

# Neurotransmitter Release

Thomas C. Südhof

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**Abstract** Neurons send out a multitude of chemical signals, called neurotransmitters, to communicate between neurons in brain, and between neurons and target cells in the periphery. The most important of these communication processes is synaptic transmission, which accounts for the ability of the brain to rapidly process information, and which is characterized by the fast and localized transfer of a signal from a presynaptic neuron to a postsynaptic cell. Other communication processes, such as the modulation of the neuronal state in entire brain regions by neuromodulators, provide an essential component of this information processing capacity. A large number of diverse neurotransmitters are used by neurons, ranging from classical fast transmitters such as glycine and glutamate over neuropeptides to lipophilic compounds

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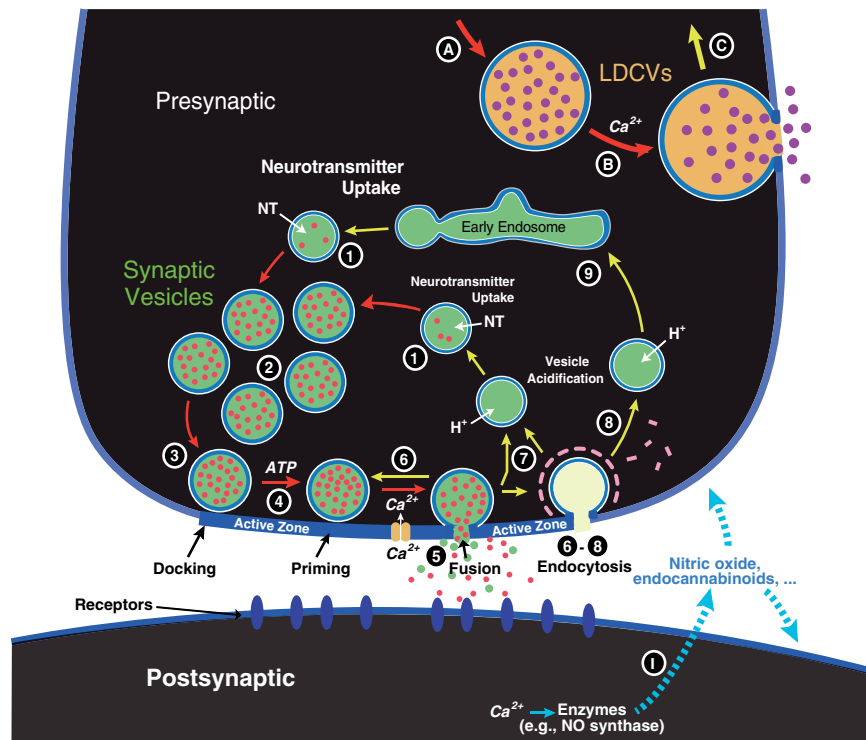
and gases such as endocannabinoids and nitric oxide. Most of these transmitters are released by exocytosis, i.e. the fusion of secretory vesicles with the plasma membrane, which exhibits distinct properties for different types of neurotransmitters. The present chapter will provide an overview of the process of neurotransmitter release and its historical context, and give a reference point for the other chapters in this book.

## 1 Principles of Neurotransmitter Release

Neurons communicate with each other and their target cells via two principal mechanisms: the secretion and reception of chemical messengers called neurotransmitters, and the direct transfer of intercellular signals via gap junctions. Communication via neurotransmitters occurs in several forms that range from classical synaptic transmission at synapses to diffuse secretion of neuromodulators which mediate volume transmission. Communication via gap junctions occurs at so-called electrical synapses. Almost all of the neuronal communication is mediated by neurotransmitters, and electrical synapses are exceedingly rare in vertebrate brain. Both types of communication are not unique to neurons. Secretion of neuromodulators and neuropeptides is also mediated by endocrine cells and even some highly differentiated cells such as adipocytes, and diffusible neurotransmitters such as nitric oxide are released by many non-neuronal cells. Only the presynaptic secretion of classical neurotransmitters in the context of a synapse is specific to neurons, although the postsynaptic cell can be either a neuron (most of the time) or an effector cell (e.g., a muscle cell). The present book will only deal with communication by neurotransmitters, and only with the release of such transmitters and the pharmacology of this release.

What is a neurotransmitter, and how many different “types” of neurotransmitter release exist? At least five types of neurotransmitter release can be defined.

1. Synaptic neurotransmitter release occurs in a classical, electron microscopically observable synapse, and is mediated by synaptic vesicle exocytosis from nerve terminals (Figure 1; Katz, 1969; Südhof, 2004; note that a “nerve terminal” is not necessarily the end of an axon, but generally is formed by axons en passant as they arborize throughout the brain). Synaptic neurotransmitter release, the first step in synaptic transmission, transfers information extremely rapidly (in milliseconds) in a highly localized manner (restricted to an area of less than a square micrometer; reviewed in Südhof, 2004). Synaptic release secretes “classical” neurotransmitters: GABA, glycine, glutamate, acetylcholine, and ATP. It has been suggested that in addition to neurons, astrocytes also secrete classical neurotransmitters by a similar mechanism (?), but this type of secretion has not been directly demonstrated.
2. Monoaminergic neurotransmitters (dopamine, noradrenaline, adrenaline, histamine, and serotonin) are released by exocytosis of small dense-core vesicles from



**Fig. 1** Secretory pathways in neurons. The drawing schematically illustrates the three major neurotransmitter release pathways. **(a)** Release of classical neurotransmitters by synaptic vesicle exocytosis (center; steps 1–9). Classical neurotransmitter release depends on an underlying synaptic vesicle cycle that starts when synaptic vesicles are filled with neurotransmitters by active transport (step 1), and form the vesicle cluster (step 2). Filled vesicles dock at the active zone (step 3), where they undergo a priming reaction (step 4) that makes them competent for  $Ca^{2+}$ -triggered fusion-pore opening (step 5). After fusion-pore opening, synaptic vesicles undergo endocytosis and recycle via three alternative pathways: local reuse (step 6; also called kiss-and-stay), fast recycling without an endosomal intermediate (step 7; also called kiss-and-run), or clathrin-mediated endocytosis (step 8) with recycling via endosomes (step 9). Steps in exocytosis are indicated by red arrows, and steps in endocytosis and recycling by yellow arrows. **(b)** Release of neuropeptides and biogenic amines by LDCV exocytosis. LDCVs are generated in the cell body by budding from the Golgi complex filled with neuropeptides (not shown). LDCVs are then transported from the cell body to the axons or dendrites (step A, as shown for nerve terminals). A  $Ca^{2+}$ -signal triggers the translocation and fusion of LDCVs with the plasma membrane outside of the active zone (step B). After exocytosis, empty LDCVs recycle and refill by transport to the cell body and recycling via the Golgi complex (step C). **(c)** Release of gaseous or lipidic neurotransmitters, which are synthesized in either the pre- or the postsynaptic neuron (only the postsynaptic synthesis is shown), and secreted by diffusion across the plasma membrane (step I) to act on local extracellular receptors (e.g., CB1 receptors for endocannabinoids) or intracellular targets (e.g., guanylate cyclase for nitric oxide). (Modified from Südhof, 2004).

