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SYNAPTIC VESICLE PROTEIN TRAFFICKING AT THE GLUTAMATE SYNAPSE

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Abstract

Expression of the integral and associated proteins of synaptic vesicles is subject to regulation over time, by region, and in response to activity. The process by which changes in protein levels and isoforms result in different properties of neurotransmitter release involves protein trafficking to the synaptic vesicle. How newly synthesized proteins are incorporated into synaptic vesicles at the presynaptic bouton is poorly understood. During synaptogenesis, synaptic vesicle proteins sort through the secretory pathway and are transported down the axon in precursor vesicles that undergo maturation to form synaptic vesicles. Changes in protein content of synaptic vesicles could involve the formation of new vesicles that either mix with the previous complement of vesicles or replace them, presumably by their degradation or inactivation. Alternatively, new proteins could individually incorporate into existing synaptic vesicles, changing their functional properties. Glutamatergic vesicles likely express many of the same integral membrane proteins and share certain common mechanisms of biogenesis, recycling, and degradation with other synaptic vesicles. However, glutamatergic vesicles are defined by their ability to package glutamate for release, a property conferred by the expression of a vesicular glutamate transporter (VGLUT). VGLUTs are subject to regional, developmental, and activity-dependent changes in expression. In addition, VGLUT isoforms differ in their trafficking, which may target them to different pathways during biogenesis or after recycling, which may in turn sort them to different vesicle pools. Emerging data indicate that differences in the association of VGLUTs and other synaptic vesicle proteins with endocytic adaptors may influence their trafficking. These observations indicate that independent regulation of synaptic vesicle protein trafficking has the potential to influence synaptic vesicle protein composition, the maintenance of synaptic vesicle pools, and the release of glutamate in response to changing physiological requirements.

Keywords

vesicular glutamate transporter; synaptic vesicle recycling; synaptic vesicle biogenesis; protein interaction; endocytosis; presynaptic

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Introduction

Calcium triggered exocytosis of neurotransmitters, such as glutamate, from synaptic vesicles underlies the quantal nature of synaptic transmission (Katz and Miledi, 1969). In addition to early studies of neuroendocrine PC12 cells and the cholinergic mammalian neuromuscular junction and *Torpedo* electric organ (Ceccarelli et al., 1973, Heuser and Reese, 1973, Kelly, 1993), analysis of glutamatergic small central synapses of the rodent hippocampus, calyx-type synapses of the brainstem, the *Drosophila* neuromuscular junction (NMJ), and ribbon synapses of retinal bipolar cells has led to fundamental insights into the properties of exocytosis and endocytosis of distinct pools of synaptic vesicles (reviewed in Murthy and De Camilli, 2003, Royle and Lagnado, 2003, Sudhof, 2004, Rizzoli and Betz, 2005). Studies in these various systems have revealed basic mechanisms of synaptic vesicle protein trafficking that may be shared by many synapses. However, the specialized properties of neurotransmitter release at these different synapses may involve differences in their mechanisms of synaptic vesicle biogenesis, recycling, and degradation (Wu et al., 2007). A detailed understanding of the similarities and differences in synaptic vesicle trafficking among synapses is needed to elucidate the mechanisms by which differences in presynaptic function affect neurotransmission at individual glutamatergic synapses.

Although the major constituents of an average synaptic vesicle have been comprehensively characterized (Sudhof and Jahn, 1991, Takamori et al., 2006), regulation of the expression, targeting, and interactions of distinct synaptic vesicle proteins has the potential to influence the capacity of individual synaptic vesicles to store and release neurotransmitter (Kelly and Grote, 1993, Valtorta et al., 2001, Bonanomi et al., 2006). Glutamatergic vesicles presumably contain many of the proteins that mediate fusion and recycling of all synaptic vesicles, but are defined by their capacity to store and release glutamate, requiring the expression of VGLUTs (Fremeau et al., 2004b). VGLUTs mediate the transport of glutamate from the cytoplasm into synaptic vesicles, driven primarily by the electrical component of the H^+ electrochemical gradient generated by the vacuolar H^+-ATPase (Maycox et al., 1988, Wolosker et al., 1996, Liu and Edwards, 1997a). VGLUT expression is used as a specific marker of the glutamatergic phenotype, and indeed is sufficient to confer the property of regulated glutamate release on synaptic vesicles (Takamori et al., 2000a, Takamori et al., 2001). Analysis of the regulation of VGLUT expression and trafficking provides a model system to understand how the targeting of individual synaptic vesicle proteins can influence neurotransmitter signaling. We review here mechanisms of the expression, biogenesis, recycling, and degradation of integral synaptic vesicle proteins studied in a variety of neurotransmitter systems, with a focus on VGLUTs and glutamatergic vesicles. Regulation of these mechanisms has the potential to alter glutamatergic neurotransmission.

PROTEIN EXPRESSION

VGLUTs comprise a family of three distinct isoforms, which display similar transport characteristics, but exhibit different patterns of expression. In the adult brain, VGLUT1 and 2 exhibit an essentially complementary pattern of expression that correlates with probability of release and potential for plasticity, with VGLUT1 the major isoform in cortex, hippocampus and cerebellar cortex, and VGLUT2 in thalamus and brainstem (Bellocchio et al., 2000, Takamori et al., 2000a, Fremeau et al., 2001, Hayashi et al., 2001, Herzog et al., 2001, Takamori et al., 2001, Fremeau et al., 2004b). VGLUT3 is expressed by many cell populations not traditionally considered glutamatergic, including cholinergic, serotoninergic and GABAergic neurons, where it may mediate co-release with other neurotransmitters (Fremeau et al., 2002, Gras et al., 2002, Takamori et al., 2002, Seal and Edwards, 2006b). In addition, VGLUT3 is expressed in somatodendritic regions of neurons, suggesting novel modes of glutamate release (Seal and Edwards, 2006a). VGLUT expression is developmentally regulated, with VGLUT2

and 3 expressed at high levels during early stages of development. VGLUT1 expression is almost undetectable at birth, but increases throughout development, replacing VGLUT2 in some brain regions (Miyazaki et al., 2003, Boulland et al., 2004). VGLUT1 and 2 segregate to different release sites (Fremeau et al., 2004a, Nakamura et al., 2005), but a fraction may be expressed on the same vesicle (Herzog et al., 2006). VGLUT3 is transiently expressed in several cell types, suggesting an important role in development (Boulland et al., 2004, Gillespie et al., 2005, Seal and Edwards, 2006a). The transcriptional regulation of VGLUT genes in response to physiological stimuli and in pathological states is beginning to be explored (Smith et al., 2001, Tordera et al., 2003, Talbot et al., 2004, Tordera et al., 2005, Wilson et al., 2005, Chung et al., 2006, Kawasaki et al., 2006, Boulland et al., 2007, Mark et al., 2007). Indeed, VGLUT1 and 2 were originally identified as cDNAs upregulated in response to NMDA and growth factors, respectively, in a family of Na^+-dependent inorganic phosphate transporters (Ni et al., 1994, Aihara et al., 2000).

