MOLECULAR DETERMINANTS OF SYNAPTIC VESICLE EXOCYTOSIS AND ENDOCYTOSIS COUPLING

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DEDICATION

I would like to thank the members of my Graduate Committee, my family, my friends, my fellow labmates, and so on and so forth.

MOLECULAR DETERMINANTS OF SYNAPTIC VESICLE EXOCYTOSIS AND ENDOCYTOSIS COUPLING

by

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Synaptic vesicle recycling is essential for maintaining normal synaptic function. The reuse of vesicles maintains the size of the presynaptic terminal and ensures the availability of synaptic vesicles for subsequent exocytosis. The coupling of exocytosis and endocytosis allows for continued rapid synaptic transmission; however, the molecular mechanisms of this process are not well understood. This coupling is assumed to be Ca^{2+}-dependent but the exact role of Ca^{2+} and its key effectors in the regulation of endocytosis are not clear. Using a

genetically encoded pH-sensitive GFP tag expressed in cultured hippocampal neurons, I analyzed synaptic vesicle trafficking in high resolution optical experiments. By manipulating the expression of various effectors of vesicle fusion I was able to dissect out the relationship between exocytic pathway and subsequent endocytic kinetics. My results showed that the slowed endocytosis phenotype previously reported after synaptotagmin 1 loss-of-function can also be triggered by other manipulations that promote asynchronous release such as Sr^{2+} substitution and complexin loss-of-function. The link between asynchronous release and slowed endocytosis was due to selective targeting of fused synaptic vesicles towards slow retrieval by the asynchronous release Ca^{2+} sensor synaptotagmin7. This divergence in Ca^{2+} sensor function supports findings that VAMP4 selectively drive asynchronous release through a population of vesicles that do not interact with synaptotagmin 1 or complexins. At the single synaptic vesicle level, synaptotagmin 1 acted as an essential determinant of synaptic vesicle endocytosis time course by increasing the kinetics of vesicle retrieval in response to increasing Ca^{2+} levels. In contrast, synaptotagmin 1 did not affect the rapid retrieval of spontaneously fused vesicles. Taken together, these results suggest that exocytic pathways dictate endocytic kinetics as asynchronously fused vesicles are retrieved slowly while spontaneously fused vesicles are rapidly retrieved. These mechanisms may diversify the molecular compositions of synaptic vesicles regenerated after fusion to provide presynaptic terminals with a wide range of synaptic vesicle populations with distinct biogenesis properties and exo-endocytosis kinetics.

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PRIOR PUBLICATIONS

Li Y.C., Chanaday N.L., Kavalali E.T. (*submitted*) Synaptotagmin-1 and synaptotagmin-7-dependent fusion mechanisms target synaptic vesicles to kinetically distinct endocytic pathways.

Li Y.C. and Kavalali E.T. (2015) How do RIM-BPs link voltage-gated Ca(2+) channels to evoked neurotransmitter release? *Neuron* **87**(6):1119-21.

Raingo J., Khvotchev M., Liu P., Darios F., Li Y.C., Ramirez D.M., Adachi M., Lemieux P., Toth K., Davletov B., Kavalali E.T. (2012) VAMP4 directs synaptic vesicles to a pool that selectively maintains asynchronous neurotransmission. *Nat Neurosci.* **15**(5):738-45.

Battefeld A., Bierwirth C., Li Y.C., Barthel L., Velmans T., Strauss U. (2010) I(h) "run-up" in rat neocortical neurons and transiently rat or human HCN1-expressing HEK293 cells. J *Neurosci Res.* **88**(14):3067-78.

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AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR – AMPA receptor

AP – action potential

AP-5 – 2-amino-5-phosphonopentanoic acid

CNS – central nervous system

CNQX – 6-cyano-7-nitroquinoxaline-2,3-dione

DIV – days in vitro

EPSC – excitatory postsynaptic current

GABA – gamma-aminobutyric acid

GFP – green fluorescent protein

Het - heterozygous

Homo - homozygous

Hz – Hertz

IPSC – inhibitory postsynaptic current

KD - knockdown

KO - knockout

mEPSC – miniature excitatory postsynaptic current

mIPSC – miniature inhibitory postsynaptic current

NMDA – N-methyl-D-aspartate

NMDAR – NMDA receptor

SV – synaptic vesicle

Syb2 – synaptobrevin 2

Syt1 – synaptotagmin 1

Syt7 – synaptotagmin 7

TTX - tetrodotoxin

WT – wild-type

CHAPTER ONE

GENERAL INTRODUCTION

Overview of the Synaptic Vesicle Cycle

Synapses are basic structural units for communication between neurons and are essential for neuronal function. Neuronal signals, specifically action potentials, travel along axons and trigger the opening of voltage-gated calcium (Ca^{2+}) channels in presynaptic terminals. The influx of Ca^{2+} 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, re-acidified 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. The coupling of exo- and endocytosis allows for continued rapid synaptic transmission; however, the molecular mechanisms of this process are not well understood.

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; Sudhof, 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 re-formed vesicles and neurotransmitter transporters utilize 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 SV recycling is still being explored, it seems that stimulation intensity and Ca^{2+} levels may induce shifts from one form to the other (Gandhi and Stevens, 2003; Zhang et al., 2009). 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. My dissertation is focused on the mechanisms of synaptic vesicle endocytosis after different modes of neurotransmitter release, specifically in excitatory hippocampal neurons.

Modes of Synaptic Vesicle Exocytosis

Synchronous Fusion

The most molecularly well-characterized pathway of vesicle fusion is synchronous fusion. Vesicles fuse and neurotransmitters are released in a precise time-locked manner with stimulation and ensuing Ca^{2+} influx. This rapid and reliable exocytosis depends on many complex protein and lipid interactions. SNARE (soluble N-

ethylmaleimide-sensitive-factor attachment protein receptor) proteins and its binding partners are essential for this fast exocytosis. The canonical SNARE complex is composed of synaptobrevin 2, on the synaptic vesicle, and syntaxin-1 and SNAP-25, both on the target plasma membrane (see reviews: Sudhof, 2004; Rizo and Rosenmund, 2008; Sudhof and Rothman, 2009). The α-helical SNARE motifs of these proteins facilitate the formation a tight complex which brings vesicles close to the presynaptic membrane. Munc18-1 is a Sec1/Munc18 (SM) protein essential for neurotransmitter release (Verhage et al., 2000). It interacts with syntaxin-1 and the SNARE complex to regulate SNARE complex assembly and consequently SV exocytosis (Rizo and Sudhof, 2012). Complexins are small, hydrophilic proteins that bind with high-affinity to assembled SNARE complexes via its α-helical motif (McMahon et al., 1995). Synaptotagmin 1 (Syt1) functions as the Ca^{2+} sensor for synchronous neurotransmission by coupling Ca^{2+} influx with SNARE-mediated SV fusion (Brose et al., 1992; Geppert et al., 1994; Fernandez-Chacon et al., 2001). Ca^{2+} binding to synaptotagmin 1 promotes its interaction with the 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.

Asynchronous Fusion

Asynchronous neurotransmission lacks the mechanistic insights of its synchronous counterpart. It is kinetically delayed release, persisting after stimulation-induced Ca^{2+} influx has ceased (Barrett and Stevens, 1972; Goda and Stevens, 1994). During trains of action potentials, intracellular Ca^{2+} builds up and asynchronous release

becomes more prominent (Hagler and Goda, 2001; Kirischuk and Grantyn, 2003; Wen et al,. 2013). It 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 studies focusing on asynchronous release and attempting to parse out its physiological 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 hippocampus interneurons, asynchronous vesicle fusion is the predominant form of neurotransmitter release (Lu and Trussell, 2000; Hefft and Jonas , 2005; Ali and Todorova et al., 2010; Daw et al., 2010). In cortical interneurons, asynchronous release may play an important role in regulating epileptoform 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 and Overstreet-Wadiche, 2011). During development, asynchronous release may allow for broadly tuned coincidence detection which 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 (SAP97) 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 us to define it beyond a simple kinetic distinction. While v-SNARE syb2 is involved in rapid Ca^{2+}-dependent synchronous neurotransmission, VAMP4 seems to selectively maintain bulk Ca^{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^{2+} signals and promote release (Bal et al., 2013; Raingo et al., 2012). Biochemical experiments demonstrated that VAMP4 forms a stable complex with syntaxin 1 and SNAP25. However, unlike syb2-driven complexes, VAMP4 complexes did not readily interact with complexins or syt1, proteins that function to synchronize vesicle fusion (Raingo et al., 2012). This biochemical insight may explain why VAMP4 preferentially affects asynchronous release.

Synaptotagmin isoform syt7 has been proposed as a Ca^{2+} sensitive regulator of asynchronous release and is discussed at length below. Doc2 proteins are also candidates for being the Ca^{2+} sensor that mediates asynchronous release. They are soluble cytosolic proteins and similar to the synaptotagmins, they have two conserved C2 domains (Sakaguchi et al., 1995; Orita et al., 2001). Some studies show that doc2 functions as a Ca^{2+} sensor that is required for asynchronous neurotransmitter release (Yao et al., 2011; Xue et al., 2015). However, two other groups have found asynchronous release unaffected by reducing doc2 levels (Groffen et al., 2010; Xu et al., 2009). Another

possible regulator of asynchronous release is synapsin II as deletion of it leads to a decrease in asynchronous GABA release and subsequent increased neuronal and network excitability (Medrihan et al., 2015).

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 AP-driven vesicles 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 syb2 vesicles that respond to stimulation are not essential for spontaneous fusion (Schoch et al., 2001; Deak et al., 2004; see also Figure 5.2). 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 Vti1a (vesicle transport through interaction with t-SNAREs homologue 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 AP-independent vesicle populations.

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 syt1 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 provide specific molecular manipulations that can be used to selectively probe the mechanism and function of spontaneous neurotransmission.

One more area where spontaneous release distinguishes itself from evoked release is in the postsynaptic receptor differentiation. Selective blockade of postsynaptic NMDA and AMPA receptors to spontaneously released neurotransmitters does not affect receptor-mediated responses after evoked release (Atasoy et al., 2008; Sara et al., 2011). This suggests that spontaneous and evoked release activate non-overlapping 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).

Mechanisms of Synaptic Vesicle Endocytosis

The Coupling of Exocytosis and Endocytosis

The coupling of exocytosis and endocytosis is assumed to be calcium dependent but the mechanism is still unknown (Beutner et al., 2001; Schweizer and Ryan, 2006; Hosoi et al., 2009). The first evidence suggesting the importance of calcium in endocytosis, independent of its role in exocytosis comes from α-latrotoxin, a neurotoxin found in black widow spider venom. It triggers calcium independent exocytosis of vesicles through activation of presynaptic receptors (Sudhof et al., 2001). At frog neuromuscular junction in the absence of extracellular calcium, the neurotoxin led to massive exocytosis and depletion of synaptic vesicles (Ceccarelli and Hurlbut, 1980). Endocytosis could not be stimulated without addition of extracellular calcium, suggesting the importance of calcium influx for endocytosis (Henkel and Betz, 1995). Extracellular calcium is required to initiate endocytosis, specifically for the formation of clathrin-coated pits while the other processes are calcium-independent (Gad et al., 1998). The calcium chelator BAPTA, but not EGTA, completely blocks endocytosis suggesting that high intracellular calcium concentrations close to calcium channels is essential for endocytosis (Augustine et al., 2003). Also, experiments with botulinum toxin which arrests exocytosis show that endocytosis proceeds even without exocytosis as long as calcium influx is intact (Neale et al., 1999).

Calcineurin and syt1 have both been proposed as potential calcium sensors for endocytosis. Calcineurin inhibitors have been shown to inhibit calcium dependent endocytosis (Marks and McMahon, 1998; Sun et al., 2010). However, some studies in

hippocampal neurons have shown that there is no requirement for calcineurin in endocytosis and others show that calcineurin slows endocytosis (Sankaranarayanan and Ryan, 2000; Leitz and Kavalali, 2011). Conflicting results on the role of calcineurin in endocytosis may be due to the diversity of preparations but there may even be developmental differences in the calcium sensor (Smillie et al., 2005). The role of syt1 in endocytosisis discussed in detail below.

Calcium channel regulation may also play an important role in endocytosis. Endophilin and AP-2, both essential proteins for endocytosis, are bound to calcium channels at rest and dissociate with increased calcium concentration (Chen et al., 2003; Watanabe et al., 2010). Peptide fragments that disrupt interaction between calcium channel, AP-2 and synaptotagmin have an inhibitory effect on endocytosis. Calcium channels associated with synaptic vesicles may mediate calcium influx after vesicle fusion (Yao et al., 2009; Xue et al., 2012).

Calcium dependent endocytosis may even be lipid dependent. Sphingosine has not only been shown to facilitate exocytosis but also calcium dependent endocytosis independent of dynamin and calmodulin, suggesting a different, more direct endocytic pathway for vesicle recycling (Darios et al., 2009; Rosa et al., 2010). Lipid forces have also been shown to drive massive endocytosis in a calcium dependent manner in baby hamster kidney and HEK293 cells that is also independent of classic endocytic proteins including clathrin, dynamin and calcineurin (Fine et al., 2011; Lariccia et al., 2011).

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 measures of membrane capacitance have provided useful real-time data on endocytosis, its application is limited to large synapses such as the calyx of Held. An optical approach has proved important for visualizing membrane trafficking in small central synapses by using fluorescent membrane dyes and more recently, genetically encoded pH-sensitive fluorescent proteins (Miesenbock et al., 1998). Tagging synaptic vesicle proteins with pH-sensitive GFP 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). It is characterized by well-defined morphological markers such as clathrin coated pits and endosomal intermediates. The 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 require

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 endocytic 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., 2009). A new form of ultrafast endocytosis has recently been characterized with flash-and-freeze electron microscopy and membrane capacitance measures (Watanabe et al., 2013; Delvendahl et al., 2016). This process is observed at physiological temperatures (~37°C) and appears to be mediated by dynamin and actin but is clathrin independent.

Studies using the *Drosophila* temperature sensitive dynamin mutant *shibire*, have provided strong support for a relationship between vesicle recycling and synaptic release during stimulation. Dynamin is a GTPase that serves to pinch vesicles off the membrane during synaptic vesicle endocytosis. A direct comparison of the rate of synaptic depression at *Drosophila* neuromuscular junction from WT and *shibire* mutant flies showed that synapses from *shibire* mutant flies at non-permissive temperatures rapidly depressed without a plateau phase in response to high frequency stimulation. The kinetic difference between this rate of depression and the depression observed with functional dynamin revealed a recycling rate of one to two vesicles per second per active zone (Delgado et al., 2000). Dynamin 1 KO also revealed a similar rapid synaptic depression

due to loss of vesicle endocytosis occurring during synaptic stimulation (Ferguson et al., 2007). Disruption of dynamin SH3 domain interactions (Shupliakov et al., 1997), genetic impairment of synaptojanin 1, an abundant presynaptic molecule which functions as a polyphosphoinositide phosphatase (Cremona et al., 1999; Luthi et al., 2001) or expression of differentially spliced isoforms of synaptotagmin 7 (Virmani et al., 2003) all lead to frequency dependent changes in the rate of short-term synaptic depression. The dephophorylation-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 has been 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 physiological significance of synaptic vesicle reuse during synaptic activity.

Molecular Determinants

Synaptotagmin 1

Synaptotagmins are a family of integral membrane proteins with two calcium binding domains, C2A and C2B. Synaptotagmin 1 (Syt1) is localized to synaptic vesicles with a small N terminal in the lumen of vesicles, a single transmembrane domain and the C2 domains in the cytoplasm (Adolfsen and Littleton, 2001). Synaptotagmin 1 serves as a calcium sensor for 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., 1994). Its function is regulated by Ca^{2+} binding and mediated by interactions with SNARE proteins and membrane lipids (Schiavo et al., 1996; Davis et al., 1999). Ca^{2+} binding to synaptotagmin 1 promotes its interaction with the 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).

Protein and lipid analysis reveals that there are about 15 syt1 molecules on a single vesicle, this high number suggests it is an essential trafficking protein and multiple copies are needed to ensure that sufficient copies are present during repetitive rounds of recycling (Takamori et al., 2006). Microscopy shows that there is minimal lateral diffusion of synaptotagmin after vesicle fusion suggesting that vesicles retain the same cluster of synaptotagmins after exocytosis (Willig et al., 2006). Syt1 molecules oligomerize at high concentrations of calcium (more than 10 μM) and this clustering may also facilitate interaction with AP-2, stonin 2 and subsequent endocytic processes (Chapman et al., 1998).

The calcium binding properties of synaptotagmin make it a key candidate for investigating its role in the regulation of endocytosis. Biochemical experiments demonstrating the interactions between synaptotagmin 1 and clathrin adaptor protein (AP-2) suggesting a role for synaptotagmin in clathrin-mediated endocytosis (Zhang et al., 1994; Haucke and De Camilli, 1999; von Poser et al., 2000). Selective mutagenesis experiments in the calcium binding domains (C2A and C2B) of synaptotagmin 1 suggest that the calcium sensing role of synaptotagmin 1 is important not only for exocytosis, but

also for endocytosis. Mutations of the calcium binding residues in the C2B domain of synaptotagmin 1 slows endocytosis rates to the same extent as in syt1-null Drosophila and was rescued by increasing the extracellular calcium concentration (Poskanzer et al., 2006). Similar mutations that inhibited the calcium binding function of C2A or C2B as well as full deletions of the domains in syt1 KO mouse neurons reveal that the calcium sensing function of either domain is sufficient to restore normal endocytic function (Yao et al., 2012).

A major challenge in studying the function of syt1 is separating the established role of syt1 in exocytosis from its putative involvement in endocytosis. Previous studies have used timed light-activated inactivation of syt1 as well as syt1 mutants to preserve the exocytic function of syt1 and analyze the effect its loss has on endocytosis (Poskanzer et al., 2003; Yao et al., 2012). These experiments have revealed the similar impairments in endocytosis as observed in straightforward synaptotagmin KO experiments (Jorgensen et al., 1995; Nicholson-Tomishima and Ryan, 2004). A mutant form of syt1 that was localized to presynaptic membranes instead of synaptic vesicles exhibited normal exocytic function but impaired endocytosis similar to syt1 loss of function (Yao et al., 2012). Taken together, these findings suggest that syt1 has an important function in endocytosis that is independent of its role in exocytosis and may serve to couple these two processes.

Synaptotagmin 7

Syt7 is localized to the plasma membrane at synapses rather than to synaptic vesicles (Sugita et al., 2001; Virmani et al., 2003). It is less abundant than syt1 (Wilhelm et al., 2004) but exhibits a higher Ca^{2+} affinity than syt1 (Li et al., 1995; Sugita et al., 2002; Wang et al., 2005**).** Syt7 C2 domains also insert more deeply into membranes and are also more sensitive to Ca^{2+} compared to syt1 (Brandt et al., 2012). Syt7 also displays slower lipid-unbinding kinetics compared to syt1 suggesting that it may be better adapted for coordinating delayed rather than rapid synchronous fusion (Hui et al., 2005; Brandt et al., 2012). This is consistent with syt7's ability to interact with SNAP-23, the t-SNARE involved in asynchronous fusion events (Chieregatti et al., 2004; Weber et al., 2014). Syt7 has multiple splice variants that are expressed in a developmentally regulated pattern and differentially modulate vesicle endocytosis (Sugita et al., 2001; Virmani et al., 2003). A short variant syt7 lacking the C2B domain accelerates endocytosis while the regular variant slows endocytosis (Virmani et al., 2003).

In functional studies of syt7, inhibitory cortical neurons from syt7 KO mice did not display consistent deficits in evoked or spontaneous neurotransmission (Maximov et al., 2008). However, in inhibitory hippocampal syt1 KO neurons, which lack synchronous release, asynchronous release was markedly reduced (Bacaj et al., 2013). Additionally, inhibitory spontaneous neurotransmission in wild type and syt1 knockout hippocampal neurons is not affected by syt7 knockdown, but overexpression of syt7 in syt1 knockout neurons reduces spontaneous neurotransmission (Bacaj et al., 2013). These data suggest that syt7 promotes asynchronous evoked release and possibly clamps

spontaneous fusion but these phenotypes are not apparent in neurons expressing an abundance of syt1. Experiments in SNAP-25 KO neurons, show that syt7 acts as a plasma membrane Ca^{2+} sensor by interacting with SNAP-23 (Weber et al., 2014). A recent study suggests that syt7 acts as a slow Ca^{2+} sensor for synaptic facilitation to enhance neurotransmitter release (Jackman et al., 2016).

Vesicular Heterogeneity

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

Clathrin-dependent endocytosis utilizes AP-2 (Di Paolo and De Camilli, 2006; Kim and Ryan, 2009) while AP-3 has been shown to be involved in the regeneration of SVs from synaptic endosomal intermediates (Faúndez et al., 1998; Blumstein et al., 2001; Voglmaier et al., 2006; Glyvuk et al., 2010). Interestingly, loss of AP-3 results in decreased asynchronous release with unaffected localization of syb2 (Scheuber et al., 2006; Evstratova et al., 2014). This suggests VAMP4 may

selectively maintain the asynchronously fusing vesicles which are retrieved through AP-3-dependent bulk endosomes after strong stimulation (Cheung and Cousin, 2012; Nicholson-Fish et al., 2015). My findings presented below and other studies further support the concept that different endocytic mechanisms can generate synaptic vesicles with diverse proteins that can endow these vesicles with distinct fusion properties.

Clinical Relevance

Defects in presynaptic function underlie a wide variety of neurological 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 exo- and endocytosis provides 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 non-synchronous forms of neurotransmitter release are important for 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 message is maintained even postsynaptically as variations in presynaptic release differentially affect receptors and downstream targets (Atasoy et al., 2008; Sara et al, 2011; Autry et al., 2011; Stepanyuk et al., 2014). Similarly, affecting mediators of

endocytosis can change kinetics of subsequent exocytosis. This differential postsynaptic signaling is enabled by presynaptic segregation of vesicle trafficking pathways that mediate spontaneous and synchronous evoked release. 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 of isolation of neurotrophic, homeostatic or other functions of released neurotransmitter substances from their critical role in precise presynaptic action potential driven information transfer.

Changes in the balance between synchronous and asynchronous release have been observed in various models of neurological dysfunction. A significant shift from synchronous to asynchronous release was seen in neurons deficient in amyloid precursor protein (APP), a protein involved in the pathogenesis of Alzheimer's disease (Yang et al., 2007). A similar increase in asynchronous release during repetitive stimulation was also observed in the neuromuscular junctions of a spinal muscular atrophy mouse model (Ruiz et al., 2010). Asynchronous release from cortical interneurons seems to play in tonic inhibition and may a role in epilepsy (Jiang et al., 2012; Medrihan et al., 2015).

Due to its crucial role in synaptic transmission, synaptotagmin 1 has been the focus of many clinical studies. Abnormal levels of synaptotagmin expression have been observed in patients with Alzeimer's disease and schizophrenia (Davidsson et al., 1996; Sokolov et al., 2000; Reddy et al., 2005; Glavan et al., 2009). A recent study shows that syt1 interacts with APP and promotes beta-amyloid generation and may play a role in the AD pathophysiology (Gautam et al., 2015). 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., 2013). 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 *C. 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 (Wu et al., 2007; McColl et al., 2003; Vaslin et al., 2007). Recently, a *de novo* human 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 faster endocytosis after stimulation.

