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Review

Differential signaling in presynaptic neurotransmitter release

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Abstract. Neuronal communication is tightly regulated by presynaptic signaling, thereby temporarily and locally secreting one or more transmitters in order to exert propagation or modulation of network activity. In the last 2 decades our insight into the molecular regulation of presynaptic transmitter vesicle traffic and fusion has exponentionally grown due to the identification of specific functional interactions between presynaptic proteins involved in these processes. In addition, a plethora of extracellular and intracellular messengers regulate neurotransmitter release, occasionally leading to short- or long-term adaptations of the synapse to altered environmental signals. Important in this respect is the ability of various nerve terminals to diverge their output by differentiation in secretion of co-localized transmitters. This divergence in presynaptic signaling may converge in the postsynaptic target neuron or spread to neighbouring cells. In this review differential presynaptic signaling mechanisms will be related to their potential divergent roles in transmitter release.

Key words. Presynaptic signaling; differential regulation; transmitter release; synaptic vesicles; dense-cored vesicles.

Introduction

Because presynaptic signaling paves the way for interneuronal communication, much interest has been and is still being focused on this topic. Since the discovery of the quantal nature of neurotransmitter release 50 years ago [1] and the electron microscopical visualization of small vesicles clustered at sites where neurons communicate with each other [2], the literature on presynaptic neuroscience has increased tremendously. The basic properties of presynaptic signaling in the central nervous system (CNS), i.e. regulated secretion of transmitters stored in synaptic vesicles at special regions in the presynaptic plasma membrane upon arrival of one or a train of action potentials, has been derived from experimentally easily accessible giant terminals from squid and amphibian neuromuscular junctions. A second burst in research of presynaptic signaling occurred when the molecular entities involved in transmitter release, i.e. a still growing number of proteins located in plasma membranes, synaptic vesicles or cytosol, were purified from nerve terminal preparations. The ability to manipulate these proteins genetically by recombinant DNA technology both in isolated cells as well as in animals expanded our knowledge of their potential roles in presynaptic signaling. An interesting concept arose, namely that vesicle exocytosis and endocytosis were mediated by changing assemblies of protein protein interactions, thereby directing transport of synaptic vesicles and their recycling inside the nerve terminal [3]. The fact that many of these proteins appeared not only to be present in presynaptic structures but in practically all cells of mammalian organisms underscored their importance in general cell biology. Interestingly, genomic analysis did show that homologues proteins were present in primitive eukaryotes such as yeast and nematodes, thereby indicating their relevance throughout evolution [4]. In the 1980s the patch-clamp technique

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became available, enabling local monitoring of transmission at proper time resolution between much smaller synapses [5].

Using these approaches, it became clear that diversity in presynaptic structures exists which closely correlates with a variety in function. It is not difficult to understand that such a diversity implicates, besides common general properties mentioned above, differences in the signaling cascades involved. In addition to this structural and functional diversity between synapses, there is differentiation in response of one and the same synapse because of secretion of multiple transmitters, which are co-localized inside the nerve terminal. This co-localization, already known in several peripheral nerves, was observed some 25 years ago in central nerve terminals as well. By combinations of advanced microscopy and cytochemistry presynaptic co-localization of classical transmitters such as amino acids, acetylcholine or catecholamines with the newer class of the neuropeptides became visible in diverse brain areas [6]. In current neuroscience the principle of co-localization is more rule than exception. Most catecholamine-releasing neurons contain various neuropeptides as well, which are co-released from synapses as well as from their varicosities, release sites which have no postsynaptic specialization associated with them [7]. And in brain areas such as the hippocampus and ventral striatum a population of synapses which all contain GABA as a classical transmitter can be sub-divided into synapses which each contain different neuropeptides, such as cholecystokinin (CCK), Met-enkephalin or dynorphin, as co-transmitter [7]. It is widely believed that such a segregation has important consequences for the function of these synapses, although the exact relationship between chemical phenotype and function is in most cases still far from understood. These co-transmitters are stored in morphologically distinct vesicles which are localized and secreted at distant sites in the synapses [8]. By this principle the nerve terminal is equipped for variation in signaling dependent on the activation pattern, thereby generating a temporal and spatial diversification of presynaptic strength. Therefore, a better understanding of the underlying presynaptic signaling is of crucial importance.

In 1935 Dale proposed that a neuron releases the same transmitter at all its synapses [9]. Over the years this statement was interpreted as that each neuron could release only one transmitter, which became known as Dale's principle, and dominated thinking until the early eighties. However, in current neuroscience the presence of multiple transmitters is a well-established general feature of neurons [6, 7]. And as neurons are usually defined by the 'classical' transmitter they release, i.e. GABAergic interneurons or dopaminergic cells, co-transmission of modulatory neuropeptides from their synapses is a generally accepted feature of most neurons as well. In this review an attempt will be made to consider diverse presynaptic elements in relationship to differential signaling in transmitter release. Since most, if not all, messenger molecules known nowadays are present in nerve terminals, their existence will not be described extensively here. Prior to the description of the chemical signals, including Ca^{2+}, cyclic nucleotides, inositol phosphates, Rab-GTPase and phosphorylation, a short overview of the diversity in presynaptic ultrastructural elements will be given. A detailed description of the involvement of the large array of proteins in presynaptic signaling is not the purpose of this review, although touching their relevance as potential targets for differential regulation of transmitter release cannot be avoided. Diverse excellent reviews on the role of presynaptic proteins in the regulation of transmitter release are available, including this journal [3, 10, 11]. Due to space restrictions, not all literature relevant in this intensively investigated field of current neuroscience can be cited, for which the authors apologize beforehand.

Differentiation in presynaptic structural elements

In contrast to neurosecretory cells, most neurons are clearly compartmentalized in different entities which all contain their own typical signal transduction. As such, axonal nerve endings are separate structures at a relatively large distance from the cell body, although both compartments function in close dependency on each other. Inside the nerve terminal the classical transmitters are mainly stored in typical membrane-surrounded structures, the synaptic vesicles (SVs). These vesicles are electron-lucent under the electron microscope and have small diameters (35–50 nm). A distinction was made between asymmetrical excitatory synapses containing round (circular) shaped SVs and symmetrical inhibitory synapses with oval (flattened) SVs, though this structure-function relationship was not absolute [12]. In the nerve terminals mitochondria are present, ensuring a proper energy supply for presynaptic activity (fig. 1A). In addition endosome-like organelles are enclosed, which may represent intrasynaptic membrane stores of invaginated SVs recycled after their fusion and waiting for a new cycle (fig. 1A) [13]. The presence of endoplasmic reticulum organelles in the nerve terminal may subserve as local supplies for Ca^{2+} delivery and/or storage site for presynaptic proteins [14].

Regularly, co-localization of larger dense-cored vesicles (LDCVs) is observed in nerve terminals which store neuropeptides or biogenic amines (fig. 1C) [8]. Their dense-cored nature is caused by condensed packaging of these transmitters inside the centre of the vesicles similar to the vesicles in neurosecretory cells and granules in chromaffin cells [15, 16]. Besides their structural differences, both vesicle types are localized at different sites inside the

