Synaptic Vesicle-Recycling Machinery Components as Potential Therapeutic Targets

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Abstract—Presynaptic nerve terminals are highly specialized vesicle-trafficking machines. Neurotransmitter release from these terminals is sustained by constant local recycling of synaptic vesicles independent from the neuronal cell body. This independence places significant constraints on maintenance of synaptic protein complexes and scaffolds. Key events during the synaptic vesicle cycle—such as exocytosis and endocytosis—require formation and disassembly of protein complexes. This extremely dynamic environment poses unique challenges for proteostasis at synaptic terminals. Therefore, it is not surprising that subtle alterations in synaptic vesicle cycle-associated proteins directly or indirectly contribute to pathophysiology seen in several neurologic and psychiatric diseases. In contrast to the increasing number of examples in which presynaptic dysfunction causes neurologic symptoms or cognitive deficits associated with multiple brain disorders, synaptic vesicle-recycling machinery remains an underexplored drug target. In addition, irrespective of the involvement of presynaptic function in the disease process, presynaptic machinery may also prove to be a viable therapeutic target because subtle alterations in the neurotransmitter release may counter disease mechanisms, correct, or compensate for synaptic communication deficits without the need to interfere with postsynaptic receptor signaling. In this article, we will overview critical properties of presynaptic release machinery to help elucidate novel presynaptic avenues for the development of therapeutic strategies against neurologic and neuropsychiatric disorders.

I. Introduction

A. Rationale Behind Targeting Presynaptic Vesicle-Recycling Machinery

Chemical synapses are the major channels of information transfer and processing in the central nervous system (CNS). They consist of two functionally and structurally distinct compartments: presynaptic terminals and postsynaptic specializations. Presynaptic terminals store and release neurotransmitter substances in membranous organelles named synaptic vesicles, whereas postsynaptic structures contain signaling molecules responsible for generation of neuronal responses to released neurotransmitters. Neurotransmission at the presynaptic terminal involves synaptic vesicle exocytosis, endocytosis, and reuse of synaptic vesicles. Synaptic vesicle recycling is essential to the function of neurons. These processes rely on the complex interactions of a multitude of synaptic proteins and lipids. In this review, we aim to highlight recent advances in our understanding of the molecular determinants of synaptic vesicle cycle, their physiologic functions, pathologic roles, and their pharmacological potential as drug targets for amelioration of disease states. Defects in presynaptic function underlie a wide variety of neurologic and psychiatric disorders. However, the synaptic vesicle-recycling machinery is an underexplored area for drug development as much focus has been placed on ion channels, G protein–coupled receptors, and other mainly postsynaptic targets.

The presynaptic machinery is an attractive therapeutic target because it allows for dynamic modulation of synaptic transmission. Targeting regulators of exocytosis and endocytosis provide a range of outputs, from complete abolition of neurotransmitter release to subtle modifications of neuronal signaling and neuronal firing. Manipulation of the diverse vesicular proteins can selectively alter different forms of neurotransmitter release. Recent studies demonstrate that nonsynchronous forms of neurotransmitter release are important to the regulation of synaptic plasticity, memory processing, and antidepressant action (Autry et al., 2011; Xu et al., 2012; Nosyreva et al., 2013; Cho et al., 2015). The precision of the synaptic message is maintained at the postsynaptic level as variations in presynaptic release differentially affect receptors and downstream targets (Atasoy et al., 2008; Autry et al., 2011; Sara et al., 2011; Stepanyuk et al., 2014). This differential postsynaptic signaling is enabled by presynaptic segregation of vesicle-trafficking pathways that mediate spontaneous and synchronous evoked release (Kavalali, 2015). In some cases, this segregation may also extend to mechanisms and signaling targets of asynchronous release. The parallel signaling by kinetically diverse release processes may enable isolation of neurotrophic, homeostatic, or other functions of released neurotransmitter substances from their critical role in precise presynaptic action potential-driven information transfer. Presynaptic terminals, therefore, present a wide spectrum of novel targets for CNS drug design where synaptic communication can be altered in subtle ways that can alleviate disorder symptoms with limited side effects.

Due to space constraints, we will focus on components of the synaptic vesicle-recycling pathway in the CNS with therapeutic potential. Although G protein–coupled...
receptors are important for regulation of presynaptic function, compounds targeting G protein–coupled receptors are some of the most widely prescribed drugs for the treatment of neurologic and psychiatric disorders and will not be discussed in this review. Similarly, vesicular transporters and voltage-gated ion channels are also well-studied targets for pharmaceuticals and have been covered in other reviews (Chaudhry et al., 2008; Wulff et al., 2009; Mantegazza et al., 2010; Zamponi et al., 2015; Bermingham and Blakely, 2016).

II. An Overview of the Synaptic Vesicle Cycle

Synapses are basic structural units for communication between neurons and are essential for neuronal function. Neuronal signals travel along axons and trigger the opening of voltage-gated calcium (Ca²⁺) channels in presynaptic terminals. The influx of Ca²⁺ initiates a series of events leading to the fusion of synaptic vesicles to the presynaptic membrane at active zones. This results in the release of neurotransmitters into the synaptic cleft and the propagation of signals downstream via the actions of various postsynaptic receptors. Synaptic vesicles in the presynaptic terminals are retrieved from the membrane, reacidified, and refilled with neurotransmitters for reuse. This dynamic process of synaptic vesicle recycling is critical for maintaining normal synaptic function. Precise release of neurotransmitters depends on the equilibrium between vesicular fusion during exocytosis and membrane retrieval during endocytosis (see Figs. 1 and 2).

In the full-collapse fusion model of synaptic vesicle recycling, vesicles fuse with the presynaptic membrane at the active zone and completely collapse onto the membrane (Heuser and Reese, 1973; Südhof, 1995; Cremona and De Camilli, 1997). Subsequently, clathrin and its adaptor proteins are recruited to the membrane and form clathrin-coated vesicles that pinch off the plasma membrane through the scissioning action of dynamin. Endocytosis of clathrin-coated vesicles may also occur through larger structures such as membrane infoldings or endosomal cisternae that form upon accumulation of fused synaptic vesicles (Koenig and Ikeda, 1996; Takei et al., 1996). Vacuolar-type ATPases pump protons into these newly formed vesicles, and neurotransmitter transporters use this gradient to refill vesicles with neurotransmitter. Kiss-and-run is an alternative model of synaptic vesicle fusion and retrieval that involves faster kinetics. In this pathway, vesicles contact presynaptic membranes and create transient pores for neurotransmitter release, but do not fully collapse (Ceccarelli et al., 1973; Alabi and Tsien, 2013). The connection between these two forms of synaptic vesicle recycling is still being explored; it seems that stimulation intensity and Ca²⁺ levels may induce shifts from one form to the other (Gandhi and Stevens, 2003; Zhang et al., 2009a; Leitz and Kavalali, 2011, 2014). In both models, the tight coupling between exocytosis and endocytosis points to the fusion machinery itself as a key mediator of the balance between exo- and endocytosis (Deak et al., 2004).

A. Modes of Synaptic Vesicle Exocytosis

1. Synchronous Fusion. Molecularly, the best-characterized pathway of vesicle fusion is synchronous fusion (Südhof, 2013). Vesicles fuse and neurotransmitters are released in a precise time-locked manner with stimulation and ensuing Ca²⁺ influx. This rapid and reliable exocytosis depends on many complex protein and lipid interactions. Soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins and its binding partners are essential for this fast exocytosis. The canonical SNARE complex is composed of synaptobrevin (syb)2, on the synaptic vesicle, and syntaxin-1 and SNAP-25, both on the target plasma membrane (see reviews: Südhof, 2004; Rizo and Rosenmund, 2008; Südhof and Rothman, 2009). The α-helical SNARE motifs of these proteins facilitate the formation of a tight complex that brings vesicles close to the presynaptic membrane. Munc18-1 is a Sec1/Munc18 protein essential for neurotransmitter release (Verhage et al., 2004).
2000). It interacts with syntaxin-1 and the SNARE complex to regulate SNARE complex assembly and consequently synaptic vesicle exocytosis (Rizo and Südhof, 2012). Complexes are small, hydrophilic proteins that bind with high affinity to assembled SNARE complexes via its a-helical motif (McMahon et al., 1995). Synaptotagmin 1 (Syt1) functions as the Ca\textsuperscript{2+} sensor for synchronous neurotransmission by coupling Ca\textsuperscript{2+} influx with SNARE-mediated SV fusion (Brose et al., 1992; Geppert et al., 1994a; Fernandez-Chacon et al., 2001). Ca\textsuperscript{2+} binding to syt1 promotes its interaction with the target-SNAREs (t-SNAREs), syntaxin-1 and SNAP-25, to facilitate membrane fusion and subsequent neurotransmitter release (Chapman et al., 1995; Davis et al., 1999; Bai et al., 2004). Its function as a regulator of endocytosis will be discussed below.

