Molecular analysis of neurotransmitter release

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Introduction

A key step in the evolution of complex pluricellular organisms was the creation of a specialized pool of cells devoted to the processing and transduction of signals arriving from the external environment, leading to a suitable co-ordinated response of the organism. These specialized cells, a primitive functional nervous system, had to solve the problem of sustaining a reliable and efficient cross-talk with neighbouring cells. The winning strategy that evolved is based on the regulated and compartmentalized secretion of ligands, a mechanism at the basis of synaptic transmission in neurons. Two distinct pathways characterize neurotransmitter release: non-quantal molecular leakage and quantal release. While the former is still a matter of debate and, in general, accounts for a minority of total synaptic transmission, the latter mechanism has been clearly demonstrated and predicts that the generation of quanta is due to the fusion of neurotransmitter-containing small synaptic vesicles (SSVs) with specialized active zones at the presynaptic plasma membrane [1].

Although the complexity of neuroexocytosis is not yet fully understood, studies from several laboratories around the world have begun to provide a unitary view of the molecular steps preceding and following SSV fusion. As represented schematically in Figure 1, a SSV, after being loaded with neurotransmitter, is transported into close proximity with the presynaptic plasma membrane via interaction with the actin cytoskeleton. This transport phase is

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followed by binding of the SSV to the active zone at the target membrane, in a process called tethering. This is followed by the functional docking of the SSV in the immediate vicinity of a Ca$^{2+}$ channel and by priming, a reversible ATP-dependent phase. Exocytosis of the primed SSV is triggered by a very rapid increase in the intracellular Ca$^{2+}$ concentration, generated by the opening of presynaptic voltage-gated Ca$^{2+}$ channels following depolarization (fusion). The release of the SSV contents is via the opening of a fusion pore or complete fusion of the SSV with the presynaptic membrane. The empty SSV undergoes rapid endocytosis and is refilled with neurotransmitter by proton-driven neurotransmitter transporters.

In the last decade, the molecular analysis of neurotransmitter release has made the transition from a mere description of proteins expressed in the nervous system to a more sophisticated investigation of the functions that these proteins perform during the SSV life cycle. One of the first soluble proteins important for SSV docking and fusion to be identified was the so-called N-ethylmaleimide-sensitive factor (NSF), an ATPase that is involved in a variety of intracellular transport pathways [4]. NSF binds to its membrane receptors via another soluble protein, SNAP (soluble NSF accessory protein); hence the name SNAREs (SNAP receptors) for the membrane receptor proteins. As SNAREs are localized to different membrane compartments, the nomenclature v-SNARE for vesicular SNARE [i.e. VAMP (vesicle-associated membrane

**Figure 1. Late steps in the life cycle of an SSV**
SSVs are mobilized from the reserve pool by the phosphorylation of synapsin, and are tied to the synaptic plasma membrane via specific protein–protein interactions. This tethering phase is followed by the functional docking of the SSV in the immediate vicinity of a Ca$^{2+}$ channel and by priming, a reversible ATP-dependent phase. Exocytosis of the primed SSV is triggered by a very rapid increase in the intracellular Ca$^{2+}$ concentration, generated by the opening of presynaptic voltage-gated Ca$^{2+}$ channels following depolarization (fusion). The release of the SSV contents is via the opening of a fusion pore or complete fusion of the SSV with the presynaptic membrane. The empty SSV undergoes rapid endocytosis and is refilled with neurotransmitter by proton-driven neurotransmitter transporters.
SNARE proteins

Three proteins of the synaptic terminal are at the centre of increasing attention as key players in neurotransmitter release. These are the SSV-specific VAMP (also known as synaptobrevin), and syntaxin and SNAP-25, both of which are localized predominantly on the plasma membrane.

VAMP is a protein of 13 kDa that is localized to SSVs, dense-core granules and synaptic-like vesicles. Ten different isoforms have been identified on the basis of structural sequence similarity, but only three isoforms have been extensively characterized: VAMP-1, VAMP-2 and cellubrevin. VAMP isoforms are present in all vertebrate tissues, but their distribution varies [5]. Structurally, VAMP is composed of an N-terminal portion that is rich in proline residues and is divergent in different isoforms, a very conserved central portion that contains coiled-coil segments which are responsible for the pairing with the corresponding t-SNAREs (syntaxin and SNAP-25), and a single membrane-spanning domain. The majority of the protein mass is exposed to the cytoplasm, while only a short and poorly conserved portion is intravesicular (Figure 2). On SSVs, VAMP-2 is associated with synaptophysin, a major component of the SSV membrane, and with subunits of the V-ATPase. VAMP-2, but not VAMP-1 or cellubrevin, interacts with a prenylated Rab acceptor (termed PRA) via sequences that are present only in the proline-rich and the transmembrane domain of VAMP-2. The binding requirements for VAMP-2 and PRA are thus distinct from those characterizing the interaction of VAMP-2 with syntaxin and SNAP-25.

