation do not allow electrophysiological approaches, usually biochemical stimulations lasting seconds to minutes are applied. In order to analyze release at a more physiologically relevant time scale we
developed a rapid mixing device. In chapter 2 it was shown that the sub-second time resolution of stimulation enabled the determination of the onset of CCK release from LDCVs, thereby indicating the difference in kinetics of transmitter release from SVs and LDCVs. The rapid mixing device provided the possibility to study regulation and modulation of the initial, fast component of endogenous neurotransmitter release in a biochemical preparation of purified nerve terminals.

Recently developed techniques, such as membrane capacitance (reviewed by von Gersdorff and Matthews, 1999) and loading of endocytosed vesicles with fluorescent dyes (reviewed by Cochilla et al., 1999) have allowed direct measurements of kinetic parameters of exocytosis and endocytosis. Although these techniques have also provided new insights in vesicle recycling, many issues concerning the individual steps in this process remain (still) unresolved by these methods. Our combined approach of short stimulations with ultrastructural analysis allowed the direct analysis of SV movement involved mainly in exocytosis and not in endocytosis, a process that probably takes seconds to minutes (chapter 5, see also section 3.3). This resulted in visualization and quantification of intrasynaptic changes in SV pools and SV cycling in central nerve terminals (chapter 3, 5). In addition, this approach provides an experimental tool to resolve some of the issues regarding the molecular mechanisms of the intrasynaptic steps of SV cycling, such as the role of rab3A in SV transport (chapter 4).

2. Differential regulation of SV and LDCV release

2.1. Sub-second release and vesicle mobilization

Mainly due to their small size, direct determinations of neurotransmitter release from central nerve terminals are still rather limited. Although the fast mixing technique does not allow measurement of transmitter release upon a depolarization equivalent to the duration of one action potential, i.e. within one millisecond, the time resolution of 50 ms that can be achieved with this method is a reasonable approximation of the physiological situation. The sub-second (≥ 50 ms) stimulations clearly showed the difference between release kinetics of CCK and of glutamate and GABA, indicating that LDCV exocytosis was at least two orders of magnitude slower than that of SVs (chapter 2).
Chapter 7

The much larger time delay between the Ca\textsuperscript{2+}-influx and the release of LDCVs as compared to the delay of SV release (chapter 2, 3) is most likely due to differences in release readiness and in localization of the vesicles within the terminal. A fraction of the SVs is docked and primed at the active zone, whereas non-docked LDCVs reside at ectopic sites (chapter 3). Furthermore, SVs at the active zone are presumably directly coupled to Ca\textsuperscript{2+}-channels, as indicated by interactions between SNARE complex proteins and Ca\textsuperscript{2+}-channel subunits (Sheng et al., 1998). Such a functional coupling allows SV release to be extremely fast, and to be triggered by a low-affinity Ca\textsuperscript{2+}-sensor. Obviously there is a larger distance between the Ca\textsuperscript{2+}-channels and the non-docked LDCVs (chapter 3) in central nerve terminals. Apparently, diffusion of Ca\textsuperscript{2+}-ions from the plasma membrane to the LDCVs and subsequent mobilization of LDCVs towards the plasma membrane accounts greatly for the delay in LDCV exocytosis from the central nerve terminals.

Activation of LDCV mobilization is necessary before exocytosis, induced by a single stimulus, can occur in the central nerve terminals. Interestingly, SV mobilization is activated upon a single short stimulation as well, however, without being coupled to immediate exocytosis (chapter 3). This mobilization probably contributes to the maintenance of maximal capacity of the readily releasable SVs, and prepares the terminals for exocytosis by subsequent stimulations (see also section 3.1).