How might differences in VGLUT expression affect glutamatergic signaling? Increasing evidence indicates that the level of VGLUT can affect the amount of neurotransmitter stored and released per vesicle, which will determine whether it saturates receptors, and/or whether it spills over to activate adjacent synapses (Liu et al., 1999). Although one transporter may be sufficient to fill a vesicle (Daniels et al., 2006), overexpression of VGLUTs results in an increase in quantal size (Daniels et al., 2004, Wojcik et al., 2004, Wilson et al., 2005), perhaps by filling faster in comparison to a non-specific leak of transmitter or by affecting vesicle volume (reviewed in Edwards, 2007). The effect of reduced levels of VGLUT expression on quantal size is less clear, with a decrease measured in autaptic cultures from VGLUT deficient mice (Wojcik et al., 2004, Moechars et al., 2006), but no effect in the hippocampal slice or at the *Drosophila* NMJ (Fremeau et al., 2004a, Daniels et al., 2006). Decreases in VGLUT expression do have significant effects on behavior. Mice that express less VGLUT1 exhibit increased anxious and depressive behaviors, and impaired memory (Tordera et al., 2007). VGLUT2 heterozygotes exhibit decreases in defensive and neuropathic pain responses (Moechars et al., 2006, Wallen-Mackenzie et al., 2006). Decreased expression of VGLUTs results in homeostatic changes in several systems, increasing the elaboration of zebrafish retinotectal axonal arbors (Smear et al., 2007) or the number of vesicles released at the *Drosophila* NMJ, thus normalizing synaptic response (Daniels et al., 2006). Interestingly, in *Drosophila*, mutation of several other proteins associated with synaptic vesicle recycling, including endophilin, synaptojanin, dynamin, AP180, and synaptotagmin, results in homeostatic increases in bouton number that may compensate for a decrease in the number of vesicles at a terminal (Verstreken et al., 2003, Dickman et al., 2005, Dickman et al., 2006). Regional differences in the expression of other proteins, such as G-protein α-subunits that regulate the biphasic chloride dependence of vesicular glutamate transport, may also differentially affect filling by VGLUT isoforms (Winter et al., 2005). An effect on the rate of neurotransmitter filling may be especially important under conditions of high frequency or prolonged stimulation, when the demand on transmitter supply is high or the time for refilling is short in comparison to the time required for recycling of the vesicle.

In addition to its role in glutamate transport, expression of VGLUT protein appears to have a direct role in the biogenesis or recycling of synaptic vesicles. Genetic deletion of VGLUT1 or 2 results in a reduced number of vesicles, especially those furthest from the active zone, traditionally associated with the reserve pool. Loss of VGLUTs also results in larger, elongated vesicles and tubulovesicular structures (Fremeau et al., 2004a, Wallen-Mackenzie et al., 2006). This effect is not observed with inhibition of vesicle filling or release (Augustin et al., 1999, Parsons et al., 1999, Verhage et al., 2000, Zhou et al., 2000, Altrock et al., 2003), or in synapses from vesicular monoamine transporter 2 (VMAT2) knockout mice, which maintain normal biogenesis and recycling of empty synaptic vesicles, in contrast to synapses from VGLUT knockout mice (Croft et al., 2005). Comparison of glutamate release in the VGLUT1

knockout mouse, at synapses expressing VGLUT2, and wild type synapses expressing VGLUT1, suggests that individual synaptic vesicle proteins might recycle differently. VGLUT2 synapses depress more rapidly and recover more slowly than VGLUT1 synapses in response to high frequency stimulation, suggesting that the two isoforms differ in their recycling, or that they localize to different synapses with distinct recycling properties (Fremeau et al., 2004a).

The expression of vesicle associated and active zone proteins may also affect the release of neurotransmitter at different synapses. Although both excitatory glutamatergic and inhibitory GABAergic synapses display a wide range of physiological properties, some general differences have been proposed, including variation in vesicle pools, probability of release, and response to homeostatic changes (Kaeser and Sudhof, 2005, Li et al., 2005b, Moulder et al., 2006, Moulder et al., 2007). Interestingly, the molecular heterogeneity observed between glutamatergic and GABAergic terminals extends beyond the proteins responsible for transmitter biosynthesis and transport, to several proteins involved in synaptic vesicle release and recycling (Craig and Boudin, 2001, Moulder et al., 2006). For example, glutamatergic, but not GABAergic, terminals express Munc13 proteins, which are involved in vesicle priming and neurotransmitter release (Augustin et al., 1999). Remarkably, differential expression of Munc13 isoforms is associated with differences in vesicle priming and short-term plasticity at individual glutamatergic synapses (Rosenmund et al., 2002). Deletion of genes encoding the three synapsin isoforms reveals different consequences on glutamatergic and GABAergic vesicles (Gitler et al., 2004, Bogen et al., 2006, Lonart and Simsek-Duran, 2006, Baldelli et al., 2007). Although their precise function is unclear, synapsins associate with the synaptic vesicle membrane and with cytoskeletal elements, and may be involved in phosphorylation dependent clustering of vesicles and the regulation of short-term plasticity. In synapsin triple knockout mice, vesicle numbers are reduced. However, while there is an overall loss of vesicles from inhibitory terminals, synaptic vesicles further from the active zone are selectively depleted in excitatory terminals. Furthermore, basal transmission is reduced selectively at inhibitory synapses, whereas defects in excitatory neurotransmission occur in a late phase of repetitive stimulation (Gitler et al., 2004). Knockout of dynamin 1 also differentially affects excitatory and inhibitory transmission, with a reduction of the peak amplitude of inhibitory, but not excitatory, postsynaptic currents (Ferguson et al., 2007).

Glutamatergic and GABAergic vesicles exhibit a different shape under certain fixation conditions (Gray, 1959), however, the only integral synaptic vesicle proteins known to differ between vesicles are their respective vesicular neurotransmitter transporters (Takamori et al., 2000b). In addition to vesicular neurotransmitter transporters, developmental, regional, and experience-dependent differences in the expression of other integral synaptic vesicle proteins may also affect the properties of glutamate release. At the *Drosophila* NMJ, different isoforms of the calcium sensor synaptotagmin confer different modes of release (Littleton et al., 1999), and different domains of synaptotagmin 1 control endocytosis rate and vesicle size (Poskanzer et al., 2006). Different isoforms of the v-SNAREs responsible for vesicle fusion may also mediate different modes of exocytosis. *Mocha* mice deficient in the brefeldin A-sensitive endocytic adaptor AP3 show a severe reduction in the amount of the tetanus toxin-insensitive v-SNARE TI-VAMP/VAMP7, which may contribute to the increased spontaneous and decreased asynchronous glutamate release observed at hippocampal mossy fiber synapses in these mice (Scheuber et al., 2006). Loss of VAMP2 results in vesicles of altered size and shape, suggesting that it is also required for the production of synaptic vesicles, apparently by recruiting endocytic machinery (Grote et al., 1995, Grote and Kelly, 1996, Salem et al., 1998, Deak et al., 2004). Since synaptic vesicles are formed in the process of endocytosis, differences in the expression or trafficking of individual proteins have the potential to affect the functional characteristics of the resulting vesicle.

SYNAPTIC VESICLE BIOGENESIS

The specialized role of synaptic vesicles relies on a restricted set of integral and associated proteins that confer competence for neurotransmitter storage and release. Lacking a common sorting motif, synaptic vesicle membrane proteins employ several strategies for targeting along the intracellular trafficking pathway. This process may involve protein-protein and protein-lipid interactions, and sorting at the level of the endoplasmic reticulum (ER), trans-Golgi network (TGN) or plasma membrane (reviewed in Hannah et al., 1999, Prado and Prado, 2002, Bonanomi et al., 2006). Synaptic vesicle membrane proteins are synthesized and inserted into the membrane of the rough ER and traffic through the Golgi complex. Some protein-protein interactions occur in the ER. For example, several subunits of the vacuolar ATPase are assembled in the ER and remain together throughout subsequent trafficking steps (Stevens and Forgac, 1997). Other interactions may occur at the level of the TGN. Synaptophysin forms homo-oligomers in the TGN of PC12 cells before constitutive secretion to the plasma membrane (Regnier-Vigouroux et al., 1991). Newly synthesized membrane proteins can exit the TGN in constitutive secretory vesicles (CSVs), which are common to all eukaryotic cells, or in some cases, in regulated secretory vesicles (RSVs) that occur mainly in cells capable of regulated secretion (Blazquez and Shennan, 2000).