CHAPTER TWO

VAMP4 SELECTIVELY MAINTAINS ASYNCHRONOUS RELEASE

BACKGROUND

SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) protein interactions are largely responsible for vesicle fusion and neurotransmitter release at synaptic terminals. The canonical synaptic SNARE complex composed of synaptobrevin 2 on the synaptic vesicle and syntaxin 1 and SNAP-25, both on the target plasma membrane, mediates rapid exocytosis. Synaptobrevin 2, also known as vesicle associated membrane protein 2 (VAMP2), is the most well characterized vesicle associated SNARE (v-SNARE), as it essential for fast synchronous neurotransmission. Deletion of synaptobrevin 2 eliminates rapid neurotransmitter release but leaves spontaneous and some asynchronous release intact (Schoch et al., 2001; Deak et al., 2004). These findings suggest that additional SNARE proteins must be involved in the remaining neurotransmitter release, possibly by diversifying vesicle populations that drive different modes of transmission. However, the vesicular release machineries that give rise to these auxiliary forms of neurotransmission are poorly understood.

The v-SNARE proteins are of particular interest because they can form distinct SNARE complexes which in turn recruit different associated proteins, such as calcium sensors, to diversify neurotransmitter release patterns. In this chapter, I examined the functional divergence the canonical v-SNARE, syb2, and vesicle associated membrane protein 4 (VAMP4). In contrast to syb2, VAMP4 has a wider subcellular distribution as is implicated in endosomal and trans-Golgi network vesicle trafficking (Steegmaier et al.,

1999; Mallard et al., 2002; Park et al., 2011; Shitara et al., 2013). In this chapter, I investigated how synaptic vesicles tagged with synaptobrevin 2 and VAMP4 were differentially trafficked in response to stimulation using a multicolor optical approach.

METHODS

Lentiviral Infection

Lentiviruses were produced in HEK293 cells (ATCC) by cotransfection of pFUGW transfer vectors and 3 packaging plasmids (pCMV-VSV-G, pMDLg/pRRE, pRSV-Rev) using Fugene 6 transfection reagent (Promega). Cell culture supernatants containing the viruses were harvested 48 hours after transfection and clarified by low speed centrifugation for infection of neurons at 4 DIV. The syb2-mOrange construct was made by replacing the pHluorin tag from syb2-pHluorin with a fluorescent DsRed variant with pH sensitivity (Shaner et al., 2004). VAMP4 was tagged with the classical super ecliptic pHluorin (Miesenbock et al., 1998) at the short luminal domain.

Cell Culture

Dissociated hippocampal cultures from postnatal day 0–3 Sprague-Dawley rats were prepared as previously described (Kavalali et al., 1999; Mozhayeva et al., 2002; Deak et al., 2006). At 4 days *in vitro* (DIV), cultures were infected with lentivirus expressing syb2-pHluorin, syb2-mOrange or VAMP4-pHluorin, and experiments were performed between 14-21 DIV. All experiments were performed following protocols approved by the UT Southwestern Institutional Animal Care and Use Committee.

Fluorescence Imaging

Cultured hippocampal neurons infected with lentivirus expressing syb2-pHluorin, syb2-mOrange or VAMP4-pHluorin were used for the imaging experiments at 14-21 *DIV* at room temperature. A modified Tyrode's solution containing 2 mM Ca^{2+} was used with 10 μM CNQX and 50 μM AP-5 to block recurrent network activity. Images were obtained using a Carl Zeiss LSM 510 META laser-scanning microscope equipped with LSM 510 Laser module (Carl Zeiss). Image acquisition was 1 Hz and cultures were stimulated with field stimulation using parallel platinum electrodes (duration, 1 ms; amplitude, 30 mA). Individual synaptic puncta were identified after NH_4Cl application to visualize the total pool of fluorescent proteins and each fluorescence traces was analyzed.

RESULTS

VAMP4 and syb2 trafficking show limited overlap

To simultaneously examine the trafficking of syb2 and VAMP4 in presynaptic terminals, I used syb2 tagged with mOrange, a red-shifted pHluorin, and VAMP4 tagged with pHluorin. The syb2-mOrange was validated against the well-utilized syb2-pHluorin (Miesenbock et al., 1998) and showed identical trafficking responses to 20 Hz stimulation (Figure 2.1A). In most terminals, the 20-Hz stimulation caused robust endocytic trafficking (internalization) of VAMP4-pHluorin and normal exocytic trafficking of syb2-mOrange (Figure 2.1B). In some terminals, the VAMP4-pHluorin did show an exocytic response (Figure 2.1B, inset), although the endocytic behavior of VAMP4 was predominant. The total amount of VAMP4 in the synaptic terminals was comparable, as determined by the fluorescence change in response to alkalinizing NH_4Cl treatment

administered at the end of each experiment. These findings suggest that VAMP4 traffics independently without dynamic overlap with syb2 present in the same synaptic boutons. Taken together with the robust rescue of evoked neurotransmission in syb2-deficient synapses (Raingo et al., 2012), this suggests that VAMP4 simultaneously traffics to and from the plasma membrane in an activity-dependent manner. In many synapses, the balance between VAMP4 exo- and endocytosis favors a net endocytic fluorescence change in pHluorin-based measurements. Notably, these experiments also indicate that vesicle populations containing VAMP4 and syb2 overlap minimally, as the SNAREs manifest distinct profiles of trafficking within individual presynaptic terminals.

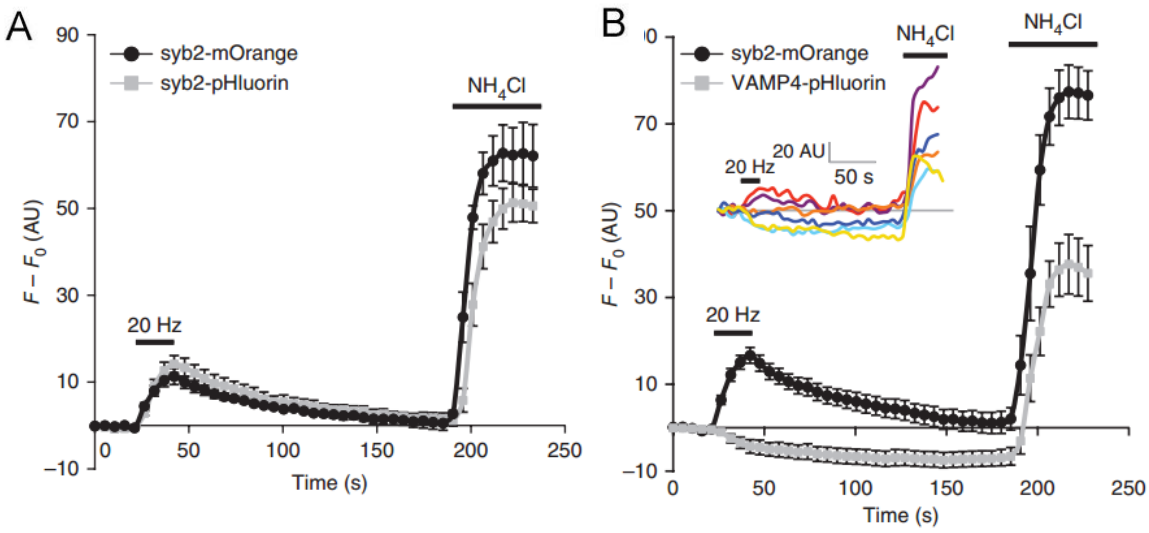

Figure 2.1. Trafficking of VAMP4 at central synapses. (A) Average traces of syb2-mOrange and syb2-pHluorin fluorescence change at 282 individual boutons in response to 400 action potentials at 20 Hz followed by NH4Cl treatment (n = 5 experiments, P > 0.05, difference between groups is not significant). **(B)** Average traces of syb2-mOrange and VAMP4-pHluorin fluorescence change at 798 individual boutons in response to 400 action potentials at 20 Hz followed by NH4Cl treatment (n = 11 experiments, P < 0.05).

Inset, VAMP4-pHluorin individual traces. VAMP4-pHluorin trafficking in response to 400 action potentials at 20 Hz at five different synapses. These traces demonstrate spectrum of stimulation-induced VAMP4-pHluorin trafficking from exo-endocytic or positive to endo-exocytic or negative (reproduced from Figure 6, Raingo et al., 2012).

DISCUSSION

Knockdown of VAMP4 in cultured hippocampal neurons reduces the asynchronous phase of inhibitory postsynaptic currents after strong stimulation and VAMP4-pHluorin tagged vesicles display bulk endocytic activity in response to stimulation (Raingo et al., 2012). These results support a model in which VAMP4 functionally diverges from the canonical v-SNARE syb2 and predominantly maintains asynchronous release. In comparison to the canonical SNARE complex formed by syb2, VAMP4-containing SNARE complexes do not readily interact with key constituents of fast synchronized release machinery, such as complexins and syt1. Therefore, inability of SNARE complexes containing VAMP4 to bind complexins or synaptotagmin1 could underlie the asynchronous nature of release sustained by VAMP4. These findings suggest an alternative pathway involving activity-dependent generation of a synaptic vesicle population enriched in VAMP4. The VAMP4-dependent SNARE complex formed after recruitment of these vesicles can then provide a molecular substrate on which an alternate Ca^{2+} sensor acts to drive asynchronous release. In this way, sustained activity could shift the proportion of vesicles enriched in VAMP4 and desynchronize the kinetics of neurotransmitter release.

Since publication Raingo et al., 2012, more recent studies have shown that VAMP4 is an essential cargo molecule for activity-dependent bulk endocytosis (ADBE) (Nicholson-Fish et al., 2015). After intense neuronal activity, activity-dependent bulk endocytosis (ADBE) is the dominant SV endocytosis mode and there is an enrichment of endogenous VAMP4 in bulk endosomes formed by ADBE. Furthermore, clathrin adaptor protein AP-3 is essential for generating SVs from bulk endosomes (Cheung and Cousin, 2012) and AP-3b2 KO neurons display large deficits in asynchronous release (Evstratova et al., 2014). Thus, ADBE may produce SVs with a distinct VAMP4 enrichment that destines them to specifically maintain a SV pool that mediates asynchronous release.

CHAPTER THREE

ASYNCHRONOUSLY RELEASED VESICLES ARE ENDOCYTOSED SLOWLY

BACKGROUND

During neuronal activity, synaptic nerve terminals release neurotransmitter via synaptic vesicle fusion with the plasma membrane in response to presynaptic action potential (AP) firing and the ensuing Ca^{2+} influx. However, the endocytic pathways that retrieve synaptic vesicles after fusion are poorly understood (Kononenko and Haucke, 2015). The coupling of exo- and endocytosis is assumed to be Ca^{2+} dependent but the mechanism is still unknown (Wu et al., 2014). Importantly, key questions on the exact role of Ca^{2+} and its effectors in regulation of endocytosis remain open as experiments assessing the impact of Ca^{2+} on synaptic vesicle retrieval provided wide ranging and often conflicting results (Leitz and Kavalali, 2015). Under certain conditions and preparations, Ca^{2+} has been shown to slow endocytic retrieval (von Gersdorff and Matthews, 1994; Leitz and Kavalali, 2011), in others, Ca^{2+} has been demonstrated to have a positive effect, facilitating synaptic vesicle retrieval (Marks and McMahon, 1998; Sankaranarayanan and Ryan, 2000; Wu et al., 2009). At the single synaptic vesicle level, Ca^{2+} has been shown to slow retrieval in a manner that is not shared by synaptic vesicles that fuse spontaneously (Leitz and Kavalali, 2014). These seemingly conflicting observations contrast with our more in-depth and precise understanding of the key molecular players that give rise to synaptic vesicle exocytosis (Sudhof, 2013). Nevertheless, it is well-established that the fidelity of synaptic transmission and the

structural homeostasis of nerve terminals rely on the efficient recycling of synaptic vesicles after neurotransmitter release (Kavalali, 2006).

Synaptic vesicle protein synaptotagmin-1 (syt1) acts as a key Ca^{2+} sensor for fast synchronous synaptic vesicle exocytosis but it is also implicated in synaptic vesicle endocytosis (Geppert et al., 1994, Fernandez-Chacon et al., 2001, Sudhof 2004; Sudhof, 2013). Early biochemical experiments have demonstrated that syt1 interacts with clathrin adaptor protein (AP-2) suggesting a role in clathrin-mediated endocytosis and coupling synaptic vesicle fusion to retrieval (Zhang et al., 1994; Haucke and De Camilli, 1999; von Poser et al., 2000). Accordingly, experiments with constitutive syt1 knockout (syt1 KO) neurons revealed slower endocytosis rate compared to wild type (WT) neurons (Nicholson-Tomishima and Ryan, 2004). In the *Drosophila* neuromuscular junction acute inactivation of syt1 function also gave rise to impaired synaptic vesicle endocytosis (Poskanzer et al., 2003). In adrenal chromaffin cells, endocytosis has been shown to switch from being Ca^{2+}-dependent to Ca^{2+}-independent in the absence of syt1 (Yao et al., 2012a). Site-directed mutagenesis experiments in the Ca^{2+} binding domains (C2A and C2B) of syt1 suggest that the Ca^{2+} sensing role of syt1 is critical not only for exocytosis, but also for endocytosis (Poskanzer et al., 2006; Yao et al., 2012b). Mutations of the Ca^{2+} binding residues in the C2B domain of syt1 slow endocytosis rates to the same level as in syt1-null *Drosophila* neuromuscular junctions (Poskanzer et al., 2006). Similar syt1 mutants that inhibited the Ca^{2+} binding function of C2A or C2B also failed to rescue rapid endocytosis in syt1 KO mouse neurons (Yao et al., 2012b). Our results demonstrated that syt1 along with other key synaptic vesicle fusion machinery

components (i.e. complexins and synaptotagmin7) determine the rate of synaptic vesicle endocytosis during repetitive activity.

METHODS

Lentiviral Infection

Lentiviruses were produced in HEK293 cells (ATCC) by contransfection of pFUGW/L307 transfer vectors and 3 packaging plasmids (pCMV-VSV-G, pMDLg/pRRE, pRSV-Rev) using Fugene 6 transfection reagent (Promega). The VGLUT1-pHluorin construct was a generous gift from Drs. R.H. Edwards and S.M. Voglmaier (University of California, San Francisco) (Voglmaier et al., 2006). Syt1, Syt7 and Cpx1/2 lentiviral knockdown and rescue constructs used were generous gifts from Dr. T.C. Südhof (Stanford University) and have been previously validated (Maximov et al., 2009; Xu et al., 2012; Bacaj et al., 2013). Cell culture supernatants containing the viruses were harvested 48 hours after transfection and clarified by low speed centrifugation for infection of neurons.

Cell Culture

Dissociated hippocampal cultures from postnatal day 0–3 Sprague-Dawley rats were prepared as previously described (Kavalali et al., 1999). Neurons were infected with lentiviruses at 4 *DIV* and experiments were performed on 14–21 *DIV* cultures when synapses were mature and lentiviral expression of constructs of interest was optimal (Mozhayeva et al., 2002; Deak et al., 2006). All experiments were performed following

protocols approved by the UT Southwestern Institutional Animal Care and Use Committee.

Western blotting

Western blots to assess knockdown efficacy of syt1, cpx1/2, syt7 were performed as described in Nosyreva and Kavalali (2010). Primary antibodies anti-syt1 mouse monoclonal, anti-syt7 rabbit polyclonal, anti-cpx1/2 rabbit polyclonal, and anti-syb2 mouse monoclonal were used at 1:1000 dilution (Synaptic Systems). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL), captured on autoradiography film and analyzed using ImageJ software. Syt1, Cpx1/2 and Syt7 protein levels were normalized to syb2 loading control.

Electrophysiology

Cultured pyramidal neurons were used between 14 to 21 DIV for experiments. Whole cell recordings were made at -70 mV holding potential using Axopatch 200B and Clampex 8.0 software (Molecular Devices), filtering at 2 kHz and sampling at 5 kHz. The cells were visualized using a Zeiss Axiovert S100 microscope. The internal pipette solution contained 115mM $CsMeSO_3$, 10mM CsCl, 5mM NaCl, 10mM HEPES, 0. mM EGTA, 20mM tetraethylammonium chloride, 4mM Mg-ATP, 0.3mM Na_2GTP and 10mM QX-314 (lidocaine N-ethyl bromide). The final solution was adjusted to pH 7.35 and 300 mOsm. Final resistance of the electrode tips was ~ 3-6 MΩ. For all experiments, the extracellular solution was a modified Tyrode's solution containing 150mM NaCl, 4mM KCl, 10mM glucose, 10mM HEPES, 2mM MgCl and 2mM $CaCl_2$, adjusted to pH 7.4 and 310 mOsm. To isolate IPSCs, postsynaptic ionotropic glutamate receptors

antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma) and aminophosphonopentanoic acid (AP-5; Sigma) were added at concentrations of 10 μM and 50 μM, respectively. To elicit evoked responses, electrical stimulation was delivered through parallel platinum electrodes with a constant current unit (WPI A385) set at 30 mA.

Fluorescence imaging

16–21 DIV cultured pyramidal neurons expressing vGlut1-pHluorin were used for the imaging experiments. The modified Tyrode's solution from above containing 2, 4, 8 mM Ca^{2+} or Sr^{2+} was used with 10 μM CNQX and 50 μM AP-5 to prevent recurrent network activity. Experiments were performed using an Andor iXon+ back-illuminated EMCCD camera (Model no. DU-897E-CSO-#BV) collected on a Nikon Eclipse TE2000-U microscope with a 100X Plan Fluor objective (Nikon). For illumination we used a Lambda-DG4 (Sutter instruments) with a FITC filter. Images were acquired at ~6 Hz with an exposure time of 120 ms and binning of 4 by 4 to optimize the signal-to-noise ratio. Neurons were stimulated using parallel bipolar electrodes (FHC) delivering 30 mA pulses at 20 Hz. Data was collected using Nikon Elements Ar software, 1.5 μm square regions of interest (ROIs) and the resulting fluorescence values were exported to Microsoft Excel for analysis.

Fluorescence analysis

Decay time constants were determined by fitting data with single exponential decay curves using Levenberg-Marquardt least sum of squares minimizations in Clampfit

(Molecular Devices). Linear correction was used for photobleaching during longer 20Hz stimulation experiments.

Statistics

For imaging experiments, n refers to the number of experiments performed with each experiment containing up to 50 regions of interest. Student's t-test (2-tailed, unpaired) was used to analyze all pairwise data sets obtained from synapses under distinct conditions. For analysis of multiple comparisons, two-way ANOVA and one-way ANOVA with Bonferroni post hoc analysis were used.

RESULTS

Syt1 knock down in hippocampal neurons slows multivesicle retrieval after repetitive stimulation

To investigate the role of syt1 in synaptic vesicle endocytosis, we used a previously characterized shRNA knock down (KD) construct to suppress syt1 expression in rat hippocampal neurons (Xu et al., 2012). Under these conditions, using whole cell voltage clamp recordings, we detected a decrease in peak evoked inhibitory postsynaptic current (IPSC) amplitudes in combination with an increase in asynchronous release compared to wild type (WT) neurons (Figure 3.1A–D). Both phenotypes could be rescued by co-expression of a shRNA resistant syt1 construct indicating that they were specific to syt1 loss-of-function (Figure 3.1A-D). Moreover, under the same conditions, we observed an increase in the frequency of spontaneous miniature IPSCs (mIPSCs), which could also be reduced back to WT levels after co-expression of the same shRNA resistant syt1 construct (Figure 5.1). These findings indicate that acute knock down of

syt1 expression completely recapitulates the neurotransmitter release phenotypes previously reported in syt1 KO neurons (Maximov and Sudhof, 2005).

To monitor the properties of synaptic vesicle retrieval, we infected hippocampal neurons with a lentivirus expressing a well-characterized probe where a pH-sensitive GFP is attached to the vesicle lumen region of vesicular glutamate transporter-1 (vGlut1-pHluorin) (Voglmaier et al., 2006), which shows low levels of surface expression and substantially improved signal-to-noise ratio compared other similar probes (Kavalali and Jorgensen, 2014) (Figure 3.1E). When we stimulated vGlut1-pHluorin expressing neurons with 100 APs at 20 Hz, we detected significantly slower decay of fluorescence back to baseline levels — consistent with slowed endocytosis — after syt1 knock down (Figure 3.1F-G). This finding also replicates the slowed endocytosis phenotype previously observed in syt1 KO neurons (Nicholson-Tomishima and Ryan, 2004).

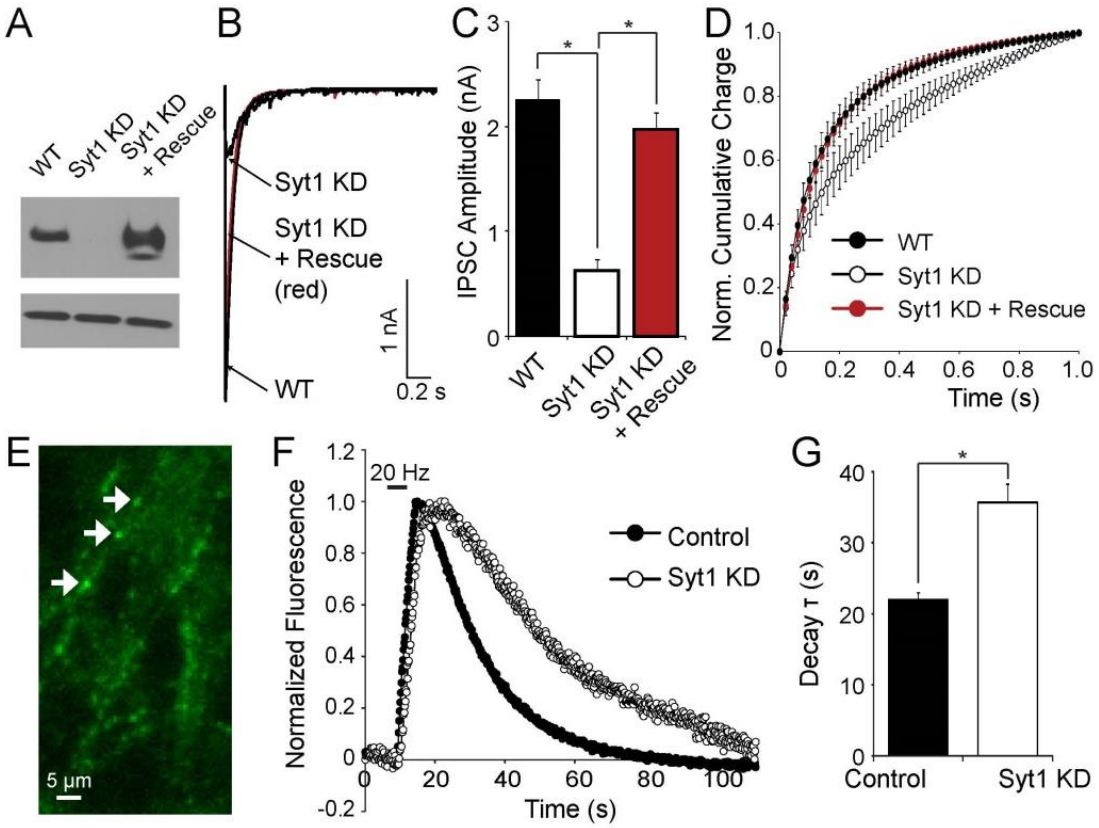

Figure 3.1. Syt1 KD effectively diminishes Ca^{2+}-sensitive synchronous release. (A) Representative immunoblot shows decreased Syt1 protein levels in neurons infected with Syt1 KD shRNA expressing lentivirus and rescue of Syt1 in neurons co-infected with Syt1 KD and shRNA-resistant Syt1 rescue expressing lentivirus. Synaptobrevin is the control below. **(B)** Representative traces of IPSCs recordings evoked by single-pulse stimuli from WT neurons and neurons infected with lentivirus expressing Syt1 shRNA (Syt1 KD) and Syt1 KD with shRNA-resistant Syt1 (Syt1 KD + Rescue). **(C)** Peak amplitudes of single-stimulus evoked IPSCs in WT (n=17), Syt1 KD (n=17) and Syt1 KD rescue (n=14) neurons. Error bars in this and in all other figures indicate SEM and asterisks (*) denote statistical significance. Statistical significance was assessed using one-way ANOVA with Bonferroni correction ($p<0.000001$ for both conditions compared to Syt1 KD). See Figure 5.1 for spontaneous IPSC events. **(D)** Normalized cumulative IPSC charge for 1 s after the stimulus reveals slower peak latency in Syt1 KD neurons consistent with previous experiments from Syt1 KO neurons. Two-way ANOVA without replication shows significant differences between the groups ($p<0.0001$). **(E)** Example frame showing numerous punctate boutons (white arrows) in neurons infected with lentivirus expressing vGlut1-pHluorin. **(F)** Average fluorescence traces of vGlut1-pHluorin in control and Syt1 KD neurons in response to 20Hz stimulation for 5s. **(G)**

Average decay time constants (τ) of the fluorescence return to baseline after 20 Hz stimulation in control (n=299 boutons from 6 coverslips) and Syt1 KD neurons (n= 275 boutons from 7 coverslips). Syt1 KD post-20Hz decay τ was significantly greater than control ($p<0.001$).