2. Asynchronous Fusion. The precise molecular mechanisms underlying asynchronous neurotransmitter release are unclear. Asynchronous neurotransmitter release is kinetically delayed release, persisting after stimulation-induced Ca\textsuperscript{2+} influx has ceased (Barrett and Stevens, 1972; Goda and Stevens, 1994). During trains of action potentials, intracellular Ca\textsuperscript{2+} builds up and asynchronous release becomes more prominent (Hagler and Goda, 2001; Kirischuk and Grantyn, 2003; Wen et al., 2013). Asynchronous release also appears to be more resistant to depression of evoked activity than synchronous release and may serve to maintain longer-lasting tonic release (Lu and Trussell, 2000; Otsu et al., 2004; Iremonger and Bains, 2016).

There are an increasing number of studies focusing on asynchronous release and attempting to parse out its physiologic significance. The balance between synchronous and asynchronous release may change depending on the output demands of different neuron types and at various developmental stages (Kaeser and Regehr, 2014). In some hippocampal interneurons, asynchronous vesicle fusion is the predominant form of neurotransmitter release (Lu and Trussell, 2000; Hefft and Jonas, 2005; Ali and Todorova, 2010; Daw et al., 2010). In cortical interneurons, asynchronous release may play an important role in regulating epileptiform activity (Manseau et al., 2010; Jiang et al., 2012; Medrihan et al., 2015). In excitatory synapses, asynchronous release can generate larger and prolonged postsynaptic responses and perhaps play a role in potentiation and plasticity (Iremonger and Bains, 2007; Peters et al., 2010; Rudolph et al., 2011). During development, asynchronous release may allow for broadly tuned coincidence detection that becomes more narrowly tuned in mature synapses for phase-locked high-fidelity synaptic transmission (Chuhma and Ohmori, 1998). Regulation of asynchronous release has also been observed retrogradely as synapse-associated protein 97 in the postsynapse can act through N-cadherin to enhance presynaptic asynchronous release (Neff et al., 2009).

A major challenge in studying the underlying mechanisms of asynchronous neurotransmission is the difficulty in separating it from its more dominant synchronous counterpart. Recent work has identified potential molecular determinants involved in asynchronous release, which has allowed a better definition of this process beyond a simple kinetic distinction. Although vesicle-SNARE (v-SNARE) syb2 is involved in rapid Ca\textsuperscript{2+}-dependent synchronous neurotransmission, vesicle-associated membrane protein 4 (VAMP4) seems to selectively maintain bulk Ca\textsuperscript{2+}-dependent asynchronous release (Raingo et al., 2012). VAMP4 did not show robust trafficking under resting conditions, although it was shown that VAMP4-enriched vesicles can respond to elevated presynaptic Ca\textsuperscript{2+} signals and promote release (Raingo et al., 2012; Bal et al., 2013). In addition, syt7 has recently emerged as a key Ca\textsuperscript{2+}-sensing synaptic protein that maintains asynchronous neurotransmitter release independently of syt1 (Wen et al., 2010; Bacaj et al., 2013; Jackman et al., 2016).

3. Spontaneous Fusion. Spontaneous neurotransmitter release was originally thought to occur due to random low-probability conformational changes in the vesicle fusion machinery. However, accumulating evidence suggests that spontaneous release has specific molecular determinants that distinguish it from action potential-driven release as well as divergent postsynaptic effects (Ramirez and Kavalali, 2011; Kaeser and Regehr, 2014; Kavalali, 2015). One form of segregation occurs at the v-SNARE level as spontaneous fusion can persist in the absence of syb2 (Deitcher et al., 1998; Schoch et al., 2001; Deak et al., 2004; Sara et al., 2005). The specific molecular mechanisms that underlie the
segregation of the evoked and spontaneous neurotransmission are beginning to be elucidated (Hua et al., 2011; Ramirez et al., 2012; Bal et al., 2013). VAMP7 (also known as tetanus-insensitive VAMP) and Vps10p-tail-indicator-1a (Vti1a; vesicle transport through interaction with t-SNAREs homolog 1A) have been identified as alternative v-SNAREs that drive spontaneous release (Ramirez et al., 2012; Bal et al., 2013). These vesicular proteins tag vesicles that display divergent trafficking activity from syb2-enriched vesicles and may function to maintain separate vesicle populations that recycle independent of presynaptic action potentials.

The Ca$^{2+}$ sensitivity of spontaneous release is another area of functional divergence from stimulation-dependent neurotransmitter release. Spontaneous release frequency is much less dependent on changes in Ca$^{2+}$ levels than evoked release. The source of Ca$^{2+}$ and its different ion transients also complicate efforts to investigate the Ca$^{2+}$ dependence of spontaneous fusion. Both syntaxin 1 and cytosolic protein doc2 have been proposed as Ca$^{2+}$ sensors for spontaneous release (Xu et al., 2009; Groffen et al., 2010). However, doc2 may also modulate spontaneous neurotransmission through a Ca$^{2+}$-independent mechanism (Pang et al., 2011). Molecular interactions of the V0a1 subunit of the vacuolar-type ATPase may also regulate Ca$^{2+}$-dependent spontaneous release (Wang et al., 2014).

Loss of complexin, a cytoplasmic protein that binds SNARE complexes, results in increased spontaneous release (Huntwork and Littleton, 2007; Yang et al., 2013; Lai et al., 2014). This growing list of molecular players that regulate spontaneous neurotransmission provides specific molecular manipulations that can be used to selectively probe the mechanism and function of spontaneous neurotransmission (Kavalali et al., 2011; Kavalali, 2015).

There is also evidence that spontaneous release distinguishes itself from evoked release in regard to its postsynaptic receptor targets. Selective blockade of postsynaptic N-methyl-D-aspartic acid (NMDA) and α-aminooxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors activated by spontaneously released glutamate does not affect receptor-mediated responses after evoked release (Atasoy et al., 2008; Sara et al., 2011; Reese and Kavalali, 2016). This suggests that spontaneous and evoked release activate nonoverlapping populations of postsynaptic receptors. Spontaneous release leads to distinct postsynaptic changes and plays a role in synaptic homeostasis and plasticity (Sara et al., 2005; Sutton et al., 2006; Atasoy et al., 2008; Nosyreva et al., 2013).

4. Endocytic Pathways. Our relative lack of insight into the mechanisms underlying endocytosis is due in part to the technical challenges involved in studying the membrane retrieval process. Much of our understanding of exocytic pathways have come from electrophysiological patch-clamp experiments, which offer high temporal resolution. However, this method does not provide any information on endocytosis as it reports integrated postsynaptic responses. Although direct measurements of membrane capacitance have provided useful real-time data on endocytosis, its application is limited to large synapses such as the calyx of Held (Sun and Wu, 2001). An optical approach has proved important for visualizing membrane trafficking in small central synapses by using fluorescent membrane dyes (Betz and Bewick, 1992) and, more recently, genetically encoded pH-sensitive fluorescent proteins (Miesenbock et al., 1998). Tagging synaptic vesicle proteins with pH-sensitive green fluorescent protein provides the ability to look at single vesicle exo- and endocytic events (Balaji and Ryan, 2007; Leitz and Kavalali, 2011).

Clathrin-mediated endocytosis is a well-studied pathway of synaptic vesicle retrieval. It involves adaptor protein recruiting clathrin triskelia, which link together around budding vesicles. Accessory protein amphiphysin is also drawn to the nascent vesicle and recruits dynamin, which pinches off the new vesicle via GTP hydrolysis (Schmid and McMahon, 2007). Clathrin-mediated endocytosis is characterized by well-defined morphologic markers such as coated pits and endosomal intermediates. Kiss-and-run refers to an alternative faster pathway of vesicle recycling during which synaptic vesicles retain their identity and do not intermix with the plasma membrane or endosomal compartments (Fesce et al., 1994). This pathway appears to be clathrin-independent and may not necessarily require the same fission machinery (Palfrey and Artalejo, 1998).