Alternative hypotheses have been proposed for the *de novo* formation of synaptic vesicles. Some studies suggest that synaptic vesicles may be formed directly from the TGN and then transported down the axon to the presynaptic terminal (Holtzman, 1977, Tixier-Vidal et al., 1988, Janetzko et al., 1989). Conversely, other studies in both cholinergic and glutamatergic systems suggest that mature synaptic vesicles are formed locally at the presynaptic terminal after constitutive recycling of membrane carriers or from endosomal intermediates (Tsukita and Ishikawa, 1980, Okada et al., 1995, Nakata et al., 1998, Yonekawa et al., 1998, Ahmari et al., 2000). In cell lines, newly synthesized synaptophysin and vesicular acetylcholine transporter exit the TGN in CSVs and undergo extensive membrane recycling. Synaptic vesicle proteins in CSVs may be delivered to the plasma membrane, and go through several rounds of constitutive exo-endocytosis that may sort and cluster proteins (Regnier-Vigouroux et al., 1991, Santos et al., 2001, Barbosa et al., 2002). When expressed in PC12 cells or cultured hippocampal neurons, VMAT2 is sorted to RSVs in somatodendritic and axonal regions. For trafficking to synaptic vesicles, VMAT2 may sort directly into CSVs, or it may undergo regulated exocytosis from RSVs and subsequent endocytosis to allow entry into synaptic vesicles at the terminal (Li et al., 2005a). Although there is no direct evidence demonstrating how newly synthesized VGLUTs are sorted to synaptic vesicles, comparison of their respective localization in mature neurons to that of other synaptic vesicle proteins may suggest pathways for sorting VGLUTs to CSVs and RSVs. For example, given its nearly exclusive presynaptic localization, similar to synaptophysin, VGLUT1 could be sorted to CSVs before reaching synaptic vesicles. On the other hand, the somatodendritic location of some VGLUT3-containing vesicles, similar to VMAT2, suggests RSVs might also be important in sorting newly synthesized VGLUTs (Fremeau et al., 2002, Boulland et al., 2004, Harkany et al., 2004, Seal and Edwards, 2006a, Duguid et al., 2007) (Figure 1).

During synaptogenesis, distinct membrane carriers deliver defined functional sets of presynaptic components to the nascent synapse by fast axonal transport along microtubules (Tsukita and Ishikawa, 1980, Kaether et al., 2000, Zhen and Jin, 2004, Hirokawa and Takemura, 2005) (Figure 2). Scaffolding proteins of the active zone, such as Piccolo, Bassoon, and RIM are delivered on ~80 nm dense core vesicles that do not contain synaptic vesicle proteins such as synaptophysin, VAMP2, or synaptotagmin (Zhai et al., 2001, Ohtsuka et al., 2002, Waites et al., 2005; Tao-Cheng, 2007). Many synaptic vesicle proteins are transported down the axon in synaptic vesicle precursors that are substantially larger than individual synaptic vesicles (Nakata et al., 1998, Ahmari et al., 2000, Sytnyk et al., 2004). There are at least two distinct

synaptic vesicle precursors, which may each carry a subset of synaptic vesicle proteins, and are transported using the KIF1A or KIF1Bβ kinesin family motor proteins (Okada et al., 1995, Yonekawa et al., 1998, Zhao et al., 2001). For example, synaptophysin, but not SV2, is transported by a carrier associated with KIF1A (Okada et al., 1995). During assembly of presynaptic boutons, dense core vesicles, synaptic vesicle precursors, and other tubulovesicular structures may cluster into mobile "transport packets" that may include a number of the proteins required for presynaptic function and undergo remodeling (Ahmari et al., 2000, Roos and Kelly, 2000, Zhai et al., 2001, Sabo et al., 2006, Tao-Cheng, 2007) (Figure 2). For example, one study in cultured hippocampal neurons demonstrates the colocalization of green fluorescent protein-VAMP2 in transport packets with markers for voltage dependent Ca^{2+} channels, synapsin, SV2, and amphiphysin (Ahmari et al., 2000). A complete molecular and structural characterization of these packets will be an interesting topic for further investigation.

Synaptogenesis engages a great deal of membrane remodeling while the nerve process grows by inserting new membrane material along axons and at the growth cone. This may require the machinery for exocytosis and endocytosis along the membrane of the growing axon (Hazuka et al., 1999, Hsu et al., 1999, Prekeris et al., 1999). Newly synthesized synaptic vesicle proteins are subject to calcium-independent recycling before their sorting to the pool of regulated vesicles (Regnier-Vigouroux et al., 1991), consistent with the hypothesis that constitutive cycles of membrane exocytosis and endocytosis along the axonal membrane contribute to the maturation of synaptic vesicle precursors. Moreover, in cultured neurons high levels of continuous recycling of synaptic vesicle precursors (Matteoli et al., 1992, Diefenbach et al., 1999) correlate with the spontaneous release of neurotransmitter that precedes synapse maturation (Young and Poo, 1983, Antonov et al., 1999). Interestingly, synaptic vesicle precursors and transport packets pause at predefined sites along the growing axons of cultured neurons. Although cell-cell contact is not absolutely necessary, transport packets may be more likely to pause at sites where neuronal processes meet (Ahmari et al., 2000, Sabo et al., 2006). At these sites, additional sorting is carried out in order to segregate proteins that reside at the axonal membrane from those belonging to the presynaptic bouton. The presence of membrane targets that signal vesicle clustering during synaptogenesis has been suggested, including the sec6/8 complex, or exocyst. The exocyst is ubiquitously expressed, concentrated at sites of active exocytosis/membrane recycling and it is indeed required for exocytosis in several cell lines (Guo et al., 1997, Kee et al., 1997, Wang and Hsu, 2006). In neurons, the exocyst localizes to the plasma membrane of the axon and nerve terminal (Hsu et al., 1996a, Kee et al., 1997, Vik-Mo et al., 2003, Wreden et al., 2003). Moreover, the exocyst is highly expressed in regions undergoing neurite outgrowth and synapse formation (Hsu et al., 1996a, Hazuka et al., 1999, Vega and Hsu, 2001).