Sr^{2+} substitution promotes asynchronous release and slows endocytosis after repetitive activity

Our experiments so far suggest that syt1 has two seemingly opposite functions in the regulation of synaptic vesicle endocytosis. In response to repetitive stimulation, as reported previously, syt1 appears to facilitate synaptic vesicle retrieval, whereas following single vesicle fusion it delays vesicle retrieval in a Ca^{2+}-dependent manner. To resolve this dichotomy we aimed to create a condition where asynchronous release is elevated mimicking the phenotype of syt1 loss-of-function by substituting Sr^{2+} for Ca^{2+}. Sr^{2+} has been widely used as a tool to promote asynchronous release (Dodge et al., 1969), a property that arises from its slower clearance from presynaptic terminals compared to Ca^{2+} (Xu-Friedman and Regehr, 2000) as well as its differential affinity for Ca^{2+} sensors such as syt1 (Shin et al., 2003; Evans et al., 2015). In whole cell recordings, substituting Sr^{2+} for Ca^{2+} resulted in substantial desynchronization of release where neurotransmitter release events returned to their baseline levels with a longer delay after application of 100 APs at 20 Hz (Figure 3.2A-C). Under the same conditions, in optical experiments we detected slower endocytosis after 20 Hz stimulation in a manner similar to our observations after syt1 KD (Figure 3.2D-E).

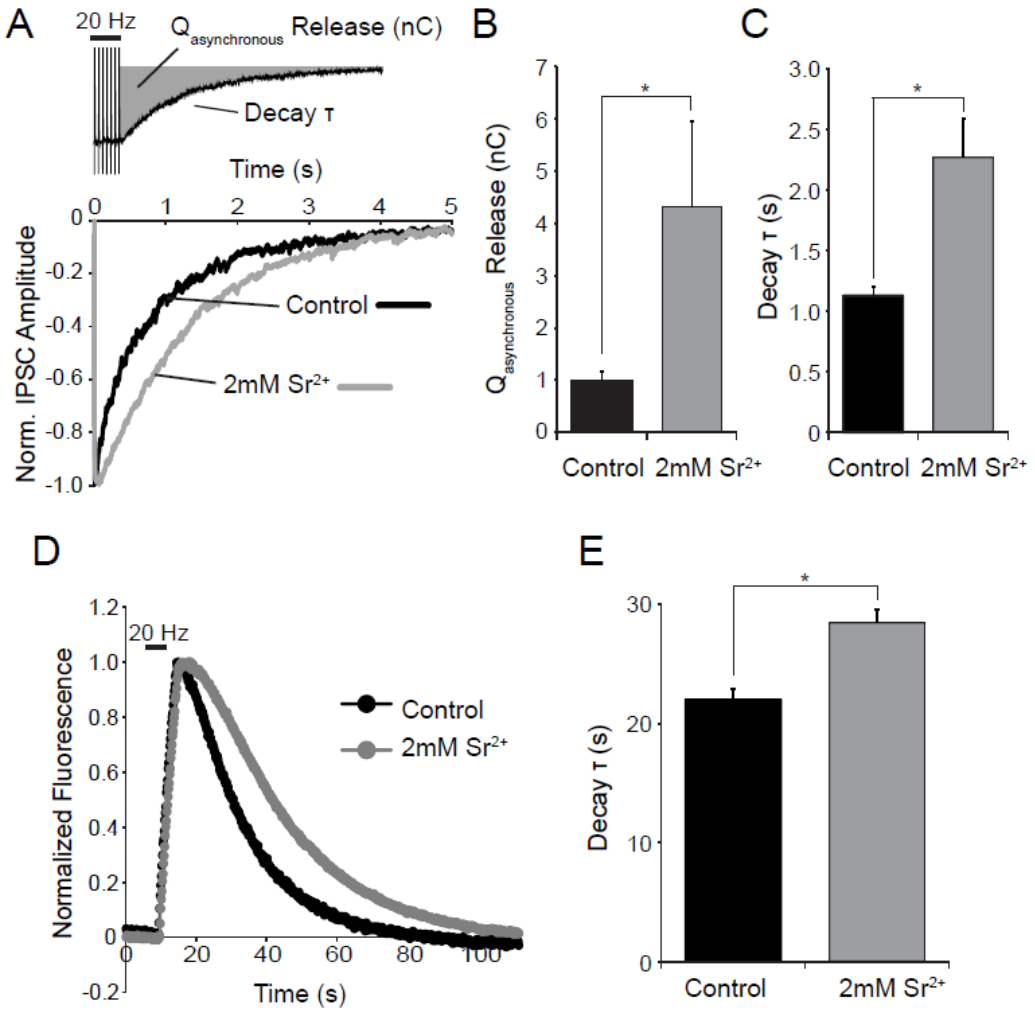

Figure 3.2. Sr^{2+} promotes asynchronous release and slows vesicle retrieval after 20 Hz stimulation. (A) Top, schematic depicting the experimental paradigm and two measures of asynchronous release after 100 APs at 20 Hz. Total asynchronous release charge ($Q_{asynchronous}$ release) was calculated as the area from the onset of the final stimulation to the baseline (gray area). Decay τ from a single-exponential fit quantified the time course of the return to baseline. Bottom, sample IPSC traces for 5 s after the final stimulation in 2 mM Ca^{2+} and 2 mM Sr^{2+}. **(B)** Average $Q_{asynchronous}$ release was significantly increased in 2 mM Sr^{2+} (n=5) compared to 2 mM Ca^{2+} control (n=10) ($p<0.05$). **(C)** Average decay τ of IPSCs back to baseline is significantly increased in 2 mM Sr^{2+} compared to 2 mM Ca^{2+} control ($p<0.0005$). **(D)** Average fluorescence traces of vGlut1-pHluorin in 2 mM Ca^{2+} (control) and 2 mM Sr^{2+} in response to 20Hz stimulation for 5s. **(E)** Average decay τ for 2 mM Sr^{2+} (n=301 boutons from 6 coverslips) was significantly greater than 2 mM Ca^{2+} (control) ($p<0.0005$).

Knock down of complexin 1/2 slows endocytosis after repetitive stimulation

Complexins comprise a family of small proteins that bind to soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes (McMahon et al., 1995; Tang et al., 2006) and as such they promote synaptic vesicle priming for fusion and cooperate with syt1 in Ca^{2+} triggering of fusion (Reim et al., 2001; Maximov et al., 2009). Loss-of-function of complexin-1 and -2 — the most abundant complexin subtypes in the CNS — in hippocampal neurons results in an impairment in synchronous release (Reim et al., 2001) and an increase in asynchronous release (Maximov et al., 2009). To evaluate the impact of complexin loss-of-function on synaptic vesicle endocytosis, we used previously characterized shRNA constructs that elicit knock-down of both complexin1 and complexin2 (Maximov et al., 2009; Yang et al., 2013). In agreement with earlier work, whole cell voltage clamp recordings from neurons infected with the complexin1-2 (Cpx1/2) KD construct revealed enhancement of asynchronous release after 100AP-20Hz stimulation (Figure 3.3A-C) (Maximov et al., 2009). Under the same conditions, optical recordings revealed a dramatic slowdown in fluorescence decay time course after repetitive stimulation indicating a decrease in the rate of synaptic vesicle retrieval (Figure 3.3D-E).

Taken together, our results so far suggest a strong link between the mechanism of fusion and the kinetics of subsequent endocytosis. Vesicles released asynchronously (or in a desynchronized manner) during repetitive stimulation after syt1 loss-of-function, Sr^{2+} substitution or complexin loss-of-function are retrieved far slower with a time course that

outlasts the delayed fusion kinetics of asynchronous release. In the next set of experiments, we investigated the role of synaptotagmin7 (syt7) which is an alternative Ca^{2+} sensor that binds Ca^{2+} with higher affinity (Sugita et al., 2002) in the regulation of endocytosis.

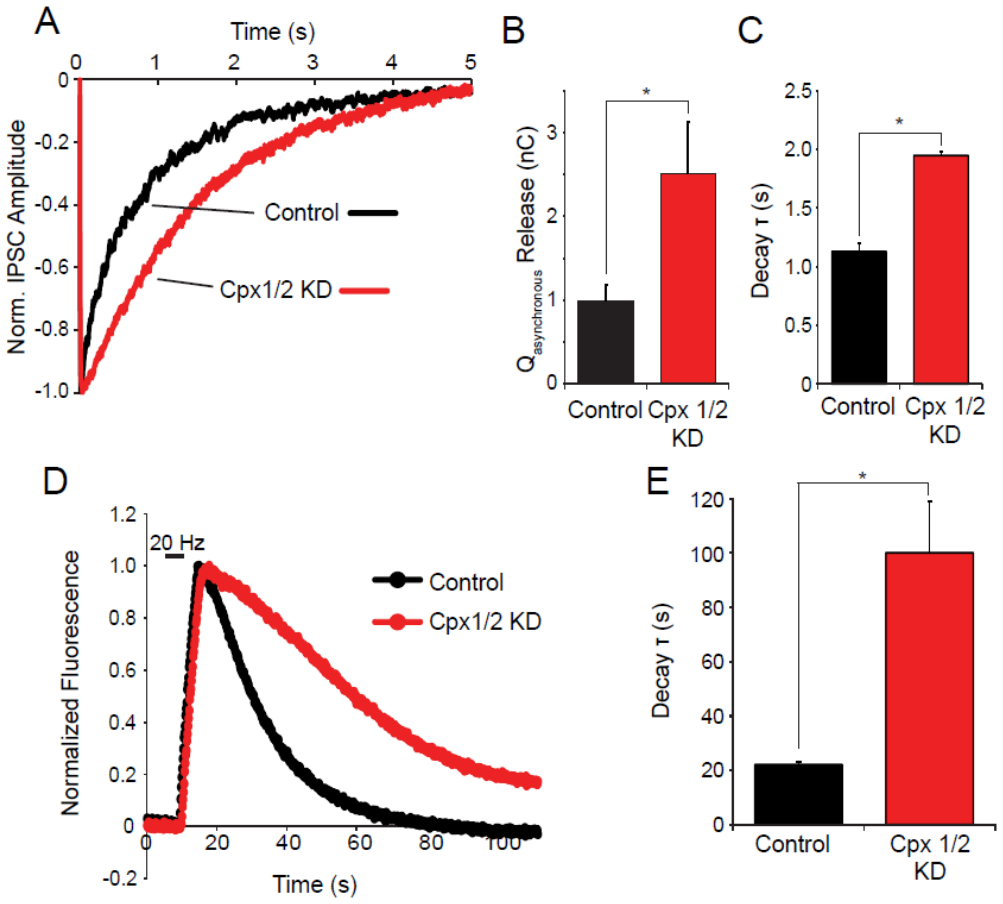

Figure 3.3. Cpx1/2 KD slows vesicle retrieval after 20 Hz stimulation but minimally affect single-vesicle retrieval times. (A) Sample IPSC traces for 5 s after the final stimulation in control and Cpx1/2 KD neurons (same experimental paradigm and analysis as explained in Figure 3A). **(B)** Average $Q_{asynchronous}$ release was significantly increased in Cpx1/2 KD neurons (n=7) compared to WT control (n=10) ($p<0.05$). **(C)** Average decay τ of IPSCs back to baseline is significantly increased in Cpx1/2 KD compared to WT

control ($p<0.05$). **(D)** Average fluorescence traces of vGlut1-pHluorin in control and Cpx1/2 KD neurons in response to 20Hz stimulation for 5s. **(E)** Average decay τ for Cpx1/2 KD (n=242 boutons from 6 coverlips) was significantly greater than control ($p<0.0005$).

Synaptotagmin7 supports asynchronous release and slows vesicle retrieval during repetitive activity

Syt7 has recently emerged as a key Ca^{2+}-sensing synaptic protein that maintains asynchronous neurotransmitter release independent of syt1 (Wen et al., 2010; Bacaj et al., 2013, Jackman et al., 2016). In addition, earlier work from our group has shown that the full-length splice variant of syt7 that retains both C2-domains decelerates recycling and preferentially targets synaptic vesicles to a slow recycling pathway (Virmani et al., 2003). To test the premise that syt7 may play a role in controlling vesicle retrieval after repetitive stimulation mediated asynchronous release, we suppressed levels of Syt7 expression during syt1 KD. In electrophysiological experiments, we detected a substantial reduction in asynchronous release (typically seen after syt1 loss-of-function), replicating earlier findings (Figure 3.4A-B) (Bacaj et al., 2013). Importantly, under the same conditions after syt1 loss-of-function, optical experiments revealed a marked facilitation of endocytic retrieval following syt7KD (Figure 3.4C-D) apparently rescuing the impact of syt1 deficiency on vesicle retrieval. Conversely, Syt7 overexpression slowed endocytosis compared to controls (Figure 3.4E-F) corroborating our earlier findings that syt7 indeed has a bona fide role in slowing endocytic retrieval during activity (Virmani et al., 2003). Interestingly, Syt7 KD alone did not appear to

significantly alter endocytosis compared to controls, suggesting that in WT conditions syt1 has a dominant role in regulation of endocytosis (data not shown).

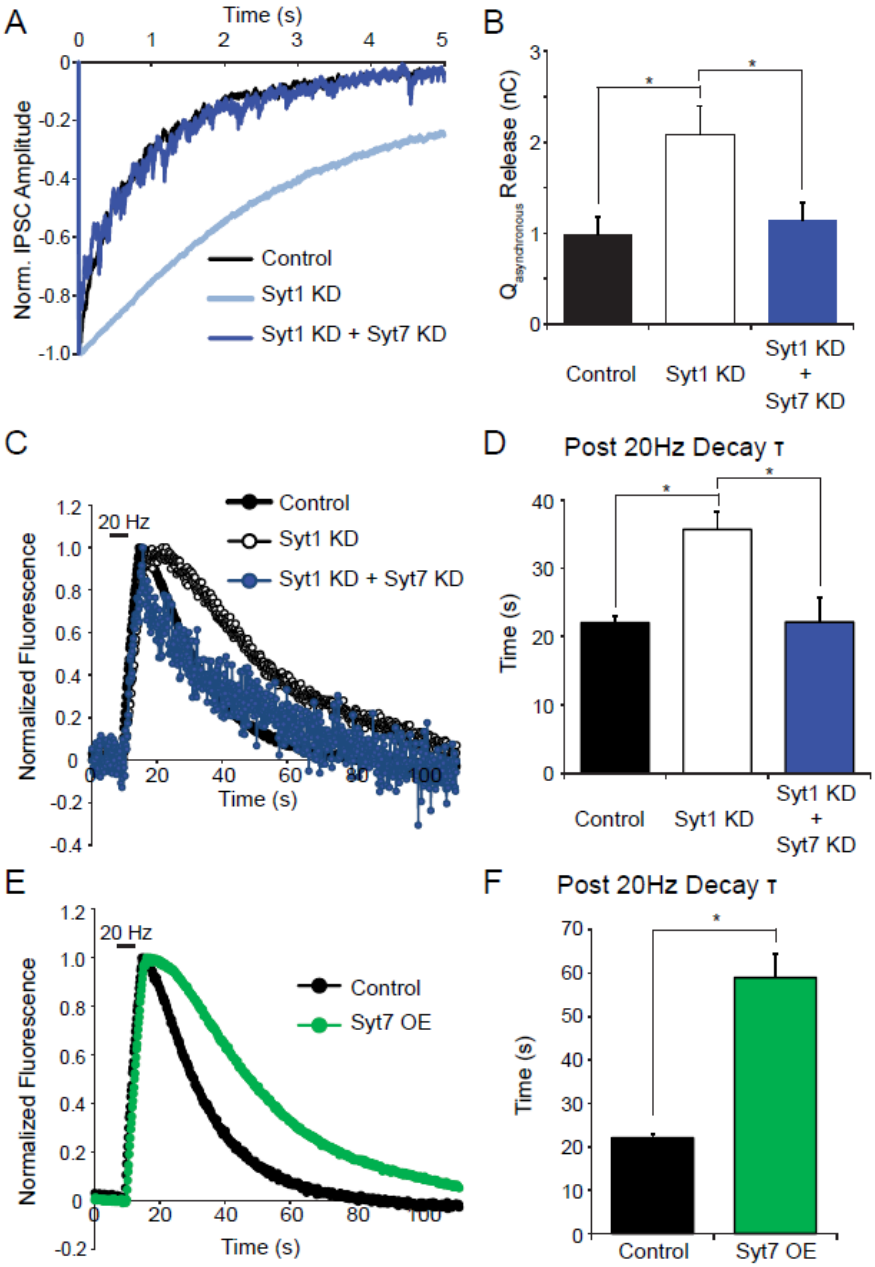

Figure 3.4. Syt7 KD rescues faster synaptic vesicle retrieval rates in Syt1 KD background. (A) Sample IPSC traces for 5 s after the final stimulation in control, Syt1 KD and Syt1 + Syt7 DKD neurons (same experimental paradigm and analysis as explained in Figure 3A). **(B)** Average $Q_{asynchronous}$ release for WT control (n=10), Syt1 KD (n=8) and Syt1 + Syt7 DKD (n=8) neurons. One-way ANOVA with Bonferroni correction ($p<0.05$ for both conditions compared to Syt1 KD) shows that Syt7 KD rescues synchronous release in the Syt1 KD background. **(C)** Average fluorescence traces of vGlut1-pHluorin in control, Syt1 KD and Syt1+Syt7 DKD neurons in response to 20Hz stimulation for 5s. **(D)** Average decay τ of the fluorescence return to baseline after 20 Hz stimulation in Syt1 + Syt7 DKD (n=393 boutons over 11 experiments) neurons is significantly decreased ($p<0.05$) compared to Syt1 KD neurons. **(E)** Average fluorescence traces of vGlut1-pHluorin in control and Syt7 OE neurons in response to 20Hz stimulation for 5s. **(F)** Average decay τ of the fluorescence return to baseline after 20 Hz stimulation in Syt1 + Syt7 DKD (n=393 boutons over 11 experiments) neurons is significantly decreased ($p<0.05$) compared to Syt1 KD neurons.

DISCUSSION

Synaptic vesicle retrieval after asynchronous release

These results demonstrated that the synaptic vesicle fusion machinery comprised of syt1, complexins as well as syt7, while dictating the timing and Ca^{2+}-dependence of neurotransmitter release, also exerts a strong influence on the properties of synaptic vesicle endocytosis. Our optical experiments — based on monitoring the trafficking of vGlut1-pHluorin tagged synaptic vesicles — replicated earlier findings and demonstrated that after repetitive stimulation syt1 facilitates the pace of synaptic vesicle retrieval (Poskanzer et al., 2003; Balaji and Ryan, 2007; Yao et al., 2012b). However, our additional experiments showed that the same "slowed endocytosis" phenotype can also be triggered by Sr^{2+} substitution as well as complexin loss-of-function, all manipulations that promote asynchronous release, thus suggesting a link between desynchronization of neurotransmitter release and the subsequent rate of synaptic vesicle retrieval. This

putative link between asynchronous release and slowed endocytosis was supported by the subsequent experiments demonstrating the role of the asynchronous release Ca^{2+} sensor syt7 in endocytosis. These experiments revealed that the slowed vesicle retrieval seen after repetitive stimulation is influenced by syt7 as syt7 KD resulted in rescue of fast endocytosis impaired after syt1 loss-of-function. In agreement with this observation, syt7 overexpression substantially slowed endocytosis consistent with the earlier proposal that syt7 selectively targets synaptic vesicles to a slow endocytic pathway (von Poser et al. 2000; Virmani et al., 2003). At the ultrastructural level, the syt7-dependent reduction in endocytosis kinetics was also associated with preferential retrieval of large endocytic structures after strong stimulation (Virmani et al., 2003). Taken together, these results highlight the key role of the Ca^{2+}-dependent fusion machinery in dictating the subsequent kinetics and pathway of synaptic vesicle endocytosis where asynchronously released vesicles are selectively targeted to a slower synaptic vesicle trafficking pathway compared to their synchronously released counterparts.

Direct impact of the fusion machinery on the properties of synaptic vesicle retrieval

Taken together, our findings reveal the strong impact of the fusion machinery in dictating the properties of synaptic vesicle retrieval. This premise agrees well with earlier work from our group as well as others which demonstrated the essential role of the vesicular SNARE protein synaptobrevin2 (also called VAMP2) in rapid synaptic vesicle retrieval (Deak et al., 2004; Hosoi et al., 2009; Xu et al., 2013). The impact of synaptobrevin2 on vesicle retrieval may also be shared with the function of target

membrane SNARE SNAP-25 in endocytosis (Xu et al., 2013, but see Bronk et al., 2007). Slowed kinetics of endocytic retrieval seen after synaptobrevin2 and SNAP-25 loss-of-function is consistent with the current observation that synapses deficient in SNARE complex binding proteins complexin1 and 2 also manifest slow endocytosis after repetitive stimulation. These findings indicate that compromising rapid canonical SNARE-mediated fusion machinery — including SNAREs, complexins and syt1— targets synaptic vesicles to an alternative slow endocytic pathway.