SNAP-25, originally described as the major palmitoylated protein in the central nervous system, is required for axonal growth during neuronal development and in nerve terminal plasticity in the mature nervous system. In the nervous system and in neuroendocrine cells, SNAP-25 is expressed in two isoforms which are developmentally regulated (SNAP-25A and B) [6]. Structurally, SNAP-25 lacks a classical transmembrane segment, and its membrane binding is mediated by the palmitoylation of cysteine residues located in the middle of the polypeptide chain and by additional, still unidentified, protein–lipid interactions. Palmitoylation is essential for correct targeting of the molecule to the plasma membrane. As with the other SNAREs, SNAP-25
contains segments with the ability to form coiled-coil helices. SNAP-25 forms a stoichiometric complex with the putative Ca\(^{2+}\) sensor synaptotagmin I, and this interaction is believed to be important for a late step of the Ca\(^{2+}\)-dependent phase of neurotransmitter release. In addition, SNAP-25 was demonstrated to interact in a Ca\(^{2+}\)-dependent manner with Hrs-2, an ATPase having a negative regulatory effect on exocytosis.

Syntaxin is a typical type II membrane protein; its N-terminal portion is exposed to the cytosol, followed by a single transmembrane domain and few residues emerging into the intersynaptic space (Figure 2). Syntaxin is associated with N-, P- and Q-type Ca\(^{2+}\) channels in the active zones, where neuro-
transmitter release takes place, and it is also present on most of the neuronal cell membrane [5]. Syntaxin undergoes, together with SNAP-25, a recycling process in organelles indistinguishable from SSVs. It interacts in a Ca\(^{2+}\)-dependent manner with some isoforms of the SSV protein synaptotagmin. The minimal segment of syntaxin required for Ca\(^{2+}\)-dependent binding to synaptotagmin was localized to amino acids 220–266 (Figure 2). A more extended portion of this same region is responsible for the interaction with VAMP and SNAP-25, to assemble the synaptic SNARE complex, and for the interaction with α-SNAP. Deletional analysis confirmed that, in the other SNAREs also, regions with a high probability of forming coiled coils are essential for SNARE-complex formation.

Syntaxins constitute a large protein family, with more than a dozen different isoforms coded for by different genes or generated by alternative splicing, and a vast syntaxin polymorphism exists within the nervous tissue. Syntaxins are essential for neuronal development and survival. Several isoforms undergo a complex pattern of alternative splicing and expression control during long-term potentiation, suggesting that syntaxins are involved in synaptic plasticity. This differential expression could be important for a direct modulation of Ca\(^{2+}\) entry via selective interactions with specific Ca\(^{2+}\) channels, in addition to the formation of distinct SNARE complexes with different SNAP-25 and VAMP isoforms.

What is the precise role of the SNARE complex and its functional ligands, α-SNAP and NSF, in neurotransmitter release? The accumulation of vesicles in an in vitro intra-Golgi assay after NSF depletion, in the yeast NSF mutant sec18 and in the corresponding comatose mutant of Drosophila, sustains the hypothesis that NSF could be involved in vesicle fusion with the target membrane [4]. The ability of NSF to bind via SNAPs to the SNARE complex and to disassemble this large 20 S particle via its ATPase activity further strengthens this proposal, and suggests that the disassembly of this large particle may represent the core of the membrane fusion mechanism. However, recent experiments have challenged this view and indicate that the action of NSF may be restricted to an earlier stage, the pre-docking and/or docking step [7]. In particular, NSF action could be restricted to the disassembly of SNARE complexes during the recycling of VAMP, SNAP-25 and syntaxin, allowing them to re-enter the docking and fusion cycle of the exocytic vesicles. Although compelling, these experiments cannot exclude a role for NSF in a post-docking stage of heterotypic membrane fusion or in modulation of the speed of neurotransmitter release [8].