2.2. Differential regulation of SV and LDCV exocytosis by Ca\textsuperscript{2+}-channels

2.2.1 Differential contributions of the P-, Q- and N-type Ca\textsuperscript{2+}-channels

Both SV and LDCV mobility and neurotransmitter release depend on Ca\textsuperscript{2+}-influx, which enters through multiple high voltage activated Ca\textsuperscript{2+}-channels. Studies regarding the contribution of the different Ca\textsuperscript{2+}-channels to release of SVs demonstrated involvement of P-, Q-, N- and R-subtype Ca\textsuperscript{2+}-channels (reviewed by Dunlap et al., 1995). Although these subtypes also appear to be involved to some extent in triggering release from LDCVs, L-type Ca\textsuperscript{2+}-channels appeared to be the major contributors to LDCV release in neuroendocrine cells (Mansvelder and Kits, 2000). In the central nerve terminals, however, this distinction in Ca\textsuperscript{2+}-channel subtypes involved in SV and LDCV release appeared to be somewhat different (chapter 3). P-, Q-type, and N-type Ca\textsuperscript{2+}-channels contributed to regulation of CCK release, whereas no involvement of L-type Ca\textsuperscript{2+}-channels was observed, indicating that LDCV release appeared to be mediated by the same subtypes of Ca\textsuperscript{2+}-channels.
responsible for SV release. The relative contributions of the Ca\(^{2+}\)-channel subtypes to SV and LDCV release, however, was not the same. Whereas SV exocytosis is regulated mainly by the P-type Ca\(^{2+}\)-channels, LDCV exocytosis is primarily regulated by the Q-type Ca\(^{2+}\)-channels. Moreover, N-type Ca\(^{2+}\)-channel contribution to LDCV exocytosis was observed only indirectly (see section 2.3.2). Ultrastructural localization of the different Ca\(^{2+}\)-channel subtypes on the individual terminals might reveal whether these differences in subtype contribution to SV and LDCV release may be explained by a non-uniform distribution of the Ca\(^{2+}\)-channel subtypes on the nerve terminals (Reid et al., 1997; Reuter, 1995) and/or by an uneven distribution of the different Ca\(^{2+}\)-channel subtypes on single nerve terminals (Wu et al., 1999). A preferential localization of Q-type Ca\(^{2+}\)-channels outside the active zone could account for their large contribution to LDCV exocytosis, in contrast with P- and N-type Ca\(^{2+}\)-channels that are thought to be localized in the active zone (Reuter, 1996).

2.3.2. Differential coupling of Ca\(^{2+}\)-channels to SV and LDCV release
The differences in distance between Ca\(^{2+}\)-channels and the two vesicle types (see section 2.2.1) might imply differences in coupling of Ca\(^{2+}\)-channels to the release from these vesicles. Indeed, such a differential regulation was shown by application of combinations of toxins (chapter 3) and by the regulation of release from nerve terminals of the tottering mice (chapter 6). Tottering mice might represent a model for human ataxia and absence epilepsy (Fletcher et al., 1996; Noebels and Sidman, 1979). Although our analysis of regulation of release in the tottering mice did not clarify how this phenotype is caused (see discussion chapter 6), it did reveal a number of interesting features regarding the contribution and coupling of Ca\(^{2+}\)-channel subtypes to the release of different neurotransmitters types (as discussed above).

In the active zone, P- and to a lesser extent N- and Q-type Ca\(^{2+}\)-channels jointly control neurotransmitter release from SVs (chapter 3, see also Dunlap et al., 1995), whereas regulation of LDCV exocytosis by these Ca\(^{2+}\)-channels could be explained by cooperativity between the P-, Q- and N-type Ca\(^{2+}\)-channels in the elevation of global intracellular Ca\(^{2+}\) above a threshold needed to trigger LDCV exocytosis. In tottering mice terminals, the reduction in the contribution of Q-, (P-) type Ca\(^{2+}\)-channels to the release of CCK was proportional to the observed reduced expression of the mutated α1A subunit. The quantitative compensation by N-type Ca\(^{2+}\)-channels
maintained CCK release from tottering mice terminals. In line with this observation, contribution of N-type Ca\(^{2+}\)-channels to CCK release in rat cortex synaptosomes was revealed only when P-, Q- type Ca\(^{2+}\)-channels were sub-maximally blocked. These results support the concept of a more or less linear coupling between Ca\(^{2+}\)-channels and neuropeptide release from LDCVs. Such a coupling would be in agreement with the linear relationship between intrasynaptic Ca\(^{2+}\)-rise and CCK release (Verhage et al., 1991a). This implies that CCK release depends indeed on the total rise in intrasynaptic Ca\(^{2+}\) irrespective of Ca\(^{2+}\)-channel subtype through which the Ca\(^{2+}\) entered, as proposed in chapter 3. In contrast, the data obtained for SV release, both in normal and tottering mice, are in agreement with the nonlinear power relationship between Ca\(^{2+}\)-influx and release of fast transmitters from SVs (Dodge and Rahamimoff, 1967; Heidelberg et al., 1994; Heinemann et al., 1994; Katz and Miledi, 1970). This indicates that at the active zone the release from SVs depends on the joint control by multiple Ca\(^{2+}\)-channel subtypes (chapter 3, Dunlap et al., 1995).