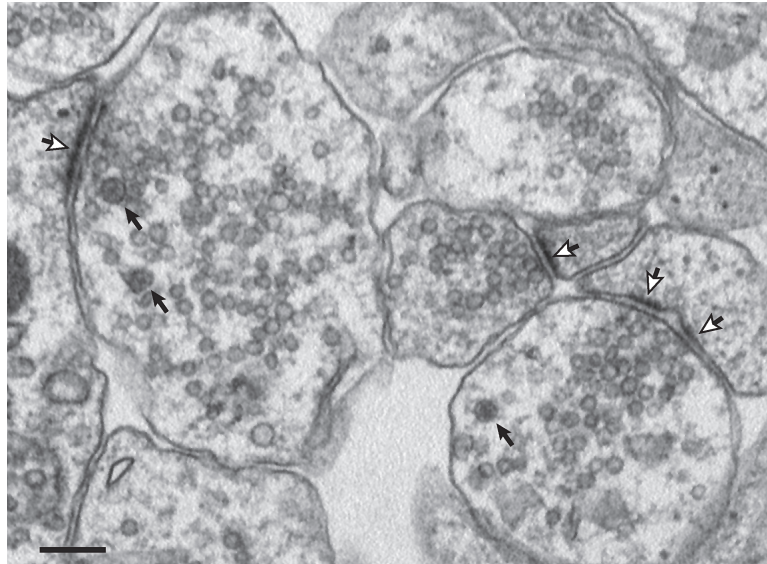
axonal varicosities that are largely not associated with a specialized postsynaptic structure (i.e., are outside of synapses; Brock and Cunnane, 1987; Stjärne, 2000). However, at least in the case of dopamine, postsynaptic specializations can occur with presynaptic small dense-core vesicles.

3. Neuropeptides are secreted by exocytosis of large dense-core vesicles (LDCVs) outside of synapses (Figure 1; Salio et al., 2006). LDCVs undergo exocytosis in all parts of a neuron, most often in axon terminals and dendrites. Monoamines are often co-stored with neuropeptides in LDCVs and co-secreted with them upon exocytosis. For all intents and purposes, LDCV-mediated secretion resembles hormone secretion in endocrine cells.
4. Classical neurotransmitters and monoamines may rarely be secreted by neurons, not by exocytosis, but by transporter reversal. This mechanism involves the transport of neurotransmitters from the cytosol to the extracellular fluid via transporters that normally remove neurotransmitters from the extracellular fluid. This mechanism appears to account for the burst of dopamine released by amphetamines (Fleckenstein et al., 2007), but its physiological occurrence remains unclear.
5. A fifth pathway, finally, is the well-established secretion of small membrane-permeable mediators by diffusion. This mechanism is used for the secretion of nitric oxide, endocannabinoids, and other important lipidic or gaseous neurotransmitters. The major point of regulation of release here is the synthesis of the respective compounds, not their actual secretion.

Only the first type of neurotransmitter release mediates the fast point-to-point synaptic transmission process at classical synapses (sometimes referred to as wiring transmission). All of the other types of neurotransmitter release effect one or another form of “volume transmission” whereby the neurotransmitter signal acts diffusely over more prolonged time periods (Agnati et al., 1995). Of these volume transmitter pathways, the time constants and volumes involved differ considerably. For example, diffusible neurotransmitters such as nitric oxide act relatively briefly in a localized manner, whereas at least some neuropeptides act on the whole brain, and can additionally act outside of it (i.e., function as hormones). There is an overlap between wiring and volume neurotransmission in that all classical neurotransmitters act as wiring transmitters via ionotropic receptors, and also act as “volume transmitters” via G-protein-coupled receptors. Moreover, neuromodulators in turn feed back onto classical synaptic transmission.

Quantitatively, synaptic transmission is the dominant form of communication between neurons. A single look at an electron micrograph reveals that synapses with their appendant organelles, especially synaptic vesicles, are abundant in brain, whereas LDCVs are only observed occasionally (Figure 2). However, this does not mean that synaptic transmission is more important than the volume transmission pathways. The two principally different signaling pathways play distinct roles in information processing by the brain, and both are essential for brain function.

With the multitude of different types of transmitters, the question arises whether a single neuron can release more than one transmitter. Dale’s principle stated that



**Fig. 2** Electron micrograph of synapses. The image shows synapses formed by cultured cortical neurons from mouse. Note abundant synaptic vesicles in nerve terminals adjacent to synaptic junctions that are composed of presynaptic active zones and postsynaptic densities (open arrows point to postsynaptic densities of synaptic junctions; synapse on the right contains two junctions). In addition to synaptic vesicles, two of the nerve terminals contain LDCVs (closed arrows). Calibration bar = 500 nm. (Image courtesy of Dr. Xinran Liu, UT Southwestern).

this is not the case, but seems to be incorrect given the fact that virtually all neurons secrete neuropeptides and either classical neurotransmitters or monoamines (Salio et al., 2006). Moreover, many neurons additionally secrete diffusible neurotransmitters. Thus, a neuron usually operates by multiple neurotransmitter pathways simultaneously. To add to the complexity of these parallel signaling pathways, the relatively small number of neurons that secrete monoamines from axonal varicosities may also secrete classical neurotransmitters in separate classical synapses (Trudeau, 2004). Despite this complexity, however, Dale has to be given credit for his principle because the multiple transmitters secreted by a given neuron generally operate in distinct secretory and effector pathways. A given neuron usually releases only one type of classical neurotransmitter (with a few exceptions), suggesting that a modified Dale principle is still correct cotransmission.

## 2 Very Short History of the Analysis of Neurotransmitter Release

Our current concept of synaptic transmission, as mediated by intercellular junctions formed by one neuron with another neuron or target cell, is fairly recent. This concept was proposed in the second half of the 19th century, and proven

only in the 20th century. It was embedded in a larger debate of whether neurons form a “reticular” network of connected cells, or a network of cells whose connections are discontinuous (the so-called neuron theory). Like with everything else in neuroscience, Ramón y Cajal is usually credited with the major discoveries in this field, but the actual concept predates him, and the development of the current view of synaptic transmission is due to a team effort. When Ramón y Cajal followed in the footsteps of scientists like Kühne, Koelliker, and His, who had formulated the first concept of synapses, even though the actual term was coined much later, Cajal’s elegant prose and the fortunate opposition of Emilio Golgi to the neuron theory enhanced the influence of his writings and somewhat obscured the fact that the actual concepts that Cajal was presenting were already well established in the literature.