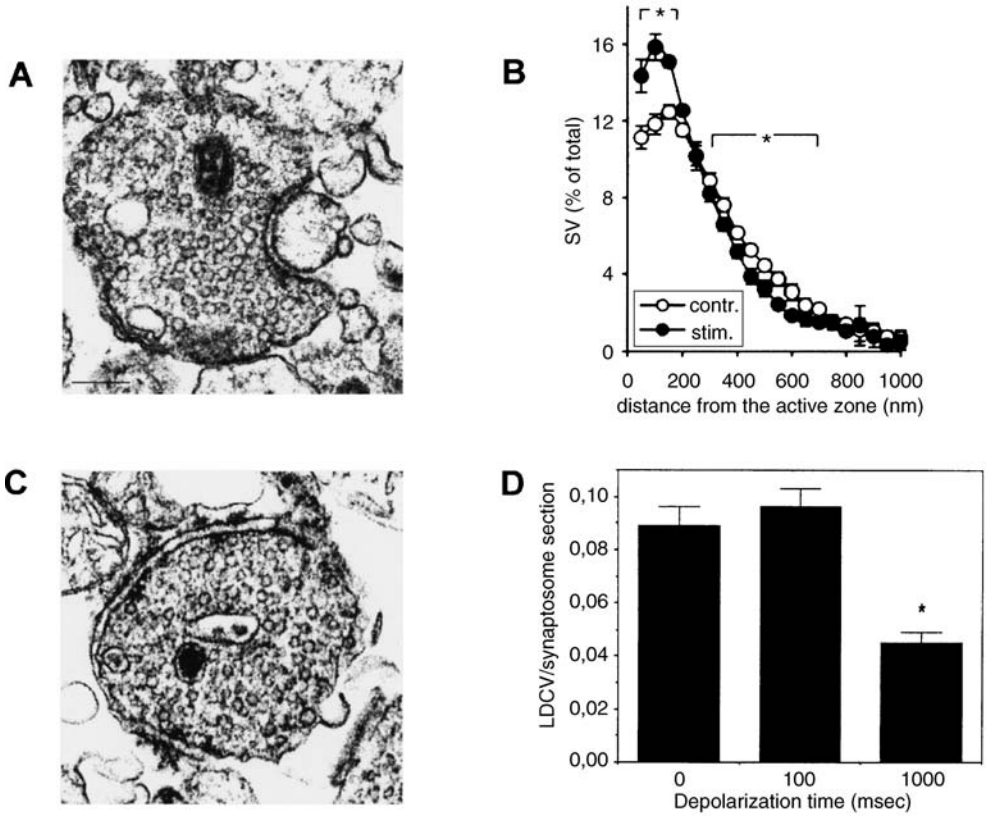

Figure 1. Electron microscopic pictures of sections of isolated nerve terminals from rat brain containing SVs (*A*) and occasionally co-localization of a LDCV (*C*). These structures contain mitochondria and endosome-like organelles, and normally have postsynaptic fragments opposing their active zone still attached. Fast redistribution of SVs in isolated nerve terminals towards the active zone by a 100 ms K^+ pulse (*B*). Slower disappearance of co-localized LDCVs by K^+ depolarization (*D*). Scale bar in *A* (left below), 100 nm. In (*B*): contr. control; stim. 100 ms K^+ depolarization; reprinted from [13], © (2002), with permission from Elsevier.

nerve terminal. The SVs are partially clustered at a special part of the presynaptic plasma membrane which abuts the postsynaptic neuron, the active zone where some of the vesicles dock directly (fig. 1A) [17]. The LDCVs are dispersed more diffusely inside the nerve terminal at remote sites from the active zone (fig. 1C) [15]. In contrast to the SVs, the LDCVs are always located at some distance from the presynaptic plasma membrane, at least in resting nerve terminals. Upon nerve stimulation, the SVs fuse with the active zone membrane and lose their transmitter content into the synaptic cleft. Since the fusion-regulating signaling elements such as Ca^{2+} channels and Ca^{2+} sensors are presumably located in immediate proximity to the clustered SVs in the active zone, as described in more detail below, transmitter release can occur within 1 ms after stimulation. The small dimension of the synaptic cleft between active zone and postsynaptic neuron, about 20 nm, enables fast postsynaptic reaction on evoked SV-derived transmitter release by activation of specific local ion channel-coupled receptors in the postsynaptic density [18]. The release of transmitters stored in LDCVs at ectopic sites inside the nerve terminal into the extracellular space requires more extensive and prolonged stimulation [19, 20], and probably takes place at parts of the presynaptic plasma membrane distant from the active zone [21]. Both features, traffic of LDCVs towards the plasma membrane at remote sites and subsequent secretion of its transmitter(s) outside the synaptic cleft, involve delayed action of these transmitters as compared with the SV ones, including possible modification of the fast signaling by the latter. By this means divergent signaling after presynaptic activation would cause convergence of synaptic signals, thereby restricting the fast signal in space and time.

An important feature of presynaptic release is that transmitter secretion can persist upon repetitive high-frequency stimulation. Essential for prolongation of release is that 'new' transmitter-containing vesicles are ready to fuse upon subsequent stimulation, i.e. they have to be recruited rapidly. For that purpose local recycling of SVs has been proposed, enabling their fast reuse [22]. The abundant presynaptic phosphoprotein synapsin I probably plays a crucial role in SV recruitment. In the dephosphorylated form synapsin I attaches SVs to short actin filaments

near the active zone. Upon stimulation of the nerve terminal synapsin is phosphorylated by, among others, Ca^{2+}/calmodulin kinase II, thereby detaching them from the cytoskeleton and facilitating their recruitment, docking and fusion [23]. In small boutons which normally have one active zone where presumably maximally one vesicle fuses upon stimulation by a single action potential, vesicle fusion after the next stimulation requires fast replenishment of readily releasable docked vesicles in the active zone and fast recruitment from other vesicle pools inside the nerve terminal. In thin electron microscopical sections of isolated nerve terminals this docked pool comprises about 4 SVs, i.e. less than 10% of the total amount (fig. 1A) [17], which would be in accordance with a total amount of 10 SVs in this pool estimated for a whole bouton [24]. Short K^+ depolarization pulses induce both recruitment of SVs in the active zone and increase docking within 100 ms, the fastest time point investigated (fig. 1B) [17]. After full fusion of SVs in the active zone their retrieval via endosome-like structures requires seconds, a period which would be too slow to follow high-frequency stimulation [13]. Recently, an additional much faster 'replenishment' mechanism in nerve terminals has been shown which would perfectly match such a rapid reuse of SVs, namely by 'kiss-and-run'. According to this mechanism, SVs fuse partially, thereby secreting only part of their transmitter content and stay associated with the active zone membrane in order to be ready for a next stimulation pulse [25]. In conclusion, current views favor the presence of several recycling pools inside the nerve terminal with different involvement in fast transmission upon repetitive stimulation and upon extensive stimulation [26]. In addition, a 'resting' pool of SVs is proposed which is not really active in the transmitter release process and from which vesicles can be recruited on demand [27].

In contrast to the established evidence for SV recycling and their reuse in order to cope with fast repetitive stimulation, these features are less clear for the LDCVs. Only after prolonged stimulation, i.e. 250 ms, do these vesicles release their dense-cored content, which may be due to their location distant from the plasma membrane and from Ca^{2+}-entry sites [17]. In accordance with the slower transmitter release from LDCVs, their disappearance from nerve terminals, presumably as a consequence of their exocytosis, is much slower compared to the SVs (fig. 1D) [17]. The mechanism of translocation of LDCVs towards the plasma membrane in order to fuse is much less clear than that for SVs mentioned above. It has been proposed that these vesicles are anchored in actin networks preventing their free mobility, as suggested for granules in chromaffin cells [28]. If so, LDCV movement to the plasma membrane would require their detachment by actin disassembly, though in a synapsin I-independent way since this protein is not present in LDCVs [23]. However, in growth cones of cultured PC12 cells neuropeptide release appeared to be primarily derived from freely diffusible cytoplasmic LDCVs rather than immobile docked vesicles [29]. Although LDCVs have been assumed to be unable for recycling and fully dependent on new synthesis of their peptide transmitter content in the cell body and subsequent transport towards the nerve terminal, recent evidence in neuroendocrine cells suggests that these larger vesicles regularly did not secrete their full peptide content during fusion and could be recycled in order to be used for a next exocytotic round [30, 31]. Extrapolation of these findings to small nerve terminals requires care, but repeated secretion of peptide from isolated nerve terminals by subsequent massive stimulatory pulses would be in agreement with such a mechanism [32].

Besides the differential transmitter release from different vesicle types as a presynaptic instrument for functional diversification common in many synapses, the whole architecture of the nerve terminal is highly determining as well. The experimentally easily accessible giant nerve terminals from the neuromuscular junction, the calyx of Held in the auditory system and the retina have highly increased our insight in that respect. These nerve terminals have in common that they have each hundreds of active zone-like areas where small SVs dock and fuse in order to secrete their contents to many clusters of postjunctional receptors. In this way these large nerve terminals are optimally equipped for fast release of vesicles from multiple sites in order to exert strong sensory or mechanical signals [33, 34]. In relatively smaller CNS nerve terminals a divergence in architecture also exists. An example is the discrimination between small one single active zone containing nerve endings projecting to CA1 pyramidal neurons in the hippocampus with sizes of <1 µm (fig. 1A) and the multiple active zones containing mossy fiber boutons from dentate gyrus granule cells which project mainly onto CA3 pyramidal neurons in that brain region, being almost 10-fold larger. Interestingly, long-term potentiation (LTP) of glutamatergic transmission in these synapses is induced differentially. Whereas LTP in CA1 synapses is induced postsynaptically, this form of plasticity is induced presynaptically in the mossy fiber synapses [35]. By these multiple active zones containing mossy fibers are optimally designed to diverge their signal to several CA3 pyramidal cells, whereas the smaller synapses transmit their signal more in a single point-to-point fashion [36]. As such, mossy fiber synapses can generate activity-dependent diversity in signaling after transmitter exocytosis, including paracrine effects by spillover to other synapses and autocrine presynaptic feedback [37]. These large irregular synapses have another interesting property with regard to differential signaling. Dynorphin, the co-localized LDCV neuropeptide in mossy fibers, is diffusely released by high-frequency

stimulation, thereby exerting heterosynaptic inhibition of neighbouring mossy fiber synapses [38].