However, limited experimental access to this process has made functional characterization and determination of the molecular mechanism difficult. Bulk endocytosis is another pathway of endocytosis that is observed after high-frequency stimulation as it allows for the retrieval of vesicles from the excess presynaptic plasma membrane (Cheung and Cousin, 2013). Deep plasma membrane infoldings form characteristic intracellular endosome-like intermediates. It is dependent on activation of calcineurin, which dephosphorylates dynamin I as well as clathrin (Takei et al., 1996; Ferguson et al., 2007; Wu et al., 2009a). In addition, recent studies have identified a new form of ultrafast endocytosis characterized with flash-and-freeze electron microscopy and membrane capacitance measures (Watanabe et al., 2013; Delvendahl et al., 2016). This process is observed at physiologic temperatures (~37°C) and appears to be mediated by dynamin and actin but is clathrin-independent.

In loss-of-function experiments, compromising synaptic vesicle endocytosis machinery gives rise to rapid depression and fatigue of synaptic responses during high-frequency stimulation. For instance, impairing the function of dynamin—a GTPase that pinches synaptic vesicles from the plasma membrane during synaptic vesicle endocytosis—using the Drosophila temperature-sensitive dynamin mutant shibire resulted in a fast decrease in neurotransmitter release with no detectable
sustained release during activity (Delgado et al., 2000). Mouse hippocampal synapses deficient in Dynamin 1 also showed strong synaptic depression presumably due to loss of vesicle endocytosis and recycling (Ferguson et al., 2007). Furthermore, interference with dynamin Src homology 3 domain interactions (Shupliakov et al., 1997), genetic deletion of synaptotagmin 1, an abundant presynaptic polyphosphoinositide phosphatase (Cremona et al., 1999; Luthi et al., 2001), or manipulation of differentially spliced isoforms of syt7 (Virmani et al., 2003) all lead to activity-dependent changes in the rate of synaptic depression. Although these observations suggest that recycled presynaptic vesicles can be rapidly reused during activity (Pyle et al., 2000; Sara et al., 2002), they may also indicate unavailability of release sites and suppression of exocytosis due to slow clearance of fused vesicles as a consequence of impaired endocytosis (Kawasaki et al., 2000).

The dephosphorylation–phosphorylation cycle of dynamin as well as other endocytic proteins has been shown to be critical for their function in activity-dependent regulation of synaptic vesicle endocytosis (Robinson et al., 1994). In particular, the dephosphorylation of dynamin is thought to be a critical trigger for endocytosis during activity (Marks and McMahon, 1998). Molecular manipulations of the synaptic vesicle-recycling machinery are important in uncovering vesicle-trafficking mechanisms as well as providing an extremely valuable setting to study the kinetics and physiologic significance of synaptic vesicle reuse during synaptic activity (Fig. 2). Furthermore, recent studies have provided several examples in which synaptic vesicle-recycling process and subsequent dynamics of neurotransmitter release can be modulated by small molecules that target dynamin-dependent endocytosis or myosin light chain kinase-dependent vesicle transport (Chung et al., 2010; Maeno-Hikichi et al., 2011; Linares-Clemente et al., 2015). From a neurotherapeutics perspective, targeting the synaptic vesicle-recycling machinery may present a key advantage as it induces frequency-dependent changes in the efficacy of neurotransmission to counter or correct disease processes, in contrast to typical blockers of neurotransmission that trigger global suppression or augmentation of neurotransmitter release that may potentially yield broader side effects (Kavalali, 2006).

**B. Vesicular Heterogeneity**

Accumulating evidence indicates that presynaptic terminals contain a molecularly heterogeneous population of vesicles that drive distinct forms of neurotransmission with different $\text{Ca}^{2+}$ dependence and divergent exo- and endocytic kinetics (Rizzoli and Betz, 2005; Sara et al., 2005; Fredj and Burrone, 2009). The route of synaptic vesicle recycling may differentially affect neurotransmission by generating vesicles with divergent propensities for fusion (Virmani et al., 2003; Voglmaier et al., 2006; Kavalali, 2007; Clayton et al., 2010). These vesicles are released in one of the different modes (synchronous, asynchronous, or spontaneous) and go through different routes of endocytosis that may lead to segregation into distinct vesicular pools (Crawford and Kavalali, 2015). Specific targeting of vesicular proteins may enable it to selectively modulate different forms of release pharmacologically (Figs. 1 and 2).

**III. Fusion Machinery**

### A. SNARE Proteins

SNARE proteins are a large family of proteins involved in intracellular vesicle trafficking and secretion. In neurons, the canonical SNARE complex, consisting of the synaptic vesicle protein syb2 and the presynaptic plasma membrane-associated proteins SNAP-25 and syntaxin-1, mediates synaptic vesicle exocytosis. Assembly of the SNARE complex is aided by a number of critical priming factors and provides a key substrate that the synaptic vesicle and presynaptic membrane close together, which allows for vesicle fusion (Südhof, 2004). Deletion of syb2 or SNAP-25 in mice leads to severely impaired neurotransmission and lethality at birth (Schoch et al., 2001; Washbourne et al., 2002).

SNAREs are the targets of bacterial clostridial neurotoxins, which inhibit neurotransmission and are responsible for botulism and tetanus (see discussion in Cleavage of SNAREs by Clostridial Toxins). Although the essential nature of the synaptic SNARE proteins in neurotransmission limits their physiology, there are few severe loss-of-function mutations in human disease. Studies show altered expression of syb2 in Alzheimer disease (AD) and Huntington’s disease (Shimohama et al., 1997; Morton et al., 2001). Deletion of the SNAP-25 gene in mice results in a hyperactive phenotype similar to attention deficit hyperactivity disorder (Hess et al., 1996; Brophy et al., 2002). Many studies have shown DNA variations in the SNAP-25 gene associated with attention deficit hyperactivity disorder (Brophy et al., 2002; Mill et al., 2002; Hawi et al., 2013). SNAP-25 reduction was also observed in the hippocampi of patients with schizophrenia (Thompson et al., 2003). Single-nucleotide polymorphisms in syntaxin-1a associated with schizophrenia (Wong et al., 2004). SNARE complex assembly is impaired in human brain tissue from patients with AD and Parkinson’s disease (Sharma et al., 2012). Importantly, SNAREs can be a key point of vulnerability in maintenance of synaptic proteostasis, as sustained activity requires continual assembly and disassembly of SNARE complexes, which in the absence of chaperone activity may yield accumulation of misfolded proteins (Chandra et al., 2005).

### B. Cleavage of SNAREs by Clostridial Toxins

Anaerobic Clostridium bacteria produce potent neurotoxins, including several botulinum toxins and tetanus toxin. The toxins cleave SNARE proteins involved...
in synaptic vesicle fusion and abolish neurotransmitter release (Schiavo et al., 2000). Botulinum toxins are taken up by motor neurons and block acetylcholine release at neuromuscular junctions and cause skeletal muscle weakness. Tetanus toxin is preferentially targeted to spinal cord interneurons, where it blocks the release of inhibitory neurotransmitters to cause hyperexcitation of skeletal muscle and tetanic contractions as a result of the loss of synaptic inhibition on spinal motor neurons. The different serotypes A–G of botulinum toxin proteolyze different components of the SNARE complex (Jahn and Niemann, 1994).

Botulinum toxin injections have a long history of being used for a variety of muscle disorders, including strabismus, blepharospasm, hemifacial spasm, cervical dystonia, and cosmetic procedures (Jankovic, 2004). Recently, there has been more evidence suggesting that the action of botulinum toxin is not limited to the peripheral nervous system. In addition to acting directly at the neuromuscular junction, peripheral botulinum toxin A injections may also alter sensory inputs to the CNS by indirectly inducing secondary central changes (Curra et al., 2004). Botulinum toxin A is retrogradely transported by central neurons and transcytosed to afferent synapses, cleaving SNAP-25 in the contralateral hemisphere after unilateral botulinum toxin A delivery, demonstrating long-distance retrograde effects of botulinum toxin A (Antonucci et al., 2008).