The low-resolution structures of the SNARE complex and of 20 S particles have recently been determined using electron microscopic and rotary shadowing techniques [9]. The SNARE complex has a rod-shaped structure containing the SNARE proteins arranged in a parallel fashion (Figure 3). NSF (present as a hexamer) and α-SNAP occupy one end of the rod, and disappear when the particle is incubated in the presence of Mg\(^{2+}\)-ATP. The geometry of
the SNARE complex, the large amount of energy liberated by its formation and the observation that the cleavage of the SNAREs by clostridial neurotoxins leads to a block in SSV fusion and their subsequent accumulation on the plasma membrane suggest that the interaction of VAMP-2, SNAP-25 and

Figure 3. Structure of the SNARE complex and mechanism of membrane fusion
(a) Low-resolution electron micrograph of the trimeric SNARE complex (modified from [9] with permission; © 1997 Cell Press). The rod-shaped SNARE complex contains VAMP-2 (light blue) and syntaxin (dark blue) arranged in a parallel fashion. (b) SNARE proteins are able to fuse two populations of artificial liposomes, one containing purified VAMP-2 and the other containing the complex between syntaxin (dark blue) and SNAP-25 (dark grey) respectively. The formation of the trimeric SNARE complex (SNARE zipping) is essential for Ca^{2+}-independent membrane fusion. (c) The Ca^{2+}-sensitivity of the fusion process could be provided by interaction of SNAREs with the Ca^{2+} sensor synaptotagmin (black), which might act by blocking the fusion reaction. This functional Ca^{2+} clamp could be removed as a result of the rise in intracellular Ca^{2+} concentration caused by the opening of Ca^{2+} channels.
syntaxin could be the basis of lipid bilayer fusion. Recent work from Rothman et al. [10] demonstrated that SNARE proteins are able to fuse artificial liposomes, in the absence of α-SNAP and NSF, following their interaction to form the trimeric complex. This complex, linking two membranes, named SNAREpins by the authors, has analogy with the viral fusogenic proteins containing hairpin-like structures and could be the basis of a general mechanism for protein-mediated lipid bilayer fusion.

Synaptotagmins as Ca\(^{2+}\) sensors at the synapse

Synaptotagmin is a type I membrane protein localized on SSVs that belongs to a growing family of proteins, with more than a dozen members present in nervous and non-nervous tissues [11]. Impairment of its function in neurons via gene knock-out approaches and by microinjection of peptides and antibodies strongly supports the possibility that synaptotagmin is the main Ca\(^{2+}\) sensor in the fast phase of neurotransmitter release [11]. Structurally, synaptotagmin is characterized by a large cytosolic domain, a single transmembrane region flanked on the cytoplasmic side by a cysteine-rich palmitoylated segment, and a short N-terminal portion in the lumen of the vesicles (Figure 2). The cytoplasmic portion of the protein is hydrophilic, contains two protein modules homologous to the C2 regulatory domain of protein kinase C and has a highly conserved C-terminus known to interact with members of the neurexin family. Moreover, synaptotagmin is able to interact with a variety of proteins and lipids via its C2 domains, both in a Ca\(^{2+}\)-dependent and in a Ca\(^{2+}\)-independent manner. Phosphatidylserine and syntaxin bind synaptotagmin via its C2A domain at Ca\(^{2+}\) concentrations similar to those observed at the synapse during neuroexocytosis. The C2B domain of synaptotagmin mediates the binding of SNAP-25, which is weakly dependent on Ca\(^{2+}\), and the binding of β-SNAP, which is Ca\(^{2+}\)-insensitive. The C2B domain is also responsible for the Ca\(^{2+}\)-dependent hetero- and homo-dimerization of synaptotagmin. In fact, the presence of multiple synaptotagmin isoforms on a single SSV suggests that a combinatorial range of Ca\(^{2+}\) sensors could be created by heterodimerization of isoforms with different Ca\(^{2+}\) affinities. As a consequence, the probability of an SSV being released at a certain Ca\(^{2+}\) concentration is directly dependent on the repertoire of synaptotagmin isoforms present on its surface (Osborne, S.L., Herreros, J., Bastiaens, P.I. and Schiavo, G., unpublished work).