Although presynaptic signaling shares basic mechanisms notwithstanding the diversity in ultrastructure and function between nerve terminals, this diversity certainly involves distinct signaling complements related to their particular physiological roles. This review will be restricted as much as possible to adult mammalian nerve terminals of small size measuring a few micrometers or less, which comprise about 95% of all synapses in CNS.

Diversity in presynaptic signals

Presynaptic signaling is the result of a tightly coordinated interplay between extracellular and intracellular messengers. Maturation of nerve terminals is accompanied by their incorporation into a dense extracellular matrix formed by diverse intercellular adhesion molecules (CAMs). These CAMs connect the nerve terminals properly with the postsynaptic target sites, and some of them may also be involved in activity-dependent remodeling of synapses [39, 40]. Particularly interesting is the junction between the presynaptic surface CAM neurexin and the postsynaptic CAM neuroligin. Intracellularly, neurexin interacts with several proteins belonging to the transmitter release machinery, including Ca^{2+}-channel clusters in the active zone [41]. In addition to these junctional proteins free diffusible extracellular messengers regulate presynaptic activity. Many of them are of postsynaptic origin, thereby acting in a retrograde fashion. The gas nitrogen oxide (NO) and the lipophilic arachidonic acid and endocannabinoids are examples of this category [42]. More recently, retrograde signaling by various neurotrophins was reported, thereby inducing presynaptic functional maturation and stabilization [43]. The brain-derived neurotrophic factor (BDNF) stimulates neurotransmitter release by activating a presynaptic tyrosine kinase receptor (Trk) [44]. Apparently, a traditional signal cascade comprising Trk and mitogen-activated protein kinase (MAPK), known as prominent regulator of nuclear transcription, can induce post-translational effects locally in nerve terminals as well.

Presynaptic receptors in the plasma membrane sense extracellular signals and convert them in order to modulate transmitter release. Originally it was thought that all presynaptic receptors belong to the relative slow acting metabotropic G-protein-coupled class only, exerting mainly inhibitory effects on the release machinery [45]. Normally, these receptors act by inhibiting voltage-gated Ca^{2+} channels or by increasing K^+-channel activity, though actions downstream of Ca^{2+} entry has been suggested as well [46]. More recently, direct activation or modulation of transmitter release by presynaptically located ionotropic receptors have been reported [47]. Since these receptors directly gate ion channels upon activation, their action on transmitter release is regularly much faster than that of their metabotropic counterparts, which makes them potential modulators of short-term plasticity events. In addition to these temporal differences, segregation of multiple presynaptic receptors at synaptic and perisynaptic localizations enables differential regulation of transmitter release in a use-dependent way [48, 49]. The excitability of the nerve terminal is determined by the balance between the voltage-dependent Na^+- and K^+-channel activities. Upon relatively strong depolarization, as exerted by action potentials, multiple presynaptic Ca^{2+} channels will be activated, some of them in close proximity to the transmitter release site(s), allowing entrance of the ultimate trigger for release and ultrafast induction of vesicle fusion [50]. In addition to receptors and ion channels, specific transporters are located in presynaptic plasma membranes which locally recycle classic transmitters, such as amino acids and catecholamines, into the nerve terminal after their secretion, thereby restricting extrasynaptic diffusion of transmitters [51]. Since these transporters are driven by ion gradients, their direction of action can change from the inward mode under resting polarized conditions towards the outward mode under strongly depolarized conditions. Depending on the relative substrate concentrations in the cytosol and extracellular space, transporter-mediated uptake of transmitter may convert to transmitter release mediated by reversal of the transporter [52]. Evidence was presented for a presynaptic N-methyl-D-aspartate (NMDA) autoreceptor-induced reversal of the glutamate transporter enabling release from a cytoplasmic pool, thereby possibly enhancing transmitter concentrations at extrasynaptic sites in order to exert transmission in a paracrine fashion [53].

It is evident that the interplay between extracellular matrix proteins, diffusible messengers and transmitters, activation of membrane-located receptors, ion channels and transporters all determine how a specific nerve terminal will respond. Intracellularly, a variety of chemical signals, including Ca^{2+}, cAMP and cGMP, GTP-binding proteins, lipid-derived messengers as well as the numerous proteins dispersed in vesicles, other intrasynaptic organelles, cytoplasm and active zones, translate these signals into triggering or modulation of differential transmitter release.

Differentiation in presynaptic calcium signaling of neurotransmitter release

As a universal trigger for secretion, Ca^{2+} is very likely an important parameter in differential regulation of presynaptic neurotransmitter release. The versatility of mechanisms which regulate intrasynaptic Ca^{2+} concentration would enable various possible signaling elements where such a

differentiation can take place, ranging from those involved in Ca^{2+} entry to those involved in Ca^{2+} sensing for vesicle fusion. Since the involvement of Ca^{2+} as a discriminator between presynaptic transmitter release from SVs and LDCVs is studied quite extensively, we will describe diverse potential mechanisms in this section separately.

Differentiation in Ca^{2+}-channel regulation

Most central nerve terminals possess the unique property that multiple high-voltage-activated Ca^{2+}-channel types belonging to the Ca_v2 family, classified according to the molecular homology between the pore-forming α_1-subunits they contain, regulate transmitter release [50]. It is now widely accepted that normally both $Ca_v2.1$ (P- and Q-type) and $Ca_v2.2$ (N-type) Ca^{2+} channels are involved. In addition, $Ca_v2.3$ (R-type) channels have been reported to trigger transmitter release [54]. However, not all nerve terminals contain all three subtypes of Ca^{2+} channels. Dependent on brain region and developmental stage, $Ca_v2.1$ or $Ca_v2.2$ channels predominate [55, 56]. Although the P and Q subtypes of Ca^{2+} channels are molecularly difficult to discriminate, pharmacological difference in sensitivity for the blocker ω-agatoxin IVA does suggest such a discrimination [57]. Whereas the P-type channels have high sensitivity to this toxin (<10 nM), the Q-type channels appear at least 10-fold less sensitive. Interestingly, in studies on differential transmitter release from synaptosomes subjected to sub-second K^+ depolarization, the release of the SV transmitters glutamate and GABA was inhibited by ω-agatoxin IVA at high affinity (~10 nM), whereas the release of the partial co-localizing LDCV peptide CCK was much less sensitive to this blocker [17]. However, such a differentiation in channel regulation of release between both classes of transmitter does not imply exclusive involvement of the respective Ca^{2+} channels. As mentioned above, N-type channels are often involved in transmitter release as well. Indeed, N-type Ca^{2+} channels regulate glutamate and GABA release, though to a different extent. Whereas glutamate release was strongly inhibited at higher concentrations of the $Ca_v2.2$-specific toxin ω-contoxin GVIA, GABA release was inhibited by only 40% [17]. Such a difference in N-type channel involvement could reflect the absence of this type of Ca^{2+} channel in a substantial part of the GABAergic terminals in the suspension [58]. Experiments where both blockers were combined showed completely non-additive effects, suggesting regulation of common glutamate or GABA-releasable SV pools in those nerve terminals, which contain both Ca^{2+}-channel types [17]. Such non-additivity is in full agreement with patch clamp recordings from individual nerve terminals [59]. Since, the amino acid SV transmitters have to be secreted within 1 ms after stimulation of the nerve terminals in order to exert the required fast communication with postsynaptic ion channel-coupled receptors, their localization in direct proximity to the SV fusion sites in the active zone is a functional prerequisite. Dense localization of Ca^{2+} channels at SV release sites directly opposite to the target receptors was suggested originally in functional Ca^{2+}-imaging studies in nerve terminals of the squid [60]. Such a highly restricted location of transmitter release-triggering Ca^{2+} channels was suggested in a more indirect way in these nerve terminals by the fact that after loading with an exogenous Ca^{2+} chelator only the fast Ca^{2+}-binding BAPTA did reduce release from SV transmitter, whereas the slower Ca^{2+} chelator EGTA did not [61]. Immunocytochemical studies in giant auditory calyx of Held terminals of the rat showed predominant staining of the active zones with antibodies against $Ca_v2.1$-Ca^{2+} channels, whereas $Ca_v2.2$ and $Ca_v2.3$ types appeared to be located at more remote sites [62]. Recently, an immunogold electron microscopy study showed a high density of the $Ca_v2.1$-Ca^{2+} channels in active zones of small parallel fiber terminals from rat cerebellum [63].