The mechanisms that underlie this syt7-driven slow endocytic pathway, however, remain poorly understood. In earlier work, our group has demonstrated that the alternative vesicular SNARE protein VAMP4 can be selectively recruited to synaptic vesicles endocytosed after strong stimulation (Raingo et al., 2012). The subsequent generation of a pool of vesicles enriched in VAMP4 selectively drives asynchronous release as VAMP4-mediated SNARE complexes do not readily interact with syt1 or complexins (Raingo et al., 2012). Interestingly, VAMP4 has recently been shown to be a specific mediator of activity-dependent bulk endocytosis, which is a non-canonical endocytic pathway activated during strong stimulation (Nicholson-Fish et al., 2015). When viewed together with our current findings, these earlier results suggest that impairing the classically defined rapid synaptic fusion machinery or delivering strong stimulation to augment asynchronous neurotransmitter release target synaptic vesicles to these alternative recycling pathways governed by distinct endocytic mediators such as VAMP4 and syt7 (Figure 6.1). These mechanisms may help diversify the molecular compositions of synaptic vesicles regenerated after fusion and provide presynaptic

terminals with a wide range of synaptic vesicle populations with distinct biogenesis properties, fusion propensities. This diversity may in turn help presynaptic terminals respond to extrinsic factors and stimuli selectively to promote specific forms of neurotransmission with dedicated signaling roles (Bal et al., 2013).

CHAPTER FOUR

SYNAPTOTAGMIN 1 SLOWS SINGLE-VESICLE ENDOCYTOSIS

BACKGROUND

A major challenge in studying the function of syt1 in endocytosis is separating its role in exocytosis from its putative involvement in synaptic vesicle retrieval. Most studies to date have relied on strong stimulation to evaluate synaptic vesicle retrieval after multi-vesicular exocytosis (Leitz and Kavalali, 2015). This work, therefore, has been unable to reconcile previously observed differences in the role of Ca^{2+} in synaptic vesicle endocytosis with syt1 function. In particular, it is plausible to expect that syt1's role in endocytosis may diverge after exocytosis of single vesicles versus vesicle retrieval following tandem release of multiple vesicles in rapid succession. In this chapter, I examined single vesicle versus multi-vesicle endocytic events using the improved signal-to-noise characteristics of vGlut1-pHluorin in syt1-deficient neurons to further probe syt1's role in Ca^{2+}-dependent synaptic vesicle recycling. Our results demonstrated that syt1 along with other key synaptic vesicle fusion machinery components (i.e. complexins and synaptotagmin7) determine the rate of synaptic vesicle endocytosis during repetitive activity. In contrast, after single synaptic vesicle fusion, syt1 acts as a key determinant of synaptic vesicle endocytosis time course in response to alterations in Ca^{2+} levels.

METHODS

Lentiviral Infection

Lentiviruses were produced in HEK293 cells (ATCC) by contransfection of pFUGW/L307 transfer vectors and 3 packaging plasmids (pCMV-VSV-G, pMDLg/pRRE, pRSV-Rev) using Fugene 6 transfection reagent (Promega). The VGLUT1-pHluorin construct was a generous gift from Drs. R.H. Edwards and S.M. Voglmaier (University of California, San Francisco) (Voglmaier et al., 2006). Syt1, Syt7 and Cpx1/2 lentiviral knockdown and rescue constructs used were generous gifts from Dr. T.C. Südhof (Stanford University) and have been previously validated (Maximov et al., 2009; Xu et al., 2012; Bacaj et al., 2013). Cell culture supernatants containing the viruses were harvested 48 hours after transfection and clarified by low speed centrifugation for infection of neurons.

Cell Culture

Dissociated hippocampal cultures from postnatal day 0–3 Sprague-Dawley rats were prepared as previously described (Kavalali et al., 1999). Neurons were infected with lentiviruses at 4 *DIV* and experiments were performed on 14–21 *DIV* cultures when synapses were mature and lentiviral expression of constructs of interest was optimal (Mozhayeva et al., 2002; Deak et al., 2006). All experiments were performed following protocols approved by the UT Southwestern Institutional Animal Care and Use Committee.

Fluorescence imaging

16–21 DIV cultured pyramidal neurons expressing vGlut1-pHluorin were used for the imaging experiments. The modified Tyrode's solution from above containing 2, 4, 8 mM Ca^{2+} or Sr^{2+} was used with 10 μM CNQX and 50 μM AP-5 to prevent recurrent network activity. Experiments were performed using an Andor iXon+ back-illuminated EMCCD camera (Model no. DU-897E-CSO-#BV) collected on a Nikon Eclipse TE2000-U microscope with a 100X Plan Fluor objective (Nikon). For illumination we used a Lambda-DG4 (Sutter instruments) with a FITC filter. Images were acquired at ~6 Hz with an exposure time of 120 ms and binning of 4 by 4 to optimize the signal-to-noise ratio. Neurons were stimulated using parallel bipolar electrodes (FHC) delivering 30 mA pulses at 10, 30, 40, 60s intervals, followed by a rest period prior to 100 APs delivered at 20 Hz. Data was collected using Nikon Elements Ar software, 1.5 μm square regions of interest (ROIs) and the resulting fluorescence values were exported to Microsoft Excel for analysis.

Fluorescence analysis

For single-vesicle analysis, successful single vesicle fusion events were determined using specific criteria. Events had to have amplitudes greater than twice the standard deviation of the baseline (average of ~0.5 s prior to the event) and occur during a stimulation time point. The amplitudes also had to be below a cutoff to ensure multi-vesicle events were not included. Decay time constants were determined by fitting data with single exponential decay curves using Levenberg-Marquardt least sum of squares minimizations in Clampfit (Molecular Devices). Dwell times were calculated as the time

between the initial fluorescence step and the start of fluorescence decay predicted by the best fit decay in Clampfit.

Statistics

For imaging experiments, n refers to the number of experiments performed with each experiment containing up to 50 regions of interest. Student's t-test (2-tailed, unpaired) was used to analyze all pairwise data sets obtained from synapses under distinct conditions. The Kolmogorov–Smirnov (K-S) test was used to determine differences in cumulative probability histograms. For analysis of multiple comparisons, two-way ANOVA and one-way ANOVA with Bonferroni post hoc analysis were used.

RESULTS

Syt1 loss-of-function accelerates single synaptic vesicle endocytosis

I aimed to detect single synaptic vesicle retrieval taking advantage of the improved signal-to-noise ratio characteristics of vGlut1-pHluorin (Balaji and Ryan, 2007; Leitz and Kavalali, 2011). For this purpose, we triggered synaptic vesicle fusion at low frequency and detected positive fluorescence fluctuations with amplitudes two standard deviations above the mean baseline noise. In the presence of 2 mM Ca^{2+} in the extracellular medium, each positive fluorescence fluctuation was followed by swift decay back to baseline fluorescence consistent with rapid vesicle re-acidification and retrieval (Figure 4.1A, inset). This setting allowed us to systematically investigate Ca^{2+}-dependence of neurotransmitter release probability (Pr) by counting events at each stimulation as failures (events within two standard deviations of baseline noise) or successes (two standard deviations above the mean baseline noise) (Figure 4.1A, inset)

and calculated the Pr as the ratio of successful events to the total number of stimuli (i.e. trials) applied to a given synapse. Single-vesicle event detection was confirmed by comparing the amplitude changes between evoked and spontaneous events (Figure 4.2A). Under these conditions we detected single evoked fusion events with a median probability of 0.1 (Figure 4.1A, also see Leitz and Kavalali, 2011) indicating that these settings enable visualization of release from single synapses (Murthy et al., 1997). However, knock down of syt1 resulted in a dramatic decrease in Pr by increasing the propensity of failures (Figure 4.1B). Syt-1 loss-of-function also suppressed the Ca^{2+}-dependent increase in Pr as rightward shifts seen in Pr distributions at 4 and 8 mM Ca^{2+} were abolished after syt1 knock down (Figure 4.1C-F). These results agree well with earlier findings on the key role of syt1 in Ca^{2+}-dependent regulation of evoked Pr (Fernandez-Chacon et al., 2001). Moreover, under the same conditions we detected a substantial decrease in the prevalence of high amplitude events seen in elevated extracellular Ca^{2+} concentrations that may reflect multivesicular fusion (Leitz and Kavalali, 2011) (Figure 4.2B-D). This result indicates that syt1 loss-of-function does not only impair Ca^{2+}-dependent increase in Pr but also diminishes multivesicular release.

Analyzing single-vesicle retrieval kinetics in syt1 KD neurons also revealed a previously unknown function of syt1. In earlier work, we have observed a profound increase in the dwell times of single vesicle fusion events in response to increasing Ca^{2+} levels (Leitz and Kavalali, 2011). Dwell times report the residency of vGlut1-pHluorin molecules at the presynaptic surface membrane prior to retrieval and range between <1s at 2 mM Ca^{2+} up to 20s at 8 mM Ca^{2+} (Figure 4.3A-F). This substantive increase in

duration seen at elevated Ca^{2+} concentrations only follows evoked fusion but does not happen during spontaneous synaptic vesicle retrieval (Leitz and Kavalali, 2014). Interestingly, following syt1 loss-of-function, we did not detect this increase in the duration of single vesicle dwell times despite a robust increase in the same parameter in control neurons (Figure 4.3A-F). Importantly, syt1 KD's effect on single vesicle dwell times was also evident at 2 mM Ca^{2+} indicating a key role for syt1 in regulating the residency of synaptic vesicle proteins at the surface membrane after fusion (Figure 4.3G-I). The impact of syt1 KD on Ca^{2+}-dependent regulation of single vesicle dwell times also extended to the fluorescence decay time courses of single vesicle events that are thought to reflect the re-acidification time course of vesicles after retrieval (Figure 4.4). Furthermore, this dramatic effect of syt1 KD on single vesicle retrieval kinetics was indeed specific to syt1 function as it could be rescued by co-expression of a shRNA-resistant syt1 (Figure 4.5).

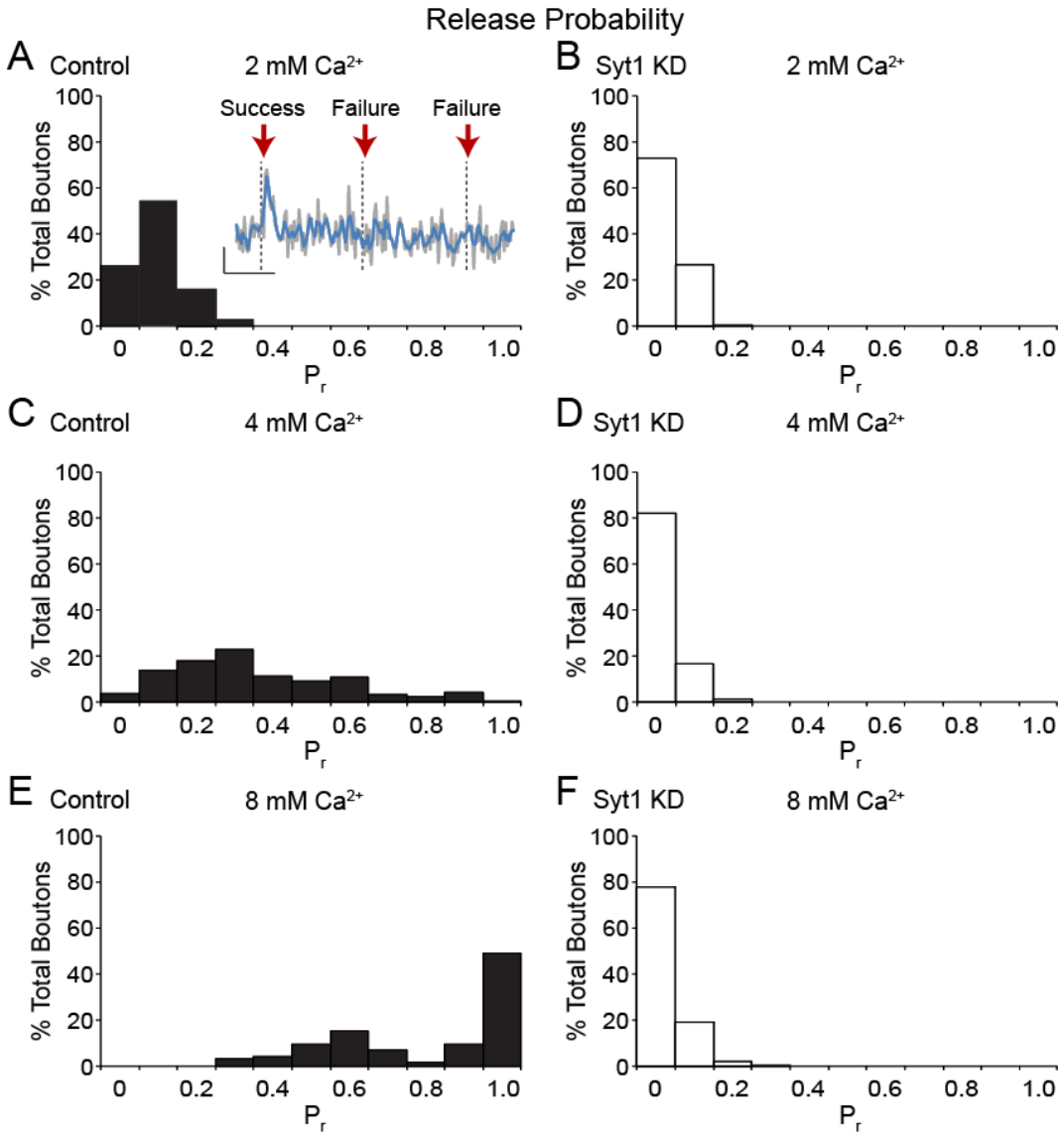

Figure 4.1. Syt1 KD diminishes Ca^{2+} sensitivity of release probability. (A) Histogram of release probability distribution by bouton for control neurons in response to low frequency single-AP stimuli in 2 mM extracellular Ca^{2+}. Inset, example trace showing a successful event and two failures where the dotted lines indicate when stimuli was applied. The raw fluorescence trace is in gray with a moving average of 4 points (~0.5 s) depicted by the blue line. Vertical scale bar represents 200 a.u. while horizontal scale bar is 5 s. **(B)** Release probability distribution for Syt1 KD neurons in 2 mM extracellular Ca^{2+}. **(C)** Release probability distribution for control neurons in 4 mM extracellular Ca^{2+}. **(D)** Release probability distribution for Syt1 KD neurons in 4 mM extracellular Ca^{2+}. **(E)** Release probability distribution for control neurons in 8 mM extracellular Ca^{2+}. **(F)** Release probability distribution for Syt1 KD neurons in 8 mM extracellular Ca^{2+}.

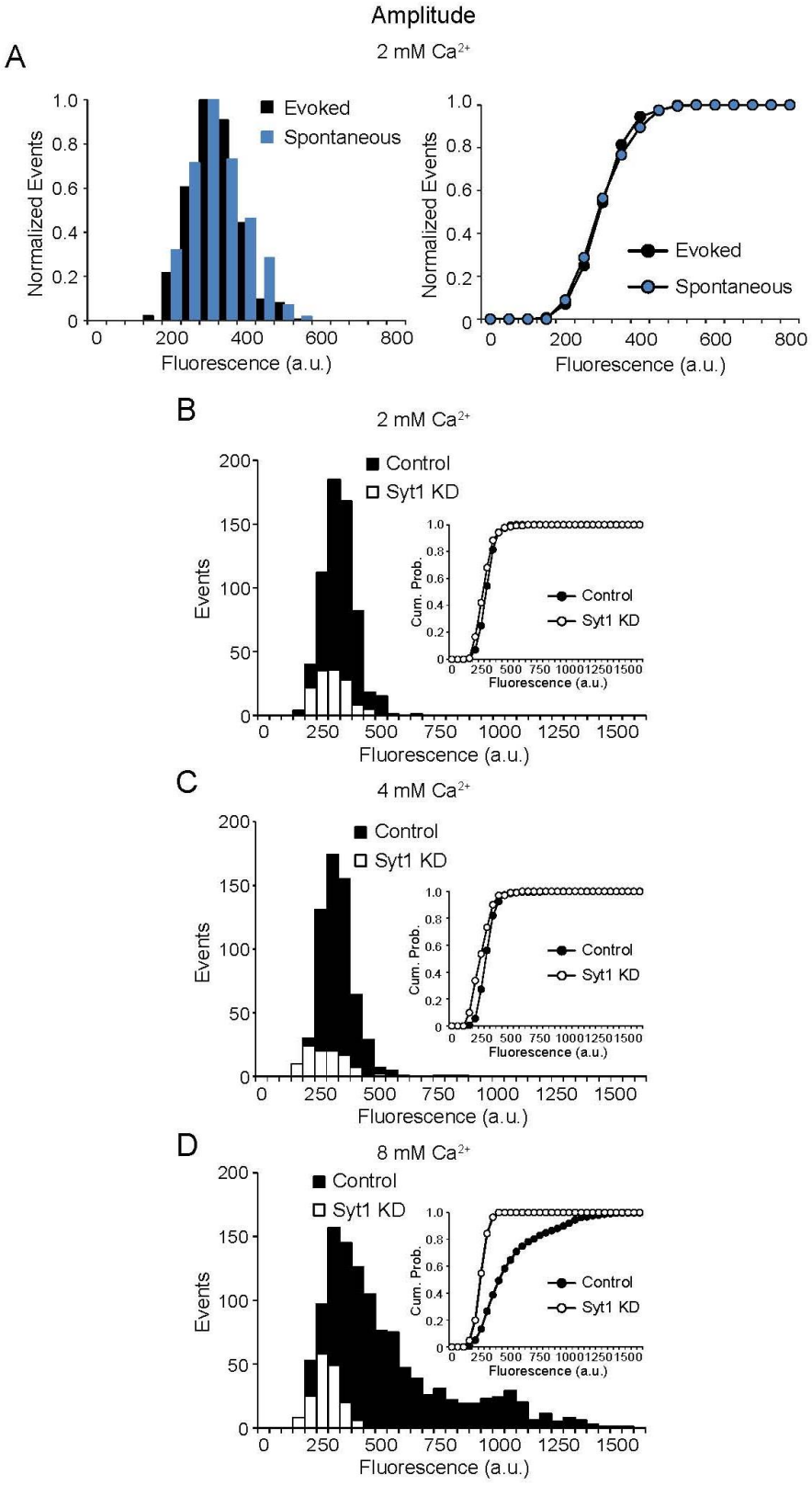
Amplitude
A
2 mM Ca2+
Normalized Events
Evoked
Spontaneous
Fluorescence (a.u.)
B
2 mM Ca2+
Control
Syt1 KD
Events
Cum. Prob.
Fluorescence (a.u.)
C
4 mM Ca2+
D
8 mM Ca2+

Figure 4.2. Syt1 KD minimizes multi-vesicle fusion events in increasing Ca^{2+}. (A) Left, histogram comparing amplitudes of fluorescence changes between single-vesicle evoked events and spontaneous events in control neurons. Right, cumulative probability distribution showing the minimal difference in the amplitudes of evoked and spontaneous events. **(B-D)** Histograms showing the amplitudes of fluorescence changes in response to low frequency single-AP stimuli (0.01–0.2 Hz) in 2, 4 and 8 mM extracellular Ca^{2+} for control and Syt1 KD neurons. Inset, cumulative probability distribution of amplitudes comparing the two conditions. Amplitudes increase with increasing Ca^{2+} consistent with previous findings (Leitz and Kavalali, 2011).

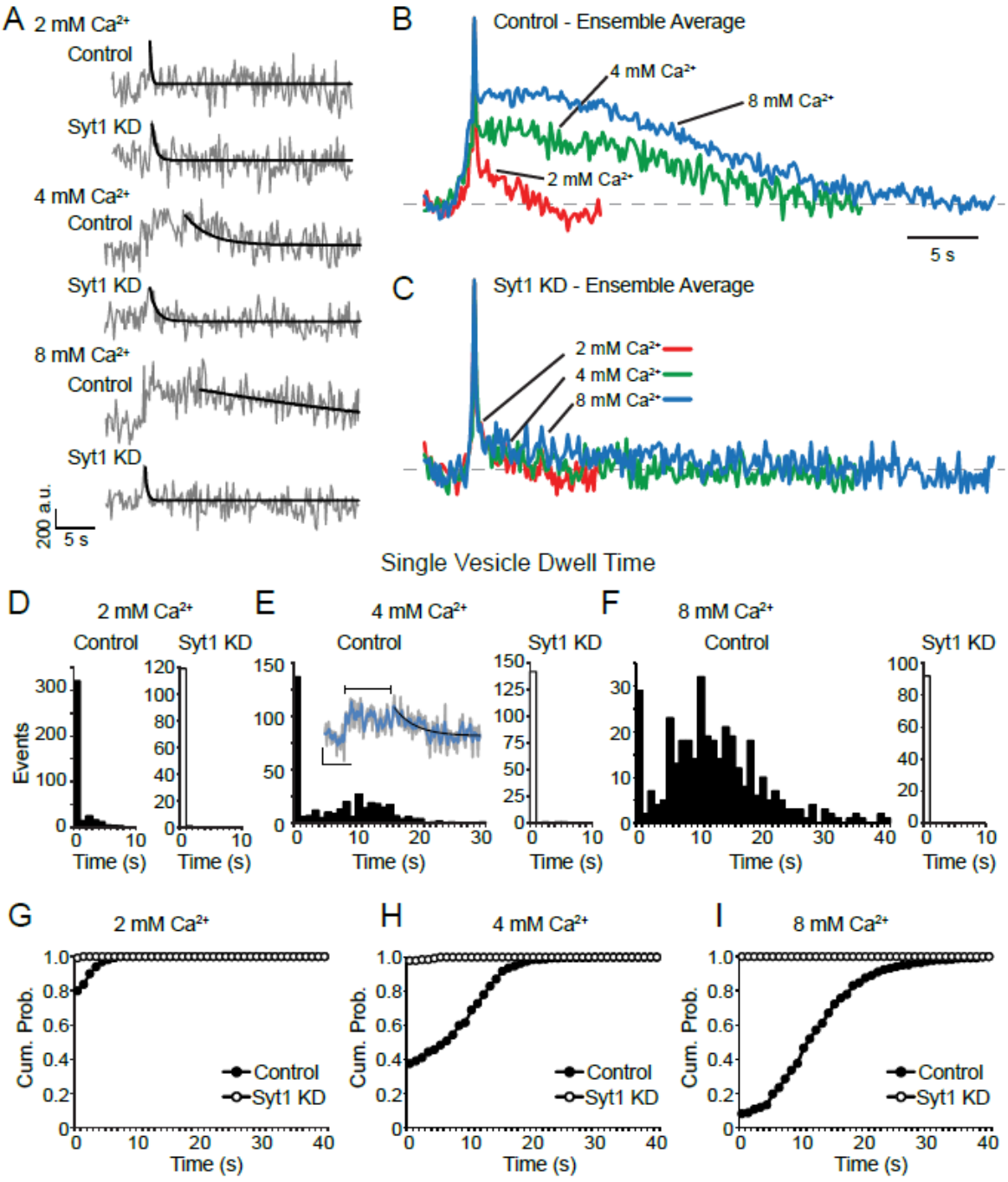
A
2 mM Ca2+
Control
Syt1 KD
4 mM Ca2+
Control
Syt1 KD
8 mM Ca2+
Control
Syt1 KD
200 a.u.
5 s
B
Control - Ensemble Average
4 mM Ca2+
8 mM Ca2+
2 mM Ca2+
5 s
C
Syt1 KD - Ensemble Average
2 mM Ca2+
4 mM Ca2+
8 mM Ca2+
Single Vesicle Dwell Time
D
2 mM Ca2+
Control
Syt1 KD
Events
Time (s)
E
4 mM Ca2+
Control
Syt1 KD
Time (s)
F
8 mM Ca2+
Control
Syt1 KD
Time (s)
G
2 mM Ca2+
Cum. Prob.
Control
Syt1 KD
Time (s)
H
4 mM Ca2+
Cum. Prob.
Control
Syt1 KD
Time (s)
I
8 mM Ca2+
Cum. Prob.
Control
Syt1 KD
Time (s)

Figure 4.3. Syt1 KD accelerates retrieval of single-vesicle fusion events. (A) Sample traces of single-vesicle events observed in control and Syt1 KD neurons in 2, 4 and 8 mM extracellular Ca^{2+}. The gray trace shows raw fluorescence data and the black trace is a single-exponential decay fit. **(B)** Average traces of single-vesicle events in control neurons in 2, 4 and 8 mM extracellular Ca^{2+}. **(C)** Average traces of single-vesicle events in Syt1 KD neurons in 2, 4 and 8 mM extracellular Ca^{2+}.**(D)** Distribution of single-vesicle dwell times from control (left, n=473 events from 7 coverslips) and Syt1 KD (right, n=120 events from 8 coverslips) neurons in 2 mM extracellular Ca^{2+}. **(E)** Distribution of single-vesicle dwell times from control (left, n=365 events from 6 coverslips) and Syt1 KD (right, n=145 events from 18 coverslips) neurons in 4 mM extracellular Ca^{2+}. Inset, example trace showing an event with dwell time marked and a single-exponential fit curve (black) for decay τ calculation (see Figure 4.2). The raw fluorescence trace is in gray with a moving average of 4 points (~0.5 s) depicted by the blue line. Vertical scale bar represents 200 a.u. while horizontal scale bar is 5 s. **(F)** Distribution of single-vesicle dwell times from control (left, n=356 events from 10 coverslips) and Syt1 KD (right, n=124 events from 14 coverslips) neurons in 8 mM extracellular Ca^{2+}. **(G)** Cumulative probability histograms of dwell times from control and Syt1 KD neurons in 2 mM extracellular Ca^{2+} are significantly different ($p<0.05$) according to the K-S test. **(H)** Cumulative probability histograms of dwell times from control and Syt1 KD neurons in 4 mM extracellular Ca^{2+} are significantly different ($p< 0.0001$) according to the K-S test. **(I)** Cumulative probability histograms of dwell times from control and Syt1 KD neurons in 8 mM extracellular Ca^{2+} are significantly different ($p< 0.0001$) according to the K-S test.