Maturation of synaptic vesicles is a dynamic process, involving multiple rounds of exo/endocytosis to remodel the vesicle membrane. The recycling of synaptic vesicle proteins and release of transmitter initially occur at multiple sites along the axon (Antonov et al., 1999). Along developing axons of the mammalian NMJ, these processes are sensitive to inhibition of the small GTP binding protein ARF1, which regulates the adaptor protein AP3 and is insensitive to tetanus toxin, which blocks most release at the presynaptic bouton (Verderio et al., 1999, Zakharenko et al., 1999). As the axon matures, vesicle recycling becomes restricted to the presynaptic bouton and the molecular mechanism may shift to a pathway that primarily uses the clathrin adaptor protein AP2 (Takei et al., 1996, Schmidt et al., 1997, Shi et al., 1998, Antonov et al., 1999, Zakharenko et al., 1999, Polo-Parada et al., 2001). In mature neurons, the AP2 and AP3 pathways may both be employed, but activated by different stimulation conditions, used to recycle different synaptic vesicle cargos, or generate vesicles from different membranes (Voglmaier et al., 2006, Newell-Litwa et al., 2007). In PC12 cells, AP3 mediates the formation of synaptic-like microvesicles from endosomes, whereas AP2 mediates vesicle formation mainly from the plasma membrane (Desnos et al., 1995, Faundez

et al., 1998, Blumstein et al., 2001). *Mocha* mice, deficient in AP3, have reduced vesicular levels of the AP3-sorted cargo zinc transporter 3 (Kantheti et al., 1998, Salazar et al., 2004). Additionally, mice deficient for the subunit μ3B of the AP3 complex show decreased expression of the vesicular GABA transporter (VGAT) and impairment of GABA release from hippocampal synaptic vesicles (Nakatsu et al., 2004), suggesting that AP3 might target VGAT to synaptic vesicles. The synaptic vesicle protein VAMP2 uses both pathways during synaptic-like microvesicle biogenesis in PC12 cells (Shi et al., 1998).

The mature presynaptic terminal is composed of three pools of synaptic vesicles, generally termed the readily releasable pool (RRP), the recycling pool, and the reserve pool. The RRP vesicles are immediately available upon stimulation, while recycling pool vesicles maintain activity-dependent recycling with moderate stimulation, and the reserve pool is defined as a depot of synaptic vesicles from which release is triggered with intense stimulation (reviewed in Rizzoli and Betz, 2005). An intriguing question is whether the establishment of these pools relates to the maturation of the synapse. To answer this, Kavalali and colleagues (2002) studied the development of vesicle pools during various states of neuronal maturation in hippocampal cultures. Optical, electrophysiological, and morphological measurements of vesicle recycling suggest a sequential model for the development of distinct vesicle pools. Younger cultures possess clusters of recycling vesicles, but exhibit no detectable electrophysiological responses upon perfusion of hypertonic sucrose, which induces release from the RRP. At an intermediate stage of neuronal maturation, most vesicles can be rapidly mobilized from the RRP. In mature neurons, synaptic vesicles are distributed into distinct pools and the total number is increased (Mozhayeva et al., 2002). Interestingly, the rate of synaptic vesicle endocytosis at the glutamatergic calyx of Held increases proportionally over the course of postnatal development of the rodent CNS, leading to an increased efficiency of recycling (Renden and von Gersdorff, 2007).

SYNAPTIC VESICLE PROTEIN RECYCLING

The release of neurotransmitter by exocytosis from pre-formed vesicles allows for rapid response to stimulation, but requires replenishment of release-ready vesicles to sustain neurotransmission, either by reuse or by recruitment from synaptic vesicle pools (Pyle et al., 2000, Murthy and De Camilli, 2003, Sudhof, 2004). Depletion of release-ready synaptic vesicles during high frequency action potential firing is one model for the mechanism of synaptic depression, a form of short-term synaptic plasticity (Schneggenburger et al., 2002, Zucker and Regehr, 2002), although other factors, such as regulation of calcium influx or the fusion machinery, may also play a role (Hsu et al., 1996b, Forsythe et al., 1998, Xu and Wu, 2005, Wolfel et al., 2007, Mochida et al., 2008). Regulation of vesicle replenishment may influence the kinetics of synaptic depression and thus synaptic output (Schneggenburger et al., 2002, Kushmerick et al., 2006, Kavalali, 2007). The local recycling of synaptic vesicle membrane and proteins at the nerve terminal is also important to maintain the morphology and protein composition of the presynaptic plasma membrane (Li and Murthy, 2001, Royle and Lagnado, 2003). Multiple mechanisms have been proposed to underlie the efficient recycling of synaptic vesicles (Ceccarelli et al., 1973, Heuser and Reese, 1973, Royle and Lagnado, 2003, Matthews, 2004), but most models assume that the vesicle components recycle together. However, differences in the trafficking of individual synaptic vesicle proteins could target them to different recycling pathways resulting in different rates of recycling, delivery to different vesicle pools, or even molecular heterogeneity of synaptic vesicles that could determine their functional characteristics (Valtorta et al., 2001, Bonanomi et al., 2006, Newell-Litwa et al., 2007, Voglmaier and Edwards, 2007).

Three alternative pathways have been proposed for the synaptic vesicle cycle, kiss-and-run, full fusion followed by classical clathrin mediated endocytosis (CME) or full fusion followed

by bulk membrane retrieval (Ceccarelli et al., 1973, Heuser and Reese, 1973, Royle and Lagnado, 2003, Matthews, 2004) (Figure 3). Fast kiss-and-run recycling remains controversial at some synapses, but is thought to involve the reversible opening of a transient fusion pore that limits diffusion, and thus does not require sorting of vesicular proteins from plasma membrane components in order to regenerate a vesicle (Ceccarelli et al., 1973, Fesce and Meldolesi, 1999, Harata et al., 2006). The molecular mechanisms underlying kiss and run recycling, including whether clathrin is involved in the process, are not yet clear (Jockusch et al., 2005, Newton et al., 2006).

During full fusion, the synaptic vesicle membrane collapses fully into the plasma membrane as the content of neurotransmitter is released. CME generally recovers small vesicles directly from the plasma membrane, but may also be involved in the generation of vesicles from endosomes or from large portions of membrane recovered by bulk retrieval. The mechanism of CME of synaptic vesicles is similar to endocytosis occurring in non-neural cells and requires the recruitment of a clathrin coat by adaptor proteins (Schmid, 1997, Zhang et al., 1998), the acquisition of curvature mediated by endophilin, epsin and other proteins (Ringstad et al., 1999, Ford et al., 2002), and the subsequent scission of the nascent vesicle from the plasma membrane orchestrated by dynamin (Brodin et al., 2000, Yamashita et al., 2005). Phosphoinositide (PI) metabolism plays an important role in synaptic vesicle recycling (reviewed in Cremona and De Camilli, 2001, Haucke, 2005). Phosphorylation of PI4P, generating $PI(4,5)P_2$, results in recruitment of clathrin and endocytic adaptors (Wenk et al., 2001, Krauss et al., 2003, Di Paolo et al., 2004). Dephosphorylation of $PI(4,5)P_2$ subsequently promotes vesicle uncoating (Cremona et al., 1999, Mani et al.). Uncoating is triggered by the phosphatidylinositol phosphatase synaptojanin acting together with Hsc70 and auxilin (Rothman and Schmid, 1986, Cremona et al., 1999, Schuske et al., 2003, Verstreken et al., 2003).

Synaptic vesicle components retrieved by bulk endocytosis may remain in contact with the plasma membrane on deep infoldings or be delivered to an endosome after first budding from the plasma membrane (Heuser and Reese, 1973, Wucherpfennig et al., 2003, Ferguson et al., 2007). Bulk endocytosis may be similar to macropinocytosis in non-neuronal cells, depend on the actin cytoskeleton, and be regulated by phosphorylation (Holt et al., 2003, Tan et al., 2003, Clayton et al., 2007, Evans and Cousin, 2007). Evidence for the involvement of clathrin in bulk membrane retrieval has been reported in some synapses (Takei et al., 1995, Takei et al., 1996, Lenzi et al., 2002, Paillart et al., 2003). A number of glutamatergic and other synapses indeed exhibit both morphologically and functionally distinct forms of membrane recycling, suggesting that alternative pathways for endocytosis of synaptic vesicle components co-exist at individual synapses (Heuser and Reese, 1973, Koenig and Ikeda, 1996, Takei et al., 1996, Gad et al., 1998, Marxen et al., 1999, Richards et al., 2000, Teng and Wilkinson, 2000, de Lange et al., 2003, Holt et al., 2003, Paillart et al., 2003, Kuromi and Kidokoro, 2005, Harata et al., 2006, Wu et al., 2007).