The Ca^{2+} channel regulation for LDCV transmitter release is completely different. Whereas CCK release was not affected by separate addition of the $Ca_v2.2$-blocker ω-contoxin GVIA, a synergistic inhibition of CCK release was performed by this blocker if combined with the $Ca_v2.1$-blocker ω-agatoxin IVA [17]. Such an involvement of $Ca_v2.2$-type Ca^{2+} channels for CCK release under reduced Ca^{2+}-entrance conditions only strongly suggests a more global relationship between the channels and LDCV transmitter release. Apparently, LDCV release is just diffusely coupled to Ca^{2+} channels with a slight preference for Q-type channels, as far as CCK release is concerned. In line with such a relationship is the gradual effect of the slow Ca^{2+}-binding chelator EGTA on dynorphin-mediated heterosynaptic depression in hippocampal mossy fiber synapses, indicating slow diffuse rather than localized fast Ca^{2+} regulation of the release of this LDCV-derived co-transmitter [64]. In contrast, the fusion of SVs is more directly coupled to P- and N-type Ca^{2+} channel subtypes. Indeed, such a tight coupling was indicated by the molecular interaction between the intracellular II-III linker region of the α_1-subunit, the so-called synprint site, of the mammalian P/Q- and N-type Ca^{2+} channels and the proteins involved in SV fusion, i.e. syntaxin1, SNAP25 and synaptotagmin1 [65]. Further studies showed strong reduction in synaptic transmission after injection of the synprint peptide into presynaptic superior ganglion neurons, suggesting the functional relevance of this molecular interaction [66]. Since these Ca^{2+}-channel types appeared also to interact with two typical presynaptic scaffolding proteins, i.e. Mint1 and CASK, in a synprint-independent manner, their interaction with the SV fusion-mediating proteins is not only considered to be of purely structural importance,

e.g. proper targeting of the channels near the SV fusion site, but also, or instead, of modulatory relevance [67]. Such a modulatory role at the synprint-binding site of the P/Q- and N-type Ca^{2+} channels is suggested by channel inactivation when syntaxin is bound [67].

In addition to the targeting of both P/Q- and N-type Ca^{2+} channels to the presynaptic active zone and their modulation by interacting with proteins involved in the SV release machinery, this diversity of SV fusion-triggering Ca^{2+} channels provides the nerve terminal with great flexibility. In nerve terminals from tottering mice, which have a mutation in the α_{1A}-subunit near the pore-forming loop of P/Q-type channels, K-induced glutamate and CCK release were not changed [68]. Pharmacological studies showed that P/Q-type channel regulation of the release of both transmitters was strongly reduced, due to a decrease in expression of this channel by 85% in nerve terminals. Instead, the contribution of N-type channels to the release was increased, resulting in full compensation of the reduced regulation by P/Q-type channels. This increased contribution of N-type channels was not accompanied by changes in their expression in the nerve terminal [68]. Such a preservation of transmitter release despite absence of clear changes in expression of other Ca^{2+} channel subtypes was also observed in nerve terminals from mice where the α_{1A}-subunit was genetically depleted [69]. Interestingly, other adaptations were apparent in these nerve terminals, including increases in Ca^{2+}-sensitivity of exocytosis and in size of the SV recycling pool [70].

Differences in Ca^{2+} sensitivity

An important, but still controversial, issue in Ca^{2+} regulation of neurotransmitter release is the actual Ca^{2+} sensitivity of the fusion of transmitter-containing vesicles. A collaborative study between Nicholls' and our laboratory suggested a difference in Ca^{2+} sensitivity of release between LDCV transmitters and SV ones [15]. Whereas the release of the neuropeptide CCK from LDCV in isolated nerve terminals required less than 0.5 μM Ca^{2+}, the Ca^{2+} sensitivity of the SV amino acid transmitters glutamate and GABA was at least 10-fold lower. These Ca^{2+} estimates were based on fura-2 fluorescence measurements of controlled homogenous Ca^{2+}-concentration elevations inside synaptosomes from 0.1 μM to >5 μM by titration with the Ca^{2+} ionophore ionomycin, which was assumed to penetrate the synaptosomal plasma membrane for Ca^{2} uniformly. This finding was attributed to a relative low Ca^{2+} affinity of transmitter release from SVs due to the relative high Ca^{2+} elevations near the mouth of the Ca^{2+} channels in the active zone, whereas buffering and other clearance mechanisms could suppress Ca^{2+} elevations at more distant sites in the nerve terminal where the LDCVs are located. This difference in Ca^{2+} sensitivity of exocytosis between SVs and LDCVs was suggested more directly in experiments comparing the Ca^{2+} dependency of release of glutamate and norepinephrine in synaptosomes with the plasma membrane permeated by streptolysin-O [71]. In this study the Ca^{2+} sensitivity for glutamate release was more than 100 µM, whereas that for norepinephrine release was around 1 µM. However, similar studies using that approach showed a comparable Ca^{2+} sensitivity of CCK, norepinephrine and glutamate release amounting to 10 µM [72, 73]. This apparent discrepancy is probably due to loss of cytosolic components by plasma membrane permeation, shown to be essential in the former study [71].

The low affinity for Ca^{2+} of SV fusion was originally suggested in studies with giant squid and goldfish retina synapses, indicating Ca^{2+} requirements of 100 µM or more [60, 74]. However, in synapses from the calyx of Held Ca^{2+} sensitivities of glutamate release between 10 and 25 µM were measured [75, 76]. A similar value was deduced from comparison between the effects of the fast-acting BAPTA and slow EGTA Ca^{2+} chelators on transmitter release in cortical synapses [77], whereas such studies in hippocampal Schaffer collateral synapses were more in line with Ca^{2+} affinities in the 100 µM range [78]. Apparently, the exact Ca^{2+} affinities for neurotransmitter release in the active zone vary between the different nerve terminals, which could be due to variations in distance between the release-triggering Ca^{2+} channels and the Ca^{2+} sensor(s) for release [79]. The SV-protein synaptotagmin I shows a Ca^{2+}-binding affinity of its C2 domains in the 10 µM range, and a reduction of this Ca^{2+}-binding affinity by mutating one of the C2 domains causes a similar reduction in transmitter release [80]. This study makes this protein one of the most probable candidates for Ca^{2+} sensing for SV fusion. Similar studies to identify the Ca^{2+}-sensing mechanism(s) for LDCV fusion are still lacking, but involvement of frequenin, a member of the neuronal calcium sensor (NCS) family, has been proposed on basis of the increase in LDCV exocytosis, when overexpressed in a rat adrenal tumour cell line [81]. Frequenin shows Ca^{2+}-binding affinities of their EF-hands as low as 0.3 µM, which would perfectly fit mediation of LDCV exocytosis mentioned above. However, in contradiction to synaptotagmin I, which interacts dynamically with the proteins comprising the soluble N-ethylmaleimide-sensitive fusion protein attachment receptor (SNARE) core complex [82], frequenin does not act directly on the LDCV exocytosis machinery, but instead facilitates $Ca_v2.1$-Ca^{2+}-channel functioning indirectly, as shown in calyx of Held terminals [83]. Interestingly, the cytosolic Ca^{2+}-dependent activator protein for secretion (CAPS), which associates specifically with LDCVs [84], selectively enhances Ca^{2+}-dependent dopamine release from LDCVs in permeated synaptosomes without affecting glutamate release [71].