2.4. Differential regulation of SV and LDCV release, In conclusion

Considering the data described in this thesis concerning SV and LDCV release and those from the literature, the following concept of differential regulation of SV and LDCV release from central nerve terminals can be put forward (fig.7.1).

At the active zone of the terminal docked and primed SVs can be released extremely fast (< 1 ms) upon localized Ca\(^{2+}\)-influx through P- and to a lesser extent Q- and N-type Ca\(^{2+}\)-channels. These different Ca\(^{2+}\)-channel subtypes are co-localized and jointly control release at individual release sites. The concurrent activation, besides SV fusion, of SV mobilization from the reserve pool to the docked SV pool ensured maintenance of a maximal capacity of the readily releasable SV pool.

When stimulation is maintained long enough (> 100 ms) bulk intracellular Ca\(^{2+}\) can be raised sufficiently to trigger LDCV release by first mobilizing the LDCVs to the plasma membrane followed by fusion with the membrane. A predominant contribution of Q-type (possibly located at ectopic sites in the terminals), with a lesser contribution by P- and N-type Ca\(^{2+}\)-channels is responsible for the total Ca\(^{2+}\)-influx necessary to trigger LDCV release. Coupling of Ca\(^{2+}\)-channels to LDCV release appeared to be linear, in agreement with the previously shown linear coupling between [Ca\(^{2+}\)] and LDCV release (Verhage et al., 1991a).
General discussion

Figure 7.1. Differential regulation of release from SVs and LDCVs

The differences in kinetics and regulation by Ca\(^{2+}\)-channels most likely arise from different distances between Ca\(^{2+}\)-channels and Ca\(^{2+}\)-sensors on the vesicles, which may reflect the functional demands for rapid and focal release of fast acting transmitters from SVs on the one hand, and a more diffuse release of slower, modulatory neuropeptides from LDCVs on the other.

3. Intrasynaptic transport of SVs

3.1. SV recruitment and docking

3.1.1 Sub-second depolarization induced SV recruitment and docking

Short depolarizations induced a shift in the SV distribution indicating the recruitment of SVs towards and docking of SVs at the active zone membrane (chapter 3, 5). The recruitment and docking of SVs, like neurotransmitter release, appeared to be regulated by Ca\(^{2+}\)-influx through high voltage activated Ca\(^{2+}\)-channels, although these processes may be interrelated with each other (chapter 3). Such a Ca\(^{2+}\)-dependent regulation, however, would be in agreement with recent studies showing
facilitation of refilling of the readily releasable pool of SVs by Ca\(^{2+}\)-influx (Pyle et al., 2000; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998). Our results indicate that small central synapses have the capability to actively and rapidly replenish releasable SV pools, by recruitment of SVs from the reserve pool. This simultaneous activation of these events, namely fusion, recruitment and docking of SVs would be a very efficient mechanism to keep the synapses at their maximal release capacity. What are the molecular mechanisms that regulate these three processes? The components involved in the SV cycle are largely characterized. Most of the SV proteins are cloned, several molecules specifically localized at active zones are known and a large number of cytosolic proteins involved in these processes have been identified (Fernández-Chacón and Südhof, 1999; Garner et al., 2000). Nevertheless, except for membrane fusion, little is known about the molecular mechanisms of the various other steps of the SV cycle within the presynaptic terminal. The Ca\(^{2+}\)-dependence of SV recruitment and docking could reflect involvement of proteins activated by elevated [Ca\(^{2+}\)]\(_i\), such as synapsin I, which can be phosphorylated by CaM-kinase II (Pieribone et al., 1995; Rosahl et al., 1995) or C\(_2\)-domain containing Ca\(^{2+}\)-binding proteins such as rabphilin, Doc2 and synaptotagmin (Goda and Südhof, 1997).