Several studies have suggested that the mode and rate of recycling may depend on the exocytic stimulus, the pool from which the vesicle is mobilized, expression of trafficking proteins, or the type of synapse. Kiss and run recycling has been implicated in the recycling of large dense core vesicles, of synaptic vesicles released during high frequency firing, or by synaptic vesicles with a lower probability of release (Klyachko and Jackson, 2002, Gandhi and Stevens, 2003, Zhang et al., 2007), although the interpretation of kinetic differences as different modes of recycling remains controversial, especially at small central glutamatergic synapses (Dittman and Kaplan, 2006, Fernandez-Alfonso et al., 2006, Granseth et al., 2006, Wienisch and Klingauf, 2006, Balaji and Ryan, 2007, He and Wu, 2007). Bulk endocytosis is presumably activated by the prolonged, high frequency stimulation that may exceed the capacity of the recycling pool and mobilize vesicles from the reserve pool (Richards et al., 2003, Royle and

Lagnado, 2003, Evans and Cousin, 2007). Synaptic vesicles may be induced to recycle at different rates. For instance, at retinal bipolar terminals, capacitance measurements indicate that synaptic vesicles released from the RRP are normally recycled by a fast mode of endocytosis and the reserve pool by a slower mode of endocytosis that involves components of the CME machinery (Jockusch et al., 2005). Inhibition of the slower (CME) mode of endocytosis results in vesicles stranded on the cell surface. However, upon inhibition of the fast mode by calcium buffering, vesicles released from the RRP can be endocytosed by CME (Neves et al., 2001). The rate of synaptic vesicle endocytosis in cultured hippocampal neurons may also be altered by expression of different splice variants of the plasma membrane localized Ca^{2+} sensor, synaptotagmin 7 (Virmani et al., 2003).

In some cases, synaptic vesicles maintain their release characteristics after recycling. A pool of vesicles that are loaded with styryl dye by spontaneous recycling can be destained only by spontaneous release, whereas vesicles loaded by evoked recycling can be destained only by stimulation (Diefenbach et al., 1999, Sara et al., 2005). Labeling of cultured hippocampal neurons with spectrally distinct dyes indicates that vesicles released by short stimuli are preferentially recycled to the front of the synapse and those released by longer stimuli, presumably mobilized from the reserve pool, are drawn from and return to the back of the synapse (Vanden Berghe and Klingauf, 2006). At the cholinergic frog NMJ, although 3-D reconstruction of electron micrographs shows that vesicles of the RRP are randomly distributed throughout the vesicle cluster, this functional pool refills specifically by fast recycling whereas the reserve pool recovers more slowly, by bulk endocytosis (Richards et al., 2000, Rizzoli and Betz, 2004)

On the other hand, vesicles may redistribute between pools with different propensities for release, and this process may be highly regulated. Vesicles in the cluster may become tethered in place after endocytosis by synapsins and actin (Shtrahman et al., 2005), but their movement can be controlled by calcium, calmodulin, cAMP, and phosphorylation (reviewed in Schweizer and Ryan, 2006). The relative size of different synaptic vesicle pools may be regulated by such factors as activity, ubiquitination, and neurotrophic factors, possibly by diversion of vesicles from other pools (Tyler et al., 2006, Virmani et al., 2006, Willeumier et al., 2006). After prolonged stimulation with hypertonic sucrose, vesicles can enter a “silent” rather than recycling pool in both glutamatergic and GABAergic terminals (Ashton and Ushkaryov, 2005). Although vesicle reuse is thought to be important for continued neurotransmitter release during high frequency action potential firing (Kavalali, 2007), the RRP may also be refilled with vesicles from a reserve pool in both excitatory and inhibitory synapses (Li et al., 2005b). Similarly, at the *Drosophila* NMJ, larger vesicles from the reserve pool are recruited for release by an actin and protein kinase A-dependent mechanism in response to changes in behavior—increased larval crawling (Steinert et al., 2006).

If synaptic vesicles can redistribute between pools, do synaptic vesicle components redistribute between vesicles? It had been assumed that synaptic vesicle components remain clustered after exocytosis, translocating *en bloc* to the periactive zone for endocytosis (Miller and Heuser, 1984, Roos and Kelly, 1999, Teng and Wilkinson, 2000, Li and Murthy, 2001, Rizzoli and Betz, 2004). However, recent experiments have shown that in the case of VAMP2 and synaptotagmin, the protein that undergoes endocytosis in response to stimulation is not necessarily the same molecule that has just undergone exocytosis (Dittman and Kaplan, 2006, Fernandez-Alfonso et al., 2006, Wienisch and Klingauf, 2006). Biochemical and optical approaches have also revealed differences in the clustering of individual synaptic vesicle proteins after exocytosis. Tagged VAMP2 (synaptopHluorin) diffuses rapidly out of synapses along the plasma membrane (Li and Murthy, 2001, Fernandez-Alfonso et al., 2006, Granseth et al., 2006), and endogenous VAMP2 is also highly expressed over the cell surface (Taubenblatt et al., 1999, Sampo et al., 2003). In contrast, synaptophysin, synaptotagmin and

VGLUT1 maintain a more synaptic localization (Granseth et al., 2006, Voglmaier et al., 2006, Willig et al., 2006). One factor that could limit the spread of synaptophysin is its binding to the cholesterol-rich membrane of synaptic vesicles, which may form a raft-like domain upon exocytosis (Deutsch and Kelly, 1981, Martin, 2000, Thiele et al., 2000, Jia et al., 2006, Wasser et al., 2007). Despite these differences, VAMP2 and synaptophysin appear to undergo endocytosis at a similar rate (Granseth et al., 2006). Recycling of synaptic vesicles through an endosomal intermediate could also provide a mechanism for intermixing and resorting of synaptic vesicle membrane cargo (Sudhof, 2004). Indeed, manipulating the GTPase rab5, involved in endosomal fusion, changes release probability at *Drosophila* NMJ, which was suggested to result from a change in the molecular composition of synaptic vesicles formed from the endosome (Wucherpfennig et al., 2003). Sorting of vesicular proteins from the plasma or endosomal membrane may involve protein-protein or protein-lipid interactions among the components of the endocytosing vesicle, and it may involve recognition of protein sorting signals by endocytic adaptors.

VAMP2, synaptotagmin, and VGLUT1 and 2 have effects on the biogenesis or recycling of synaptic vesicle membrane in addition to their established functional roles. Mutation or deletion of these proteins result in changes in vesicle size, shape, number, or endocytic rate in several glutamatergic systems (Deak et al., 2004, Fremeau et al., 2004a, Poskanzer et al., 2006, Wallen-Mackenzie et al., 2006). These proteins all contain sorting signals that direct them to the endocytic machinery, which may mediate their roles in synaptic vesicle recycling (Grote and Kelly, 1996, Blagoveshchenskaya et al., 1999, Haucke and De Camilli, 1999, Grass et al., 2004, Poskanzer et al., 2006, Voglmaier et al., 2006). Some proteins may be targeted to synaptic vesicles by interacting with other proteins that contain sorting signals, but many synaptic vesicle proteins undergo endocytosis when expressed alone in non-neural cells (Cameron et al., 1991, Grote and Kelly, 1996, Tan et al., 1998, Voglmaier et al., 2006). In contrast to other organelles, there does not seem to be a universal determinant for sorting proteins to synaptic vesicles, and little is known about the signals required for sorting and endocytosis of synaptic vesicle proteins (Prado and Prado, 2002, Voglmaier and Edwards, 2007). However, many synaptic vesicle proteins contain dileucine or tyrosine-based sequences thought to interact with adaptor proteins such as AP2 (Figure 4). Binding to protein cargo and phospholipids at the plasma membrane by AP2 leads to recruitment of a clathrin coat and endocytosis. Synaptic vesicle proteins may also contain additional sorting sequences. For example, some synaptotagmin isoforms also interact with a cargo-specific endocytic adaptor, stonin 2, that contributes to AP2-mediated internalization (Diril et al., 2006).