The range of synaptotagmin interactions with negatively charged lipids was recently expanded by the finding that the C2B domain binds phosphoinositides. In particular, PtdIns(3,4,5)\(P_3\) binds to synaptotagmin in the absence of Ca\(^{2+}\), whereas PtdIns(4,5)\(P_2\) binds at micromolar Ca\(^{2+}\) concentrations. This equilibrium constitutes a Ca\(^{2+}\)-dependent switch that has the potential to localize the cytoplasmic portion of synaptotagmin to phosphoinositide-rich domains of the lipid bilayer. In addition, the interaction of phosphoinositides with synaptotagmin is the first direct linkage between a member of the protein...
exocytosis apparatus and these tightly regulated and essential lipid components of the membrane (see below).

Recent evidence suggests another important role for synaptotagmin in nerve-terminal physiology through its interaction with voltage-gated Ca\(^{2+}\) channels. N-type Ca\(^{2+}\) channels bind synaptotagmin directly via a cytosolic loop, and this binding fully restores the current amplitude and inactivation kinetics of these syntaxin-modulated channels. Immunoprecipitation experiments indicate that synaptotagmin also associates with P- and Q-type Ca\(^{2+}\) channels and that these complexes recruit the SNARE complex. At equilibrium, a large percentage of presynaptic Ca\(^{2+}\) channels is engaged in this association. Taken together, these results suggest that these proteins may constitute an isolated exocytic complex in which the Ca\(^{2+}\) channel interacts tightly with the SSV docking site.

**Rab3 and neurotransmitter release**

All trafficking steps throughout the secretory pathway are regulated by members of the Rab family of small GTP-binding proteins. Rab proteins do not contain a transmembrane region, but interact directly with the lipid bilayer through a C-terminal geranylgeranyl modification (Figure 2). Two members of this family (Rab3A and Rab3C) are suggested to play a role in regulated secretion, based on their exclusive expression in neurons and neuroendocrine cells. However, despite massive efforts to define the precise molecular action of Rabs in this process, the details of their action remain unknown, and have been assigned to the phase of docking and fusion of vesicles with the target membranes [11]. The function(s) of the Rab proteins is thought to be dependent on their GTPase activity as well as their effectors. The cycling of Rabs from the GDP- to the GTP-bound state determines their translocation to the target membranes. At least three types of activities are required for the Rab3 cycle: (1) a GTPase-activating factor triggering GTP hydrolysis; (2) a GDP-dissociation inhibitor promoting the removal of the Rab3–GDP from SSVs after exocytosis; and (3) a GDP-exchange protein catalysing GDP–GTP exchange. In this cycle, a pool of soluble Rab3 is maintained by the interaction with the GDP-dissociation inhibitor to shield the geranylgeranyl group [12].

Rab3A interacts *in vitro* with SNARE proteins and with the SNARE complex, but a direct effect on SNARE complex assembly has been clearly identified only in an early step of the secretory pathway in yeast. The lack of validation of this finding in other trafficking steps, and in neurotransmitter release in particular, is complicated by the absence of a severe phenotype in Rab3A-deficient mice. The only observed anomaly in hippocampal neurons lacking Rab3A is an increase in evoked quantal release after a single stimulation. This result was interpreted as an inhibition of multivesicular fusion mediated by Rab3A. Thus the function of Rab3A at the nerve terminal would be to restrict exocytosis to a single vesicle per releasing site [11], possibly through the interaction with a new Rab effector, termed Rim (Rab-interacting protein).
in mammals. Rim binds only to GTP–Rab3 and not to GDP–Rab3, and is localized to presynaptic active zones in conventional synapses. Rim overexpression has a stimulatory effect on regulated exocytosis, possibly by reversing the inhibitory function of Rab3A.

Many putative Rab effectors have been described, but the lack of functional data makes speculation premature. The first Rab3A effector discovered was rabphilin 3A, which is specific for GTP–Rab3A. This soluble protein is associated with SSVs and contains two C2 homology domains and a zinc finger. Rabphilin 3A also interacts with cytoskeletal proteins and could be important for actin remodelling [11].