Contribution of intracellular Ca^{2+} stores

Presynaptic endoplasmic reticulum Ca^{2+} stores would be an excellent mechanism to differentiate between release of SV- and LDCV-derived transmitters. Whereas the former primarily relies on fast triggering by local Ca^{2+} entry through voltage-operated Ca^{2+} channels in the active zone as outlined above, the latter would require slower and lower Ca^{2+} elevations at more distant sites in the nerve terminals. Indeed, the presence of endoplasmic-like Ca^{2+} deposits in isolated presynaptic nerve terminals has been shown [85]. Involvement of Ca^{2+} stores in LDCV-like exocytosis is widely shown in various neurosecretory cells and sympathetic nerve terminals, but their participation in LDCV secretion in nerve terminals remains to be established [14, 86]. In addition, Ca^{2+}-store-mediated transmitter release from SVs in presynaptic terminals has been observed as well [87, 88]. Apparently, Ca^{2+} stores are not exclusively involved in LDCV exocytosis but are more generally destined for prolongation of transmitter release after initial action potential triggering. As such they may be an important source of (remote) Ca^{2+} elevations enabling vesicle translocation and short-term plasticity. In addition, spontaneous, action-potential-independent release of SVs can be evoked by presynaptic receptors in a Ca^{2+}-store-mediated manner [88]. The exact molecular mechanisms of presynaptic Ca^{2+} mobilization are still elusive, but most studies indicate involvement of ryanodine-type receptors in the endoplasmic reticulum [86, 87, 89].

Differentiation in Ca^{2+} buffering

Time and locus of stimulation-induced presynaptic Ca^{2+} elevation are critically dependent on fixed and mobile Ca^{2+}-buffering elements as well as Ca^{2+}-extrusion mechanisms in the plasma membrane. Prolonged Ca^{2+} elevations would sustain transmitter release in a needless way or would evoke eventual unwanted release of co-localized transmitter distant from the active zone. In addition, activation of potential Ca^{2+}-dependent deteriorating processes in the nerve terminal should be avoided. In full accordance with such prevention, Ca^{2+} ions, which enter through channels in the active zone, are rapidly buffered by more than 99.9% [90]. With regard to the presynaptic Ca^{2+}-buffering capacity one has to take into account whether and in what concentration fast, such as calbindin, or slow, such as parvalbumin, Ca^{2+}-binding proteins are present. Furthermore, the actual distance between the release-triggering Ca^{2+} channels and the Ca^{2+} sensor for release determines to what extent Ca^{2+}-buffering proteins interfere with the release process. Especially in central synapses where channels and sensor are located in the direct vicinity of each other in the active zone, slow Ca^{2+} buffering is hardly expected to affect initial bursts of transmitter release. However, except the original studies in giant nerve terminals of squids mentioned above, exogenously added EGTA inhibits SV fusion in active zones as well, though regularly at higher concentration and to a lesser extent than BAPTA [79]. In fact, one has to discriminate between the docked SVs which are very close (<20 nm) to the release-triggering Ca^{2+} channels, so ready to fuse, and the docked SVs at the borders of the active zone at larger distances from the channels [91]. The release from LDCVs would be expected to be more equally affected by exogenous Ca^{2+} chelators with different binding kinetics, in agreement with observations in neurosecretory cells [92]. However, similar studies on LDCV release in small central nerve terminals are not yet done. As mentioned above, in addition to the velocity of Ca^{2+} binding, the concentration of Ca^{2+} binding proteins is determining in (differential) transmitter release. Saturation of calbindin is decisive for facilitation of SV fusion upon repetitive stimulation in mossy fiber terminals, which contain high concentrations of this protein [93]. Similarly, it has been proposed that local saturation of Ca^{2+} binding proteins should facilitate LDCV fusion at remote sites in the nerve terminal [15]. Besides mobile Ca^{2+}-buffering proteins, organelles such as mitochondria and endoplasmic reticulum may accumulate Ca^{2+} inside their lumen. Since their location in nerve terminals is presumably distant from the active zones, involvement of these organelles in Ca^{2+} regulation will occur at relatively high residual Ca^{2+} concentrations and will be determined by competition with mobile Ca^{2+} buffers. In addition, local accumulation of Ca^{2+} inside both SVs as well LDCVs has been proposed [94, 95], but this mechanism was recently debated [96]. Interestingly, recent evidence indirectly indicates that SV2 proteins, present in both SVs and LDCVs, could have such a regulatory role [97].

Differences in Ca^{2+} extrusion

Finally, the plasma membrane located Ca^{2+}-ATPase and/or Na^+/Ca^{2+}-exchanger extrude Ca^{2+} from the nerve terminal, thereby keeping or restoring the pre-stimulation basal Ca^{2+} concentration to 70–80 nM. Whereas the low-capacity Ca^{2+}-ATPase with its sub-μM Ca^{2+} affinity has been proposed to exert a primary 'house-keeping' role, the high-capacity Na^+/Ca^{2+} exchanger with its supra-micromolar affinity has been proposed to function at stages where Ca^{2+} is elevated, i.e. during nerve terminal activation [98]. An immunohistochemical study in giant calyx synapses suggested localization of Ca^{2+}-ATPase near the release sites and more distant localization of the Na^+/Ca^{2+} exchanger [99]. This difference in distribution between both extrusion proteins would enable low Ca^{2+} thresholds near the SV fusion site, and fast suppression of elevated Ca^{2+} concentrations at remote sites and/or rapid exchange with Ca^{2+}-sequestering organelles situated there. Since the Na^+/Ca^{2+} exchanger can operate also in

the reversed direction under conditions where the transmembrane Na^+ gradient is strongly reduced, i.e. during extensive or prolonged depolarization, the protein can potentially induce transmitter release as well [98]. Whether such a release-inducing action does occur only during pathological conditions remains to be established.
In figure 2 the differential mechanisms of presynaptic Ca^{2+} regulation in relationship to neurotransmitter release, as mentioned above, are summarized.

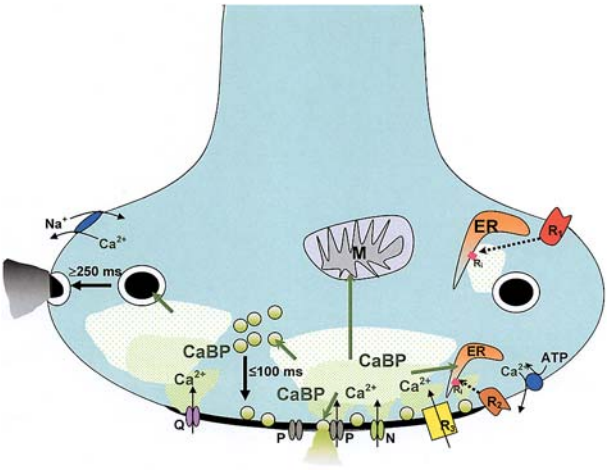

Figure 2. Model of differentiation in presynaptic Ca^{2+} signaling of neurotransmitter release. Depicted are the diverse Ca^{2+}-signaling elements involved in SV and LDCV exocytosis and their intrasynaptic spatial organization. The Q-, P- and N-type Ca^{2+} channels locally trigger transmitter release from SVs in the active zone at Ca^{2+} concentrations >5 µM. (Note: only pharmacological discrimination between P- and Q-type channels exists; R-channels are not shown.) Replenishment of SVs from the reserve pool occurs in a Ca^{2+}-dependent way within 100 ms. The Ca^{2+}concentration near the mouth of the activated channels is buffered by mobile high-affinity Ca^{2+}-binding proteins (CaBPs). During strong stimulation CaBPs are presumably saturated with Ca^{2+}, allowing delayed Ca^{2+}-induced translocation of LDCVs from remote sites towards the plasma membrane and their exocytosis at 0.5 µM Ca^{2+} (≥250 ms). Ca^{2+} can be mobilized by ryanodine or inositol-triphosphate (R_i) receptors from endoplasmic reticulum (ER) stores by receptors at a distant side (R_1) or near the active zone (R_2), thereby inducing local exocytosis of LDCVs and/or SVs. Alternatively, direct triggering of exocytosis can take place by Ca^{2+} entry through ion channel-coupled receptors (R_3). In addition, metabotropic receptors can modulate exocytosis by influencing Ca^{2+}- or K^+-channel activity (not shown). Besides via CaBPs, rapid clearance of Ca^{2+} from the cytosol can take place by accumulation into SVs, LDCVs, ER and mitochondria (M) (indicated by green arrows). Finally, Ca^{2+} is secreted from the nerve terminal by the Ca^{2+}-ATPase near the active zone and/or the Na^+/ Ca^{2+} exchanger at distant sites.