VGLUTs contain dileucine-like internalization motifs, but the C-terminus of VGLUT1 also contains two polyproline domains not found in VGLUT2 or 3. At one of these polyproline domains, VGLUT1 interacts with endophilin, a protein that mediates membrane curvature and the recruitment of other endocytic machinery (De Gois et al., 2006, Vinatier et al., 2006, Voglmaier et al., 2006). The interaction of VGLUT1 with endophilin has no effect on recycling of tagged VGLUT1 under moderate stimulation conditions. During prolonged, high frequency stimulation, however, deletion of the endophilin binding domain slows endocytosis of VGLUT1. Surprisingly, inhibition of the AP3 recycling pathway with brefeldin A rescues this defect, suggesting that during prolonged high frequency stimulation, the interaction with endophilin redistributes VGLUT1 from a slower AP3-dependent pathway to a fast recycling pathway, presumably mediated by AP2. In addition, inhibition of the AP3 pathway after strong stimulation results in a substantial fraction of both wild-type and mutant VGLUT1 stranded on the cell surface, indicating activity dependent differences in targeting of VGLUT1 to the two pathways (Voglmaier et al., 2006). Since AP3 mediates internalization of VGLUT1 after the prolonged stimulation associated with bulk endocytosis, and bulk retrieval has been implicated in recycling of the reserve pool, it is possible that competition of AP2 and AP3 for recycling of VGLUT1 during stimulation diverts the transporter to different pools.

Pharmacologic and genetic inhibition of AP3 indeed lessens synaptic depression, suggesting an increased amount of VGLUT1 in the recycling pool (Voglmaier et al., 2006). Synapses expressing VGLUT2, which lacks any polyproline motifs, depress more rapidly and recover more slowly in response to high frequency stimulation than VGLUT1 synapses (Fremeau et al., 2004a). Differences in trafficking of VGLUT isoforms thus have the potential to alter neurotransmitter release.

Other synaptic vesicle proteins may use the AP2 and AP3 pathways to differing extents. Indeed zinc transporter 3, VGAT, and TI-VAMP/VAMP7 are among a number of proteins that are preferentially sorted by AP3 (Kantheti et al., 1998, Nakatsu et al., 2004, Salazar et al., 2005, Scheuber et al., 2006). In addition, other synaptic vesicle proteins may contain sorting signals that bind other cargo-specific adaptors, or that regulate the mode of exo- or endocytosis based on other signals. These differences could affect the rate or pathway of recycling, or targeting to different vesicle pools. Differential sorting of proteins during recycling also raises the possibility of their activity-dependent redistribution, generating synaptic vesicles with varying protein compositions and functional characteristics, which could influence neurotransmitter release (Valtorta et al., 2001, Bonanomi et al., 2006, Voglmaier and Edwards, 2007).

SYNAPTIC VESICLE PROTEIN DEGRADATION

The main protein synthetic and degradative machinery of the neuron is located in the perikaryon, distant from the presynaptic terminal. Although it is clear that presynaptic proteins synthesized in the cell body are carried down the axon by anterograde transport, very little is known about the degradation of synaptic vesicle proteins in the mature CNS. Synaptic vesicle protein levels are controlled by transcription and distinct post-transcriptional mechanisms. For instance, in developing hippocampal neurons in culture, the levels of synaptophysin are regulated primarily by the rate of translation, whereas the levels of synaptotagmin 1, synapsin 1 and VAMP2 are controlled primarily by protein half-life (Daly and Ziff, 1997). Moreover, cellular homeostasis in neurons requires that the synthesis of protein and membrane be balanced by their degradation. Recently, protein degradation has attracted attention as a mechanism to control synaptic protein levels.

Eukaryotic cells have two major systems for the regulated degradation of proteins: the lysosomal pathway and the ubiquitin-proteasome system (Gray et al., 2003). Addition of ubiquitin in growing chains tags proteins for degradation by the proteasome or by trafficking to the lysosomal pathway. On the other hand, addition of a single ubiquitin moiety can regulate protein trafficking and activity (Hicke, 2001). Consistent with the absence of lysosomes at the presynaptic terminal, it has been shown that integral synaptic vesicle proteins are retrogradely transported in large membranous carriers, presumably for degradation in the cell body (Tsukita and Ishikawa, 1980, Li et al., 1996a, Li et al., 1996b, Li and Dahlstrom, 1997, Li et al., 1997). Pioneering morphological studies of dorsal root ganglion cell axons demonstrated retrograde trafficking of multivesicular bodies (MVBs) in close spatial relationship with microtubules (Tsukita and Ishikawa, 1980). Much of the information about retrograde transport of MVBs comes from the study of neurotrophin signaling (Weible et al., 2001). Once bound to receptors at the presynaptic terminal, neurotrophins are internalized and subsequently incorporated into MVBs. Synaptic vesicle proteins and other presynaptic components could also be trafficked in MVBs, however, the mechanisms for their entry, trafficking, and subsequent fate are unknown.

The ubiquitin-proteasome system is known to participate in the processes of neuronal development such as axon growth, axon guidance, and synapse formation and elimination (reviewed in Yi and Ehlers, 2007). Ubiquitination and de-ubiquitination of several synaptic proteins are implicated in the regulation of synaptic transmission and vesicle recycling (Wilson

et al., 2002, Chen et al., 2003, Ehlers, 2003, Speese et al., 2003, Zhao et al., 2003, Willeumier et al., 2006, Yao et al., 2007b). The levels of the synaptic vesicle protein synaptophysin and the presynaptic membrane proteins syntaxin and SNAP-25 can be regulated by proteasomal inhibition (Chin et al., 2002, Wheeler et al., 2002, Ma et al., 2005). The E3 ligase Siah (Sina-seven in absentia homolog) binds both synaptophysin and the neuronal E2 ubiquitin-conjugating enzyme UbcH8, promoting the degradation of synaptophysin (Wheeler et al., 2002). Mice with altered expression of Usp14, a de-ubiquitinating enzyme, exhibit decreased paired-pulse facilitation and post-tetanic potentiation in hippocampus, and decreased quantal content at the NMJ, suggesting an effect on neurotransmitter release (Wilson et al., 2002). RIM 1, a synaptic scaffolding molecule that regulates vesicle priming, is ubiquitinated and targeted for degradation by the E3 ligase SCRAPPER. Mice lacking SCRAPPER have increased levels of RIM and show an increased frequency of spontaneous glutamate release and altered paired-pulse facilitation (Yao et al., 2007a). Interestingly, the levels of several presynaptic proteins, including synaptophysin, synaptotagmin, synaptogyrin, CASK and Munc13 are also elevated in SCRAPPER knockout mice (Yao et al., 2007a).