Although botulinum toxin can cause a deadly disease, utilization of certain serotypes has therapeutic potential. Intrahippocampal injection of botulinum toxin E resulted in inhibition of seizure activity in rat models of epilepsy (Costantin et al., 2005; Bozzi et al., 2006). Botulinum toxin A2 reduces incidence of seizures in mouse models of temporal lobe epilepsy (Kato et al., 2013). Botulinum toxin A2 injection into rat striatum ameliorates pathologic behavior in a rat Parkinson’s disease model (Itakura et al., 2014). In contrast, rat intrahippocampal infusion of botulinum toxin B is proconvulsant and can be used as a focal epilepsy model. This may be due to serotype B targeting syb2 and inhibiting GABA release (Broer et al., 2013). The different botulinum serotypes have different receptor and affinities that determine neuronal specificity and can be used for therapeutic benefit. The nontoxic recombinant heavy chain of botulinum neurotoxin A coupled to dextran could be an efficient drug delivery vehicle for botulinum countermeasure. The atoxic part helps to target botulinum neurotoxin-sensitive cells and promotes internalization of the complex (Zhang et al., 2009b).

Tetanus toxin binds to peripheral neurons and undergoes retrograde and trans-synaptic transfer to central inhibitory neurons, where it cleaves syb2, and blocks neurotransmitter release. Tetanus toxin entry is mediated through synaptic vesicle glycoprotein 2 (SV2) binding and synaptic vesicle endocytosis (Matteoli et al., 1996; Yeh et al., 2010). This endogenous pathway can be exploited to bypass the blood brain barrier for drug delivery. The nontoxic C-terminal domains of tetanus toxin can be used to penetrate the nervous system; a fragment of tetanus toxin has been shown to inhibit 5-HT reuptake and prevent serotonin from being transported through the presynaptic membrane (Najib et al., 2000). Recombinant atoxic mutants, made by Escherichia coli, can be engineered quickly and efficiently as useful vehicles for delivery to central neurons (Li et al., 2001). Using protein stapling technology, a chimera of botulinum neurotoxin A and the tetanus binding domain was created to specifically target CNS function and spare the neuromuscular junction (Ferrari et al., 2013). By limiting muscle paralysis, this may be useful for the future treatment of epilepsy or chronic pain.

C. Effects of Other Drugs on SNARE Proteins

Small cell-permeable peptides patterned after the N terminus domain of SNAP-25 inhibit SNARE complex assembly and regulate exocytosis. They protected against glucose deprivation–induced neurodegeneration and may attenuate dysfunctional exocytosis (Blanes-Mira et al., 2003). Plant extracts and peptide library screens are being used to identify compounds that alter SNARE complex formation (Blanes-Mira et al., 2004; Riley et al., 2006; Jung et al., 2009). An extract from Albizia julibrissin was found to reduce the level of SNARE complex formation; further studies are required to determine whether this is responsible for the observed effects of A. julibrissin extract in alleviating stress, insomnia, and depression (Riley et al., 2006). It did produce anxiolytic effects in elevated plus maze in rats, possibly due to upregulation of 5-HT1A receptors (Kim et al., 2004; Jung et al., 2005).

D. SNARE-Associated Proteins

1. Munc18-1 (STXBP1). The efficient functioning of SNARE complexes in synaptic transmission relies on interactions with a variety of other proteins. Munc18-1 is a neuron-specific protein of the Sec1/Munc18-like family of membrane-trafficking proteins. It serves as a key component of the synaptic vesicle fusion machinery, as deletion of Munc18-1 leads to complete loss of neurotransmitter release (Verhage et al., 2000). Munc18-1 binds to the closed form of syntaxin-1 and blocks SNARE complex formation. Syntaxin-1 is opened by Munc13, and Munc18-1 translocates to bind the formed SNARE complex and prevent its dissociation (Rizo and Südhof, 2012).

Rare mutations in the gene that encodes Munc18-1, syntaxin-binding protein 1 (STXBP1), have been identified in patients with various types of epilepsy. Loss-of-function heterozygous de novo mutations in STXBP1 have been linked to neonatal focal seizures, early onset epileptic encephalopathy with suppression bursts, and infantile spasms (Vatta et al., 2012; Barcia et al., 2014). Some of the patients also have mental retardation, ataxia, and dyskinetic movements (Deprez et al., 2010). Treatment with levetiracetam in patients effectively.
managed seizures that were refractory to other antiepileptic drugs (Vatta et al., 2012; Dilena et al., 2016). This may be due to levetiracetam’s unique mechanism of action involving SV2A. Modulation of SV2A may compensate for the haploinsufficiency of STXBP1 by affecting the synaptic vesicle-recycling pathway that Munc18-1 is part of. The interaction between levetiracetam and SV2A will be discussed in more detail below; however, more research is needed to further explore the specific mechanism of action of levetiracetam in Munc18-1-related epilepsies.

Development of a protein–protein interface inhibitor is a potential therapy for STXBP1 haploinsufficiency-associated epileptic disorders. Advances in our structural understanding of SNARE protein complexes and drug design techniques have paved the way for targeting Munc18-1–binding partners (Hussain, 2014). However, understanding Munc18-1’s specific function in the pathophysiology of these epilepsies would be a necessary first step.

2. Synaptotagmin. Syts are a family of integral membrane proteins with two calcium binding domains, C2A and C2B. Syt1 is an abundant isoform that is localized to synaptic vesicles and serves as a calcium sensor for vesicle exocytosis. It is required for the calcium triggering of synchronous neurotransmitter release, but is not essential for asynchronous release (Brose et al., 1992; Geppert et al., 1994a). Calcium binding to syt1 promotes its interaction with the t-SNAREs, syntaxin-1 and SNAP-25, and to phospholipids, thereby facilitating synaptic vesicle and presynaptic membrane fusion (Davis et al., 1999; Bai et al., 2004; Pang et al., 2006).

Recently, a de novo syt1 missense mutation has been identified in an individual with severe motor and cognitive impairments (Baker et al., 2015). Expression of this mutant syt1 in mouse neurons revealed slowed synaptic vesicle fusion kinetics and faster endocytosis. Therefore, syt-specific protein–protein interaction inhibitors may be an effective way to target the synchronous neurotransmitter release pathway selectively.

3. Complexin. Complexin I and II are highly homologous small, hydrophilic proteins enriched in neurons. They bind with high affinity to assembled SNARE complexes via an α-helical motif (McMahon et al., 1995). Deletion of complexin I and II in mouse neuron results in reduced neurotransmitter release efficiency (Reim et al., 2001). Complexin is an important regulator of synaptic vesicle exocytosis; however, the specific molecular mechanism of complexin function remains controversial. It appears to have a dual function as both a promoter and inhibitor of vesicle fusion (Xue et al., 2010; Yang et al., 2010).

Changes in complexin I and II expression are seen in several neurodegenerative and psychiatric disorders. Progressive and selective loss of complexin II was observed in a transgenic mouse model for Huntington’s disease (Morton and Edwardson, 2001). Expression of mutant huntingtin caused a decrease in complexin II levels and defects in neurotransmission, which were rescued by overexpression of complexin II in vitro (Edwardson et al., 2003). Additionally, decreases in complexin I and II were observed in postmortem brain tissue from patients with AD, schizophrenia, and bipolar disorder (Harrison and Eastwood, 1998; Tannenberg et al., 2006).

Complexin’s important role in regulation of synaptic vesicle fusion and its association with a multitude of neurologic and psychiatric disorders make it an attractive target for pharmacotherapy. Manipulating phosphorylation of complexin may be one way to modulate its function in synaptic vesicle fusion. In vitro phosphorylation of complexin I and II by protein kinase CK2 has been shown to enhance complexin binding to SNARE complexes (Shata et al., 2007). Activity-dependent phosphorylation of complexin by protein kinase A (PKA) enhances spontaneous neurotransmitter release and affects synaptic structural plasticity in Drosophila (Cho et al., 2015).