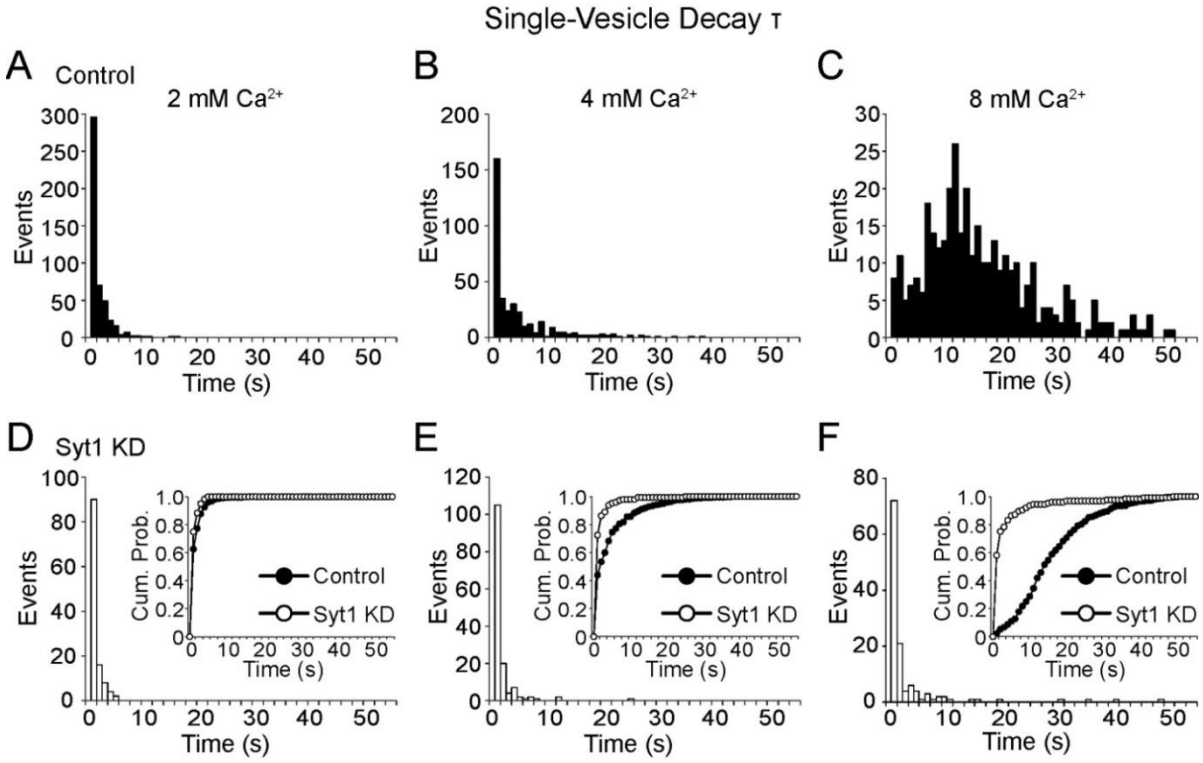

Figure 4.4. Syt1 KD decreases decay τs in increasing Ca^{2+}. (A) Single-exponential decay τs for single-vesicle events in control neurons in 2 mM extracellular Ca^{2+} (n=473 events from 7 coverslips). **(B)** Single-exponential decay τs for single-vesicle events in control neurons in 4 mM extracellular Ca^{2+} (n=365 events from 6 coverslips). **(C)** Single-exponential decay τs for single-vesicle events in control neurons in 8 mM extracellular Ca^{2+} (n=356 events from 10 coverslips). **(D)** Single-vesicle decay τs for Syt1 KD neurons at 2 mM extracellular Ca^{2+} (n=120 events from 8 coverslips). Inset, cumulative probability distribution of decay times from both control and Syt1 KD neurons show a significant difference ($p<0.05$) according to the K-S test. **(E)** Single-vesicle decay τs for Syt1 KD neurons at 4 mM extracellular Ca^{2+} (n=145 events from 18 coverslips). Inset, cumulative probability distribution of decay times from both control and Syt1 KD neurons show a significant difference ($p<0.0001$) according to the K-S test. **(F)** Single-vesicle decay τs for Syt1 KD neurons at 8 mM extracellular Ca^{2+} (n=124 events from 14 coverslips). Inset, cumulative probability distribution of decay times from both control and Syt1 KD neurons show a significant difference ($p<0.0001$) according to the K-S test.

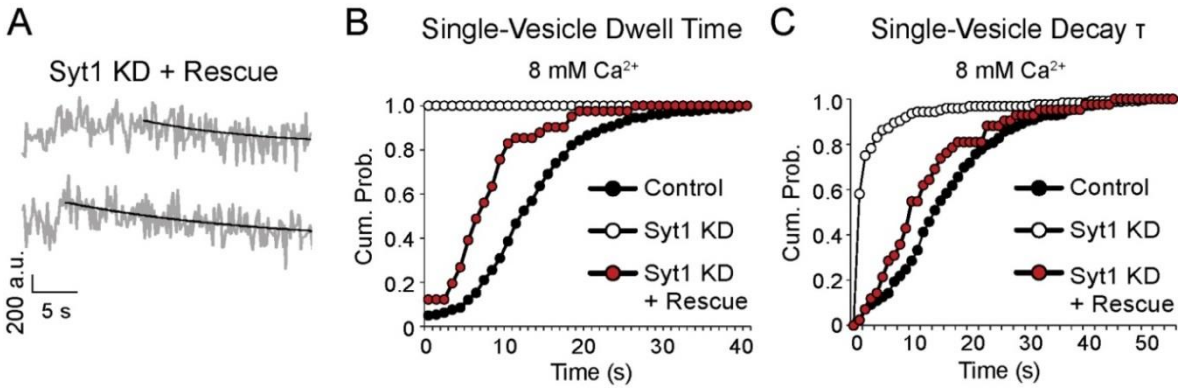

Figure 4.5. Syt1 KD rescue restores single-vesicle dwell time and decay τ. (A) Representative traces of single-vesicle events from Syt1 KD rescue neurons. **(B)** Cumulative probability distributions of single-vesicle dwell time comparing the Syt1 KD rescue to control and Syt1 KD at 8 mM extracellular Ca^{2+}, providing support for the effectiveness and specificity of the Syt1 KD shRNA. **(C)** Cumulative probability distributions of single-vesicle decay τs show Syt1 KD rescue restoring the slowed single-vesicle retrieval times to an intermediate level observed in 8 mM extracellular Ca^{2+}.

Sr^{2+} substitution slows single vesicle retrieval in the same manner as Ca^{2+}

Although substituting Sr^{2+} for Ca^{2+} mimicked the repetitive stimulation-driven slowed endocytosis phenotype seen after syt1 KD, single vesicle events detected during sparse low frequency stimulation (0.01-0.2 Hz) remained susceptible to increasing Sr^{2+} concentrations (Figure 4.6A-C). Here, increasing Sr^{2+}— like Ca^{2+}— prolonged single vesicle retrieval kinetics (Figure 4.6D-F and Figure 4.7). Moreover, this slowdown in single vesicle retrieval kinetics, as in the case of Ca^{2+}, strictly depended on syt1 expression (Figure 4.6G-I). This result demonstrates that both Ca^{2+} and Sr^{2+} interact with syt1 to delay single synaptic vesicle retrieval. However, after repetitive stimulation Sr^{2+} substitution simply mimics syt1 loss-of-function. As indicated above, both syt1 loss-of-

function and Sr^{2+} substitution desynchronizes neurotransmitter release especially during repetitive stimulation. Therefore, the Sr^{2+} mediated uncoupling of the mechanisms of endocytosis after repetitive stimulation versus single vesicle retrieval may indicate that asynchronously released vesicles are preferentially targeted to a slower endocytic pathway. To test this premise, in the next set of experiments, we investigated the impact of alternative means to desynchronize neurotransmission and promote asynchronous release on subsequent endocytosis.

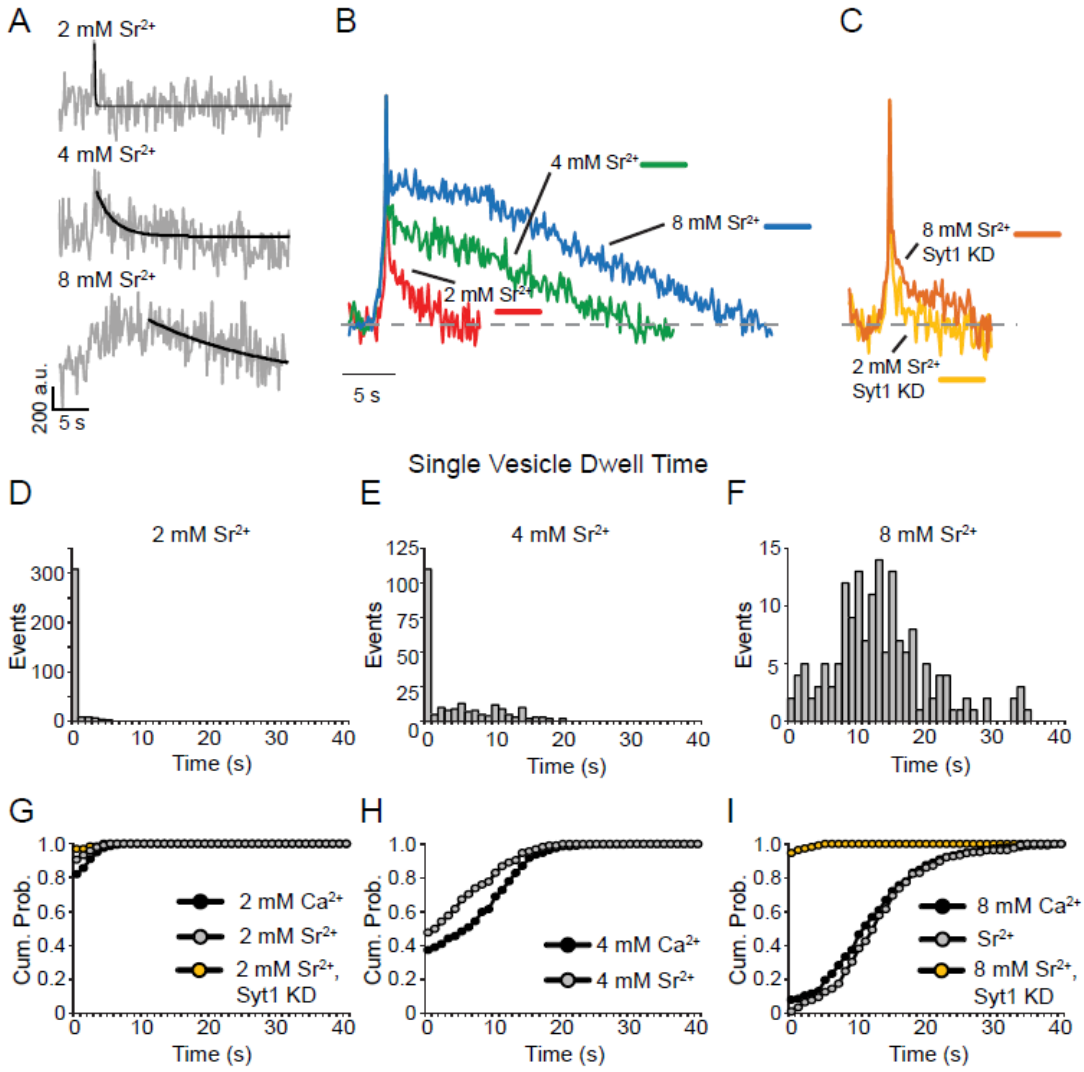

Figure 4.6. Sr^{2+} can substitute for Ca^{2+} in regulation of single-vesicle retrieval kinetics. (A) Sample traces of single-vesicle events observed in neurons in 2, 4 and 8 mM Sr^{2+}. The gray trace shows raw fluorescence data and the black trace is a single-exponential decay fit. **(B)** Average traces of single-vesicle events from neurons in 2, 4, and 8 mM extracellular Sr^{2+}. **(C)** Average traces of single-vesicle events from Syt1 KD neurons in 2 and 8 mM extracellular Sr^{2+}. **(D)** Distribution of single-vesicle dwell times from neurons in 2 mM extracellular Sr^{2+} (n=340 events from 6 coverslips). **(E)** Distribution of single-vesicle dwell times from neurons in 4 mM extracellular Sr^{2+} (n=230 events from 5 coverslips). **(F)** Distribution of single-vesicle dwell times from neurons in 8 mM extracellular Sr^{2+} (n=164 events from 5 coverslips). **(G)** Cumulative probability histograms of single-vesicle dwell times for neurons in 2 mM Ca^{2+} and 2 mM

Sr^{2+} and Syt1 neurons in 2 mM Sr^{2+} (n=65 events from 6 coverslips). No significant difference between conditions according to the K-S test. **(H)** Cumulative probability histograms of single-vesicle dwell times for neurons in 4 mM Ca^{2+} and 4 mM Sr^{2+} show a significant difference ($p<0.0001$) according to the K-S test. **(I)** Cumulative probability histograms of single-vesicle dwell times for neurons in 8 mM Ca^{2+} and 8 mM Sr^{2+} and Syt1 KD neurons in 8 mM Sr^{2+} (n=112 events from 6 coverslips). K-S tests show no significant difference between dwell times in 8 mM Ca^{2+} and 8 mM Sr^{2+} but dwell times in 8 mM Sr^{2+} in Syt1 KD neurons were significantly different from both other conditions ($p<0.0001$).

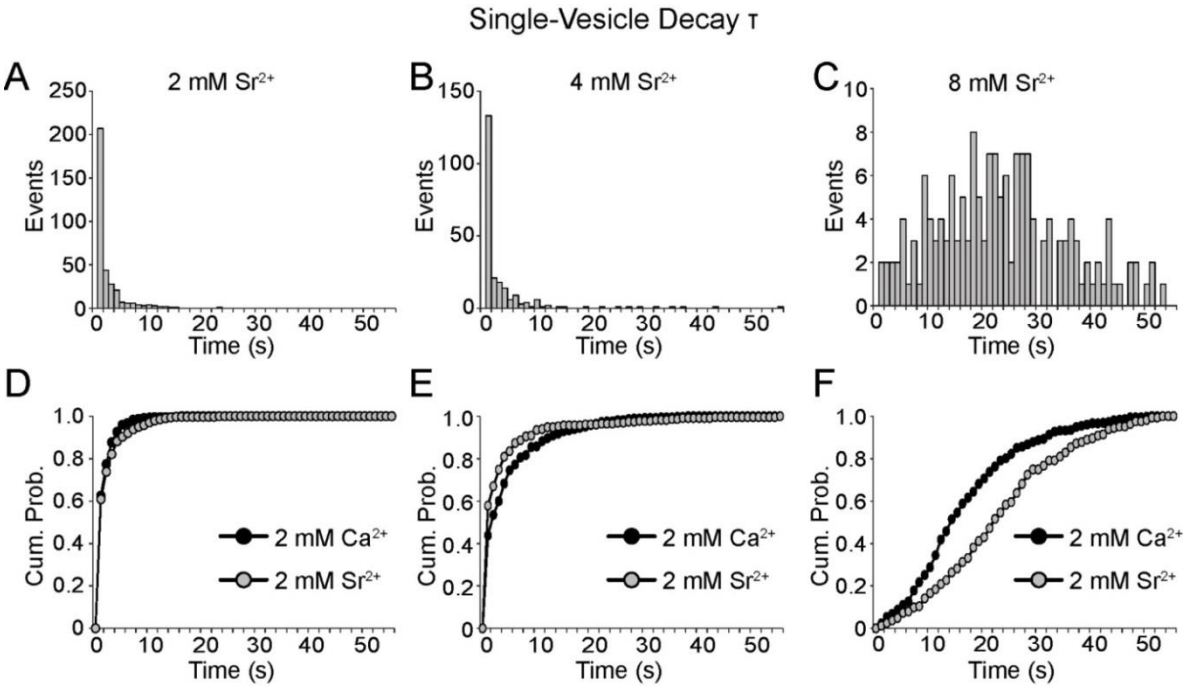

Figure 4.7. Sr^{2+} mimics Ca^{2+} for single-vesicle decay τs. (A) Single-exponential decay τs for single-vesicle events in control neurons in 2 mM extracellular Sr^{2+} (n=340 events from 6 coverslips). **(B)** Single-exponential decay τs for single-vesicle events in control neurons in 4 mM extracellular Sr^{2+} (n=230 events from 5 coverslips).Single-exponential decay τs for single-vesicle events in control neurons in 8 mM extracellular Sr^{2+} (n=164 events from 5 coverslips). **(C)** Cumulative probability distribution of decay times from neurons in 2 mM Ca^{2+} and Sr^{2+} show no significant difference between the conditions according to K-S test. **(D)** Cumulative probability distribution of decay times from neurons in 4 mM Ca^{2+} and Sr^{2+} show no significant difference between the conditions according to K-S test. **(E)** Cumulative probability distribution of decay times from neurons in 8 mM Ca^{2+} and Sr^{2+} show a significant difference ($p<0.0001$) according to the K-S test.

When I examined the impact of complexin loss-of-function on single vesicle retrieval, I detected an increase in single vesicle retrieval time course in response to elevated Ca^{2+} concentrations as in control neurons (Figure 4.8). However, in cumulative histograms of single vesicle dwell times, synapses deficient in complexins compared to control synapses showed a slightly reduced ability to respond to elevated Ca^{2+} concentrations (Figure 4.9). At the single vesicle fusion level, Ca^{2+} and Sr^{2+} elevation could prolong retrieval in a manner that strictly required syt1 but did not show substantial dependence on complexin expression suggesting a key role for syt1 in regulation of single synaptic vesicle retrieval kinetics.

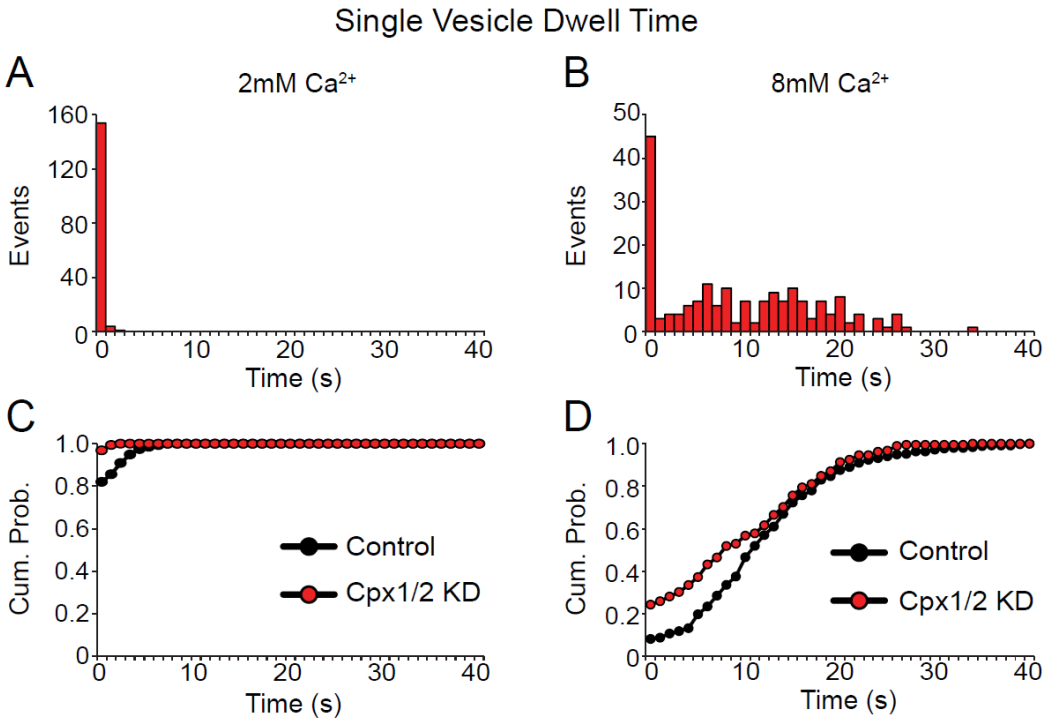

Figure 4.8. Cpx1/2 KD minimally affects single-vesicle retrieval times. (A) Distribution of single-vesicle dwell times from Cpx1/2 KD neurons in 2 mM extracellular Ca^{2+} (n=159 events from 6 coverslips). **(B)** Distribution of single-vesicle dwell times from Cpx1/2 KD neurons in 8 mM extracellular Ca^{2+} (n=185 events from 7 coverslips). **(C)** Cumulative probability histograms of single-vesicle dwell times for control and Cpx1/2 KD neurons in 2 mM Ca^{2+} show a significant difference ($p<0.05$) according to the K-S test. **(D)** Cumulative probability distribution of single-vesicle dwell times for control and Cpx1/2 KD neurons in 8 mM Ca^{2+} show a significant difference ($p<0.0001$) according to the K-S test.