The term synapse was coined in 1897 by the physiologist Charles Sherrington in M. Foster’s *Textbook of Physiology*, but the idea of the chemical synapse was developed almost half a century earlier in studies on the neuromuscular junction. As always in science, technical advance spawned conceptual breakthroughs. The three technical advances that fueled the progress in neuroscience in the second half of the 19th century were the improvements in light microscopy, chiefly due to Lister’s invention of apochromatic lenses, the continuous development of staining methods culminating in Golgi’s eponymous stain, and the application of more precise electrical recordings, allowing the emergence of electrophysiology to complement anatomy. Each historical stage in the discovery process is coupled to a particular preparation and technical approach, and major progress was usually achieved when a new technique was applied to a new preparation. This pattern also applies to the discovery of the synapse which was first described, without naming it, at the neuromuscular junction.

In the middle of the 19th century, it was known from the work of Volta, Galvani, and others that the nerve stimulates muscle contractions at the neuromuscular junction, and that electrical signals were somehow involved. Using the tools of cellular neuroanatomists, Kühne (1862) and Krause (1863) first demonstrated that the neuromuscular junction is not composed of a direct cellular connection between nerve and muscle as had been believed, but is discontinuous. Fifteen years later, the electrophysiologist Emil du Bois-Reymond (1877) proposed that the transmission of a synaptic signal is chemical. Subsequent work by Koelliker, Cajal, and Sherrington generalized this concept of a discontinuous synaptic connection that mediates intercellular signaling to the interneuronal synapses. Although the concept of the synapse continued to be disputed until well into the 20th century (e.g., see Golgi’s Nobel lecture), the very existence of these disputes should not prevent us from recognizing that the actual description of synaptic transmission, and at least its proof for one particular synapse, the neuromuscular junction, had been established 50 years earlier.

The next major step forward in deciphering the mechanisms of synaptic transmission occurred in the neuropharmacological studies of Henry Dale, Otto Loewi, Wilhelm Feldberg, and their colleagues. Although, as in the discovery of the synapse as an intercellular noncontinuous junction, many individuals contributed, Loewi is generally credited with the single decisive experiment. This is probably fair, since

Loewi demonstrated directly that a chemical mediator (acetylcholine) is responsible for the transmission of the signal from the vagus nerve to the heart (Loewi, 1921). Despite Loewi's, Dale's, and Feldberg's advances, however, doubts lingered as to whether a chemical signal could be fast enough to account for the speed of synaptic transmission. Many scientists, with John Eccles (one of Sherrington's last pupils) as the most vocal protagonist, continued to espouse the view that fast synaptic transmission is essentially electrical, whereas chemical signaling serves only as a slow modulatory event. In other words, these views proposed a clean division of transmission into fast synaptic wiring transmission that is electrical, and slow volume transmission that is chemical. The doubts about the speed of chemical neurotransmission, and its general validity, were only definitively laid to rest by Bernhard Katz's seminal experiments on the frog neuromuscular junction, demonstrating that synaptic transmission operates as a quantal chemical event (Katz, 1969). It is remarkable that from Kühne's to Katz's studies, the major contributions to establishing synaptic transmission as the major mechanism by which neurons communicate came from the neuromuscular junction. The concept of the synapse was first postulated at the neuromuscular junction, the first genuine neurotransmitter was identified with acetylcholine as the neuromuscular junction neurotransmitter, and the chemical quantal nature of synaptic transmission was revealed at the neuromuscular junction.

The findings of Katz and colleagues raised two major questions: what are the mechanisms that allow the fast secretion of neurotransmitters from presynaptic terminals in response to an action potential? What molecules mediate the fast recognition of these neurotransmitters by the postsynaptic cell? The elucidation of the basic mechanisms of release again started with the cholinergic system in the description and isolation of synaptic vesicles as the central organelle, chiefly by Victor Whittaker (Whittaker and Sheridan, 1965). The progress in the field, however, then shifted to central synapses, with the identification of the major molecules involved in release of neurotransmitters, and the description of the mechanism by which  $\text{Ca}^{2+}$ -influx into nerve terminals achieves the fast triggering of release via binding to synaptotagmins (reviewed in Südhof, 2004). The discovery of neurotransmitter receptors and their properties was initiated by classical pharmacological approaches dating back to the British school founded by Langley (1921), but the definitive description of these receptors was enabled by the simultaneous development of patch clamping by Neher and Sakmann (1976) and of molecular cloning of these receptors by S. Numa (Noda et al., 1982).

### 3 Basic Mechanisms of Release by Exocytosis

Most neurotransmitter release occurs by exocytosis of secretory vesicles, which involves the fusion of the secretory vesicles (synaptic vesicles and LDCVs) with the plasma membrane. All intracellular membrane fusion (except for mitochondrial fusion) is thought to operate by the same fundamental mechanism that involves a core machinery composed of four classes of proteins: SNARE-proteins, SM-proteins (for

Sec1/Munc18-like proteins), Rab-proteins, and Rab-effectors (Jahn et al., 2003). The specific isoforms of these proteins that are being used vary tremendously between fusion reactions, but the general principle by which these proteins act seems to be always similar: Rab and Rab-effector proteins appear to proofread the docking and fusion reaction between the two target membranes and may even mediate the docking at least in part, whereas SNARE- and SM-proteins catalyze the actual fusion reaction.

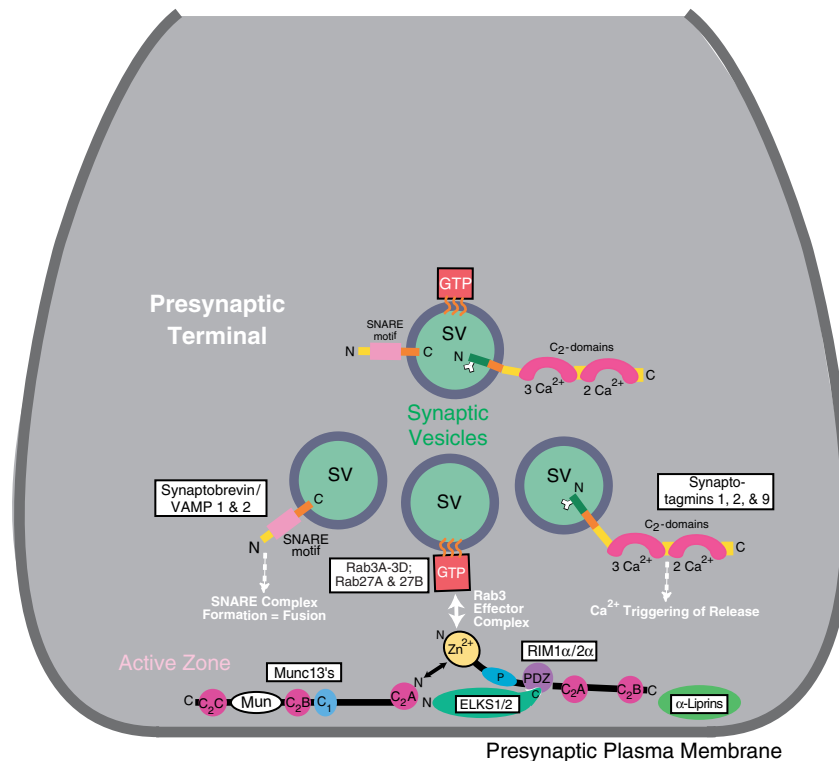
### ***3.1 Rab-Proteins and Rab-Effectors***