Cyclic nucleotides and differential neurotransmitter release

The increase in norepinephrine and glutamate release from neocortex and hippocampal slices by direct activation of adenylate cyclase strongly suggests involvement of cAMP in presynaptic transmitter release from both SVs and LDCVs [100, 101]. In most cases cAMP acts in synergy with depolarization-induced Ca^{2+} elevation to stimulate exocytosis, suggesting a mainly modulatory role of this second messenger [102]. Although G-protein-coupled receptor-mediated adenylate cyclase activation may be involved in these facilitatory effects on transmitter release, it is generally thought that the initial Ca^{2+} elevation upon Ca^{2+}-channel activation would stimulate adenylate cyclase above a certain threshold in conjunction with calmodulin [103]. Such a mechanism is for instance relevant for sustained enhancement of glutamate release as occurs during NMDA glutamate subtype receptor-independent LTP in hippocampal mossy fibers [103, 104]. In addition, a long-lasting cAMP-mediated and protein synthesis-dependent activation of previously silent presynaptic boutons in hippocampal neuronal cultures shows another important role of this second messenger in presynaptic plasticity [105]. Regularly, cAMP exerts its effects via protein kinase A (PKA), which phosphorylates one or more protein substrates inside the nerve terminal [106]. Another possibility is direct action of cAMP on target proteins, such as the recently identified cAMP-sensor cAMP-GEFII, which has been shown to be responsible for PKA-independent effects on exocytosis in PC12 cells and calyx of Held synapses [107, 108].
Receptor-coupled suppression of transmitter release due to a decrease in cAMP concentration has been related to long-term depression (LTD) in hippocampal mossy fibers [109]. In these synapses activation of metabotropic glutamate subtype 2 receptors decreases cAMP, synthesis, thereby reversing presynaptic LTP mediated by elevated cAMP, mentioned above, to presynaptic LTD. Evidently, cAMP-mediated regulation of transmitter release is a relatively slow and modulatory process which is highly determining in plasticity of certain synapses. Knowledge about the importance of cAMP in regulating the release of LDCV-peptide transmitters is mainly derived from studies with neurosecretory cells, and extrapolation of these findings to central nerve terminals requires further investigation [102].
The role of the other cyclic nucleotide, cGMP, in neurotransmitter release received special attention after the discovery that NO mediates long-term potentiation in the hippocampal CA1 region possibly by retrograde action on synaptic nerve terminals in a cGMP-dependent way [110]. However, later studies in isolated nerve terminals showed that this NO action on presynaptic glutamate release was independent of cGMP and rather involved direct biochemical modification of the docking and fusion protein machinery [111, 112]. Instead, several studies showed cGMP-mediated reduction of glutamate release from isolated nerve terminals, possibly in a protein kinase G-dependent way [113–115]. The role of cGMP in presynaptic LDCV transmitter release remains to be established.

Inositol phosphate signaling and differential neurotransmitter release

The role of inositol phospholipids, a relatively small fraction of plasma membrane phospholipids, in transmitter release has gained considerable interest since it has been shown in neurosecretory cells that ATP-dependent LDCV priming near the release sites involves binding of the vesicles to plasma membrane-bound phosphatidyl inositol-4,5-diphosphate [PtdIns(4,5)P_2] [116]. Recently, it was shown in PC12 cells that the LDCV transmitter release-stimulating protein CAPS-1, mentioned above, binds to PtdIns (4,5)P_2 by its pleckstrin homology domain [117]. Since this CAPS protein can associate selectively with LDCVs, mentioned above [84], this binding probably represents docking or priming of the donor to the acceptor membrane prior to LDCV fusion. In accordance with such a phosphoinositide-mediated release from LDCV transmitters in nerve terminals, blockade of inositol phosholipid phosphorylation in synaptosomes strongly reduced norepinephrine release but did not affect glutamate release from SVs [118]. However, in this study the release of the SV transmitter GABA was sensitive to blockade of inositol phosholipid phosphorylation as well. Moreover, a similar study in another laboratory showed attenuation of Ca^{2+}-dependent glutamate release by SV-associated PtdIns4-kinase inhibition, which was partially reversed after removal of the blockade [119]. These conflicting results question whether PtdIns(4,5)P_2 is selectively involved in LDCV transmitter release. Moreover, Ca^{2+}-sensitive PtdIns(4,5)P_2 binding to C2 domain-containing SV-associated proteins such as synaptotagmin and rabphilin would indicate a role of this phosphoinositide in SV release as well [116]. Notwithstanding their identity, binding of the transmitter-containing SVs and/or LDCVs to PtdIns(4,5)P_2 would target the release of their transmitters to specific microdomains in the plasma membrane. A role of other phosphorylated phosphoinositide recruitment sites in differential regulation of LDCV and SV exocytosis was proposed by experiments in rat spinal cord [120]. Pharmacological blockade of phosphatidylinositol 3-kinase selectively reduced release of substance-P peptide without affecting the release of glutamate and GABA. However, simple extrapolation of these findings to central nerve terminals is not allowed since inhibition of this kinase, which binds to synapsin I, suppressed recruitment of SVs from the reserve pool and glutamate release in isolated nerve terminals [121].

In contradiction to the reasonable consensus about the importance of phosphorylated inositol phospholipids in priming of vesicle-associated proteins involved in transmitter release, the role of the soluble phospholipase C-generated Ins(1,4,5)P_3 in presynaptic Ca^{2+} regulation is a matter of much debate. In a study with synaptosomes and its subfractions evidence was presented for a primarily plasma membrane-located Ins(1,4,5)P_3-gated Ca^{2+} channel coupled to neuropeptide receptor-activated phospholipase C [122]. In studies with neurosecretory cells the Ins(1,4,5)P_3-gated Ca^{2+} channel has been shown to interact with the chromogranin proteins present in secretory granules [123]. The functional role of presynaptic Ins(1,4,5)-P_3-receptors in neurotransmitter release and, if so, the extent of involvement in release from presynaptic SVs and LDCVs has yet to be determined.

Rab3A-GTPase and neurotransmitter release

The importance of GTP-binding proteins in the secretory machinery of a wide range of cells became clear through the direct effects of non-hydrolyzable GTP-analogs such as GTP-γ-S on the release of diverse hormones from permeabilized cells [102]. Injection of GTP-γ-S into squid giant nerve terminals drastically inhibited transmitter release and decreased the amount of SVs at remote sites, effects which were ascribed to involvement of small monomeric GTP-binding proteins such as Rab [124]. Rab proteins comprise the largest family within the Ras superfamily of small GTPases, and have been reported to be involved in membrane transport in all eukaryotes including yeast, nematodes and mammals, indicating their importance during eukaryotic evolution. Currently, more than 60 different Rab proteins have been identified in mammals, which are involved in diverse steps of intracellular membrane transport in different cellular compartments ranging from budding of vesicles from the endoplasmic reticulum donor membrane to docking at the acceptor membrane [125]. Among these Rab proteins, the Rab3A- and Rab3C-GTPase isoforms have attracted particular interest since they are specially involved in regulated exocytosis in neurons. Rab3A is the most abundant Rab protein in the brain, where it can associate with SVs in the GTP-bound state in nerve terminals at late stages just prior to exocytosis [126]. Biochemical studies in which synaptosomal transmitter release was correlated with the amount of Rab3A in the SV subfraction revealed that K-induced glutamate release was accompanied by dissociation of Rab3A from SVs in a Ca^{2+}-dependent way [127]. Rab3A dissociates from SVs after GTP hydrolysis, binds to the GDP dissociation inhibitor protein (GDI) and can recycle to prepare other SVs for exocytosis (see fig. 3). A role of Rab3 in SV replenishment and/or docking was suggested in *Caenorhabditis elegans* synapses, since Rab3 mutants caused depletion of SVs at their fusion sites in neuromuscular junctions, whereas at distant sites this depletion was compensated by an increase in amount of SVs [128].