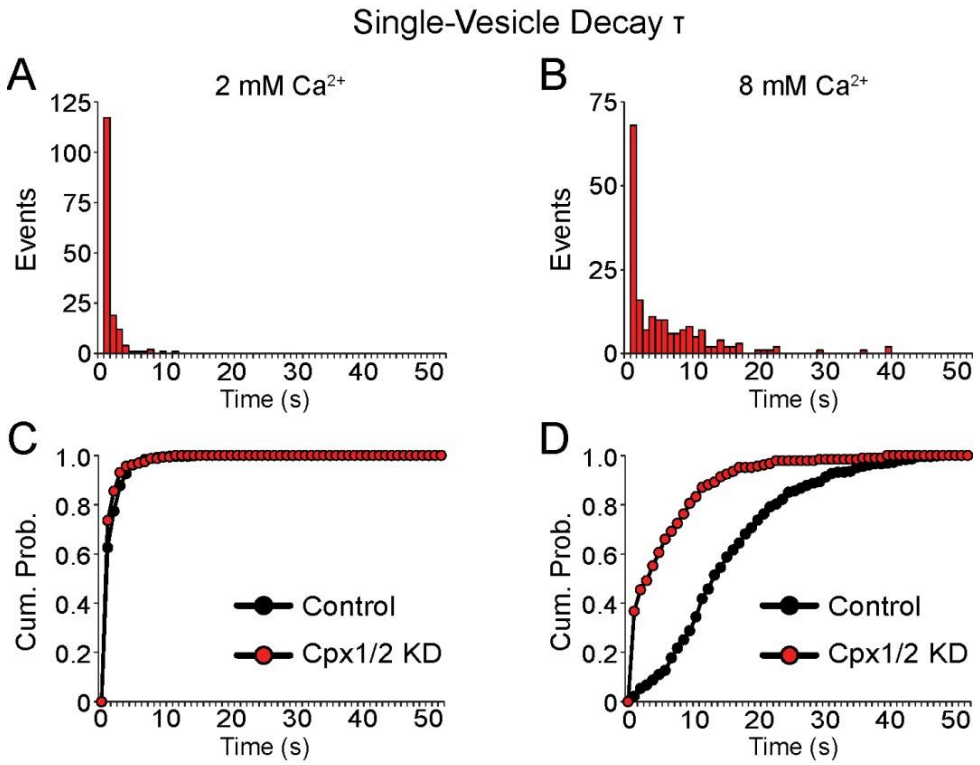

Figure 4.9. Cpx1/2 KD single-vesicle decay kinetics are slowed in high Ca^{2+}. (A) Single-exponential decay τs for single-vesicle events in Cpx1/2 KD neurons in 2 mM extracellular Ca^{2+} (n=159 events from 6 coverslips). **(B)** Single-exponential decay τs for single-vesicle events in Cpx1/2 KD neurons in 8 mM extracellular Ca^{2+} (n=185 events from 7 coverslips).**(C)** Cumulative probability distribution of decay times from control and Cpx1/2 KD neurons in 2 mM Ca^{2+} shows no significant difference according to the K-S test. **(D)** Cumulative probability distribution of decay times from control and Cpx1/2 KD neurons in 8 mM Ca^{2+} show a significant difference ($p<0.0001$) according to the K-S test.

Syt7 KD or syt7 overexpression did not significantly alter the kinetics of single synaptic vesicle retrieval during sparse stimulation (Figure 4.10). This result bolsters the notion that Ca^{2+}-dependent regulation of single synaptic vesicle endocytosis is a genuine function of syt1, whereas multivesicular retrieval after repetitive stimulation may depend on multiple factors besides syt1 such as complexins and syt7.

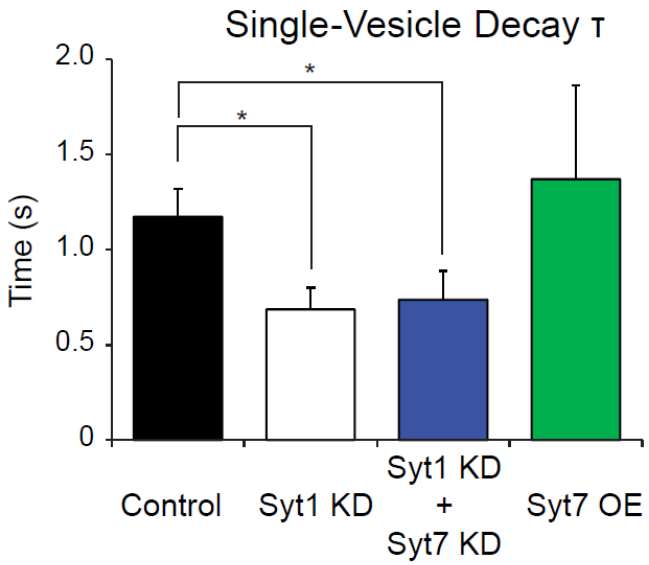

Figure 4.10. Average single-vesicle decay τs for control, Syt1 KD, Syt1+Syt7 DKD, and Syt7 OE neurons. One-way ANOVA with Bonferroni correction shows that events in Syt1 KD and Syt1 + Syt7 KD neurons decay faster than those in control and Syt7 OE neurons ($p<0.005$).

DISCUSSION

Mechanisms underlying the kinetics of single synaptic vesicle retrieval

In contrast to the experiments conducted using repetitive stimulation, single synaptic vesicle retrieval kinetics assessed using sparse low frequency stimulation remained unaffected by syt7 gain- or loss-of-function. In these experiments, properties of single synaptic vesicle retrieval strictly depended on syt1 expression and was relatively unaffected by complexin loss-of-function. Moreover, substituting Sr^{2+} for Ca^{2+} did not result in a significant alteration of single vesicle retrieval properties nor their dependence on syt1. These manipulations provides strong evidence that syt1's genuine function is to slow synaptic vesicle retrieval.

What is the syt1-mediated mechanism that underlies the Ca^{2+}dependent prolongation of single synaptic vesicle retrieval? In elevated baseline Ca^{2+} conditions, syt1 may continue its engagement with its effectors after fusion thus slowing vesicle retrieval. These effectors may either consist of the SNARE complex or membrane lipids (Sudhof, 2013). The preservation of this effect after Sr^{2+} substitution implies that syt1-lipid interactions rather than syt1's engagement with the SNARE complex may determine the time course of single vesicle retrieval as Sr^{2+} — unlike Ca^{2+}— has been shown to be a poor mediator of syt1-SNARE complex interactions (Shin et al., 2003).

CHAPTER FIVE

SPONTANEOUSLY RELEASE VESICLES ARE RAPIDLY ENDOCYTOSED

BACKGROUND

Action potential-independent synaptic vesicle exocytosis or spontaneous release has been a challenge to characterize as its stochastic nature resists manipulation. 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). 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). The endocytic pathway that mediates synaptic vesicle retrieval after spontaneous fusion also appear to be distinct from those that regulate endocytosis after stimulation (Chung et al., 2010; Peng et al., 2012; Meng et al., 2013).

Syt1 has been established as the Ca^{2+} sensor for fast synchronous vesicle fusion but its role in spontaneous fusion is unclear. Loss of syt1 in cultures involving three of more neurons increases spontaneous release while autaptic syt1 KO neurons grown in isolation show no change in spontaneous activity frequency (Geppert et al., 1994; Maximov and Sudhof, 2005; Liu et al., 2009). This may be due to differences in GABAergic inputs that regulate the clamping effect of syt1 (Wierda and Sorensen, 2014). Syt1 may simultaneously act as a Ca^{2+}-dependent activator of evoked release and clamp of spontaneous exocytosis (Xu et al., 2009). In this chapter, I used the well-characterized

vGlut1-pHluorin tag described in Chapter 3 to analyze the trafficking of spontaneous quantal events in a syt1 loss-of-function model.

METHODS

Lentiviral Infection

Lentiviruses were produced in HEK293 cells (ATCC) by contransfection of pFUGW/L307 transfer vectors and 3 packaging plasmids (pCMV-VSV-G, pMDLg/pRRE, pRSV-Rev) using Fugene 6 transfection reagent (Promega). The VGLUT1-pHluorin construct was a generous gift from Drs. R.H. Edwards and S.M. Voglmaier (University of California, San Francisco) (Voglmaier et al., 2006). Syt1 lentiviral knockdown and rescue constructs used were generous gifts from Dr. T.C. Südhof (Stanford University) and have been previously validated (Xu et al., 2012). Cell culture supernatants containing the viruses were harvested 48 hours after transfection and clarified by low speed centrifugation for infection of neurons.

Cell Culture

Dissociated hippocampal cultures from postnatal day 0–3 Sprague-Dawley rats and embryonic day 18-20 syb2 heterozygous mice were prepared as previously described (Kavalali et al., 1999; Schoch et al., 2001). Neurons were infected with lentiviruses at 4 *DIV* and experiments were performed on 14–21 *DIV* cultures when synapses were mature and lentiviral expression of constructs of interest was optimal (Mozhayeva et al., 2002; Deak et al., 2006). All experiments were performed following protocols approved by the UT Southwestern Institutional Animal Care and Use Committee.

Electrophysiology

Cultured pyramidal neurons were used between 14 to 21 DIV for experiments. Whole cell recordings were made at -70 mV holding potential using Axopatch 200B and Clampex 8.0 software (Molecular Devices), filtering at 2 kHz and sampling at 5 kHz. The cells were visualized using a Zeiss Axiovert S100 microscope. The internal pipette solution contained 115mM $CsMeSO_3$, 10mM CsCl, 5mM NaCl, 10mM HEPES, 0. mM EGTA, 20mM tetraethylammonium chloride, 4mM Mg-ATP, 0.3mM Na_2GTP and 10mM QX-314 (lidocaine N-ethyl bromide). The final solution was adjusted to pH 7.35 and 300 mOsm. Final resistance of the electrode tips was ~ 3-6 MΩ. For all experiments, the extracellular solution was a modified Tyrode's solution containing 150mM NaCl, 4mM KCl, 10mM glucose, 10mM HEPES, 2mM MgCl and 2mM $CaCl_2$, adjusted to pH 7.4 and 310 mOsm. To isolate spontaneous IPSCs, 1 μM TTX, postsynaptic ionotropic glutamate receptors antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma) and aminophosphonopentanoic acid (AP-5; Sigma) were added at concentrations of 10 μM and 50 μM, respectively. Mini IPSC events were identified with a 4-pA detection threshold and analyzed with MiniAnalysis.

Fluorescence imaging

16–21 DIV cultured pyramidal neurons expressing vGlut1-pHluorin were used for the imaging experiments. The modified Tyrode's solution from above containing 2 mM Ca^{2+} was used with 10 μM CNQX and 50 μM AP-5 to prevent recurrent network activity. Experiments were performed using an Andor iXon+ back-illuminated EMCCD camera (Model no. DU-897E-CSO-#BV) collected on a Nikon Eclipse TE2000-U microscope with a 100X Plan Fluor objective (Nikon). For illumination we used a Lambda-DG4

(Sutter instruments) with a FITC filter. Images were acquired at ~6 Hz with an exposure time of 120 ms and binning of 4 by 4 to optimize the signal-to-noise ratio. Data was collected using Nikon Elements Ar software, 1.5 µm square regions of interest (ROIs) and the resulting fluorescence values were exported to Microsoft Excel for analysis.

Fluorescence analysis

For single-vesicle analysis, successful single vesicle fusion events were determined using specific criteria. Events had to have amplitudes greater than twice the standard deviation of the baseline (average of ~0.5 s prior to the event) but below a cutoff to ensure multi-vesicle events were not included. Decay time constants were determined by fitting data with single exponential decay curves using Levenberg-Marquardt least sum of squares minimizations in Clampfit (Molecular Devices).

RESULTS

I used the well-characterized shRNA KD construct to suppress syt1 expression in rat hippocampal neurons to investigate the effects of syt1 on spontaneous release. Whole cell voltage clamp recordings show an increase in the frequency of spontaneous miniature excitatory and inhibitory postsynaptic currents in the syt1 KD neurons (Figure 5.1). Co-expression of an shRNA-resistant syt1 construct reduced mini frequency back to WT levels, again confirming the specificity of the syt1 KD. No significant differences were observed in the amplitudes of these spontaneous events, indicating that the effect of the syt1 KD is limited to the presynaptic terminal.

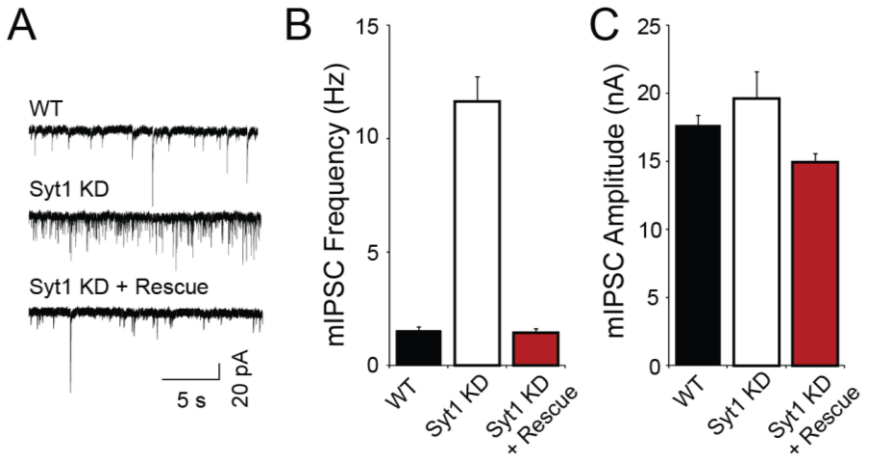

Figure 5.1. Syt1 KD increases miniature event frequency but not amplitude. (A) Representative mIPSC traces from WT, Syt1 KD and Syt1 KD rescue neurons. **(B)** Average frequency of spontaneous events from WT (n=30), Syt1 KD (n=27) and Syt1 rescue (n=25) neurons. Error bars in this and all other figures indicate SEM and asterisks (*) denote statistical significance. Statistical significance was assessed using one-way ANOVA with Bonferroni correction ($p<0.000001$ for both conditions compared to Syt1 KD). **(C)** Average amplitudes of spontaneous events from WT, Syt1 KD and Syt1 KD rescue neurons. Data from mEPSC recordings show the same results.

To determine whether the syt1 KD regulated increase in mini frequency was syb2 dependent, I infected syb2 KO cultured neurons with syt1 KD-expressing lentivirus. The syb2 KO neurons displayed a significantly decreased mini frequency with no change in mini amplitude consistent with previous studies (Schoch et al., 2001; Deak et al., 2006). Syb2 KO and syt1 KD neurons displayed the same significant decrease in mini frequency.

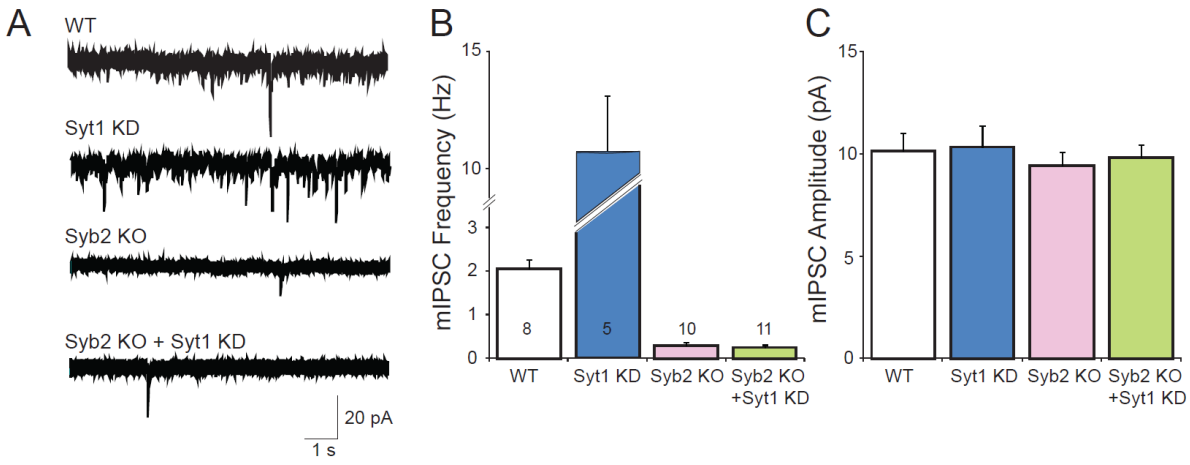

Figure 5.2. Syb2 is required for the increase in miniature event frequency in Syt1 KD neurons. (A) Representative mIPSC traces from WT, Syt1 KD, Syb2 KO and Syb2 KO+ Syt1 KD neurons. **(B)** Average frequency of spontaneous events from WT, Syt1 KD, Syb2 KO and Syb2 KO+ Syt1 KD neurons (n values for each condition within the bars). **(C)** Average amplitudes of spontaneous events from WT, Syt1 KD, Syb2 KO and Syb2 KO+ Syt1 KD neurons. Data from mEPSC recordings show the same results.

Using VGLUT1-pHluorin as a precise vesicular fluorescent tag, I analyzed the presynaptic trafficking of spontaneously fused vesicles in control and syt1 KD neurons. There was no significant difference between the amplitudes of evoked and spontaneous events (Figure 5.3). This validates the single-vesicle data presented above as it confirms the quantal nature of these fluorescent imaging studies.

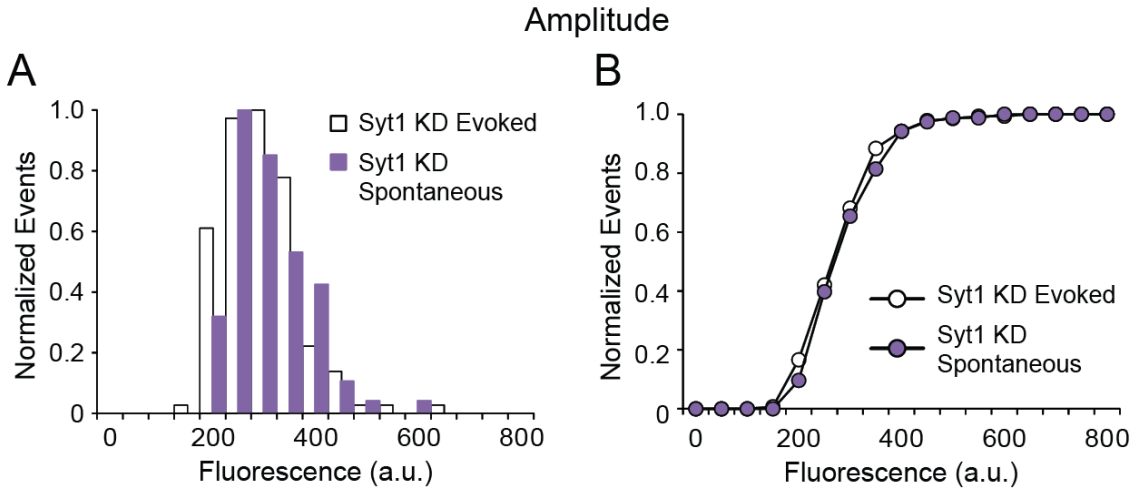

Figure 5.3. Similar fluorescence amplitude changes for evoked and spontaneous events in Syt1 KD neurons. (A) Histogram comparing amplitudes of fluorescence changes between single-vesicle evoked events and spontaneous events in control and syt1 KD neurons in 2 mM Ca^{2+}. **(B)** Cumulative probability distribution showing the minimal difference in the amplitudes of evoked and spontaneous events.

To assess whether there would be a corresponding increase in presynaptic events, I calculated the frequency of spontaneous fusion events at each region of interest (ROI), corresponding to a physiological bouton. The number of events per ROI per minute was higher in the syt1 KD neurons (Figure 5.4), though not as dramatic an increase as the postsynaptic patch clamp data (Figure 5.1).

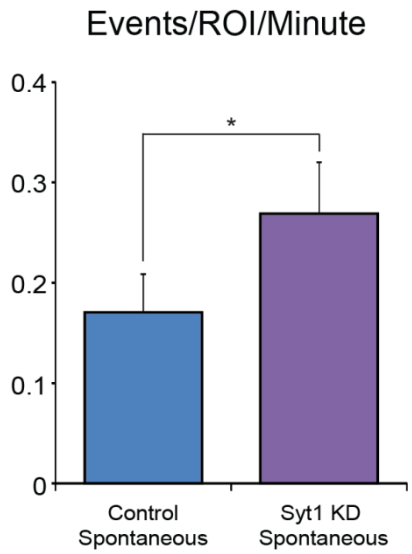

Figure 5.4. Syt1 KD increases the release probability of spontaneous events. The average number of events per ROI per minute for control and syt1 KD neurons (0.17±0.03 and 0.27±0.07, respectively).

To determine whether syt1 affects the endocytosis of spontaneously fused vesicles, I measured decay τs for fluorescence return to baseline in spontaneous events in syt1 KD and control neurons. Analyzing the kinetics of these spontaneous events reveals that syt1 does not appear to affect spontaneous retrieval times (Figure 5.5). As shown in Chapter 4, evoked single-vesicle retrieval is faster in syt1 KD neurons. However, spontaneous events are retrieved even faster as characterized in Leitz and Kavalali, 2014 (<370 ms). These results confirm the rapid retrieval of spontaneously fused vesicles and also show that syt1 does not seem to regulate this endocytosis.