E. Noncanonical SNAREs

Vesicle molecular heterogeneity makes it possible to target different vesicular proteins to selectively regulate spontaneous or asynchronous neurotransmitter release without significantly altering fast synchronous neurotransmitter release. Fast synchronous release is critical for information coding and processing in the brain; any manipulation sparing this type of synaptic transmission would be expected to have fewer side effects compared with changes of global regulation of neurotransmission (Ramirez and Kavalali, 2012; Crawford and Kavalali, 2015). This has important implications for the development of novel treatment strategies as suggested by recent work implicating spontaneous neurotransmission in mediating the fast antidepressant effects of NMDA receptor antagonists (Autry et al., 2011).

1. Vti1a. Vti1a is localized to synaptic vesicles and participates in a novel SNARE complex, independent of syntaxin-1 and SNAP-25 (Antonin et al., 2000). Vti1a coimmunoprecipitates with VAMP4, syntaxin-6, and syntaxin-16 (Kreykenbohm et al., 2002). Vti1a exhibits robust trafficking under resting conditions, and loss of vti1a function selectively reduced high-frequency spontaneous release. Taken together, these data suggest that vti1a is localized to a vesicle pool that maintains spontaneous neurotransmission (Ramirez et al., 2012).

2. VAMP7. VAMP7, also known as tetanus toxin–insensitive VAMP, forms SNARE complexes with SNAP-23 and syntaxin-3 (Coco et al., 1999). VAMP7 is localized to synaptic vesicles that recycle spontaneously but are unresponsive to stimulation (Hua et al., 2011). Mice lacking VAMP7 do not exhibit any striking developmental or neurologic defects, but behavioral characterization revealed an increased anxiety phenotype (Danglot et al., 2012).

The heterogeneity of synaptic vesicle-associated SNAREs allows for the selective modulation of action...
potential-independent neurotransmission, providing an attractive target for future therapeutic development. Glycoprotein reelin selectively augments spontaneous synaptic transmission by mobilizing a VAMP7-dependent vesicle pool (Bal et al., 2013). However, VAMP7 is not a direct target of Reelin, as the effect requires calcium, phosphatidylinositol 3-kinase, apolipoprotein E receptor 2 (ApoER2), and very-low-density-lipoprotein receptor (VLDLR). As we begin to elucidate the molecular mechanisms underlying these different vesicle pools, we can develop new tools for specific manipulation of different forms of neurotransmitter release.

IV. Synaptic Vesicle Endocytosis

A. Synaptotagmin

Syt has a recognized role as the calcium sensor for fast synaptic vesicle exocytosis, but recent evidence implicates its role in endocytosis. Biochemical experiments show that syt1 interacts with a variety of endocytosis-associated proteins, including clathrin adaptor protein (Zhang et al., 1994; Haucke and De Camilli, 1999; von Poser et al., 2000). Multiple syt1 loss-of-function models show impaired endocytosis after stimulation (Marek and Davis, 2002; Poskanzer et al., 2003; Nicholson-Tomishima and Ryan, 2004; Yao et al., 2011).

Various studies have shown changes in syt1 expression in animal stroke models; however, it is unclear how syt1 is involved in the pathophysiology of stroke-related brain injury (Yokota et al., 2001; Chen et al., 2013a). In vivo knockdown of syt1 in a rat model of ischemic stroke prevented much of the ischemic damage of hippocampal neurons, making syt1 an attractive target for neuroprotective therapy (Iwakuma et al., 2003). This effect may be related to syt1’s exocytic function contributing to excitotoxicity; however, the endocytic function of syt1 in relation to the massive increase in presynaptic calcium may also play a role. Specific deletion of syt1 and other endocytic machinery components such as AP-2 and dynamin protected Caenorhabditis elegans neurons from hypoxia-induced necrotic cell death (Troulinaki and Tavernarakis, 2012). Neuronal endocytic pathways are clearly disrupted in stroke models, and further investigation is needed to understand how this pathway functions in both normal and disease states (McColl et al., 2003; Vaslin et al., 2007).

B. Calcineurin

Calcineurin is a calcium/calmodulin-dependent protein phosphatase that regulates synaptic transmission and plasticity (Rusnak and Mertz, 2000; Groth et al., 2003). Calcineurin dephosphorylates a set of proteins, in a calcium-dependent manner, involved in synaptic vesicle endocytosis, including dynamin, amphiphysin, and synaptopjanin (Cousin and Robinson, 2001). Calcineurin inhibitors abolish synaptic vesicle endocytosis (Marks and McMahon, 1998; Cousin et al., 2001). Calcineurin inhibitor, FK506, treatment increases dendritic branching and dendritic spine density and ameliorates dendritic spine loss in an Alzheimer mouse model in mouse brains (Rozkalne et al., 2011; Spires-Jones et al., 2011).

Schizophrenia is associated with a genetic variation in the 8p21.3 gene, PP3CC, which encodes the calcineurin γ subunit, leading to decreased calcineurin expression (Gerber et al., 2003; Eastwood et al., 2005). Calcineurin knockout (KO) mice exhibit multiple abnormal behaviors related to schizophrenia, such as increased locomotor activity, decreased social interaction, and working memory impairment (Zeng et al., 2001; Miyakawa et al., 2003; Cottrell et al., 2013). Biochemically, there is increased hyperphosphorylation of synaptic vesicle-recycling proteins known to be necessary for high-frequency firing. These findings support a model in which impaired synaptic vesicle recycling represents a critical node for disease pathologies underlying the cognitive deficits in schizophrenia.

The current Food and Drug Administration–approved calcineurin inhibitors, FK506 and cyclosporine A, are used for immunosuppression. Meta-analysis of treatment of FK506 as a neuroprotective drug in animal models of stroke suggests that it is effective and safe (Macleod et al., 2005). However, these existing drugs are nonspecific, and long-term use has undesirable side effects. Therefore, new drugs need to be developed with better blood brain penetration and higher specificity. High-throughput screens for novel calcineurin inhibitors are already underway (Margassery et al., 2012; Mukherjee et al., 2015). There is also a patent filed for identifying calcineurin activators for the diagnosis and treatment of schizophrenia (Gerber et al., 2011).

C. Dynamin I

Dynamin is a GTPase that is important for endocytic membrane fission in eukaryotic cells. Dynamin I and III are predominantly expressed in the brain, whereas dynamin II is ubiquitously expressed (Ferguson and De Camilli, 2012). Dynamin I KO mice appear normal at birth, but die within 2 weeks. Synaptic vesicle endocytosis was severely impaired during strong stimulation, but resumed efficiently after the end of stimulation (Ferguson et al., 2007). Dynamin III KO mice do not have an obvious pathologic phenotype, but dynamin I and III KO mice have a more severe phenotype than the dynamin I KO mice (Raimondi et al., 2011). Inhibitory neurons appear to be more sensitive to the loss of dynamin 1 and experience endocytic defects. Silencing transmission relieves the endocytic defect, suggesting that the high intrinsic level of tonic activity in inhibitory neurons makes them more vulnerable to lack of dynamin 1 (Hayashi et al., 2008).

In humans, de novo mutations in dynamin 1 have been shown to cause epileptic encephalopathies
Expression of these epileptic encephalopathy-causing dynamin 1 mutations in vitro leads to decreased endocytosis activity (Dhindsa et al., 2015). Spontaneous dynamin 1 missense mutation in mice confers seizure susceptibility that could arise from greater sensitivity of GABAergic interneurons to endocytic perturbations. The mutant dynamin 1 does not efficiently self-assemble and results in defective synaptic vesicle recycling and slower recovery from depression after trains of stimulation (Boumil et al., 2010). Furthermore, this mutation differentially affects splice variants of dynamin, interfering with the function of dynamin 1a, but upregulating the expression of dynamin 1b, providing an additional layer of complexity to future therapeutic modulation of dynamin function (Asinof et al., 2016). In another study, upregulation of dynamin 1 was observed in an acute seizure model in rats and in human patients with temporal lobe epilepsy. Pharmacological inhibition of dynamin 1 in the rat model decreased the frequency and severity of seizures, suggesting a potential role for dynamin modulators in seizure treatment (Li et al., 2015). More investigation is needed to reconcile the apparent differences of dynamin function in the etiology of epilepsy. Altered dynamin expression has also been detected in schizophrenia patients (Pennington et al., 2008; Focking et al., 2011; Cottrell et al., 2013).