In the regulated secretory pathway of bovine chromaffin cells, newly synthesized large dense core vesicles are released preferentially over older vesicles from the functional readily releasable pool upon nicotine stimulation. In contrast, barium stimulation, which is thought to release the reserve pool, results in the preferential release of older vesicles (Duncan et al., 2003). This indicates that newly synthesized vesicles are preferentially delivered to the readily releasable pool, and the vesicles may undergo some time-dependent changes that target them to different pools. Whether this occurs in glutamatergic synaptic vesicle regulation is unknown. The fate of older synaptic vesicles and synaptic vesicle proteins has remained relatively unexplored. Regulation of the degradation of synaptic vesicle proteins could be as important as the regulation of their synthesis. Advances in tools and techniques to study protein degradation will increasingly lead to novel insights into this potentially important mechanism for regulating synaptic vesicle protein expression.

CONCLUSIONS

Excitatory transmission involves the transformation of electrical into chemical signals by the regulated release of neurotransmitters such as glutamate from synaptic vesicles. The ability of synaptic vesicles to store glutamate is conferred by VGLUTs, which undergo differential regulation during development and in response to activity. Different VGLUT isoforms are expressed in synapses with differing physiological properties. In addition, distinct sorting signals in VGLUT1 regulate its recycling after exocytosis. Although general properties of neurotransmitter release and the components of the protein trafficking machinery have been investigated, less is known about the signals and interactions required for sorting individual synaptic vesicle proteins. Like VGLUTs, many synaptic vesicle proteins have their own distinct patterns of expression and trafficking signals, which may regulate their sorting, affecting synaptic vesicle protein composition. The coordinated or independent regulation of individual synaptic vesicle protein expression, trafficking, and degradation mechanisms may determine the ability of the resulting synaptic vesicle to release transmitter in response to a range of stimuli. This may in turn determine physiological properties of a synapse, with implications for information processing and synaptic plasticity.

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Abbreviations

AP
Adaptor protein

ATPase
Adenosine triphosphatase

CME
Clathrin mediated endocytosis

CNS
Central nervous system

ER
Endoplasmic reticulum

KIF
Kinesin superfamily protein

MVB
Multivesicular body

NMJ
neuromuscular junction

PC12
Pheochromocytoma 12

PI
Phosphoinositide

RRP
Readily releasable pool

SH3
Src homology 3

SV2
Synaptic vesicle protein 2

TGN
Trans-Golgi network

VAMP
Vesicle associated membrane protein

VGAT
Vesicular GABA transporter

VGLUT
: Vesicular glutamate transporter

VMAT
: Vesicular monoamine transporter

v-SNARE
: Vesicular soluble N-ethylmalemide-sensitive factor attachment protein receptor

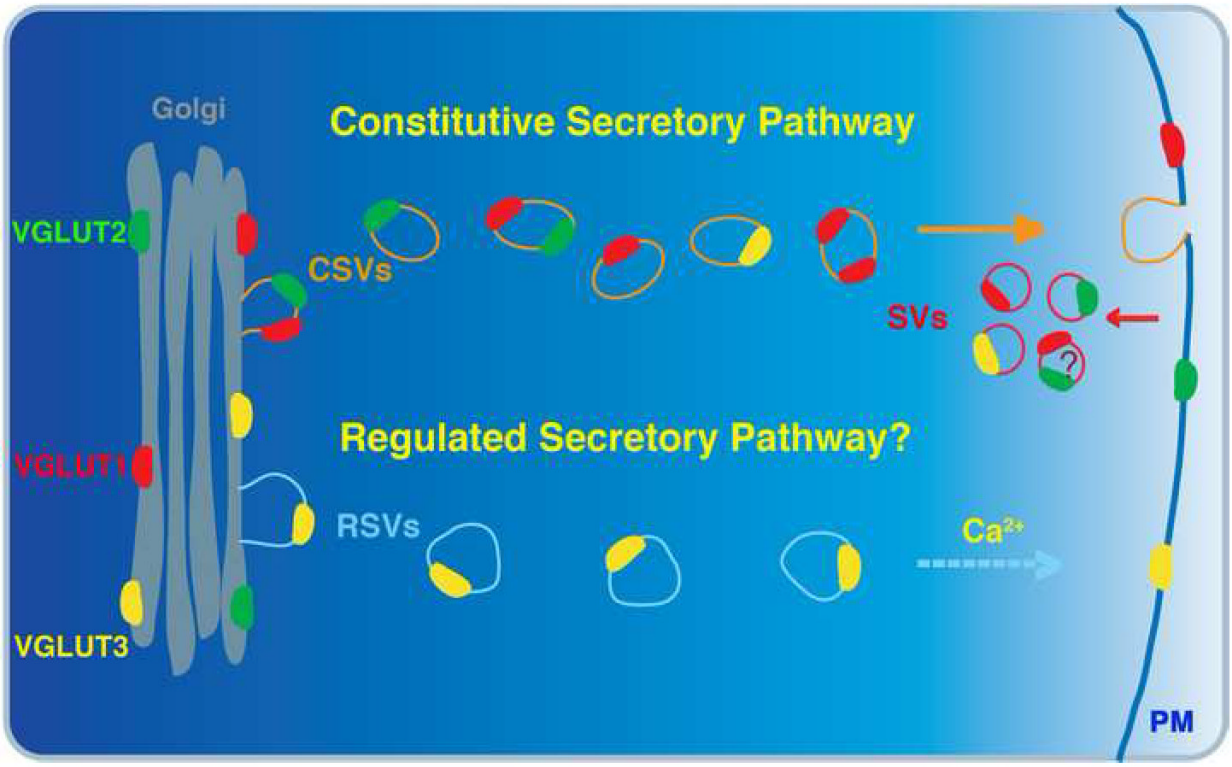

Figure 1. VGLUT trafficking in neurons

A schematic model for VGLUT sorting in neurons, based on current models of secretory vesicle trafficking, is depicted. During biogenesis, all three isoforms of VGLUTs may be delivered to the axon by trafficking to constitutive secretory vesicles (CSVs) at the level of the TGN. Constitutive exocytosis of CSVs could deliver VGLUTs to the cell surface in axons, where they could be incorporated into synaptic vesicles (SVs). In contrast, VGLUT3 is also observed in somatodendritic vesicles, suggesting that it may also sort to regulated secretory vesicles (RSVs), similarly to VMAT2. RSVs bud directly from the TGN as part of the regulated secretory pathway, with a full complement of the proteins that confer competence for regulated neurotransmitter release. RSVs could transport VGLUT3 to appropriate sites of release, either in dendrites or axons. In this case, VGLUT3-containing RSVs would fuse with the plasma membrane (PM) only upon stimulation, such as increased cytoplasmic Ca^{2+}.