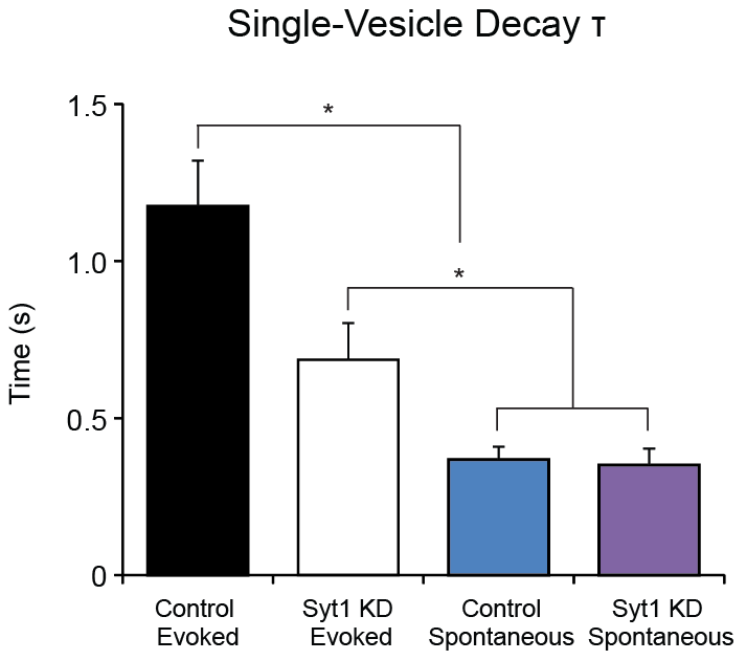

Figure 5.5. Syt1 KD does not alter fluorescence decay τs for spontaneous events. Average decay τs for control evoked (1.17±0.15), syt1 KD evoked (0.69±0.12), control spontaneous (0.37±0.04), and syt1 KD spontaneous events (0.35±0.05). Spontaneous events are retrieved more rapidly than evoked events and syt1 KD does not change retrieval times compared to control.

DISCUSSION

These results suggest that syt1 does not have the same dramatic effect on spontaneous retrieval kinetics as it does on evoked vesicle retrieval rates. Using an optical approach to monitor the trafficking of presynaptic vGlut1-pHluorin, I have recapitulated findings from earlier electrophysiological experiments. There is a small but significant increase in presynaptically monitored spontaneous event frequency in syt1 KD neurons that corresponds to increases in mIPSC and mEPSC frequency measured

postsynaptically. The less dramatic increase I observed optically is probably due to the integration of multiple inputs onto neurons that amplify the changes in mini frequency.

While syt1 has a big effect on the retrieval rates of AP-evoked vesicles as shown in Chapter 4, it does not appear to alter the retrieval rates of vesicles fusing spontaneously. And although evoked vesicles are retrieved more rapidly in syt1 KD neurons, spontaneous vesicles are retrieved even faster. One caveat of these experiments is that the rapid retrieval time course of spontaneously fused vesicles (~360 ms) approaches the limits of the temporal resolution of the imaging setup used. Given the image acquisition rate of 6 Hz, I am not be able to visualize events that decay fast than ~200 ms. We need more advanced equipment to image at a faster rate while maintaining the same spatial resolution in order to more resolutely determine the syt1-independence of endocytosis of spontaneously fusing vesicles. However, these results as they stand now further support the concept of vesicular heterogeneity. The increase in mini frequency in syt1 KD neurons is syb2-dependent but a small fraction of spontaneous activity remains suggesting that alternate mediators of spontaneous fusion exist. Syt1's function endocytosis appears to be limited to vesicles that fuse in response to stimulation while regulation of spontaneously fused vesicles may be due to a yet unidentified molecule.

CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

The results presented here attempt to further our understanding of the mechanisms that underlie exo- and endocytosis coupling. By analyzing endocytosis after different forms of synaptic vesicle fusion, I have shown that the exocytic pathway influences the rate of subsequent endocytosis. A proposed model with the key regulators of exocytosis-determined endocytosis schematically illustrates the segregation of these vesicle populations (Figure 6.1). In synchronous activity, syt1 drives Ca^{2+} triggered synaptic vesicle fusion and facilitates endocytosis. However, at the single-vesicle level syt1 slows vesicle retrieval perhaps in an effort to shunt vesicles towards a faster multi-vesicle pathway. Vesicles that fuse asynchronously are regulated by v-SNARE VAMP4 and Ca^{2+} sensor syt7 and are confined to a slower endocytic pathway. In spontaneous release, syt1 appears to affect the probability of release but does not influence the kinetics of vesicle retrieval. This separation of exo- and endocytic recycling into distinct pathways may be a mechanism for the biogenesis of different heterogeneous pools of vesicles.

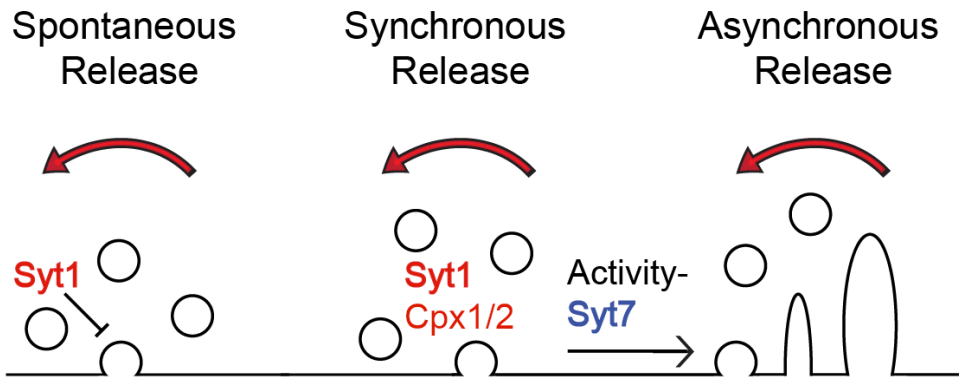

Figure 6.1. Proposed model for exocytic pathway dictated endocytosis. A model emerging from our results suggest that syt1 and complexin-dependent synchronous release is associated with rapid synaptic vesicle recycling pathways. In contrast, promoting asynchronous release via stimulated activity targets synaptic vesicles towards slower recycling pathways in a syt7-dependent manner. The red arrows represent the recycling process of each mode of neurotransmitter release as the exocytic form of fusion dictates the specific endocytic retrieval kinetics to create distinct populations of vesicles.

Molecular determinants of synchronous release have been well-elucidated with the main players being the Ca^{2+} sensor syt1, v-SNARE syb2 and t-SNAREs syntaxin-1 and SNAP-25. The endocytosis following synaptic vesicle fusion through this pathway is presumed to be dependent on AP-2, perhaps through syt1 binding. Asynchronous release regulators are still under debate but recent research supports syt7 as a Ca^{2+} sensor and VAMP4 as a specific v-SNARE. SNAP-23 interacts with syt7 in the absence of predominant t-SNARE SNAP-25. AP-3 may be involved in the re-use of vesicles in the asynchronous pathway (see Table 6.1). Although modulators of spontaneous activity frequency have been identified, absolute determinants of spontaneous fusion are still not clear. More experiments on the functional determinants of vesicle exo- and endocytosis will undoubtedly provide new insight into the molecular mechanisms of synaptic vesicle recycling.

Mode of neurotransmission	**Synchronous**	**Asynchronous**
Ca^{2+} sensor	Syt1	Syt7 (Bacaj et al., 2013; Weber et al., 2014)
v-SNARE	Syb2	VAMP4 (Raingo 2012; Nicholson-Fish et al., 2015)
t-SNARE	SNAP-25	SNAP-23 (Chieregatti et al., 2004; Weber et al., 2014)
Clathrin adaptor	AP-2	AP-3? (Voglmaier 2006; Evstratova et al., 2014)

Table 6.1. Segregation of protein isoforms into divergent functional roles. Proteins in the synchronous release column are well supported with numerous studies (see reviews: Sudhof, 2004; Rizo and Rosenmund, 2008; Sudhof and Rothman, 2009). Those listed under the asynchronous pathway are still being characterized with references to recent supporting studies included. However, the connections between the rows within the asynchronous column have not been established, with the exception of syt7 interactions with SNAP-23 (Chieregatti et al., 2004; Weber et al., 2014). Not enough is known about direct regulators of spontaneous release to populate a third column in the table.

Continued research is needed to further our understanding of the mechanisms that underlie these different forms of synaptic transmission. As we uncover more of the molecular players involved in regulating these various pathways, we will develop a clearer picture of the inner workings of the presynaptic terminal. This will also provide interesting targets for molecular manipulation in experiments as well as future therapeutics for neurological and psychiatric disorders.

BIBLIOGRAPHY

Adolfsen, B., and Littleton, J.T. (2001). Genetic and molecular analysis of the synaptotagmin family. Cell Mol Life Sci *58*, 393-402.

Alabi, A.A., and Tsien, R.W. (2013). Perspectives on kiss-and-run: role in exocytosis, endocytosis, and neurotransmission. Annu Rev Physiol *75*, 393-422.

Ali, A.B., and Todorova, M. (2010). Asynchronous release of GABA via tonic cannabinoid receptor activation at identified interneuron synapses in rat CA1. Eur J Neurosci *31*, 1196-1207.

Atasoy, D., Ertunc, M., Moulder, K.L., Blackwell, J., Chung, C., Su, J., and Kavalali, E.T. (2008). Spontaneous and evoked glutamate release activates two populations of NMDA receptors with limited overlap. J Neurosci *28*, 10151-10166.

Augustine, G.J., Santamaria, F., and Tanaka, K. (2003). Local calcium signaling in neurons. Neuron *40*, 331-346.

Bacaj, T., Wu, D., Yang, X., Morishita, W., Zhou, P., Xu, W., Malenka, R.C., and Sudhof, T.C. (2013). Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release. Neuron *80*, 947-959.

Bai, J., Wang, C.T., Richards, D.A., Jackson, M.B., and Chapman, E.R. (2004). Fusion pore dynamics are regulated by synaptotagmin*t-SNARE interactions. Neuron *41*, 929-942.

Baker, K., Gordon, S.L., Grozeva, D., van Kogelenberg, M., Roberts, N.Y., Pike, M., Blair, E., Hurles, M.E., Chong, W.K., Baldeweg, T., *et al.* (2015). Identification of a human synaptotagmin-1 mutation that perturbs synaptic vesicle cycling. J Clin Invest *125*, 1670-1678.

Bal, M., Leitz, J., Reese, A.L., Ramirez, D.M., Durakoglugil, M., Herz, J., Monteggia, L.M., and Kavalali, E.T. (2013). Reelin mobilizes a VAMP7-dependent synaptic vesicle pool and selectively augments spontaneous neurotransmission. Neuron *80*, 934-946.

Balaji, J., and Ryan, T.A. (2007). Single-vesicle imaging reveals that synaptic vesicle exocytosis and endocytosis are coupled by a single stochastic mode. Proc Natl Acad Sci U S A *104*, 20576-20581.

Barrett, E.F., and Stevens, C.F. (1972). The kinetics of transmitter release at the frog neuromuscular junction. J Physiol *227*, 691-708.

Beutner, D., Voets, T., Neher, E., and Moser, T. (2001). Calcium dependence of exocytosis and endocytosis at the cochlear inner hair cell afferent synapse. Neuron *29*, 681-690.

Blumstein, J., Faundez, V., Nakatsu, F., Saito, T., Ohno, H., and Kelly, R.B. (2001). The neuronal form of adaptor protein-3 is required for synaptic vesicle formation from endosomes. J Neurosci *21*, 8034-8042.

Brandt, D.S., Coffman, M.D., Falke, J.J., and Knight, J.D. (2012). Hydrophobic contributions to the membrane docking of synaptotagmin 7 C2A domain: mechanistic contrast between isoforms 1 and 7. Biochemistry *51*, 7654-7664.

Bronk, P., Deak, F., Wilson, M.C., Liu, X.R., Sudhof, T.C., and Kavalali, E.T. (2007). Differential effects of SNAP-25 deletion on Ca2+-dependent and Ca2+-independent neurotransmission. J Neurophysiol *98*, 794-806.

Brose, N., Petrenko, A.G., Sudhof, T.C., and Jahn, R. (1992). Synaptotagmin: a calcium sensor on the synaptic vesicle surface. Science *256*, 1021-1025.

Ceccarelli, B., and Hurlbut, W.P. (1980). Ca2+-dependent recycling of synaptic vesicles at the frog neuromuscular junction. J Cell Biol *87*, 297-303.

Ceccarelli, B., Hurlbut, W.P., and Mauro, A. (1973). Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J Cell Biol *57*, 499-524.

Chapman, E.R., Hanson, P.I., An, S., and Jahn, R. (1995). Ca2+ regulates the interaction between synaptotagmin and syntaxin 1. J Biol Chem *270*, 23667-23671.

Chen, G., Hu, T., Wang, Z., Li, Q., Li, J.K., Jia, Y., and Xu, W.H. (2013). Down-Regulation of Synaptotagmin 1 in Cortex, Hippocampus, and Cerebellum after Experimental Subarachnoid Hemorrhage. Ann Clin Lab Sci *43*, 250-256.

Chen, Y., Deng, L., Maeno-Hikichi, Y., Lai, M., Chang, S., Chen, G., and Zhang, J.F. (2003). Formation of an endophilin-Ca2+ channel complex is critical for clathrin-mediated synaptic vesicle endocytosis. Cell *115*, 37-48.

Cheung, G., and Cousin, M.A. (2013). Synaptic vesicle generation from activity-dependent bulk endosomes requires calcium and calcineurin. J Neurosci *33*, 3370-3379.

Chieregatti, E., Chicka, M.C., Chapman, E.R., and Baldini, G. (2004). SNAP-23 functions in docking/fusion of granules at low Ca2+. Mol Biol Cell *15*, 1918-1930.

Cho, R.W., Buhl, L.K., Volfson, D., Tran, A., Li, F., Akbergenova, Y., and Littleton, J.T. (2015). Phosphorylation of Complexin by PKA Regulates Activity-Dependent Spontaneous Neurotransmitter Release and Structural Synaptic Plasticity. Neuron *88*, 749-761.

Chuhma, N., and Ohmori, H. (1998). Postnatal development of phase-locked high-fidelity synaptic transmission in the medial nucleus of the trapezoid body of the rat. J Neurosci *18*, 512-520.

Chung, C., Barylko, B., Leitz, J., Liu, X., and Kavalali, E.T. (2010). Acute dynamin inhibition dissects synaptic vesicle recycling pathways that drive spontaneous and evoked neurotransmission. J Neurosci *30*, 1363-1376.

Cremona, O., and De Camilli, P. (1997). Synaptic vesicle endocytosis. Curr Opin Neurobiol *7*, 323-330.

Cremona, O., Di Paolo, G., Wenk, M.R., Luthi, A., Kim, W.T., Takei, K., Daniell, L., Nemoto, Y., Shears, S.B., Flavell, R.A., *et al.* (1999). Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell *99*, 179-188.

Darios, F., Wasser, C., Shakirzyanova, A., Giniatullin, A., Goodman, K., Munoz-Bravo, J.L., Raingo, J., Jorgacevski, J., Kreft, M., Zorec, R., *et al.* (2009). Sphingosine facilitates SNARE complex assembly and activates synaptic vesicle exocytosis. Neuron *62*, 683-694.

Davidsson, P., Jahn, R., Bergquist, J., Ekman, R., and Blennow, K. (1996). Synaptotagmin, a synaptic vesicle protein, is present in human cerebrospinal fluid: a new biochemical marker for synaptic pathology in Alzheimer disease? Mol Chem Neuropathol *27*, 195-210.

Davis, A.F., Bai, J., Fasshauer, D., Wolowick, M.J., Lewis, J.L., and Chapman, E.R. (1999). Kinetics of synaptotagmin responses to Ca2+ and assembly with the core SNARE complex onto membranes. Neuron *24*, 363-376.

Daw, M.I., Pelkey, K.A., Chittajallu, R., and McBain, C.J. (2010). Presynaptic kainate receptor activation preserves asynchronous GABA release despite the reduction in synchronous release from hippocampal cholecystokinin interneurons. J Neurosci *30*, 11202-11209.

Deak, F., Schoch, S., Liu, X., Sudhof, T.C., and Kavalali, E.T. (2004). Synaptobrevin is essential for fast synaptic-vesicle endocytosis. Nat Cell Biol *6*, 1102-1108.

Delgado, R., Maureira, C., Oliva, C., Kidokoro, Y., and Labarca, P. (2000). Size of vesicle pools, rates of mobilization, and recycling at neuromuscular synapses of a Drosophila mutant, shibire. Neuron *28*, 941-953.

Delvendahl, I., Vyleta, N.P., von Gersdorff, H., and Hallermann, S. (2016). Fast, Temperature-Sensitive and Clathrin-Independent Endocytosis at Central Synapses. Neuron *90*, 492-498.

Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. Nature *443*, 651-657.

Dodge, F.A., Jr., Miledi, R., and Rahamimoff, R. (1969). Strontium and quantal release of transmitter at the neuromuscular junction. J Physiol *200*, 267-283.

Evans, C.S., Ruhl, D.A., and Chapman, E.R. (2015). An Engineered Metal Sensor Tunes the Kinetics of Synaptic Transmission. Journal of Neuroscience *35*, 11769-11779.

Evstratova, A., Chamberland, S., Faundez, V., and Toth, K. (2014). Vesicles derived via AP-3-dependent recycling contribute to asynchronous release and influence information transfer. Nat Commun *5*, 5530.

Faundez, V., Horng, J.T., and Kelly, R.B. (1998). A function for the AP3 coat complex in synaptic vesicle formation from endosomes. Cell *93*, 423-432.

Ferguson, S.M., Brasnjo, G., Hayashi, M., Wolfel, M., Collesi, C., Giovedi, S., Raimondi, A., Gong, L.W., Ariel, P., Paradise, S., *et al.* (2007). A selective activity-dependent requirement for dynamin 1 in synaptic vesicle endocytosis. Science *316*, 570-574.

Fernandez-Chacon, R., Konigstorfer, A., Gerber, S.H., Garcia, J., Matos, M.F., Stevens, C.F., Brose, N., Rizo, J., Rosenmund, C., and Sudhof, T.C. (2001). Synaptotagmin I functions as a calcium regulator of release probability. Nature *410*, 41-49.

Fesce, R., Grohovaz, F., Valtorta, F., and Meldolesi, J. (1994). Neurotransmitter release: fusion or 'kiss-and-run'? Trends Cell Biol *4*, 1-4.

Fine, M., Llaguno, M.C., Lariccia, V., Lin, M.J., Yaradanakul, A., and Hilgemann, D.W. (2011). Massive endocytosis driven by lipidic forces originating in the outer plasmalemmal monolayer: a new approach to membrane recycling and lipid domains. J Gen Physiol *137*, 137-154.

Fredj, N.B., and Burrone, J. (2009). A resting pool of vesicles is responsible for spontaneous vesicle fusion at the synapse. Nat Neurosci *12*, 751-758.

Gad, H., Low, P., Zotova, E., Brodin, L., and Shupliakov, O. (1998). Dissociation between Ca2+-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. Neuron *21*, 607-616.

Gandhi, S.P., and Stevens, C.F. (2003). Three modes of synaptic vesicular recycling revealed by single-vesicle imaging. Nature *423*, 607-613.

Gautam, V., D'Avanzo, C., Berezovska, O., Tanzi, R.E., and Kovacs, D.M. (2015). Synaptotagmins interact with APP and promote Abeta generation. Molecular neurodegeneration *10*, 31.

Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F., and Sudhof, T.C. (1994). Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. Cell *79*, 717-727.

Glavan, G., Schliebs, R., and Zivin, M. (2009). Synaptotagmins in neurodegeneration. Anat Rec (Hoboken) *292*, 1849-1862.

Glyvuk, N., Tsytsyura, Y., Geumann, C., D'Hooge, R., Huve, J., Kratzke, M., Baltes, J., Boening, D., Klingauf, J., and Schu, P. (2010). AP-1/sigma1B-adaptin mediates endosomal synaptic vesicle recycling, learning and memory. Embo J *29*, 1318-1330.

Goda, Y., and Stevens, C.F. (1994). Two components of transmitter release at a central synapse. Proc Natl Acad Sci U S A *91*, 12942-12946.

Groffen, A.J., Martens, S., Diez Arazola, R., Cornelisse, L.N., Lozovaya, N., de Jong, A.P., Goriounova, N.A., Habets, R.L., Takai, Y., Borst, J.G., *et al.* (2010). Doc2b is a high-affinity Ca2+ sensor for spontaneous neurotransmitter release. Science *327*, 1614-1618.

Hagler, D.J., Jr., and Goda, Y. (2001). Properties of synchronous and asynchronous release during pulse train depression in cultured hippocampal neurons. J Neurophysiol *85*, 2324-2334.

Haucke, V., and De Camilli, P. (1999). AP-2 recruitment to synaptotagmin stimulated by tyrosine-based endocytic motifs. Science *285*, 1268-1271.

Hefft, S., and Jonas, P. (2005). Asynchronous GABA release generates long-lasting inhibition at a hippocampal interneuron-principal neuron synapse. Nat Neurosci *8*, 1319-1328.

Heuser, J.E., and Reese, T.S. (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol *57*, 315-344.

Hosoi, N., Holt, M., and Sakaba, T. (2009). Calcium dependence of exo- and endocytotic coupling at a glutamatergic synapse. Neuron *63*, 216-229.

Hosoi, N., Sakaba, T., and Neher, E. (2007). Quantitative analysis of calcium-dependent vesicle recruitment and its functional role at the calyx of held synapse. Journal of Neuroscience *27*, 14286-14298.

Hua, Z., Leal-Ortiz, S., Foss, S.M., Waites, C.L., Garner, C.C., Voglmaier, S.M., and Edwards, R.H. (2011). v-SNARE composition distinguishes synaptic vesicle pools. Neuron *71*, 474-487.

Hui, E., Bai, J., Wang, P., Sugimori, M., Llinas, R.R., and Chapman, E.R. (2005). Three distinct kinetic groupings of the synaptotagmin family: candidate sensors for rapid and delayed exocytosis. Proc Natl Acad Sci U S A *102*, 5210-5214.

Iremonger, K.J., and Bains, J.S. (2007). Integration of asynchronously released quanta prolongs the postsynaptic spike window. J Neurosci *27*, 6684-6691.

Iremonger, K.J., and Bains, J.S. (2016). Asynchronous presynaptic glutamate release enhances neuronal excitability during the post-spike refractory period. J Physiol *594*, 1005-1015.

Iwakuma, M., Anzai, T., Kobayashi, S., Ogata, M., Kaneda, Y., Ohno, K., and Saji, M. (2003). Antisense in vivo knockdown of synaptotagmin I and synapsin I by HVJ-liposome mediated gene transfer modulates ischemic injury of hippocampus in opposing ways. Neurosci Res *45*, 285-296.

Jackman, S.L., Turecek, J., Belinsky, J.E., and Regehr, W.G. (2016). The calcium sensor synaptotagmin 7 is required for synaptic facilitation. Nature *529*, 88-91.

Jiang, M., Zhu, J., Liu, Y., Yang, M., Tian, C., Jiang, S., Wang, Y., Guo, H., Wang, K., and Shu, Y. (2012). Enhancement of asynchronous release from fast-spiking interneuron in human and rat epileptic neocortex. PLoS biology *10*, e1001324.

Jorgensen, E.M., Hartwieg, E., Schuske, K., Nonet, M.L., Jin, Y., and Horvitz, H.R. (1995). Defective recycling of synaptic vesicles in synaptotagmin mutants of Caenorhabditis elegans. Nature *378*, 196-199.

Kaeser, P.S., and Regehr, W.G. (2014). Molecular mechanisms for synchronous, asynchronous, and spontaneous neurotransmitter release. Annu Rev Physiol *76*, 333-363.

Kavalali, E.T. (2006). Synaptic vesicle reuse and its implications. Neuroscientist *12*, 57-66.

Kavalali, E.T. (2015). The mechanisms and functions of spontaneous neurotransmitter release. Nat Rev Neurosci *16*, 5-16.