Rab-proteins are GTP-binding proteins that interact with effectors in a GTP-dependent manner. Rab3A, 3B, 3C, and 3D represent a family of Rab-proteins that are highly enriched on synaptic vesicles and other secretory organelles throughout the body. In addition, Rab27A and 27B are also generally found on secretory vesicles, although it is unclear whether they are present on synaptic vesicles (Südhof, 2004). Rab3/27 proteins together function in exocytosis, and mediate vesicle docking at least in part. Two classes of Rab3/27 effectors were described: rabphilins and RIMs. Both effector classes include multiple members encoded by distinct genes. Rabphilins are cytosolic proteins that are recruited to secretory vesicles by Rab3/27, but their function has remained largely obscure. RIMs are components of the detergent-insoluble protein complex that makes up the active zone, the part of the presynaptic plasma membrane where synaptic vesicles dock and fuse (Figure 3). The active zone is composed of the RIM-containing protein complex that includes several other large proteins, in particular Munc13s, piccolo/bassoon, ELKS, and  $\alpha$ -liprins, all of which are crucial for normal synaptic vesicle exocytosis. It is noticeable that in most intracellular fusion reactions, Rab-effectors are composed of large complexes that do more than just bind the Rab-protein, but perform several functions in the fusion process, with the Rab-protein often being involved in the docking of the membranes for fusion and in the regulation of the other activities of the complex during the fusion reaction. The same appears to be true for Rab3/27 binding to the RIM-containing active zone protein complex. The whole active zone complex could be considered as a single large Rab-effector complex (Figure 3), and is likely involved not only in the docking of synaptic vesicles, but also in organizing the actual fusion reaction and in synaptic plasticity (see below).

### ***3.2 SNARE Proteins***

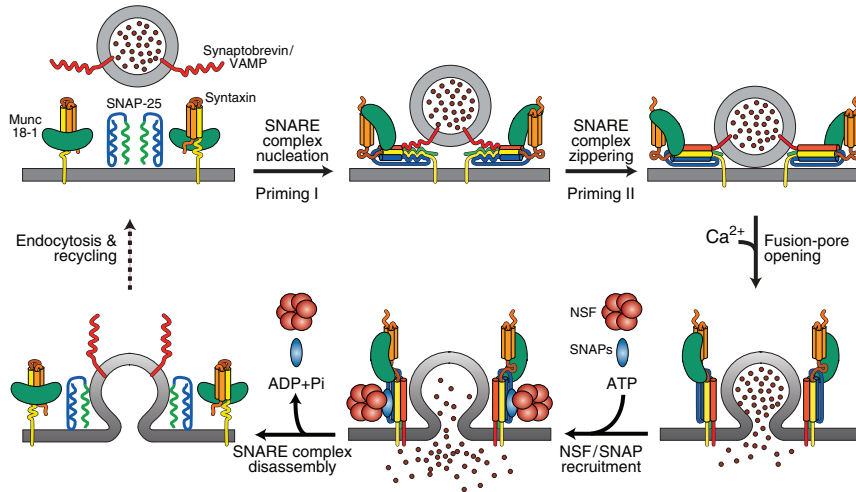
Membrane fusion consists of merging two negatively charged phospholipid bilayers, and thus requires overcoming a major energy barrier (Jahn et al., 2003). SNARE proteins represent a family of membrane proteins that are present on opposing membranes destined to fuse. As first proposed by Jahn, Heuser, Rothman and colleagues





**Fig. 3** Interaction of Rab3 and Rab27 on synaptic vesicles with the active zone protein complex containing Munc13s, RIMs, ELKS, and liprins. The schematic drawing depicts a nerve terminal with a few synaptic vesicles containing the three vesicle proteins that mediate exocytosis: the SNARE protein synaptobrevin/VAMP that participates in fusion (see Figure 4), the Rab-proteins Rab3 and Rab27 that attach synaptic vesicles to the active zone protein complex as shown, and the Ca<sup>2+</sup>-sensor protein synaptotagmin that translates the Ca<sup>2+</sup>-signal into release (Figure 5). The active zone protein complex is composed of Munc13, RIM, ELKS, and liprins, so that RIM binds to all of the three other active zone proteins, and additionally interacts with Rab3/27 via its N-terminal domain. The active zone protein complex likely contains other protein components that are not shown, in particular piccolo/bassoon. (Modified from Südhof, 2004).

(Hanson et al., 1997; Weber et al., 1998), formation of a “trans-complex” by SNARE proteins on opposing membranes forces these membranes together, thereby overcoming the energy barrier (Figure 4). SNARE proteins contain a characteristic 60-residue sequence, the so-called SNARE motif. SNARE complexes are assembled from four types of SNARE motifs (called R, Qa, Qb, and Qc, classified based on sequence homologies and the central residue) that fold into a tight four-helical bundle which always contains one copy for each type of SNARE motif. The close approximation of two membranes by SNARE-complex assembly destabilizes their negatively charged surfaces, thereby initiating the intermixing of their hydrophobic lipid interiors. This is thought to provide the energy for membrane fusion.



**Fig. 4** Schematic diagram of the SNARE protein/Munc18 cycle. Docked synaptic vesicles (top left) may be attached to the active zone via the Rab/RIM interaction (see Figure 3) but contain SNARE proteins that have not yet formed a complex with each other (synaptobrevin/VAMP on synaptic vesicles and SNAP-25 and syntaxin-1 on the plasma membrane; note that syntaxin-1 is thought to be complexed to the SM-protein Munc18-1). Priming is envisioned to occur in two steps that involve the successive assembly of SNARE-complexes (priming I and II). During priming, Munc18-1 is thought to be continuously associated with syntaxin-1, shifting from a heterodimeric binding mode in which it was attached to syntaxin-1 alone to a heteromultimeric binding mode in which it is attached to the entire SNARE complex (top right). After priming,  $\text{Ca}^{2+}$  triggers fusion-pore opening to release the neurotransmitters by binding to synaptotagmin (see Figure 5). After fusion-pore opening, SNAPs (no relation to SNAP-25) and NSF (an ATPase) bind to the assembled SNARE complexes, disassemble them with ATP-hydrolysis, thereby allowing synaptic vesicles to undergo re-endocytosis and to recycle with synaptobrevin on the vesicle, while leaving SNAP-25 and syntaxin-1/Munc18-1 on the plasma membrane. Note that the overall effect is that SNARE/Munc18-proteins undergo a cycle of association/dissociation that fuels the membrane fusion reaction which underlies release. (Modified from Rizo and Südhof, 2002).