3.1.2 Role of rab3A in SV recruitment and docking

Another protein implicated in SV transport is rab3A, a member of the large family of rab GTP-binding proteins. Like SNARE proteins, rabs are conserved from yeast to humans and are present in multiple isoforms, each associated to a specific membrane compartment. Rabs are thought to act as facilitators of transport steps, i.e., upstream of SNARE-complexes and fusion (Aridor and Balch, 1996; Gonzalez and Scheller, 1999; Schimmöller et al., 1998). In chapter 4 we showed that rab3A, the major rab protein in brain synapses, indeed has a facilitatory function in the transport of SVs towards the active zone in central nerve terminals. This phenotype of Rab3A null-mutant mice resulted in reduced vesicle availability at the active zone after a period of prolonged stimulation (chapter 4), which would be in line with synaptic transmission being more susceptible to fatigue during high frequency stimulation (Geppert et al., 1994a) and the absence of LTP in CA3 mossy fiber terminals (Castillo et al., 1997). Although we uncovered this function of rab3A in SV transport, our data do not exclude a separate role of rab3A such as a negative
regulator of release probability acting downstream of SNARE-complex formation (Geppert et al., 1997). Since at least two specific rab3 effectors have been characterized (Shirataki et al., 1994; Wang et al., 1997) and additional effectors may be discovered, it is conceivable that rab3A exerts multiple functions at distinct steps in the SV cycle (Gonzalez and Scheller, 1999).

How rab3A exerts its role in SV transport still remains to be determined. Interestingly, rab3A appeared to be specifically involved in depolarization induced SV recruitment, and not in SV recruitment induced by hyperosmotic sucrose solutions. This latter method very effectively releases the readily releasable SV pool, but in a Ca\textsuperscript{2+}-independent way (Rosenmund and Stevens, 1996). The sucrose-induced SV recruitment observed in our experiments did neither depend on extracellular Ca\textsuperscript{2+} (chapter 4), suggesting that rab3A might exert its function via a Ca\textsuperscript{2+}-dependent process. The rab3A effectors, rabphilin and RIM, contain C\textsubscript{2}-domains, suggesting Ca\textsuperscript{2+}-dependent regulation of these proteins. The reduced expression of rabphilin in rab3A knock-out mice (Geppert et al., 1994a) would be in agreement with the regulation of SV recruitment by the interaction between rab3A and rabphilin. However, no phenotype has been observed in rabphilin3A knock-out animals (Schluter et al., 1999). The localization of the other rab3A effector, RIM, at the active zone membrane suggests the possibility of the involvement of the rab3A-RIM interaction in docking of SVs at the zone (Wang et al., 1997). Interestingly, recent analysis of RIM-1 null-mutants revealed that these mice exhibit the same phenotype in mossy fibers, a lack of LTP, as observed in rab3A null-mutants (Castillo et al., 2000).