Initial electrophysiological studies in brain slices from Rab3A knockout mice were in accordance with such an impaired recruitment of SVs because of enhanced presy-

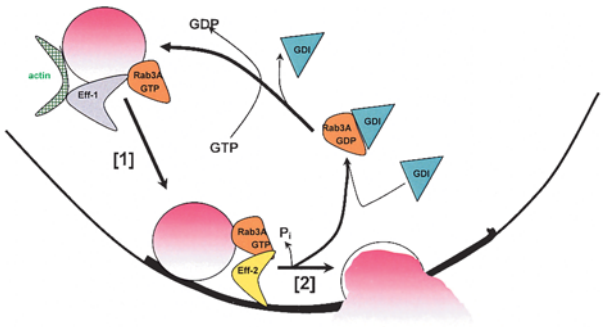

Figure 3. Possible mechanism of facilitation of synaptic vesicle exocytosis by Rab3A-GTPase acting at diverse steps. An SV anchors to actin filaments in the reserve pool by interaction of vesicle-bound Rab3A-GTP with an effector protein (Eff-1, synapsin I or rabphilin?), after which the vesicle is liberated for recruitment towards the active zone where it docks by interacting with another effector (Eff-2) [step 1]. Docked RAb3A-GTP-bound-SVs presumably inhibit SV fusion. After dissociation of Rab-GTP from the SV by GTP hydrolysis (P_i) and binding to GDP dissociation inhibitor (GDI), SVs can fuse [step 2], and Rab3A can, after replacement of GDP by GTP, recruit new SVs for another SV exocytotic round.

naptic fatigue during prolonged stimulation [129]. However, a later study with these animals indicated subtle effects more downstream in the SV cycle, controlling SV fusion rather than their recruitment [130]. Later ultrastructural and biochemical studies on SV distribution and transmitter release in isolated nerve terminals from these Rab3A-null mutant mice revealed evidence for an important role of this protein in depolarization-evoked recruitment, docking and replenishment of SVs for secretion after repetitive depolarization [131]. In this study it was shown that deletion of Rab3A expression did not affect the morphometric properties of the nerve terminals, such as their size, active zone length and the total number of SVs. Similarly, SV distribution with respect to the active zone did not differ from wild-type nerve terminals under resting conditions. However, recruitment of SVs towards the active zone upon short (100 ms) K^+ depolarization observed in wild-type nerve terminals (see fig. 1B) was completely absent in Rab3A-deficient nerve terminals. Consequently, the increase in SVs docked at the active zone by depolarization in control terminals did not occur in the absence of Rab3A. In contrast to these changes in intraterminal SV distribution, 100 ms K^+ evoked release of glutamate and GABA did not decrease, but remained the same (glutamate) or was even slightly increased (GABA), indicating that Rab3A was not really essential for SV exocytosis. Similarly, the total release capacity of both transmitters, estimated by exhaustive (3 min) depolarization of the nerve terminals, was not affected by Rab3A depletion. However, the recovery of release upon short K^+ pulses in wild-type nerve terminals after subsequent repolarization was reduced by 50% in the mutants. The replenishment of docked SVs after this repeated depolarization protocol was reduced by 50% as well [131]. Evidently, release from the readily releasable SV pool in the active zone of Rab3A-depleted nerve terminals was not affected by a single short depolarization despite disturbance of SV replenishment, but became visible after SV recycling only. This phenotype was critically dependent on depolarization-like stimulation, since sucrose-induced SV recruitment and transmitter release were not affected in Rab3A-deficient nerve terminals, indicating a strictly Ca^{2+}-dependent function of this protein [131]. Apparently, the role of Rab3A in facilitating SV transport towards the active zone and their subsequent docking in that region is highly conserved throughout eukaryotic evolution [4]. The 'new'evolutionary role of Rab3A in SV fusion indicated by the electrophysiological studies mentioned above, may lead to the hypothesis that this protein may act at diverse exocytotic steps in the SV cycle, which could explain the diversity of Rab3 subforms in higher mammals. Since Rab proteins do not interact functionally with proteins of the SNARE complex involved in vesicle fusion [132], it has been proposed that they should act via their effectors such as Rabphilin recruited to the SVs by Rab3-GTP and/or the active zone scaffolding protein RIM in order to prime the SVs in the active zone to make them ready for fusion [133]. Indeed, Rabphilin-3 levels in brain tissue were drastically reduced in Rab3A knockout mice [129]. However, studies with brain tissue from knockout mice lacking Rabphilin3a did not yet show any phenotype [134]. Similarly, RIM1α can regulate transmitter release in a manner which is independent of its interaction with Rab3A [135]. Interestingly, recently it was discovered that the SV-actin anchoring protein synapsin I, mentioned above, can act as a potential Rab3A effector as well [136]. Although not yet shown, such an interaction could regulate SV storage in the reserve pool prior to recruitment towards the active zone where fusion occurs. In conclusion, several, yet unknown, Rab3A effectors may mediate SV storage, targeting and fusion, respectively. Such a hypothetical regulation of SV exocytosis by Rab3A at diverse sequential steps is depicted in figure 3.

Since Rab-GTPases are involved in the intracellular transport of several membrane-surrounded organelles, it is not surprising that Rab3A can associate with synaptic-like microvesicles and LDCV-like organelles as secretory granules in (neuro)endocrine cells as well [137]. Overexpression of Rab3A in its active, GTP-bound form inhibited transmitter release in various secretory cells, including PC12 and chromaffin cells [102, 138]. The role of effector proteins in this process is still obscure, since, unexpectedly, overexpression of the effector rabphilin led to enhanced secretion in chromaffin cells [139]. However, Rab3A has been reported to associate exclusively with SVs in nerve terminals, making a possible role in presynaptic LDCV exocytosis rather unlikely [140].

Differential phosphorylation and transmitter release

It is obvious that phosphorylation of specific substrates by kinases is an important instrument to orchestrate the special response of a particular nerve terminal, especially over the long term, where phosphorylation can mediate processes such as presynaptic plasticity. In vitro studies revealed phosphorylation sites on a plethora of presynaptic proteins which are part of the central release machinery localized in the cytosol (SNAPs, NSF), on SVs (synapsin I, rabphilin, synaptobrevin, synaptotagmin) as well as in the membrane (syntaxin, SNAP-25) of nerve terminals. Diverse kinases have been reported to regulate phosphorylation, of which Ca^{2+}/calmodulin kinase II, PKA, protein kinase C (PKC), tyrosine kinase and MAPK are the most important ones [102]. Interestingly, phosphorylation regularly alters interactions between these proteins, providing a potential molecular mechanism to control transmitter release directly. For instance, PKC-activated phosphorylation of Munc-18 drastically reduces its binding affinity for the SNARE protein syntaxin, thereby making this latter protein available for assembly with the other SNARE partners, which is essential for vesicle fusion [141]. Furthermore, Ca^{2+}/calmodulin kinase II-induced phosphorylation of synaptotagmin increases the interaction with the target SNARE proteins syntaxin and SNAP-25 and would couple the Ca^{2+} sensor to the vesicle fusion complex [142]. Synapse-selective phosphorylation would be of potential importance in presynaptic plasticity, since the cAMP-elevating forskolin enhances rabphilin phosphorylation in hippocampal mossy fibers only which show presynaptic cAMP-dependent LTP, but not in nerve terminals isolated from the hippocampal CA1 region [143]. In addition to the proteins which belong to the transmitter release machinery, phosphorylation of plasma membrane-located Ca^{2+} channels by PKA or PKC has an important impact in modulating presynaptic activity [54]. The functional role of phosphorylation in presynaptic transmitter release became apparent in correlative studies where activation of transmitter release was coupled to phosphorylation of certain proteins. In this respect, the phosphorylation of the SV-actin cross-linking synapsin I protein is particularly interesting. This protein can be phosphorylated and dephosphorylated by several protein kinases (Ca^{2+}/calmodulin kinase II, PKA, MAPK) and phosphatases (phosphatase 2A, calcineurin) at distinct sites, and these enzymes are differentially involved in glutamate release from isolated nerve terminals [144]. A later study indicates stimulation frequency-dependent phosphorylation of synapsin I by different kinases in order to control liberation of SVs from the actin anchor for exocytosis [145]. Since synapsins are selectively associated with SVs, its phosphorylation is a potent differentiating mechanism between regulation of SV- and LDCV-derived transmitter release.

With regard to the role of phosphorylation in presynaptic LDCV exocytosis, inhibitory actions of SNARE protein-selective cleavage by tetanus and botulinum toxins similar to SV exocytosis would predict involvement of common protein kinase actions as well [146]. In addition, a common PKC-mediated potentiation of GABA release from SVs and partial co-localized CCK from LDCVs by inactivation of K^+ channels was suggested [147], though the phorbol ester and diacylglycerol effects observed could be mediated by Munc13, a SV-priming protein, as well [148]. However, since Munc13 is mainly located at active zones where only SVs would fuse, its role in presynaptic LDCV exocytosis is not yet clear.