Synaptic exocytosis involves three SNARE proteins: the R-SNARE synaptobrevin/VAMP (isoforms 1 and 2) on the vesicle, and the Q-SNAREs syntaxin (isoforms 1 and 2) and SNAP-25 on the plasma membrane (Figure 4). Since SNAP-25 has two SNARE-motifs, synaptobrevin, syntaxin, and SNAP-25 together have four SNARE-motifs. Synaptobrevins and SNAP-25 are relatively simple SNARE proteins that are composed of little else besides SNARE motifs and membrane-attachment sequences (a transmembrane region for synaptobrevin, and a cysteine-rich palmitoylated sequence for SNAP-25). Syntaxins, in contrast, are complex proteins. The N-terminal two-thirds of syntaxins include a separate, autonomously folded domain (the so-called  $H_{abc}$ -domain), while the C-terminal third is composed of a SNARE motif and transmembrane region just like synaptobrevin.

### 3.3 SM Proteins

Genes for SM-proteins were discovered in genetic screens in *C. elegans* (unc18) and yeast (sec1), and their connection to membrane fusion was identified when the SM-protein Munc18-1 was found to directly bind to syntaxin-1 (Brenner, 1974; Novick et al., 1980; Hata et al., 1993). SM-proteins are composed of a conserved ~600 amino acid sequence that folds into an arch-shaped structure. With seven members in mammals and four in yeast, SM-proteins constitute a small family of highly homologous proteins. SM proteins have essential roles in all fusion reactions tested. Three SM proteins (Munc18-1, -2, and -3) are involved in exocytosis, where they are at least as essential as SNARE proteins. For example, deletion of Munc18-1 in mice has more severe consequences for synaptic vesicle exocytosis than deletion of synaptobrevin or SNAP-25 (Verhage et al., 2000).

Initially, Munc18-1 was found to bind only to monomeric syntaxin-1 in a manner that is incompatible with SNARE-complex formation. Puzzlingly, however, other SM proteins were subsequently found to bind to assembled SNARE complexes. This puzzle was resolved with the discovery that Munc18-1 (and presumably -2) participates in two distinct modes of SNARE interactions: the originally defined binding to monomeric syntaxins, and a novel mode of direct binding to assembled SNARE complexes (Dulubova et al., 2007; Shen et al., 2007). These results suggested that all SM-proteins directly or indirectly interact with assembled SNARE complexes in fusion. The additional binding of Munc18-1 to the closed conformation of syntaxin prior to SNARE complex formation renders  $\text{Ca}^{2+}$ -triggered exocytosis unique among fusion reactions, possibly in order to achieve a tighter control of the fusion reaction.

### 3.4 Mechanism of SNARE and SM Protein Catalyzed Fusion

Both SNARE and SM proteins are required as components of the minimal fusion machinery. At the synapse, for example, deletion of Munc18-1 leads to a loss of all synaptic vesicle fusion, revealing Munc18-1 as an essential component of the fusion machine (Verhage et al., 2000). It is likely that SNARE proteins first force membranes together by forming trans-complexes, thereby creating a fusion intermediate that at least for synaptic vesicles appears to consist of a hemifusion stalk (Figure 4). Since the unifying property of SM proteins is to bind to assembled SNARE complexes, they likely act after such a fusion intermediate has formed, but their exact role remains unknown.

Each intracellular fusion reaction exhibits characteristic properties, and involves a different combination of SM and SNARE proteins. The specificity of fusion reactions appears to be independent of SNARE proteins because SNARE complex formation is nonspecific as long as the Q/R-rule is not violated (i.e., the fact that SNARE complexes need to be formed by SNARE proteins containing R-, Qa-, Qb-, and Qc-SNARE motifs), and of SM proteins because SM proteins often function in

multiple fusion reactions. Fusion specificity must be determined by other mechanisms, possibly GTP-binding proteins of the rab family.

## **4 Mechanism of $\text{Ca}^{2+}$ -Triggering: $\text{Ca}^{2+}$ -Channels, $\text{Ca}^{2+}$ -Buffering, and Synaptotagmin**

Neurotransmitter release is triggered by  $\text{Ca}^{2+}$  when an action potential invades the nerve terminal and gates the opening of voltage-sensitive  $\text{Ca}^{2+}$ -channels. Thus, there are two determinants of neurotransmitter release: (1) The  $\text{Ca}^{2+}$ -dynamics in the nerve terminal that are dictated by the properties and location of the  $\text{Ca}^{2+}$ -channels; the concentration, affinities, and kinetics of local  $\text{Ca}^{2+}$ -buffers; and the  $\text{Ca}^{2+}$ -extrusion mechanisms and (2) the action of the  $\text{Ca}^{2+}$ -receptors that translate the  $\text{Ca}^{2+}$ -signal into release, with most release being mediated by  $\text{Ca}^{2+}$ -binding to synaptotagmins (see below).

### **4.1 $\text{Ca}^{2+}$ -Dynamics**

The  $\text{Ca}^{2+}$ -concentration in a nerve terminal depends on the number and temporal pattern of action potentials, the effectiveness of these action potentials to open  $\text{Ca}^{2+}$ -channels, and the properties and concentrations of  $\text{Ca}^{2+}$ -buffers. Not only the time course of changes in  $\text{Ca}^{2+}$ -concentrations, but also the spatial distribution of  $\text{Ca}^{2+}$ , is important because  $\text{Ca}^{2+}$  is not uniformly distributed in a nerve terminal. Moreover, the  $\text{Ca}^{2+}$ -dynamics of a nerve terminal differ between nerve terminals, and play a central role in synaptic plasticity (e.g., see Rozov et al., 2001; Zucker and Regehr, 2002).

$\text{Ca}^{2+}$ -channels are well investigated, have proven to be great drug targets, and will be discussed at length in the chapter by Kisilevsky and Zamponi. Two types of  $\text{Ca}^{2+}$ -channels, the so-called P/Q- and N-type channels (referred to as Cav2.1 and 2.2) account for the vast majority of releases. These  $\text{Ca}^{2+}$ -channels are located in the active zone of the presynaptic terminal (Llinas et al., 1992), although their precise location is unknown.  $\text{Ca}^{2+}$ -channels are – not surprisingly – tightly regulated by several signaling systems. As a result of their non-uniform localization and their stringent regulation, the  $\text{Ca}^{2+}$ -signal produced by the opening of  $\text{Ca}^{2+}$ -channels by a given action potential cannot be predicted, but varies greatly between synapses in amplitude, space and time (Rozov et al., 2001). This variation is increased by differences in  $\text{Ca}^{2+}$ -buffering between synapses.  $\text{Ca}^{2+}$ -buffers are much less understood than  $\text{Ca}^{2+}$ -channels because of the large number of different types of buffers, the difficulty in manipulating them pharmacologically or genetically, and the problems in measuring them. The most important nerve terminal  $\text{Ca}^{2+}$ -buffer likely is ATP, which has a relatively low  $\text{Ca}^{2+}$ -affinity but a high concentration and is highly mobile, rendering it an effective buffer at peak  $\text{Ca}^{2+}$ -concentrations (Meinrenken et al.,

2003). In addition, a number of  $\text{Ca}^{2+}$ -binding proteins of the EF-hand class probably function as nerve terminal  $\text{Ca}^{2+}$ -buffers, including parvalbumin and calbindin. Interestingly, these proteins exhibit a highly selective distribution in the brain, with inhibitory interneurons generally expressing much higher concentrations of selected  $\text{Ca}^{2+}$ -buffer proteins than excitatory neurons, a difference that may account for the distinct short-term plasticity properties of GABA release (Silberberg et al., 2005).  $\text{Ca}^{2+}$ -extrusion, finally, is mediated by multiple mechanisms. Although uptake of nerve terminal  $\text{Ca}^{2+}$  into mitochondria and endoplasmic reticulum has been widely discussed, the most important step is the transport of  $\text{Ca}^{2+}$ -ions from the cytosol into the extracellular space against a vast concentration gradient. Several mechanisms for this transport exist, of which the  $\text{Na}^+/\text{Ca}^{2+}$ -exchanger and the plasma membrane  $\text{Ca}^{2+}$ -ATPase appear to be the most important (Meinrenken et al., 2003).