**Phosphoinositide biosynthesis and turnover at the nerve terminal**

Several lines of evidence support the pivotal role of phosphoinositides and the enzymes regulating their turnover in membrane-trafficking events. Three proteins involved in phosphoinositide biosynthesis were demonstrated to be essential for priming in Ca\(^{2+}\)-dependent exocytosis in neuroendocrine cells. The first of these factors is the phosphatidylinositol transfer protein, a soluble protein responsible for the mobilization of PtdIns and its correct presentation to lipid kinases. PtdIns transfer protein was found to be an essential cofactor in the maintenance of a primed ATP-dependent state in PC12 cells, together with PtdIns 4-phosphate 5-kinase, another soluble cytosolic protein. The activity of PtdIns 4-kinase, an integral membrane protein of secretory granules, is also necessary to sustain vesicle priming, thus strongly supporting a role for PtdIns(4,5)\(P_2\) during the terminal phases of secretory granule exocytosis [13]. Although similar studies on SSVs are still lacking, the reversible inhibitory activity of inositol polyphosphates on neurotransmitter release in giant squid axons suggests that PtdIns(4,5)\(P_2\) plays an essential role in both of these regulated exocytic events. The molecular basis of its action is still unclear. Direct involvement of PtdIns(4,5)\(P_2\) in lipid bilayer fusion is unlikely, based on the high bivalent cation concentration needed to promote aggregation and fusion of PtdIns(4,5)\(P_2\)-containing liposomes. In addition, the high curvature caused by this phospholipid in the membrane bilayer would be expected to destabilize the vesicular fusion intermediates. Several proteins of the exocytic machinery interact with PtdIns(4,5)\(P_2\), including CAPS (a novel Ca\(^{2+}\)-binding protein) [14] and synaptotagmins [15]. This latter binding is localized to the C2B domain and is blocked by Ins\(P_6\). Microinjection of a C2B-specific antibody into the giant squid axon reverses the Ins\(P_6\)-evoked inhibition of neurotransmitter release, suggesting a direct action of Ins\(P_6\) on synaptotagmin–PtdIns(4,5)\(P_2\) binding and a functional connection between phosphoinositides and synaptotagmin at the nerve terminal.
Cytoskeleton and exocytosis

Several lines of evidence suggest a central role for the cytoskeleton in the mobilization of SSVs and, more generally, the existence of a cross-talk between the cytoskeleton and members of the exocytosis machinery. At the synaptic bouton in mature synapses, the SSV distribution is determined by the interaction of synapsins with the cytoskeleton. The four members of this family of phosphoproteins (synapsins Ia, Ib, IIa and IIb) are generated by differential mRNA splicing from two distinct genes [16]. Synapsins have two domains: an N-terminal hydrophobic head region and a C-terminal tail region that is rich in polar residues (Figure 2). Crystallographic studies revealed that the N-terminus of synapsin shares structural similarities with a family of ATP-utilizing enzymes and binds ATP in a Ca\(^{2+}\)-dependent manner. The C-terminal region is responsible for the binding of synapsin to SSVs, an event which is regulated by phosphorylation. Synapsins are excellent substrates for cAMP-dependent protein kinase and Ca\(^{2+}\)/calmodulin-dependent protein kinase I. In the dephosphorylated state, synapsin I is able to bind actin filaments and to promote actin polymerization in vitro. These findings suggest that synapsins modulate the interaction of SSVs with the cytoskeleton at the nerve terminal [17]. The rapid phosphorylation of synapsins, triggered by the rise in Ca\(^{2+}\) concentration in the region proximal to the active site, disassembles the ternary complex formed by synapsin, SSV and actin, allowing the released SSV to enter the tethering phase of neurotransmitter release. This is supported by gene knock-out experiments, which demonstrated that synapsins are essential for accelerating SSV availability during repetitive stimulation [18]. Synapsin I- or synapsin II-null mice are viable and fertile, but experience seizures with a frequency proportional to the number of mutant alleles. Synapsin II/I double knock-outs exhibit decreased post-tetanic potentiation and severe synaptic depression upon repetitive stimulation [18].

Another connection between the cytoskeleton and SSVs is provided by the molecular interaction of brain myosin V, a member of the family of unconventional myosins, and the binary complex formed between VAMP-2 and synaptophysin. Brain unconventional myosins are localized within the presynaptic terminals and are capable of generating mechanochemical force and moving actin filaments. In particular, myosin V appears to be involved in the transport of axoplasmic organelles. The interaction between myosin V and the synaptophysin–VAMP-2 complex is disrupted by Ca\(^{2+}\), suggesting that a remodelling of this association occurs during the Ca\(^{2+}\)-dependent phase of neurotransmitter release [19]. The importance of this complex for Ca\(^{2+}\)-regulated exocytosis was confirmed in embryonic sea urchin cells by a microinjection approach that also revealed the involvement of members of the kinesin family.
Proteins involved in synaptic vesicle endocytosis