Differential signaling in presynaptic neurotransmitter release

The main purpose of this review is to summarize the presynaptic signals potentially involved in differential regulation of transmitter release. Special emphasis is given to the discrimination between SVs which release classical, fast acting, transmitters at special regions in the presynaptic plasma membrane, the active zones, and LDCVs which release the catecholamine and neuropeptide transmitters at remote sites in the nerve terminal. Due to the limited experimental accessibility of small nerve terminals, which constitute the majority of the terminals in the mammalian brain, most structural and physiological information was derived from giant nerve terminals such as the calyx of Held and retina. Similarly, large nerve terminals from invertebrate species have been investigated for this purpose. Although undoubtedly common signaling mechanisms are involved in directing the differential routes of both vesicle types for exocytosis in the diverse nerve terminals, our current knowledge is primarily based on the processing of SVs and their cycling. As far as LDCVs are concerned, normally (neuro)endocrine cells are taken as a model. However, extrapolation of findings in these cells to central nerve terminals deserves caution. In fact, as mentioned here, signaling molecules such as the small GTPase RAb3A have been reported to bind exclusively to SVs in nerve terminals [140], whereas in adrenal chromaffin cells they can associate with LDCV-like granules [137]. In addition, cell culture systems are often applied in order to (over)express or suppress certain signaling proteins thought to be important for regulation of secretion. These experiments have enormously enriched our insight in their potential relevance for this process, but their validity for similar functions in central nerve terminals has yet to be proven.

In addition to the variety of model systems applied for studying differential regulation of presynaptic transmitter secretion, the large variation in structure and function of nerve terminals within the central nervous system has to

Table 1. Summary of signaling elements which regulate presynaptic SV and LDCV exocytosis or secretion from LDCV-like granules in (neuro)endocrine cells, described in this review.

	SVs	LDCVs	LDCV-like granules
Structural and Dynamical features	neurocrine	paracrine and neurocrine	neurocrine, paracrine endocrine
	present in distinct pools (resting, reserve, readily releasable)	not present in pools but dispersed inside cytosol.	presence of docked pool where fusion occurs
	directed traffic towards active zone	diffuse transport in cytosol	(partial) directed traffic
	fast recruitment (ms)	slow recruitment (s)	intermediate recruitment (sub-s)
	docking at active zone	no pre-docking	docking
	fusion in active zone	fusion at remote sites	fusion in certain microdomains
	full fusion or kiss-and-run	full (and partial?) fusion	full and partial fusion, kiss-and-run
	ultrafast fusion (<1 ms)	slow exocytosis (sub-s to min)	slow exocytosis (sub-s to min)
	fast and slow recycling	no (or partial?) recycling	fast and slow recycling
Ca^{2+}-regulation	localized regulation by (P-,Q- and N-) Ca^{2+} channels	diffuse regulation by Ca^{2+} channels (pref. Q?)	localized regulation by Ca^{2+} channels
	low sensitivity (>5 µM)	high sensitivity (sub-µM)	intermediate sensitivity (µM)
	none or partial effects of slow Ca^{2+}-chelators	equal effects of fast and slow Ca^{2+} chelators	equal effects of fast and slow Ca^{2+} chelators
	involvement of Ca^{2+} mobilization from internal stores	involvement of internal Ca^{2+} mobilization not yet shown	involvement of Ca^{2+} mobilization from internal stores
	possibly Ca^{2+}-accumulating role	ND	possibly Ca^{2+}-accumulating role
Cyclic nucleotides	regulated by both cAMP and cGMP	regulated by cAMP	regulated by cAMP
Phosphorylated inositides	targeting to PtdIns(4,5)P_2 in the plasma membrane?	(selective?) mediation by phosphatidyl-inositol 4-kinase	targeting via CAPS to PtdIns(4,5)P_2 in membrane
	mediated by phosphatidyl-inositol 3-kinase via synapsin I	ND	not regulated by phophatidyl-inositol 3-kinase
Rab3A-GTPase	facilitates recruitment and docking, controls fusion	not involved	controls exocytosis
Phosphorylation	regulation by Ca^{2+}/CaM-kinase II, PKA, PKC, mitogen-activated protein kinase	regulation by PKC (and possibly other kinases which regulate SNARE proteins?)	regulation by PKC, PKA and other kinases which regulate SNARE proteins

ND, not determined; PtdIns(4,5)P_2, phosphatidyl Inositol-4,5-diphosphate; CAPS, calcium-dependent activator protein for secretion; Ca^{2+}/CaM-kinase II, Ca^{2+}/calmodulin kinase II; PKA, protein kinase A; PKC, protein kinase C. For references see the respective sections in this review.

be taken into account. Giant nerve terminals are extremely well designed for ultrafast synchronous release from SVs, which fuse in multiple active zones in order to exert their, generally sensory, tasks. Such a function requires tight control of these short-lasting presynaptic bursts, presumably by interactive modulation via slower from LDCV-secreted co-transmitters (see also below). But even in relatively small synapses and within one vesicle class, differentiation in signaling is apparent. In certain GABAergic hippocampal neurons the native absence of the presynaptic membrane SNARE protein SNAP-25, generally considered essential for regulated SV fusion, indicates that this protein is dismissible for exocytosis and that its role may be taken over by another, yet unknown, protein [149].

In table 1 important signaling factors mentioned in this review are summarized, including ultrastructurcal and chemical features, with regard to their role in SV and/or LDCV exocytosis. Taking into account the precautions mentioned above, LDCVs in nerve terminals are discriminated from LDCV-like granules in (neuro)secretory cells. It is clear that especially the structural features and Ca^{2+}-regulatory properties are important factors in differential transmitter release from SVs and LDCVs in the central nerve terminal. Structurally, the storage of the transmitters in morphologically distinct vesicles, their localization at different sites, their difference in targeting to the plasma membrane in order to fuse as well as the difference in retrieval after exocytosis all contribute to the divergence in presynaptic signaling of co-localized transmitters. As an important consequence, the regularly ultrafast release of SV transmitters in the active zone is followed by a slower release of LDCVs at remote sites, and both signals may interact with each other to restrict the fast signal in space and time. The lateral secretion of LDCV transmitters may influence activity of neighbouring synapses, thereby contributing significantly to the efficiency and direction of action of the fast transmitter. The versatility of Ca^{2+}-regulatory mechanisms provides the nerve terminal with perfect instruments to differentiate between SV and LDCV exocytosis. It is remarkable how Ca^{2+} as ubiquitous trigger for secretion in all eukaryotic cells is adapted for this task in small central nerve terminals. The close interplay between multiple high-voltage activated channels, Ca^{2+} sensors for vesicle fusion, intracellular Ca^{2+} mobilization and subsequent Ca^{2+} buffering, guarantees timely and locally highly restricted drives for exocytosis of co-localizing transmitters.

In the literature, the hypothesis about common signaling mechanisms which underlie regulation of transmitter release from SVs and LDCVs has repeatedly been put forward, mainly based on proposed similarities between the various vesicle and plasma membrane proteins. However, our current knowledge about differential signaling of presynaptic LDCV exocytosis is still very limited and largely derived from studies of their counterparts in (neuro)endocrine cells mentioned above. Selective LDCV-targeted proteins such as Ca^{2+}-sensitive CAPS would be extremely important molecular regulators of differential routing of this vesicle category [71, 117]. In addition, temporally and spatially distinct signals such as receptor-induced cyclic nucleotide elevation and/or Ca^{2+} mobilization, which take place regularly more distally inside the nerve terminal, would have relatively more impact on LDCV exocytosis. As mentioned in the introduction, the function of release of co-localized transmitters from nerve terminals is hardly known. In the light of their co-localization, it is not unlikely that following high-frequency stimulation conditions where both SV and LDCV exocytosis will take place, these transmitters could interact with each other either presynaptically and/or postsynaptically to control transsynaptic activity in time and space. Furthermore, independent paracrine signaling by secreted LDCV transmitters would supply the presynapse with an opportunity to spread its signal to neighbouring synapses or other cells [150]. In conclusion, continuation of research on differential signaling of presynaptic transmitter release will significantly broaden our insight in presynaptic contribution to versatility in neuronal communication.

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