There are many new classes of dynamin inhibitors being developed that represent a potential field for antiepileptic drug development (Gordon et al., 2013; McCluskey et al., 2013; McGeeachie et al., 2013; MacGregor et al., 2014; Robertson et al., 2014; Abdel-Hamid et al., 2015). Practically, these agents may need to act on specific isoforms or specifically target excitatory synapses and minimize effects on inhibitory interneurons. There is also a patent application for methods and agents that inhibit dynamin-dependent endocytosis for the development of treatment of epilepsy and other neurologic disorders (Hill et al., 2005). Control of dynamin phosphorylation may be a potential method of modulating synaptic vesicle endocytosis. Dephosphomimetic mutants of dynamin 1 regulate activity-dependent acceleration of endocytosis, providing a possible target for therapeutic intervention (Armbruster et al., 2013). The future challenge will be to ensure the specificity of action and limit side effects as dynamin I is involved in many cellular processes separate from its role in synaptic vesicle endocytosis.

**D. Amphiphysin**

Amphiphysin I is a hydrophilic phosphoprotein abundant in presynaptic terminals that binds various endocytic proteins and is involved in regulating clathrin-mediated endocytosis (Lichte et al., 1992; Wu et al., 2009b). Amphiphysin I KO mice have learning deficits and increased susceptibility to seizures. Neurons of these mice only revealed defects under stimulated conditions where inhibition of synaptic vesicle endocytosis was observed (Di Paolo et al., 2002).

Autoantibodies against amphiphysin and glutamic acid decarboxylase cause stiff-person syndrome, which is characterized by progressive stiffness and muscle spasms (De Camilli et al., 1993). Inhibitory GABAergic synapses are more vulnerable than glutamatergic synapses to the impaired clathrin-mediated endocytosis induced by anti-amphiphysin antibodies (Geis et al., 2010; Werner et al., 2016).

Phosphorylation of amphiphysin I by casein kinase 2 and minibrain kinase/dual-specificity tyrosine phosphorylation-regulated kinase (Mnb/Dyrk1A) inhibits its binding to other proteins involved in endocytosis, clathrin, and endophilin, respectively (Doring et al., 2006; Murakami et al., 2006). High-frequency stimulation reduces phosphorylation at the Mnb/Dyrk1A site, demonstrating a possible mechanism for activity-dependent suppression of amphiphysin function. Ubiquitous protease calpain cleaves amphiphysin I after strong stimulation, resulting in nonfunctional truncated amphiphysin I and inhibited synaptic vesicle endocytosis (Wu et al., 2007). Calpain-dependent cleavage of amphiphysin I attenuated kainate-induced seizures in mice by inhibiting excessive excitatory output. Existing kinase and calpain inhibitors can be used for further research into their potential therapeutic use.

**E. Synaptojanin**

Synaptojanin is a presynaptic lipid phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-triphosphate to phosphatidylinositol (4,5)-biphosphate (Guo et al., 1999). Its C-terminal domain interacts with many proteins involved in clathrin-mediated endocytosis. Genetic disruption of synaptojanin 1 leads to stimulation-dependent accumulation of clathrin-coated vesicles, implicating synaptojanin 1 in the dissociation of clathrin adaptors and the uncoating of nascent synaptic vesicles. Synaptojanin 1-deficient mice exhibit neurologic defects and die shortly after birth. The functional recycling pool is also smaller in synaptojanin 1-deficient neurons; synaptic depression was enhanced during high-frequency stimulation, and recovery was delayed (Cremona et al., 1999; Kim et al., 2002).

Changes in synaptojanin 1 expression have been linked to Down’s syndrome and AD (Voronov et al., 2008; Di Paolo and Kim, 2011; Martin et al., 2014). Mutations in synaptojanin have also been linked to bipolar disorder and autosomal recessive, early-onset Parkinsonism (Saito et al., 2001; Krebs et al., 2013; Quadri et al., 2013).

Mnb/Dyrk1A phosphorylation of synaptojanin 1 alters its binding to the Src homology 3 domains of amphiphysin, intersectin, and endophilin (Bauerfeind et al., 1997). Mnb/Dyrk1A is encoded in the Down syndrome critical region of chromosome 21 (Shindoh et al., 1996; Song et al., 1996) and is an excellent target for future
drug development. Reduction of synaptojanin 1 ameliorates synaptic and behavioral impairments in a mouse model of AD (McIntire et al., 2012). A screening assay for small-molecule inhibitors of synaptojanin I demonstrates that its pharmaceutical potential is already being explored (McIntire et al., 2014).

V. Stability and Maintenance of the Synaptic Vesicle-Recycling Machinery

A. Cysteine-String Protein \(\alpha\)

Cysteine-string protein (CSP\(\alpha\)) is predominately localized to synaptic vesicles (Mastrogiacomo et al., 1994). CSP\(\alpha\) associates with ATPase heat shock protein 70 (hsc70) and small glutamine-rich tetratricopeptide repeat-containing protein to form a complex that functions as a synaptic chaperone (Tobaben et al., 2001). This CSP\(\alpha\)-hsc70-small glutamine-rich tetratricopeptide repeat-containing protein complex binds to SNAP-25 and prevents aggregation to enable SNARE complex assembly. Deletion of CSP\(\alpha\) results in structurally abnormal SNAP-25 that inhibits SNARE complex formation and is more rapidly degraded (Sharma et al., 2011). CSP\(\alpha\) KO mice begin to exhibit locomotor defects at about 2–3 weeks of age and do not survive beyond 3 months. Their synapses show major changes in structure consistent with a presynaptic degenerative process (Fernandez-Chacon et al., 2004). This activity-dependent neurodegeneration is more severe in GABAergic synapses and can be partially rescued by decreasing network excitability with pharmacological blockers of AMPA receptors (García-Roy, 2012).

Heterozygous mutations in the CSP\(\alpha\) gene in humans cause autosomal-dominant adult-onset neuronal ceroid lipofuscinosis, a neurodegenerative disorder characterized by lysosomal accumulation of misfolded proteins (Noskova et al., 2011). A palmitoyltransferase inhibitor could be a possible way to regulate CSP\(\alpha\) function as palmitoylation of CSP\(\alpha\) increases aggregation seen in neuronal ceroid lipofuscinosis (Greaves et al., 2012).

Quercetin, a plant flavonoid, targets the unique cysteine string region of CSP\(\alpha\) and impairs synaptic transmission (Xu et al., 2010). Quercetin promotes formation of stable CSP\(\alpha\) dimerization and inhibits assembly of the active chaperone complex by reducing hsc70 association. The next step would be to identify other compounds that take advantage of the unique binding site to enhance rather than inhibit CSP\(\alpha\) function.

B. Synucleins

Synucleins are a family of small, soluble proteins primarily expressed in neural tissue with a highly conserved amphiphilic \(\alpha\)-helical lipid-binding motif (George, 2002). Synuclein protein is localized to presynaptic terminals and nuclei (Maroteaux et al., 1988). Synuclein binds directly to syb2 and promotes SNARE complex assembly (Burre et al., 2010). Synuclein KO mice have functional deficits in the nigrostriatal dopamine system, resulting in increased dopamine release and reduced reserve pool size (Abeliovich et al., 2000; Cabin et al., 2002). Behaviorally, the synuclein KO mice have cognitive impairments and reduced working and spatial memory (Kokhan et al., 2012). Overexpression of synuclein, below toxic levels, decreased the readily releasable pool size and led to decreased neurotransmitter release (Nemani et al., 2010). Synuclein’s function may be to maintain vesicle pool homeostasis by regulating the size of synaptic vesicle pools (Cabin et al., 2002; Nemani et al., 2010; Scott and Roy, 2012). Synuclein may also promote clathrin-mediated endocytosis and regulate the kinetics of synaptic vesicle endocytosis (Ben Gedalya et al., 2009; Vargas et al., 2014). Synuclein can also inhibit vesicle membrane fusion independent of SNARE proteins through direct interactions with lipid bilayers (Darios et al., 2010; DeWitt and Rhoades, 2013).

Mutations in synuclein are associated with rare familial and sporadic forms of Parkinson’s disease. Toxic synuclein accumulates abnormally in Parkinson’s disease, AD, dementia with Lewy bodies, and other neurodegenerative diseases (Goedert, 2001; Stefanis, 2012). The functional role of synuclein in the pathogenesis of these neurodegenerative diseases is unclear. There is growing evidence to suggest the synuclein–mediated aberrant synaptic vesicle recycling precedes overt neuro-pathology and may contribute to the pathophysiology of these synucleinopathies (Galvin et al., 2001; Nakata et al., 2012; Yasuda et al., 2013).