Kavalali, E.T., and Jorgensen, E.M. (2014). Visualizing presynaptic function. Nat Neurosci *17*, 10-16.

Kim, S.H., and Ryan, T.A. (2009). Synaptic vesicle recycling at CNS snapses without AP-2. J Neurosci *29*, 3865-3874.

Kirischuk, S., and Grantyn, R. (2003). Intraterminal Ca2+ concentration and asynchronous transmitter release at single GABAergic boutons in rat collicular cultures. J Physiol *548*, 753-764.

Koenig, J.H., and Ikeda, K. (1996). Synaptic vesicles have two distinct recycling pathways. J Cell Biol *135*, 797-808.

Kononenko, N.L., and Haucke, V. (2015). Molecular mechanisms of presynaptic membrane retrieval and synaptic vesicle reformation. Neuron *85*, 484-496.

Lariccia, V., Fine, M., Magi, S., Lin, M.J., Yaradanakul, A., Llaguno, M.C., and Hilgemann, D.W. (2011). Massive calcium-activated endocytosis without involvement of classical endocytic proteins. J Gen Physiol *137*, 111-132.

Leitz, J., and Kavalali, E.T. (2011). Ca(2)(+) influx slows single synaptic vesicle endocytosis. J Neurosci *31*, 16318-16326.

Leitz, J., and Kavalali, E.T. (2014). Fast retrieval and autonomous regulation of single spontaneously recycling synaptic vesicles. Elife *3*, e03658.

Leitz, J., and Kavalali, E.T. (2015). Ca2+ Dependence of Synaptic Vesicle Endocytosis. Neuroscientist.

Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G., Brose, N., and Sudhof, T.C. (1995). Ca(2+)-dependent and -independent activities of neural and non-neural synaptotagmins. Nature *375*, 594-599.

Liu, H., Dean, C., Arthur, C.P., Dong, M., and Chapman, E.R. (2009). Autapses and networks of hippocampal neurons exhibit distinct synaptic transmission phenotypes in the absence of synaptotagmin I. J Neurosci *29*, 7395-7403.

Lu, T., and Trussell, L.O. (2000). Inhibitory transmission mediated by asynchronous transmitter release. Neuron *26*, 683-694.

Luthi, A., Di Paolo, G., Cremona, O., Daniell, L., De Camilli, P., and McCormick, D.A. (2001). Synaptojanin 1 contributes to maintaining the stability of GABAergic transmission in primary cultures of cortical neurons. J Neurosci *21*, 9101-9111.

Mallard, F., Tang, B.L., Galli, T., Tenza, D., Saint-Pol, A., Yue, X., Antony, C., Hong, W., Goud, B., and Johannes, L. (2002). Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. J Cell Biol *156*, 653-664.

Manseau, F., Marinelli, S., Mendez, P., Schwaller, B., Prince, D.A., Huguenard, J.R., and Bacci, A. (2010). Desynchronization of neocortical networks by asynchronous release of GABA at autaptic and synaptic contacts from fast-spiking interneurons. PLoS biology *8*.

Marks, B., and McMahon, H.T. (1998). Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. Curr Biol *8*, 740-749.

Maximov, A., Lao, Y., Li, H., Chen, X., Rizo, J., Sorensen, J.B., and Sudhof, T.C. (2008). Genetic analysis of synaptotagmin-7 function in synaptic vesicle exocytosis. Proc Natl Acad Sci U S A *105*, 3986-3991.

Maximov, A., and Sudhof, T.C. (2005). Autonomous function of synaptotagmin 1 in triggering synchronous release independent of asynchronous release. Neuron *48*, 547-554.

Maximov, A., Tang, J., Yang, X., Pang, Z.P., and Sudhof, T.C. (2009). Complexin controls the force transfer from SNARE complexes to membranes in fusion. Science *323*, 516-521.

McColl, B.W., Graham, D.I., Weir, C.J., White, F., and Horsburgh, K. (2003). Endocytic pathway alterations in human hippocampus after global ischemia and the influence of APOE genotype. Am J Pathol *162*, 273-281.

McMahon, H.T., Missler, M., Li, C., and Sudhof, T.C. (1995). Complexins: cytosolic proteins that regulate SNAP receptor function. Cell *83*, 111-119.

Medrihan, L., Ferrea, E., Greco, B., Baldelli, P., and Benfenati, F. (2015). Asynchronous GABA Release Is a Key Determinant of Tonic Inhibition and Controls Neuronal Excitability: A Study in the Synapsin II-/- Mouse. Cereb Cortex *25*, 3356-3368.

Meng, J., Wang, J., Lawrence, G.W., and Dolly, J.O. (2013). Molecular components required for resting and stimulated endocytosis of botulinum neurotoxins by glutamatergic and peptidergic neurons. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *27*, 3167-3180.

Miesenbock, G., De Angelis, D.A., and Rothman, J.E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature *394*, 192-195.

Mozhayeva, M.G., Sara, Y., Liu, X., and Kavalali, E.T. (2002). Development of vesicle pools during maturation of hippocampal synapses. J Neurosci *22*, 654-665.

Murthy, V.N., Sejnowski, T.J., and Stevens, C.F. (1997). Heterogeneous release properties of visualized individual hippocampal synapses. Neuron *18*, 599-612.

Neale, E.A., Bowers, L.M., Jia, M., Bateman, K.E., and Williamson, L.C. (1999). Botulinum neurotoxin A blocks synaptic vesicle exocytosis but not endocytosis at the nerve terminal. J Cell Biol *147*, 1249-1260.

Neff, R.A., 3rd, Conroy, W.G., Schoellerman, J.D., and Berg, D.K. (2009). Synchronous and asynchronous transmitter release at nicotinic synapses are differentially regulated by postsynaptic PSD-95 proteins. J Neurosci *29*, 15770-15779.

Nicholson-Fish, J.C., Kokotos, A.C., Gillingwater, T.H., Smillie, K.J., and Cousin, M.A. (2015). VAMP4 Is an Essential Cargo Molecule for Activity-Dependent Bulk Endocytosis. Neuron *88*.

Nicholson-Tomishima, K., and Ryan, T.A. (2004). Kinetic efficiency of endocytosis at mammalian CNS synapses requires synaptotagmin I. Proc Natl Acad Sci U S A *101*, 16648-16652.

Nosyreva, E., Szabla, K., Autry, A.E., Ryazanov, A.G., Monteggia, L.M., and Kavalali, E.T. (2013). Acute Suppression of Spontaneous Neurotransmission Drives Synaptic Potentiation. Journal of Neuroscience *33*, 6990-7002.

Orita, S., Sasaki, T., and Takai, Y. (2001). Doc2 alpha as modulator of Ca(2+)-dependent exocytosis. Methods in enzymology *329*, 83-90.

Otsu, Y., Shahrezaei, V., Li, B., Raymond, L.A., Delaney, K.R., and Murphy, T.H. (2004). Competition between phasic and asynchronous release for recovered synaptic vesicles at developing hippocampal autaptic synapses. J Neurosci *24*, 420-433.

Palfrey, H.C., and Artalejo, C.R. (1998). Vesicle recycling revisited: rapid endocytosis may be the first step. Neuroscience *83*, 969-989.

Park, J.J., Gondre-Lewis, M.C., Eiden, L.E., and Loh, Y.P. (2011). A distinct trans-Golgi network subcompartment for sorting of synaptic and granule proteins in neurons and neuroendocrine cells. J Cell Sci *124*, 735-744.

Peng, A., Rotman, Z., Deng, P.Y., and Klyachko, V.A. (2012). Differential motion dynamics of synaptic vesicles undergoing spontaneous and activity-evoked endocytosis. Neuron *73*, 1108-1115.

Poskanzer, K.E., Fetter, R.D., and Davis, G.W. (2006). Discrete residues in the c(2)b domain of synaptotagmin I independently specify endocytic rate and synaptic vesicle size. Neuron *50*, 49-62.

Poskanzer, K.E., Marek, K.W., Sweeney, S.T., and Davis, G.W. (2003). Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. Nature *426*, 559-563.

Raingo, J., Khvotchev, M., Liu, P., Darios, F., Li, Y.C., Ramirez, D.M., Adachi, M., Lemieux, P., Toth, K., Davletov, B., *et al.* (2012). VAMP4 directs synaptic vesicles to a pool that selectively maintains asynchronous neurotransmission. Nat Neurosci *15*, 738-745.

Ramirez, D.M., and Kavalali, E.T. (2011). Differential regulation of spontaneous and evoked neurotransmitter release at central synapses. Curr Opin Neurobiol *21*, 275-282.

Ramirez, D.M.O., Khvotchev, M., Trauterman, B., and Kavalali, E.T. (2012). Vti1a Identifies a Vesicle Pool that Preferentially Recycles at Rest and Maintains Spontaneous Neurotransmission. Neuron *73*, 121-134.

Reddy, P.H., Mani, G., Park, B.S., Jacques, J., Murdoch, G., Whetsell, W., Jr., Kaye, J., and Manczak, M. (2005). Differential loss of synaptic proteins in Alzheimer's disease: implications for synaptic dysfunction. J Alzheimers Dis *7*, 103-117; discussion 173-180.

Reim, K., Mansour, M., Varoqueaux, F., McMahon, H.T., Sudhof, T.C., Brose, N., and Rosenmund, C. (2001). Complexins regulate a late step in Ca2+-dependent neurotransmitter release. Cell *104*, 71-81.

Rizo, J., and Rosenmund, C. (2008). Synaptic vesicle fusion. Nat Struct Mol Biol *15*, 665-674.

Rizzoli, S.O., and Betz, W.J. (2005). Synaptic vesicle pools. Nat Rev Neurosci *6*, 57-69.

Robinson, P.J., Liu, J.P., Powell, K.A., Fykse, E.M., and Sudhof, T.C. (1994). Phosphorylation of dynamin I and synaptic-vesicle recycling. Trends Neurosci *17*, 348-353.

Rosa, J.M., Gandia, L., and Garcia, A.G. (2010). Permissive role of sphingosine on calcium-dependent endocytosis in chromaffin cells. Pflugers Arch *460*, 901-914.

Rudolph, S., Overstreet-Wadiche, L., and Wadiche, J.I. (2011). Desynchronization of multivesicular release enhances Purkinje cell output. Neuron *70*, 991-1004.

Ruiz, R., Casanas, J.J., Torres-Benito, L., Cano, R., and Tabares, L. (2010). Altered intracellular Ca2+ homeostasis in nerve terminals of severe spinal muscular atrophy mice. J Neurosci *30*, 849-857.

Sakaguchi, G., Orita, S., Maeda, M., Igarashi, H., and Takai, Y. (1995). Molecular cloning of an isoform of Doc2 having two C2-like domains. Biochem Biophys Res Commun *217*, 1053-1061.

Sankaranarayanan, S., and Ryan, T.A. (2000). Real-time measurements of vesicle-SNARE recycling in synapses of the central nervous system. Nat Cell Biol *2*, 197-204.

Sara, Y., Virmani, T., Deak, F., Liu, X., and Kavalali, E.T. (2005). An isolated pool of vesicles recycles at rest and drives spontaneous neurotransmission. Neuron *45*, 563-573.

Schiavo, G., Gu, Q.M., Prestwich, G.D., Sollner, T.H., and Rothman, J.E. (1996). Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin. Proc Natl Acad Sci U S A *93*, 13327-13332.

Schmid, E.M., and McMahon, H.T. (2007). Integrating molecular and network biology to decode endocytosis. Nature *448*, 883-888.

Schoch, S., Deak, F., Konigstorfer, A., Mozhayeva, M., Sara, Y., Sudhof, T.C., and Kavalali, E.T. (2001). SNARE function analyzed in synaptobrevin/VAMP knockout mice. Science *294*, 1117-1122.

Schweizer, F.E., and Ryan, T.A. (2006). The synaptic vesicle: cycle of exocytosis and endocytosis. Curr Opin Neurobiol *16*, 298-304.

Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N., Palmer, A.E., and Tsien, R.Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nature biotechnology *22*, 1567-1572.

Shin, O.H., Rhee, J.S., Tang, J., Sugita, S., Rosenmund, C., and Sudhof, T.C. (2003). Sr2+ binding to the Ca2+ binding site of the synaptotagmin 1 C2B domain triggers fast exocytosis without stimulating SNARE interactions. Neuron *37*, 99-108.

Shitara, A., Shibui, T., Okayama, M., Arakawa, T., Mizoguchi, I., Sakakura, Y., and Takuma, T. (2013). VAMP4 is required to maintain the ribbon structure of the Golgi apparatus. Molecular and cellular biochemistry *380*, 11-21.

Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., DeCamilli, P., and Brodin, L. (1997). Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. Science *276*, 259-263.

Smillie, K.J., and Cousin, M.A. (2005). Dynamin I phosphorylation and the control of synaptic vesicle endocytosis. Biochem Soc Symp, 87-97.

Sokolov, B.P., Tcherepanov, A.A., Haroutunian, V., and Davis, K.L. (2000). Levels of mRNAs encoding synaptic vesicle and synaptic plasma membrane proteins in the temporal cortex of elderly schizophrenic patients. Biol Psychiatry *48*, 184-196.

Steegmaier, M., Klumperman, J., Foletti, D.L., Yoo, J.S., and Scheller, R.H. (1999). Vesicle-associated membrane protein 4 is implicated in trans-Golgi network vesicle trafficking. Mol Biol Cell *10*, 1957-1972.

Stepanyuk, A.R., Borisyuk, A.L., Tsugorka, T.M., and Belan, P.V. (2014). Different pools of postsynaptic GABAA receptors mediate inhibition evoked by low- and high-frequency presynaptic stimulation at hippocampal synapses. Synapse *68*, 344-354.

Sudhof, T.C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature *375*, 645-653.

Sudhof, T.C. (2001). alpha-Latrotoxin and its receptors: neurexins and CIRL/latrophilins. Annu Rev Neurosci *24*, 933-962.

Sudhof, T.C. (2004). The synaptic vesicle cycle. Annu Rev Neurosci *27*, 509-547.

Sudhof, T.C. (2013). Neurotransmitter Release: The Last Millisecond in the Life of a Synaptic Vesicle. Neuron *80*, 675-690.

Sudhof, T.C., and Rothman, J.E. (2009). Membrane fusion: grappling with SNARE and SM proteins. Science *323*, 474-477.

Sugita, S., Han, W., Butz, S., Liu, X., Fernandez-Chacon, R., Lao, Y., and Sudhof, T.C. (2001). Synaptotagmin VII as a plasma membrane Ca(2+) sensor in exocytosis. Neuron *30*, 459-473.

Sugita, S., Shin, O.H., Han, W.P., Lao, Y., and Sudhof, T.C. (2002). Synaptotagmins form a hierarchy of exocytotic Ca2+ sensors with distinct Ca2+ affinities. Embo J *21*, 270-280.

Sutton, M.A., Ito, H.T., Cressy, P., Kempf, C., Woo, J.C., and Schuman, E.M. (2006). Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. Cell *125*, 785-799.

Takei, K., Mundigl, O., Daniell, L., and De Camilli, P. (1996). The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. J Cell Biol *133*, 1237-1250.

Tang, J., Maximov, A., Shin, O.H., Dai, H., Rizo, J., and Sudhof, T.C. (2006). A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell *126*, 1175-1187.

Troulinaki, K., and Tavernarakis, N. (2012). Endocytosis and intracellular trafficking contribute to necrotic neurodegeneration in C. elegans. Embo J *31*, 654-666.

Vaslin, A., Puyal, J., Borsello, T., and Clarke, P.G.H. (2007). Excitotoxicity-related endocytosis in cortical neurons. J Neurochem *102*, 789-800.

Virmani, T., Han, W., Liu, X., Sudhof, T.C., and Kavalali, E.T. (2003). Synaptotagmin 7 splice variants differentially regulate synaptic vesicle recycling. Embo J *22*, 5347-5357.

Voglmaier, S.M., Kam, K., Yang, H., Fortin, D.L., Hua, Z.L., Nicoll, R.A., and Edwards, R.H. (2006). Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. Neuron *51*, 71-84.

von Gersdorff, H., and Matthews, G. (1994). Inhibition of endocytosis by elevated internal calcium in a synaptic terminal. Nature *370*, 652-655.

von Poser, C., Zhang, J.Z., Mineo, C., Ding, W., Ying, Y., Sudhof, T.C., and Anderson, R.G. (2000). Synaptotagmin regulation of coated pit assembly. J Biol Chem *275*, 30916-30924.

Wang, D., Epstein, D., Khalaf, O., Srinivasan, S., Williamson, W.R., Fayyazuddin, A., Quiocho, F.A., and Hiesinger, P.R. (2014). Ca2+-Calmodulin regulates SNARE assembly and spontaneous neurotransmitter release via v-ATPase subunit V0a1. J Cell Biol *205*, 21-31.

Wang, P., Chicka, M.C., Bhalla, A., Richards, D.A., and Chapman, E.R. (2005). Synaptotagmin VII is targeted to secretory organelles in PC12 cells, where it functions as a high-affinity calcium sensor. Mol Cell Biol *25*, 8693-8702.

Watanabe, H., Yamashita, T., Saitoh, N., Kiyonaka, S., Iwamatsu, A., Campbell, K.P., Mori, Y., and Takahashi, T. (2010). Involvement of Ca2+ channel synprint site in synaptic vesicle endocytosis. J Neurosci *30*, 655-660.

Watanabe, S., Rost, B.R., Camacho-Perez, M., Davis, M.W., Sohl-Kielczynski, B., Rosenmund, C., and Jorgensen, E.M. (2013). Ultrafast endocytosis at mouse hippocampal synapses. Nature *504*, 242-247.

Weber, J.P., Toft-Bertelsen, T.L., Mohrmann, R., Delgado-Martinez, I., and Sorensen, J.B. (2014). Synaptotagmin-7 is an asynchronous calcium sensor for synaptic transmission in neurons expressing SNAP-23. PLoS One *9*, e114033.

Wen, H., Linhoff, M.W., McGinley, M.J., Li, G.L., Corson, G.M., Mandel, G., and Brehm, P. (2010). Distinct roles for two synaptotagmin isoforms in synchronous and asynchronous transmitter release at zebrafish neuromuscular junction. Proc Natl Acad Sci U S A *107*, 13906-13911.

Wierda, K.D., and Sorensen, J.B. (2014). Innervation by a GABAergic neuron depresses spontaneous release in glutamatergic neurons and unveils the clamping phenotype of synaptotagmin-1. J Neurosci *34*, 2100-2110.

Willig, K.I., Rizzoli, S.O., Westphal, V., Jahn, R., and Hell, S.W. (2006). STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. Nature *440*, 935-939.

Wu, L.G., Hamid, E., Shin, W., and Chiang, H.C. (2014). Exocytosis and Endocytosis: Modes, Functions, and Coupling Mechanisms. Annu Rev Physiol *76*, 301-331.

Wu, X.S., McNeil, B.D., Xu, J., Fan, J., Xue, L., Melicoff, E., Adachi, R., Bai, L., and Wu, L.G. (2009). Ca(2+) and calmodulin initiate all forms of endocytosis during depolarization at a nerve terminal. Nat Neurosci *12*, 1003-1010.

Wu, Y., Liang, S., Oda, Y., Ohmori, I., Nishiki, T.I., Takei, K., Matsui, H., and Tomizawa, K. (2007). Truncations of amphiphysin I by calpain inhibit vesicle endocytosis during neural hyperexcitation. Embo J *26*, 2981-2990.

Xu-Friedman, M.A., and Regehr, W.G. (2000). Probing fundamental aspects of synaptic transmission with strontium. J Neurosci *20*, 4414-4422.

Xu, J., Pang, Z.P., Shin, O.H., and Sudhof, T.C. (2009). Synaptotagmin-1 functions as a Ca2+ sensor for spontaneous release. Nat Neurosci *12*, 759-766.

Xu, J.H., Luo, F.J., Zhang, Z., Xue, L., Wu, X.S., Chiang, H.C., Shin, W., and Wu, L.G. (2013). SNARE Proteins Synaptobrevin, SNAP-25, and Syntaxin Are Involved in Rapid and Slow Endocytosis at Synapses. Cell Rep *3*, 1414-1421.

Xu, W., Morishita, W., Buckmaster, P.S., Pang, Z.P., Malenka, R.C., and Sudhof, T.C. (2012). Distinct Neuronal Coding Schemes in Memory Revealed by Selective Erasure of Fast Synchronous Synaptic Transmission. Neuron *73*, 990-1001.

Xue, L., Zhang, Z., McNeil, B.D., Luo, F., Wu, X.S., Sheng, J., Shin, W., and Wu, L.G. (2012). Voltage-dependent calcium channels at the plasma membrane, but not vesicular channels, couple exocytosis to endocytosis. Cell Rep *1*, 632-638.

Xue, R., Gaffaney, J.D., and Chapman, E.R. (2015). Structural elements that underlie Doc2beta function during asynchronous synaptic transmission. Proc Natl Acad Sci U S A *112*, E4316-4325.

Yang, L., Wang, B., Long, C., Wu, G., and Zheng, H. (2007). Increased asynchronous release and aberrant calcium channel activation in amyloid precursor protein deficient neuromuscular synapses. Neuroscience *149*, 768-778.

Yang, X.F., Cao, P., and Sudhof, T.C. (2013). Deconstructing complexin function in activating and clamping Ca2+-triggered exocytosis by comparing knockout and knockdown phenotypes. Proc Natl Acad Sci U S A *110*, 20777-20782.

Yao, C.K., Lin, Y.Q., Ly, C.V., Ohyama, T., Haueter, C.M., Moiseenkova-Bell, V.Y., Wensel, T.G., and Bellen, H.J. (2009). A synaptic vesicle-associated Ca2+ channel promotes endocytosis and couples exocytosis to endocytosis. Cell *138*, 947-960.

Yao, J., Gaffaney, J.D., Kwon, S.E., and Chapman, E.R. (2011). Doc2 is a Ca2+ sensor required for asynchronous neurotransmitter release. Cell *147*, 666-677.

Yao, J., Kwon, S.E., Gaffaney, J.D., Dunning, F.M., and Chapman, E.R. (2012a). Uncoupling the roles of synaptotagmin I during endo- and exocytosis of synaptic vesicles. Nat Neurosci *15*, 243-249.

Yao, L.H., Rao, Y., Varga, K., Wang, C.Y., Xiao, P., Lindau, M., and Gong, L.W. (2012b). Synaptotagmin 1 is necessary for the Ca2+ dependence of clathrin-mediated endocytosis. J Neurosci *32*, 3778-3785.

Yokota, N., Uchijima, M., Nishizawa, S., Namba, H., and Koide, Y. (2001). Identification of differentially expressed genes in rat hippocampus after transient global cerebral ischemia using subtractive cDNA cloning based on polymerase chain reaction. Stroke *32*, 168-174.

Zhang, J.Z., Davletov, B.A., Sudhof, T.C., and Anderson, R.G. (1994). Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. Cell *78*, 751-760.

Zhang, Q., Li, Y., and Tsien, R.W. (2009). The dynamic control of kiss-and-run and vesicular reuse probed with single nanoparticles. Science *323*, 1448-1453.