#### ***4.2 Synaptotagmins as $\text{Ca}^{2+}$ -Sensors for Fast Neurotransmitter Release***

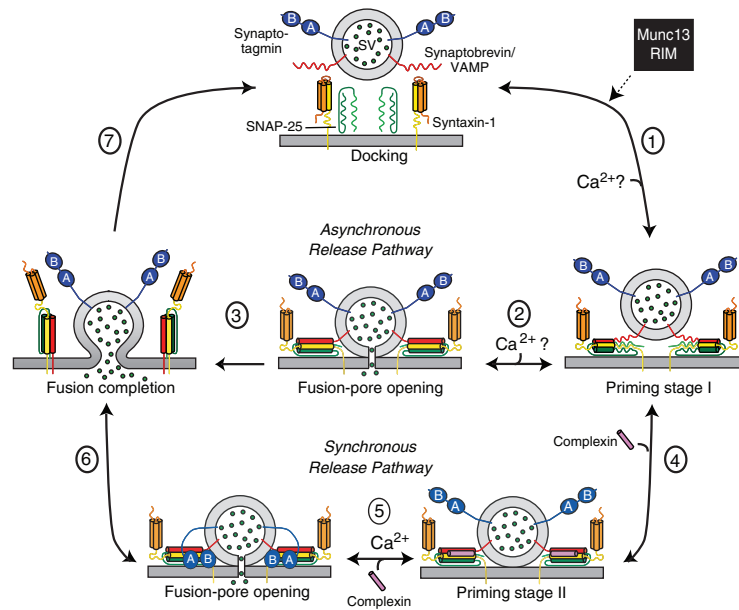
At resting  $\text{Ca}^{2+}$ -concentrations, release occurs spontaneously at a low rate;  $\text{Ca}^{2+}$  increases this rate  $>10,000$ -fold in less than a millisecond, faster than most diffusion-controlled chemical reactions.  $\text{Ca}^{2+}$  thus increases the probability of a rare event that normally occurs all the time. Studies in the Calyx of Held synapse – a large vertebrate synapse that allows simultaneous recordings from pre- and postsynaptic neurons – showed that  $\text{Ca}^{2+}$  triggers release at micromolar concentrations with an apparent cooperativity of 5 (Meinrenken et al., 2003). For each active zone, the  $\text{Ca}^{2+}$ -signal produced by an action potential has a finite probability of triggering exocytosis (usually between 0.05 and 0.30) that changes as a function of the previous use of a synapse (i.e., is subject to plasticity), and additionally depends on signaling mediated by neuromodulators. In addition to this fast synchronous type of neurotransmitter release, synapses exhibit a second slower, more asynchronous type of release that is also triggered by  $\text{Ca}^{2+}$ . Both types are likely important, but under most physiological conditions the fast synchronous type predominates.

The speed of  $\text{Ca}^{2+}$ -triggered exocytosis suggests that  $\text{Ca}^{2+}$  acts at a stage at which part of the fusion reaction has already been completed, most likely on vesicles in a hemifusion state that is presumably created by the combined action of SNARE proteins and Munc18-1 (i.e., after priming II in Figure 4). Much of synaptic vesicle exocytosis likely operates in the “kiss-and-run” mode (Harata et al., 2006). Kiss-and-run exocytosis predominates during low-frequency stimulation, while full exocytosis is more important during high-frequency stimulus trains. The kiss-and-run mode allows fast recycling of synaptic vesicles for reuse after exocytosis, but probably has only a minimal effect on the kinetics or amount of release because the total size of synaptic vesicles ( $\sim 20\text{nm}$  radius) is only 5- to 10-fold larger than that of the fusion pore.

The definition of the primary structure of synaptotagmin-1, composed of an N-terminal transmembrane region and two C-terminal  $\text{C}_2$ -domains (Figure 3), led

to the hypothesis that synaptotagmin-1 functions as the  $\text{Ca}^{2+}$ -sensor for fast neurotransmitter release (Perin et al., 1990). Subsequent studies revealed that synaptotagmin-1 and two other synaptotagmin isoforms, synaptotagmin-2 and -9, act as  $\text{Ca}^{2+}$ -sensors in exocytosis by a strikingly simple mechanism:  $\text{Ca}^{2+}$  flowing into the nerve terminal when  $\text{Ca}^{2+}$ -channels open during an action potential binds to the two  $\text{C}_2$ -domains of synaptotagmins (Xu et al., 2007).  $\text{Ca}^{2+}$ -binding to the  $\text{C}_2$ -domains induces the interaction of synaptotagmins with phospholipids and with SNARE proteins with a micromolar apparent  $\text{Ca}^{2+}$ -affinity, consistent with the affinity of release, which in turn opens the fusion pore mechanically by pulling the SNARE complexes apart (Südhof, 2004). This model was supported by a host of biochemical and genetic experiments, most importantly the finding that point mutations in the endogenous synaptotagmin gene in mice that alter the apparent  $\text{Ca}^{2+}$ -affinity of synaptotagmin also alter the apparent  $\text{Ca}^{2+}$ -affinity of release in an identical manner (Fernandez-Chacon et al., 2001). However, these findings also raised a crucial question that is only now beginning to be answered: Why doesn't the fusion pore just pop open after priming II (Figure 4)? Recent results suggest that another nerve-terminal protein, a soluble protein called complexin, plays a central role here (Giraudo et al., 2006; Tang et al., 2006). Complexins bind in a  $\text{Ca}^{2+}$ -independent manner only to the C-terminal part of assembled SNARE complexes where the SNARE complexes are anchored in the membrane (Figure 5). Deletion of complexins in mice revealed a selective impairment of fast synchronous  $\text{Ca}^{2+}$ -triggered release, whereas other forms of fusion were unchanged (Reim et al., 2001). This phenotype resembles the synaptotagmin-1 knockout phenotype in its selectivity, but was milder because the complexin knockout phenotype can be rescued by boosting  $\text{Ca}^{2+}$ -influx into the nerve terminal, whereas the synaptotagmin-1 knockout phenotype cannot. Thus complexins function as activators of SNARE complexes for subsequent synaptotagmin-1 action. The mechanism by which the functions of synaptotagmin-1 and complexins interact was revealed in biochemical experiments demonstrating that  $\text{Ca}^{2+}$ -induced binding of synaptotagmin-1 to SNARE complexes displaces complexin from the SNARE complexes (Tang et al., 2006), indicating that synaptotagmin-1 serves as the trigger of a complexin-cocked gun.