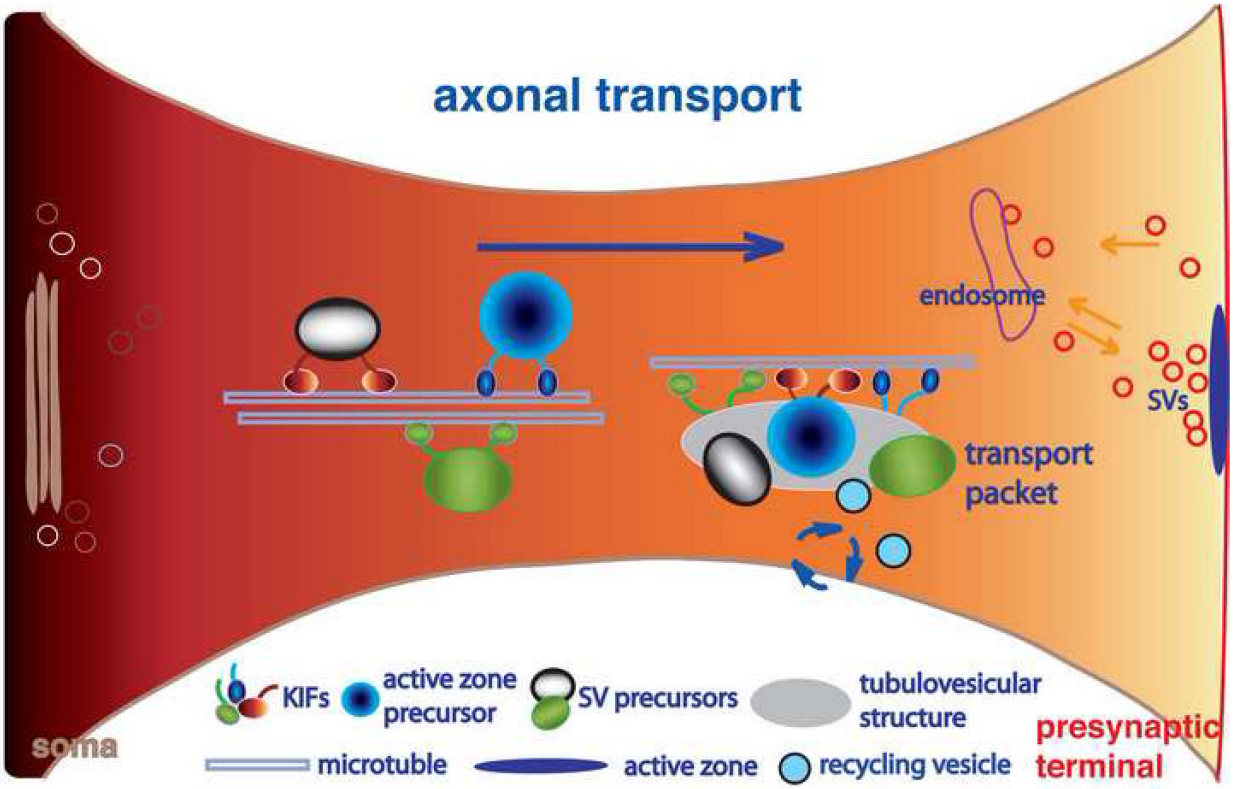

Figure 2. Axonal transport of synaptic vesicle membrane proteins

A schematic model of the microtubule-based transport of synaptic vesicle membrane proteins within an axon is depicted. Specific kinesin family motor proteins (KIFs) deliver distinct cargoes from the soma toward the axon terminal. Active zone proteins, such as Piccolo and Bassoon, are transported on dense core vesicles (blue dense core circles). Synaptic vesicle membrane proteins are transported by distinct membrane carriers (synaptic vesicle precursors, black and green ovals). Recent evidence suggests that both dense core vesicles and synaptic vesicle precursors may cluster into transport packets that contain VAMP2, SV2, amphiphysin, and synapsins. Transport vesicles may undergo dynamic membrane exchange and remodeling by recycling of membrane vesicles along the axon (blue circles).

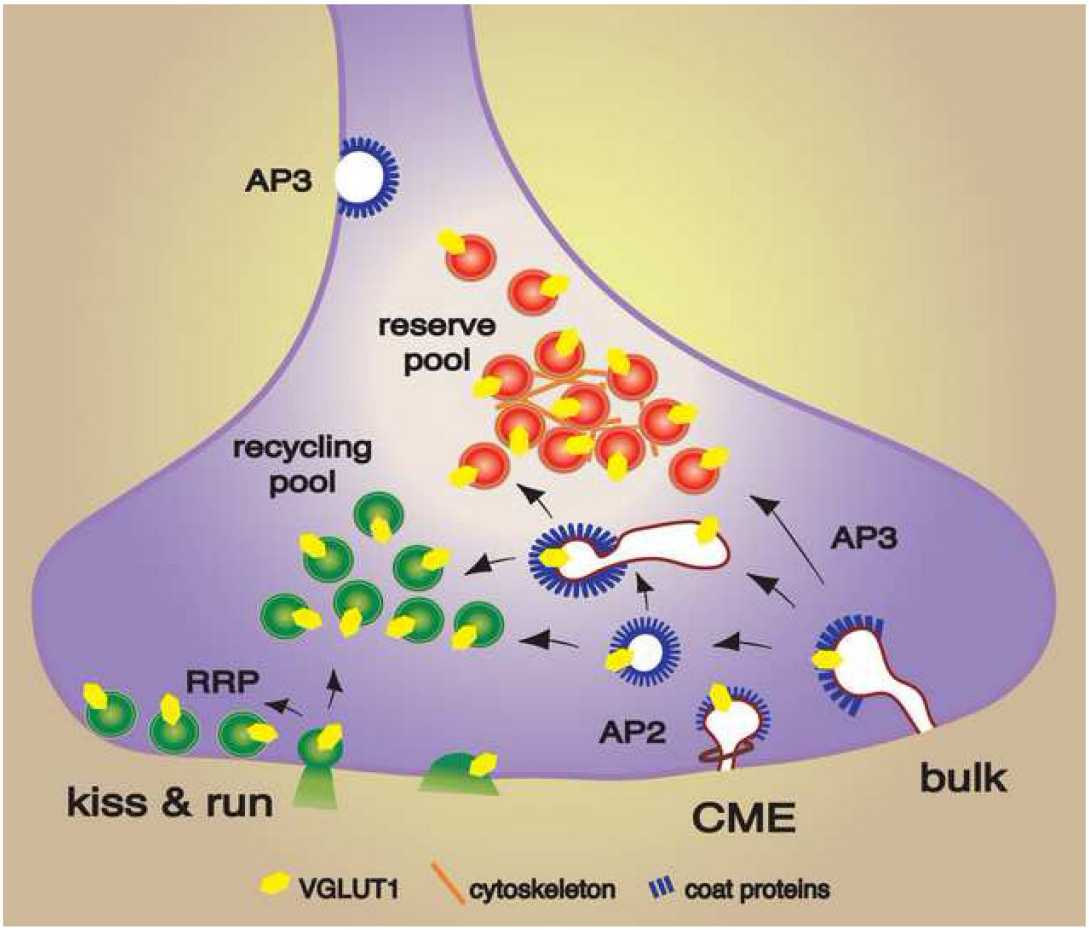

Figure 3. Model of synaptic vesicle protein recycling

Synaptic vesicles comprise three functional pools. The readily releasable pool (RRP) is released with mild stimulation. With more intense stimulation, vesicles are also recruited from the recycling pool and then the reserve pool, where vesicles are tethered by cytoskeletal elements. Three proposed mechanisms for the recycling of synaptic vesicle components are depicted. A fast kiss and run mechanism involves the reversible opening of a fusion pore without full collapse into the plasma membrane. In contrast, after full fusion, synaptic vesicle proteins recognized by the clathrin adaptor protein AP2 are recycled by clathrin-mediated endocytosis (CME). Strong stimulation activates bulk membrane retrieval into large membrane compartments from which synaptic vesicles are generated. Since strong stimulation activates both bulk endocytosis and the AP3 pathway, they might be related processes. AP3 may recognize protein cargoes in plasma membrane invaginations or endosomal membranes and function in their incorporation into synaptic vesicles.

Figure 4.
Alignment of sequences from the VGLUTs with similar targeting sequences from other synaptic vesicle proteins (bold). The dileucine-like sequences are underlined. “r”: rat; “m”: mouse; “STG”: synaptotagmin.