3.2. SV pools

SVs are usually divided into two pools: a small pool of docked vesicles, i.e. vesicles associated with the active zone membrane, and a large reserve pool, in the cytosol of the terminal. In sections of synaptosomes usually 4 SVs docked at the active zone membrane were observed (chapter 3; in mice synaptosomes the average number of docked SVs was 2, chapter 4, 5). This number is compatible with the morphologically observed docked pool of about 10 SVs in a whole bouton of cultured hippocampal neurons (Schikorski and Stevens, 1997). Interestingly, in functional assays a readily releasable pool of about 5 to 10 SVs was observed in these neurons (Rosenmund and Stevens, 1996; Murthy and Stevens, 1999). These observations suggest that this
functional readily releasable pool corresponds to the morphological docked pool of vesicles. Besides the 4 docked SVs, an average amount of 51 SVs (46 in mice synaptosomes) was found in the cytosol of the synaptosomes sections. This number also corresponds well with the total amount of about 200 SVs observed in a whole bouton (Schikorski and Stevens, 1997). Usually this cytosolic pool is referred to as the reserve pool. However, our analysis of depolarization-induced changes in SV distributions revealed that this cytosolic pool could be divided in two functionally distinct pools. Recruitment of SVs from the reserve pool resulted not only in an increased docked pool of SVs, but also in an increased fraction of SVs located in the immediate vicinity of the active zone membrane, i.e. within 150 nm distance (chapter 3, 5). Apparently, these SVs represent an intermediate vesicle pool, located between the docked and reserve pools, acting as a highly dynamic reservoir from which SVs can be recruited to enter the docked pool and which itself is replenished by SVs from the reserve pool. Based on this observation, two distinct cytosolic vesicle pools can be discriminated, the pool of recruited SVs near the active zone and the reserve pool located at more distant sites in central nerve terminals. Several independent studies also indicate the existence of functionally distinct cytosolic SV pools (Kuromi and Kidokoro, 1998; Murthy and Stevens, 1999; Pieribone et al., 1995; Richards et al., 2000). The two cytosolic pools appear to be differentially involved in sustaining neurotransmitter release. Whereas the more proximal pool is readily available to sustain normal release, the reserve pool appeared to be recruited only during high frequency stimulations. A recent study by Pyle et al. (2000) even suggested that each pool retained its identity during activity by using distinct recycling pathways. Our results, however, showed that trafficking of SVs in a one-way direction from the reserve, via recruited, to the docked pool was activated upon short stimulations. Indicating that in small central synapses movement of SVs between pools can take place during activity.

3.3. SV recycling

After fusion of the SVs with the plasma membrane the excess of membrane is retrieved by endocytosis. However, the mechanisms and location of endocytosis are still a matter of debate, and several recycling pathways appear to exist. Ultrastructural studies in neuromuscular junctions provided evidence for the “classical” endosomal recycling pathway (Heuser and Reese, 1973). Later
morphological observations also identified endosome intermediates in central nerve terminals (De Camilli and Takei, 1996; Fried and Blaustein, 1978). Over the past decade most of the proteins involved in the clathrin mediated endocytosis and several endosome related proteins have been identified in these terminals as well (Brodin et al., 2000). The membrane quantifications that we made during and after depolarizations in purified nerve terminals are in agreement with this "classical" form of recycling (chapter 5). In addition, our study showed that this form of endocytosis does occur near the active zone, unlike earlier studies (Fried and Blaustein, 1978; Heuser and Reese, 1973), that suggested that recycling via endosome intermediates occurs mainly in terminal regions away from the active zone (Heuser and Reese, 1973).

Studies using fluorescent dyes indicate, however, that SVs can be recycled much faster than was previously thought, in the order of several seconds (Betz and Bewick, 1992; Ryan et al., 1993; Thomas et al., 1994), and that the vesicles can be recycled without passing through an endosomal compartment (Klingauf et al., 1998; Murthy and Stevens, 1998). Based on their morphological data DeCamilli and colleagues (1996) also proposed a model of recycling, where SVs are directly reformed from the endocytosed vesicles after shedding their clathrin-coat. Besides this rapid endocytosis, in neuroendocrine cells an even shorter pathway, referred to as "kiss and run", was observed (Alvarez de Toledo et al., 1993; Monck and Fernandez, 1994). In "kiss and run", a fusion pore has been proposed to form between vesicle and plasma membrane that allows transmitter release without full fusion of the vesicle, and the vesicle is retrieved intact (Ceccarelli et al., 1973; Fesce et al., 1994). Very recent quantitative fluorescence measurements indicated that this "kiss and run"-like mechanism also exists in hippocampal synapses (Pyle et al., 2000; Stevens and Williams, 2000). This fast "kiss and run" preferentially refills the readily releasable pool of SVs, whereas the reserve pool of SVs is restored via classical endocytosis, consistent with similar data obtained at the neuromuscular junction (Richards et al., 2000). Our observation that after 100 ms depolarization the total amount of SVs was not decreased, while fusion of several SVs must have occurred (see chapter 3, 5), could be explained by means of this rapid "kiss and run" mechanism. However, since we cannot discriminate in our electron microscopical pictures whether a SV, filled with transmitter, is awaiting fusion or if it is just retrieved from the membrane and devoid of transmitter, detection of this form of rapid
endocytosis was not possible in our study. Labeling of endocytosed membranes upon short depolarizations by endocytic markers such as HRP or by FM-dyes, which can be visualized by photoconversion (Henkel et al., 1996), could be helpful tools to uncover the existence of rapid endocytosis at the ultrastructural level in these small terminals.