These observations suggest a model for how synaptotagmin-1 triggers exocytosis (Figure 5): By binding to SNARE complexes during assembly, complexins force completion of SNARE-complex assembly that creates an activated, frozen fusion intermediate, likely a hemifusion state. When  $\text{Ca}^{2+}$  binds to synaptotagmin, this induces binding of synaptotagmin to both fusing membranes and to the SNARE complexes. We envision that this binding pulls open the fusion pore by two independent mechanisms: a mechanical force on the membrane by coupling phospholipids to SNARE complexes via the simultaneous binding, and a disinhibition of fusion-pore opening by displacing from the SNARE complexes the complexins that at the same activate and stabilize the complexes. Note that the complexin/synaptotagmin-dependent fusion reaction mediates fast synchronous release, and is distinct from the second type of release, slower asynchronous release which also depends on SNARE-proteins and Munc18-1 but is independent of complexins and synaptotagmin.



**Fig. 5** Model for the interplay between SNARE, complexin, and synaptotagmin function in  $Ca^{2+}$ -triggered neurotransmitter release. Docked vesicles containing unassembled SNARE complexes (top) are attached to the active zone via the Rab3/27 interaction with RIMs (Figure 3), and are primed in a two-stage reaction by SNARE-complex assembly (step 1; see also Figure 4). The resulting primed vesicles form the substrate for two release pathways: asynchronous release that is synaptotagmin- and complexin-independent (steps 2 and 3), and synchronous release that depends on synaptotagmin and complexins (steps 4–6). Note that quantitatively, the asynchronous pathway is less important for release than the synchronous pathway which mediates >90% of all release under most conditions. Asynchronous release is triggered by  $Ca^{2+}$  by a poorly understood mechanism that, like synchronous release, requires assembly of SNARE complexes, but differs from synchronous release in that it does not appear to stop fusion-pore opening by a “clamp” after SNARE complexes are fully assembled. Asynchronous release becomes important when the  $Ca^{2+}$ -concentration rises for prolonged time periods to intermediate levels (0.1–1.0  $\mu M$ ) that are too low to trigger synchronous release, but sufficient to trigger asynchronous release. This occurs, for example, during the accumulation of residual  $Ca^{2+}$  during high-frequency stimulus trains (Zucker and Regehr, 2002). Synchronous release involves “superpriming” of synaptic vesicles by binding of complexins to assembled SNARE complexes (step 4). Complexin binding activates and freezes SNARE complexes in a metastable state (priming stage II). Superprimed vesicles in which the SNARE complexes have been clamped by complexin are then substrate for fast  $Ca^{2+}$ -triggering of release when  $Ca^{2+}$ -binding to synaptotagmin triggers the simultaneous interaction of synaptotagmin with phospholipids and SNARE complexes, with the latter reaction displacing complexin and resulting in fusion-pore opening (step 5). Opened fusion pores can then dilate to complete fusion (step 6), although both steps 2 and 5 are potentially reversible, i.e., lack of dilation of the fusion pore could lead to “kiss-and-stay” or “kiss-and-run” exocytosis in these pathways. Note that steps 1 and 4 are also probably reversible, with a much faster forward than backward speed. It is likely that step 1 is  $Ca^{2+}$ -dependent, but it is unclear whether or not step 2 is  $Ca^{2+}$ -dependent, since it is possible that asynchronous release is  $Ca^{2+}$ -dependent solely because  $Ca^{2+}$  accelerates step 1, and step 2 has a finite probability. Thus the nature of  $Ca^{2+}$ -triggering of asynchronous release could operate either at the priming or at the actual fusion step. Note that the function of Munc18-1 is not included in this diagram, but is thought to be central to the actual fusion reaction (see Figure 4). (Modified from Tang et al., 2006).

In addition to functioning as  $\text{Ca}^{2+}$ -sensors for vesicle exocytosis, synaptotagmins may be involved in vesicle endocytosis, particularly the decision between kiss-and-run versus full exocytosis. Such a role would be economical in linking fusion-pore opening (which is triggered by  $\text{Ca}^{2+}$ -binding to synaptotagmin) to fusion-pore expansion or contraction, but the precise mechanisms involved have not yet been explored.

As mentioned above, three different synaptotagmin isoforms mediate fast synchronous release: synaptotagmin-1, -2, and -9. Why does the vertebrate brain express three different proteins to do the same job? As it turns out, these three different synaptotagmins mediate the  $\text{Ca}^{2+}$ -triggering of release with distinct properties, and are differentially distributed (Xu et al., 2007). Of the three synaptotagmins, synaptotagmin-2 is the fastest, and synaptotagmin-9 the slowest, corresponding with the selective presence of synaptotagmin-2 in the calyx synapse, one of the fastest synapses in brain involved in sound localization, and synaptotagmin-9 in the limbic system involved in emotional reactions. Thus, the properties of the synapses formed by a neuron depend among others on the isoform of synaptotagmin which that neuron expresses, adding additional complexity to the neural circuits formed by synaptic networks.

## 5 Regulation of Release Beyond $\text{Ca}^{2+}$ -Triggering

We already alluded to the fact that release is not constant, but is highly plastic, i.e., regulated by extrinsic signals and by the intrinsic previous activity of a nerve terminal. Both forms of regulation constitute a type of synaptic memory: the history of the activity of other surrounding neurons and of the neuron to which a terminal belongs strongly influence to what extent this terminal translates an action potential into a neurotransmitter release signal (Zucker and Regehr, 2002). Many different forms of synaptic plasticity exist. Although traditionally postsynaptic forms of plasticity have been appreciated more than presynaptic forms, recent studies have revealed the existence of purely presynaptic forms of synaptic plasticity, and have moreover shown that many forms of plasticity are mediated by the combined action of pre- and postsynaptic mechanisms.

A complete discussion of presynaptic modulation and plasticity of release is beyond the scope of this chapter. Instead, three exemplary forms of presynaptic plasticity will be discussed because of their importance for the pharmacology of neurotransmitter release.

### ***5.1 Acetylcholine-Receptor-Mediated $\text{Ca}^{2+}$ -Influx into Presynaptic Nerve Terminals***

Nicotine is an addictive drug that activates a diverse subset of ionotropic acetylcholine receptors. Most cholinergic actions in brain, both by ionotropic and metabotropic receptors, are modulatory, and very few fast synapses exist that utilize



acetylcholine as a transmitter. Interestingly, although some ionotropic acetylcholine receptors are postsynaptic, a relatively large proportion, much larger than observed for other neurotransmitters, appears to be presynaptic. A possible pathway accounting for the addictive actions of nicotine was discovered in the observation that presynaptic nicotinic acetylcholine receptors, when activated, mediate the influx of  $\text{Ca}^{2+}$  into presynaptic terminals, and thereby stimulate the release of neurotransmitters (Wannacott, 1997). Interestingly, this effect appears to operate via a class of nicotinic acetylcholine receptors containing  $\alpha 6$  subunits that are relatively enriched in the nigrostriatal pathway, suggesting that nicotine may be addictive by increasing dopamine release (Quik and McIntosh, 2006). Thus the presynaptic facilitation of neurotransmitter release by cholinergic nicotinic receptors is one of the best and physiologically most important systems illustrating how presynaptically acting receptors can regulate release.