Synuclein ser125 is phosphorylated by many different kinases (Okochi et al., 2000; Pronin et al., 2000; Ishii et al., 2007; Inglis et al., 2009). Experiments in yeast show that blocking synuclein phosphorylation significantly increased trafficking defects and synuclein toxicity (Sancenon et al., 2012). This hints at a role for dynamic phosphorylation on synuclein’s function in synaptic vesicle recycling and disease progression. A small-molecule screen yielded a compound that targets vesicle-bound synuclein and inhibits its aggregation (Fonseca-Ornelas et al., 2014). It is yet to be seen how this affects synuclein’s effect on synaptic vesicle recycling; however, similar methods can be used to find other modulations of synuclein function.

VI. Active Zone Proteins

A. Munc13

Munc13, a homolog of unc-13 in C. elegans, is a large protein localized to presynaptic terminals (Brose et al., 1995). Deletion of munc13-1, the most abundant isoform, in mice leads to signification reductions in the
readily releasable pool size and neurotransmitter release in glutamatergic neurons only (Richmond et al., 1999; Varoqueaux et al., 2002). Munc13-1 interacts with the N terminus of syntaxin and primes synaptic vesicles for exocytosis (Betz et al., 2001). Munc13 also interacts with Rab3-interacting molecules (RIM) to tether and prime synaptic vesicles and modulate long-term synaptic plasticity (Betz et al., 2001; Yang and Calakos, 2011).

In synapses, munc13-1 is a key target for diacylglycerol signaling in addition to the well-described impact of this signaling pathway on protein kinase C activity (Brose and Rosenmund, 2002). In experimental settings, this function of munc-13 is typically probed with application of phorbol esters as phorbol ester binding (to the C1 domain) induces membrane association and application of phorbol esters as phorbol ester binding (to the C1 domain) induces membrane association and enhances neurotransmitter release (Betz et al., 1998).

**B. Rab3-Interacting Molecule**

RIM are large scaffolding proteins localized to presynaptic active zones. The major isoforms RIM1α and RIM2α are made up of an N-terminal zinc-finger domain, a central PDZ domain, and multiple C2 domains, allowing for binding to a multitude of synaptic proteins (Südhof, 2004). The N-terminal of RIM1α binds to synaptic vesicle GTPase rab3 and active zone protein Munc13 to regulate neurotransmitter release (Wang et al., 1997; Betz et al., 2001; Schoch et al., 2002). The GTP-dependent interaction between rab3A and the N-terminal of RIM1α is implicated in tethering of synaptic vesicles at the active zone (Wang et al., 1997). RIM interaction with munc13 is thought to be critical for synaptic vesicle priming (Betz et al., 2001). RIM1α KO mice have defects in synaptic transmission, short-term synaptic plasticity, and long-term synaptic plasticity in the hippocampus (Castillo et al., 2002; Schoch et al., 2002). In addition to their key role in synaptic vesicle priming, RIM proteins also form a close synaptic vesicle–voltage-gated Ca2+ channel scaffold to ensure rapid release (Kaeser et al., 2011).

**VII. Other Synaptic Vesicle Proteins**

**A. Rab3**

Small GTPase rab3 is an abundant synaptic vesicle protein. Mice lacking the rab3A isoform are viable and fertile with mostly normal synaptic function, except for increased synaptic depression after repetitive stimuli (Geppert et al., 1994b). Quadruple rab3A, rab3B, rab3C, rab3D KO mice do not survive; however, cultured hippocampal neurons from these embryos have normal spontaneous release with decreased evoked vesicle release probability (Schlüter et al., 2004). Experiments with rab3 single-, double-, and triple-KO mice show that the four rab3 isoforms appear to be functionally redundant. These studies suggest that rab3 is not an essential component of neurotransmission, but participates in the subtle regulation of synaptic vesicle fusion and activity-dependent recruitment of vesicles to the active zone (Leenders et al., 2001).

Changes in rab3A levels were observed in Alzheimer patients and in a mouse model for Huntington's disease (Davidsson et al., 2001; Morton et al., 2001). Rab3A also associates with pathologic α-synuclein in a GTP-dependent manner (Dalfó et al., 2004; Dalfó and Ferrer, 2005; Chen et al., 2013b). Specific GTPase inhibitors may be a possible mechanism to modulate rab3A activity.

**B. Synaptic Vesicle Glycoprotein 2A**

SV2 is a membrane glycoprotein localized to synaptic vesicles and neuroendocrine secretory granules (Buckley and Kelly, 1985). SV2 has significant amino acid sequence identity to bacterial transporters (Bajjalieh et al., 1999). SV2A, SV2B, and SV2C isoforms are highly homologous proteins with differential expression throughout the brain (Janz and Südhof 1999). SV2A KO and SV2ASV2B double-KO mice exhibit severe seizures and die postnatally (Crowder et al., 1999; Janz et al., 1999). Neurons lacking both SV2A and SV2B exhibited sustained increases in calcium-dependent synaptic transmission after repetitive stimulation due to presynaptic calcium accumulation (Janz et al., 1999). SV2 may function as regulators of presynaptic calcium and influence synaptic vesicle dynamics (Wan et al., 2010).

Decreased SV2A was observed in surgically removed temporal lobe tissue and postmortem hippocampal tissue from patients with temporal lobe epilepsy (Feng et al., 2009; van Vliet et al., 2009). Levetiracetam is a unique antiepileptic drug that targets a synaptic vesicle protein instead of other antiepileptics that block voltage-gated sodium channels or modulate GABA receptors (Fig. 1). SV2A is both necessary and sufficient for levetiracetam binding and consequent antiepileptic action (Lynch et al., 2004). Low-frequency stimulation facilitates entry of levetiracetam into neurons perhaps through binding exposed SV2A on the intravesicular surface. Increased synaptic activity leads to corresponding increases in levetiracetam’s efficacy, which may explain the antiepileptic effect of levetiracetam (Meehan et al., 2011). A possible mechanism for levetiracetam’s antiepileptic action may be due to SV2A-dependent acceleration of synaptic depression in excitatory neurons during epileptiform activity (García-Pérez et al., 2015). Brivaracetam (UCB 34714) is an analog of levetiracetam with 10-fold greater binding affinity for SV2 and more potent antiepileptic effects in animal models of epilepsy (Kenda et al., 2004; Matagne et al., 2008). A phase III study shows that brivaracetam treatment was associated with statistically significant reductions in seizure frequency compared with patients that received placebo treatment (Biton et al., 2014). These results confirm SV2 as the target of levetiracetam’s antiepileptic effect and establish the importance of targeted small-molecule screens.

SV2 also interacts with botulinum and tetanus neurotoxins and facilitates their entry into central nerve
terminals (Dong et al., 2006; Yeh et al., 2010). Tetanus neurotoxin is unable to cleave syb2 in SV2 KO neurons, further implicating SV2 as a new target that can be exploited to prevent tetanus or for uptake of modified clostridial toxins into the synapse.

C. Synapsin

Synapsins are a family of four synaptic vesicle phosphoproteins with conserved phosphorylation sites at the N-terminal domain and a high-affinity ATP-binding module (Südhof et al., 1989; Hosaka and Südhof, 1998; Südhof, 2004). Most synapses express abundant levels of synapsin I and II, but they are not essential for neurotransmitter release (Rosahl et al., 1993). Synapsin I/II KO mice are viable and fertile, but suffer from seizures (Rosahl et al., 1995). Disrupting synapsin function results in a reduction of reserve pool of vesicles and failure to sustain neurotransmitter release in response to high-frequency stimulation (Pieribone et al., 1995; Rosahl et al., 1995). Further studies with synapsin KO mice show that synapsins maintain the reserve pool of vesicles in excitatory synapses, but regulate the size of the readily releasable pool in inhibitory synapses (Gitler et al., 2004). These results implicate synapsin in the regulation of network excitability and may explain the underlying cause of epileptic seizures in synapsin KO mice (Chiappalone et al., 2009). Rab3A deletion in synapsin II KO mice appears to restore the excitatory/inhibitory balance, suggesting that the two synaptic vesicle proteins coregulate synaptic activity (Feliciano et al., 2013).