Synaptic vesicle endocytosis is strictly coupled to exocytosis. The most widely accepted model of synaptic vesicle recycling proposes that the SSV membrane is retrieved from the plasma membrane through both clathrin-mediated endocytosis [20] and a clathrin-independent process termed ‘kiss and run’ [21]. In the latter, the fusion of the vesicle with the synaptic plasma membrane is restricted to a fusion pore, with very limited, if any, intermixing between vesicle and plasma membrane components. The ‘kiss and run’ model can account for the high speed and specificity of membrane retrieval under physiological conditions. In clathrin-dependent recycling, the budding of the SSV from the plasma membrane is followed by excision that is dependent on dynamin. Dynamin binds GTP and oligomerizes into rings at the stalks of endocytic pits, closing the neck to release the recycling clathrin-coated SSV [20]. In addition, dynamin interacts with cytosolic factors essential for the coating as well as for the fission reaction. These are clathrin heavy and light chains, the clathrin adaptor complex AP2, AP180 and amphiphysins. Recent evidence indicates a crucial role for dynamin and amphiphysin in the recruitment of an inositol 5-phosphatase called synaptojanin [20]. The presence of synaptojanin on the recycling SSV strongly supports the hypothesis that, in addition to exocytosis, endocytosis is also tightly coupled with phosphoinositides, and offers a link between the cycle of phosphorylation/dephosphorylation of these lipids and SSV turnover.

Perspectives

The cloning of the major components of SSVs, together with genetic analysis in yeast, Caenorhabditis elegans and Drosophila and modern electrophysiological techniques, has provided us with invaluable insights into the molecular mechanism of neurotransmitter release. Despite these efforts, several points remain to be clarified. One of these is how synaptotagmin is able to confer Ca$^{2+}$ sensitivity to the basic and Ca$^{2+}$-independent fusion mediated by VAMP-2, SNAP-25 and syntaxin. Experimental evidence suggests that synaptotagmin binds individual SNAREs both in a Ca$^{2+}$-dependent and a Ca$^{2+}$-independent manner, but the precise dynamics of the SNARE complex interaction with synaptotagmin in relation to the lipid membrane remain unclear. The experimental system used by Rothman and co-workers [10] offers the potential to investigate this important mechanism with purified components, and it could be extremely useful in verifying the hypothesis illustrated in Figure 3, which envisages synaptotagmin acting as a Ca$^{2+}$-dependent clamp. In this model, synaptotagmin would arrest the SNARE-induced fusion of the two lipid bilayers by stabilizing the fusion intermediate via a series of protein–protein and lipid–protein interactions, awaiting Ca$^{2+}$ in order to progress to the fully competent fusion state.
A further level of complexity that remains largely unexplored is the large number of isoforms characterizing the protein machinery for neurotransmitter release. Although it is clear that the abundance of isoforms and splice variants is related to the fine regulation of the exocytic process in the central nervous system, their importance in relation to physiological processes, such as long-term potentiation and depression and synaptic plasticity, are presently unknown and deserve a much broader investigation.

In addition to the protein complexes described in the present review, a new large particle has been described recently in both yeast and mammals [22,23]. This large protein complex, termed exocyst, contains a new repertoire of proteins and is localized specifically at the site of vesicular fusion. In yeast, this particle localizes to sites of polarized exocytosis and its distribution is independent of the integrity of the actin cytoskeleton. In neuroendocrine cells, it is found in vesicle-rich processes of growing neurites and near sites of granule exocytosis. These features, which differ from those characterizing the synaptic SNARE proteins, makes the mammalian homologue of exocyst a very interesting candidate for the machinery controlling neurite elongation and for an alternative pathway for regulated exocytosis.

**Summary**

- The synaptic vesicle cycle can now be subdivided into a series of defined steps. These are the tethering of the SSV at the active site of the presynaptic membrane, followed by docking and by an ATP-dependent phase, termed priming. Fusion of the primed SSV with the plasma membrane is triggered by Ca^{2+} entry through specific Ca^{2+} channels.
- All the steps in the SSV life cycle are regulated through a cascade of protein–protein and lipid–protein interactions.
- The SNARE proteins VAMP-2, SNAP-25 and syntaxin are essential for membrane fusion.
- Synaptotagmin is the major Ca^{2+} sensor for Ca^{2+}-regulated exocytosis at the synapse.
- Rab3A and Rab3C regulate SSV neurotransmitter release by restricting exocytosis to a single vesicle per releasing site.
- The mobilization and the availability of SSVs are regulated by interactions with the actin cytoskeleton.
- Specific phospholipids, such as phosphoinositides, are essential in order to sustain both exocytosis and endocytosis.

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