### ***5.2 $\text{Ca}^{2+}$ -Channel Modulation by Presynaptic Receptors***

Presynaptic G-protein coupled receptors for a large number of neurotransmitters, both autoreceptors and receptors for extrinsic signals, suppress  $\text{Ca}^{2+}$ -channel gating in response to an action potential. This mechanism of action appears to be the dominant mechanism involved in short-term plasticity mediated by presynaptic receptors. A typical example is depolarization-induced suppression of inhibition (DSI), which is the short-term suppression of presynaptic GABA-release induced by the depolarization of the postsynaptic cell (Diana and Marty, 2004). DSI is caused when the postsynaptic depolarization causes the release of endocannabinoids from the postsynaptic cell, and the endocannabinoids then bind to presynaptic CB1 receptors whose activation suppresses presynaptic  $\text{Ca}^{2+}$ -channels. Like many other forms of presynaptic suppression mediated by activation of presynaptic receptors, this effect is short-lasting (in the millisecond range). The precise mechanisms by which  $\text{Ca}^{2+}$ -channels are suppressed appear to vary between receptors, but the outcome is always a very effective short-term decrease in synaptic signaling.

### ***5.3 Presynaptic Long-Term Plasticity Mediated by cAMP-Dependent Protein Kinase A (PKA)***

PKA-dependent long-term potentiation and depression was initially discovered in the mossy-fiber synapses of the hippocampus, and later demonstrated in parallel fiber synapses of the cerebellum and corticothalamic synapses of the forebrain (Malenka and Siegelbaum, 2001). This widespread form of plasticity does not involve changes in  $\text{Ca}^{2+}$ -influx, but operates via a direct increase or decrease, respectively, of the amount of vesicle exocytosis that can be triggered by a given  $\text{Ca}^{2+}$ -signal. Interestingly, this form of plasticity appears to depend on the interaction

of the synaptic vesicle GTP-binding protein Rab3 with its effectors, RIM-proteins (Figure 3), since deletion of either Rab3A or of RIM1 $\alpha$  abolishes LTP in the hippocampal mossy fibers (Castillo et al., 2002). Moreover, recently it was revealed that a special form of endocannabinoid-dependent long-term depression of GABAergic synapses in the hippocampus also operates as a presynaptic process that requires PKA activation and RIM1 $\alpha$ . This form of LTD, referred to as i-LTD for inhibitory LTD, is distinct from the DSI discussed above; indeed, DSI is normal in mice lacking RIM1 $\alpha$ . Thus, endocannabinoids can trigger two different forms of plasticity depending on the precise conditions of the postsynaptic depolarization. Overall, these results highlight the role of the core release machinery in presynaptic long-term plasticity.

## 6 $\text{Ca}^{2+}$ -Induced Exocytosis of Small Dense-Core Vesicles and LDCVs

Biogenic amines are generally secreted from small dense-core vesicles (SDCVs) in varicosities of axons that are not associated with classical synapses, and additionally from LDCVs, whereas neuropeptides are secreted – usually together with biogenic amines – only from LDCVs. Different from synaptic vesicle exocytosis where  $\text{Ca}^{2+}$  is thought to trigger fusion of predocked and primed vesicles, a significant part of SDCV and LDCV exocytosis involves the  $\text{Ca}^{2+}$ -dependent mobilization of vesicles before  $\text{Ca}^{2+}$ -triggered fusion. As a result, dense-core vesicle exocytosis requires more sustained  $\text{Ca}^{2+}$ -transients, but is also slower and longer lasting than synaptic vesicle exocytosis (Stjarne, 2000).

$\text{Ca}^{2+}$ -triggered exocytosis of SDCVs, LDCVs, and their endocrine equivalents has been examined in several systems (e.g., chromaffin cells, cultured melanotrophic neurons, pancreatic  $\beta$ -cells, and PC12 cells), but the best-studied system is that of chromaffin cells because they allow the highest measurement resolution, and are the only system that has been investigated extensively by a loss-of-function approach.  $\text{Ca}^{2+}$ -triggered chromaffin granule exocytosis is  $\sim 10$ -fold slower than synaptic vesicle exocytosis, but otherwise the two systems are similar. Chromaffin granule exocytosis, like synaptic vesicle exocytosis, involves SNARE and Munc18-proteins. Knockout experiments revealed, however, that  $\text{Ca}^{2+}$ -triggering of chromaffin granule and synaptic vesicle exocytosis are mechanistically distinct. Specifically, chromaffin cell exocytosis is mediated by two different synaptotagmins: synaptotagmin-1, which is shared with synaptic vesicle exocytosis, and synaptotagmin-7, which does not function in synaptic vesicle exocytosis. The synaptotagmin-7 selectively functions as a major  $\text{Ca}^{2+}$ -sensor for chromaffin granule but not for synaptic vesicle exocytosis, indicating a fundamental difference between the two types of exocytosis. However, beyond this difference, little is known about the other molecular components that direct SDCV and LDCV exocytosis and make it different from synaptic vesicle exocytosis, a question that is of obvious importance in understanding the release of biogenic amines and neuropeptides in brain.

## 7 Presynaptic Drug Targets

This introductory chapter discussed the fundamental mechanisms of release because understanding these fundamental mechanisms is crucial for insight into the actions of drugs that act on release. Overall, the neurotransmitter release machinery is a poor drug target, as judged by the number of successful drugs that act on it, and in contrast to the neurotransmitter reception machinery (i.e., neurotransmitter receptors) that is the target of many different drugs. In fact, the quantitatively largest number of drugs that influence neurotransmitter release act on presynaptic neurotransmitter receptors. Nevertheless, several well-established drugs exist that act on the release machinery: reserpine on the vesicular catecholamine transporter, leviracetam on the vesicle protein SV2, and cocaine on the presynaptic re-uptake of dopamine.

It is clear that the presynaptic machinery is an underutilized target for drugs. The actual membrane-trafficking components of that machinery, such as the SNARE, SM, or Rab-proteins or synaptotagmins, are probably not good drug targets because they operate via protein-protein interactions that are difficult to influence with small molecules. However, several synaptic proteins with receptor, transport, or enzyme functions are likely drug targets, but have not yet been explored. These proteins include synapsins, which are ATP-binding proteins (Hosaka and Südhof, 1998), CSP $\alpha$  as a synaptic vesicle co-chaperone (Tobaben et al., 2001), and vesicular GABA- and glutamate transporter (Gasnier, 2000; Takamori, 2006). We hope that the material presented in this book will not only help understand the actions of current drugs, but also stimulate the development of new ones.

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