Genetic studies in humans with epilepsy have implicated synapsin in disease etiology. A nonsense mutation in the synapsin I gene was identified in a type of familial X-linked (Garcia et al., 2004). Most epilepsies have been linked to mutations in voltage-gated channels; however, this is the first time a synaptic vesicle protein has been recognized. Single-nucleotide polymorphisms in the synapsin II gene were found to be associated with sporadic epilepsy (Cavalleri et al., 2007; Lakhan et al., 2010). Postmortem brain studies show significant decreases in synapsin II mRNA in prefrontal cortex of subjects with schizophrenia (Mirmics et al., 2001). Chronic treatment with the antipsychotic haloperidol, a dopamine D2 receptor antagonist, leads to increases in synapsin II mRNA and protein levels in rats and humans (Chong et al., 2006; Tan et al., 2014). Synapsin II KO mice exhibit behavioral abnormalities commonly seen in preclinical animal models of schizophrenia, including prepulse inhibition deficits, decreased social behavior, and locomotor hyperactivity (Dyck et al., 2009). These results implicate synapsin II in the pathophysiology of schizophrenia and as a possible target for novel therapeutics with less severe side effects. Abnormal phosphorylation of synapsin I has been linked to AD and Huntington’s disease (Parks et al., 1991; Lievens et al., 2002). However, additional research is needed to deepen our understanding of synapsin’s role in the synaptic vesicle-recycling pathway and its involvement in pathogenic mechanisms of disease states.

Phosphorylation of synapsin may be a useful mechanism to regulate synaptic vesicle activity. PKA phosphorylation of synapsin I promotes its dissociation from synaptic vesicles, enhancing the rate of stimulation-driven exocytosis and accelerating recovery from synaptic depression by recruiting vesicles from the reserve pool to readily releasable pool (Menegon et al., 2006). Plant extract forskolin has anticonvulsant effects in drug-induced seizures in mice (Sano et al., 1984; Borges Fernandes et al., 2012), possibly through downstream activation of PKA by the cAMP pathway and consequent regulation of synapsin activity in synaptic vesicle recycling. Small-molecule screens for modulators of synapsin I phosphorylation in primary neurons have already identified more potential compounds (Chan et al., 2014). Cyclin-dependent kinase-5 phosphorylation of synapsin I regulates the resting and recycling pools of synaptic vesicles in an activity-dependent manner and may be another pathway for pharmacological intervention (Verstegen et al., 2014). Four protein kinase inhibitors (staurosporine, quercetagetin, roscovitin, 70159800251) were found to compete against ATP for binding to the ATP binding site of synapsin I, highlighting one more potential mechanism to alter synapsin-mediated activity for therapeutic advantage (Defranchi et al., 2010). The challenge for drug development in this avenue is to find compounds that are specific to synapsin sites to limit off-target or systemic effects.

D. Synaptophysin

Synaptophysin is an abundant synaptic vesicle glycoprotein that binds syb2 (Johnston and Südhof, 1990; Calakos and Scheller, 1994). This interaction regulates the distribution of available syb2 and prevents formation of the SNARE complex (Edelmann et al., 1995; Pennuto et al., 2003). Dissociation of synaptophysin from syb2 allows it to form a complex with dynamin and potentially regulate quantal size and duration of exocytic events (Daly and Ziff, 2002; Gonzalez-Jamett et al., 2012). Synaptophysin KO mice reveal that synaptophysin is not essential for neurotransmitter release; however, they exhibit behavioral alterations and learning deficits (McMahon et al., 1996; Schmitt et al., 2009). More recent studies have found that synaptophysin is required for syb retrieval and affects the kinetics of synaptic vesicle endocytosis (Gordon et al., 2011; Kwon and Chapman, 2011). Taken together, this suggests synaptophysin may modulate the efficiency of the synaptic vesicle cycle and have effects on higher-order brain functions.

In normal states, synaptophysin is one of the most abundant synaptic vesicle proteins, but its levels are reduced
in AD and schizophrenia human brains (Shimohama et al., 1997; Eastwood et al., 2000; Masliah et al., 2001). These changes in synaptophysin in neurons of the hippocampus and association cortices correlate with changes in the cognitive decline in AD patients (Terry et al., 1991; Sze et al., 1997). Amyloid-β (Aβ) peptide, which is elevated in AD brains, disrupts the interaction between synaptophysin and syb2 in vitro, leading to an increased amount of primed vesicles and exocytosis (Russell et al., 2012). The effect on synaptic vesicle endocytosis was not examined; however, other studies show that Aβ disrupted synaptic vesicle endocytosis perhaps due to dynamin 1 depletion (Kelly et al., 2005; Kelly and Ferreira, 2007). It is possible that Aβ disruption of synaptophysin binding to syb2 results in more syb2 available to form SNARE complexes, leading to increased synaptic vesicle exocytosis. The defective endocytosis observed may be due to both lack of synaptophysin-mediated syb2 retrieval and also increased synaptophysin-dynamin 1 complexes. This deregulation of synaptic transmission may contribute to the pathogenesis of AD and perhaps explain the early cognitive loss preceding significant synapse loss seen in AD patients. Syb2–synaptophysin interactions are also disrupted in familial X-linked intellectual disability. Human synaptophysin X-linked intellectual disability mutants expressed in synaptophysin KO mice were dysfunctional in their retrieval of syb2 during endocytosis, providing a possible mechanistic basis for the disorder (Gordon and Cousin, 2013).

Synaptophysin may be a viable therapeutic target through disruption of its interactions with syb2 and dynamin 1. Its function may be regulated by phosphorylation at its C-terminal domain by calcium/calmodulin-dependent protein kinase II and tyrosine kinase (Alder et al., 1995; Daly and Ziff, 2002; Mallozzi et al., 2013).

**E. Rab5**

Rab5 GTPase is found in high concentration on synaptic vesicles and mediates synaptic vesicle endocytosis as well as early endosome trafficking (de Hoop et al., 1994; Fischer von Mollard et al., 1994; Stenmark, 2009). In *Drosophila* studies, a loss-of-function rab5 mutant reduced exo- and endocytosis rates and decreased the recycling synaptic vesicle pool size (Wucherpfennig et al., 2003).

Rab5 is associated with a few neurodegenerative diseases, although not much is known about its role in disease pathology. Isoform rab5b has been shown to colocalize with leucine-rich repeat kinase 2, a defective gene causing an autosomal dominant form of Parkinson’s disease, resulting in impaired synaptic vesicle endocytosis, and overexpression of rab5b rescued the defect (Shin et al., 2008). In a *Drosophila* model of Huntington’s disease, mutant huntingtin protein interacts indirectly with rab5 and inhibits its function. Rab5 overexpression then attenuated aggregation and toxicity (Ravikumar et al., 2008). Elevated rab5 levels were observed in brains from AD patients, suggesting that endocytic dysfunction may underlie the pathogenesis of AD (Ginsberg et al., 2010).

Although rab5’s functions are not confined specifically to synaptic vesicle recycling, it may be a viable therapeutic target, as much is known about GTPase inhibitors and may help in the development of modulators of rab5 function. The Research Foundation for the State University of New York recently filed a patent for small molecules that modulate rab5 activity by preventing conversion of rab5-GTP to rab5-GDP, thereby enhancing rab5-mediated activity (Zong, 2014).

**VIII. Conclusion**

Studies in the last decade have greatly advanced molecular characterization of synaptic vesicle proteins and our understanding of the mechanisms underlying synaptic vesicle recycling. These efforts have also elucidated the key roles played by the presynaptic vesicle-recycling machinery in the pathogenesis of several neurologic and neuropsychiatric disorders. However, despite these advances, systematic investigation of the presynaptic machinery components as drug targets received very little attention. Many presynaptic proteins function in larger complexes and may have multiple roles in the synaptic cycle process, making specific pharmacological intervention challenging. Nevertheless, the availability of increasingly sophisticated and specific functional assays makes identification of novel drugs acting on presynaptic targets highly feasible. A combination of the currently available approaches to dynamically monitor presynaptic function and our advanced understanding of the molecular details of neurotransmission will help us uncover how pathologic mutations alter presynaptic function and also better equip us for presynaptically targeted drug development.

**Authorship Contributions**

Wrote or contributed to the writing of the manuscript: Li, Kavalali.

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