3.4. Intrasynaptic SV transport, In conclusion

The ultrastructural analysis of depolarization induced changes in SV distributions revealed three distinct SV pools in the small presynaptic terminals: I) the docked pool, II) the recruited pool and III) the reserve pool (see fig. 7.2). Short depolarizations (100 ms) induced trafficking of SVs, in a one-way direction, with recruitment (1, fig. 7.2) from the reserve to the recruited pool and docking (2, fig. 7.2) of recruited pool SVs at the active zone membrane, thereby increasing the docked pool. The small GTP-binding protein rab3A has a facilitatory role in the regulation of

![Figure 7.2. The SV cycle.](image)

these steps, which probably are, like fusion, mediated by Ca^{2+}-dependent processes. Several distinct recycling pathways appear to co-exist in the small nerve terminals, which are proposed to be used by different SV pools and under different stimulation conditions (Südhof, 2000). Under mild stimulation conditions, SVs from the readily releasable pool release their transmitter content via a fusion-pore through the plasma membrane, i.e. “kiss and run” mode (5, fig.7.2). The empty SVs remain at or near the active zone and can be rapidly re-used after refilling with neurotransmitter. Under stronger stimulation, the readily releasable pool is depleted and SVs have to be mobilized from the reserve pool in order to maintain neurotransmitter release. However, these newly recruited SVs from the reserve pool release their content by full fusion with the plasma membrane. The vesicle membrane is thereafter retrieved via clathrin and dynamin mediated endocytosis, and recycled back to the reserve pool, either directly (4, fig.7.2) or via endosome intermediates (3, fig.7.2).

Several distinct pathways appear to be nested within the SV cycle. However, trafficking of SVs between pools also occurs (chapter 3, 5), indicating inter-relations between the pathways. Altogether these pathways enable the synapses to respond adequately to a wide range of stimuli, and thus display considerable plasticity. How the pathways are related, and which pathways are activated, and to what extent when different stimulation protocols are applied requires further investigations. Furthermore, the molecular mechanisms defining these different pathways are largely unknown. For example, it is unclear whether clathrin and dynamin are only involved in endocytosis of SV membranes fully collapsed into the plasma membrane or if they are also involved in the fast “kiss and run” pathway.

4. Perspectives

Presynaptic terminals are highly specialized neuronal compartments designed for rapid and efficient neurotransmission by SV exocytosis. The co-localized LDCVs in these terminals allow multiple signaling by releasing different neurotransmitter types. Differences between neurotransmitter release from the two vesicle types concern the localization of vesicles within the terminal, the kinetics of release and mobilization of the vesicles, the Ca^{2+}-sensitivity and the coupling between Ca^{2+}-channels and vesicles. These differences are most likely determined by distinct components
depending on the secretory pathways involved. However, as already mentioned above, although most of the proteins that regulate SV release have been characterized, we are only beginning to understand their function in the entire SV cycle. And even less is known about the protein machinery that mediates the LDCV release. It is likely that the exocytosis of LDCVs proceeds through docking/fusion steps similar to those of SVs, involving the same core complex of proteins. However, specificity might be generated by expression of vesicle type specific isoforms or by the exclusive presence of accessory proteins, such as for instance CAPS (Ca\(^{2+}\)-activator protein for secretion). This protein is selectively localized to LDCVs and implicated in LDCV release (Berwin et al., 1998; Tandon et al., 1998). Therefore the greatest challenge in the next years will be to couple distinct molecular protein-protein interactions to specific trafficking steps of both SVs and LDCVs. The ongoing development of sophisticated techniques to visualize vesicles in different phases of the cycle in living cells and in real time, combined with gene-technology will provide powerful experimental tools to unravel the exact sequence of steps and the exact roles of all the proteins involved.