Synaptotagmin 1 is the Ca^{2+} Sensor for the Endocytic Kinetics of Clathrin-Mediated Endocytosis

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THESIS
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To my committee, thank you for all that you have contributed to my project, my story and my journey. Simon, thank you for providing for me the opportunity to find my way and encourage me throughout; that incredible mind of yours still blows mine. Dave, thank you for always giving me a fresh new insight into how science should be approached- keep it simple and sell it straightforward; champagne will never again be as much fun to open without you present. Mark, I have appreciated your insight and calmness towards developing my story. Lastly, Janet, without your guidance at times, myself and my science would have been far too overwhelming

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CONTRIBUTION OF AUTHORS

Chapter 1 entails a literary review of the work described within the context of this thesis. It provides the foundation from which this work was generated and provides the means of understanding how my work contributes to the larger scope of this field. Chapter 2 represents the employed methodology utilized to generate the work that is described within the thesis itself. Some of this methodology was described in a published manuscript (Yao et al., 2012), in which Li-Hua Yao was the primary author. Li-Hua Yao, in addition to my mentor Dr. Liang-Wei Gong, provided for me the groundwork of the experimental procedure known as the cell-attached capacitance measurement, which was the primary methodology I employed for the work described in the context of this thesis. Chapter 3 represents a published manuscript (Yao et al., 2012) for which I was the third author. I assisted in generating the synaptotagmin 1 (Syt1) mouse colony utilized for the purposes of that research in addition to contributing towards the genetic analysis utilized to ensure proper identification of Syt1 mouse genotypes for cell-attached recordings; this was an important component for the context and conclusions of that publication that knock-out of the Syt1 gene is critical to proper clathrin-mediated endocytic retrieval. Chapter 4 represents my own unpublished experimental work, in which I developed, with the help and guidance of Dr. Hua Jin, a new technique in the lab; lentiviral application in the mouse adrenal chromaffin cell. This work was previously not established in field and proved to be an effective tool to utilize in answering some of the major components of this thesis. Chapter 5 represents my own unpublished work and is a continuation of the (Yao et al., 2012) publication. This work is a first step in directly answering if Syt1serves as the Ca^{2+} sensor for the endocytic fission-pore kinetics of clathrin-mediated endocytosis. I anticipate that this research will be continued in the laboratory after I leave and will ultimately be published as a co-authored

manuscript. Chapter 6 represents a different conceptual understanding of the protein Syt1 and its interactions during vesicle recycling. It also represents my own unpublished work and has contributed to an understanding that phosphatidylserine (PS) is critical for vesicle fission in clathrin-mediated endocytosis and that this may be due to a direct interaction between Syt1-PS during vesicle fission. This work is currently being revised and edited for submission as a publication in which I will be the first author. Chapter 7 represents the synthesis of my thesis/dissertation. Additionally, it describes the future directions of this work and the field itself.

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LIST OF ABBREVIATIONS

CME	Clathrin-mediated endocytosis
Syt1	Synaptotagmin 1
Ca^{2+}	Calcium
PS	Phosphatidylserine
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
WT	Wild-type
HT	Heterozygous
KO	Knock-out
GFP	Green-fluorescent protein
TH	Tyrosine hydroxylase
SNAREs	*N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors
µl	Microliter
µg	Microgram
ml	Milliliter
µM	Micromolar
mM	Milimolar
M	Molar
NMJ	Neuromuscular junction
AP-2	Activating protein 2
BoNT/A	Botulinum neurotoxin A

SUMMARY

After neurotransmitter release via exocytosis, synaptic vesicles are locally retrieved, re-filled and made readily available for the next round of release at the synapse (Heuser & Reese, 1973; Miller & Heuser, 1984). While the role of Ca^{2+} has long been established to be essential in triggering exocytosis (Neher and Sakaba, 2008), recent studies indicate a direct role of Ca^{2+} in endocytosis as well (Wu et al., 2007). Along this line, synaptotagmin 1 (Syt1), a synaptic vesicular protein, , is well established as the Ca^{2+} sensor to trigger vesicle exocytosis (Chapman, 2008) and recent evidence indicates that this protein may also play Ca^{2+} dependent roles in endocytosis (Nicholson-Tomishima & Ryan, 2004, please recite more papers for this). However, the molecular details for the role of Ca2+ and Syt1 in vesicle endocytosis remains poorly understood.

In the present study, by taking advantages of millisecond time resolution of cell-attached capacitance measurements to monitor clathrin-mediated endocytosis (CME), the classic retrieval pathway at synaptic terminals (Murthy & De Camilli, 2003; Granseth et al., 2006), I demonstrate for the first time that: 1) the dynamics of vesicle fission during CME is both Ca^{2+}- and Syt1-dependent; 2) Syt1 may serve as the biochemical Ca^{2+}sensor of CME and 3) that the interaction between Syt1 and phosphatidylserine, a putative Ca^{2+} dependent interaction, may be critical for the Ca2+ sensing role of Syt1 in CME kinetics

CHAPTER I: INTRODUCTION

1.1 Statement of problem and purpose of study

Synaptic transmission, which is mediated by exocytosis of synaptic vesicles, underlies the predominant communication between neurons within the brain. In parallel, endocytosis is occurring to form new synaptic vesicles to maintain synaptic transmission and to retrieve excess membrane to sustain the overall integrity of the synaptic structure within presynaptic terminals. The synaptic vesicle cycle, including both exocytosis and endocytosis, is important in supporting signal transduction between neurons and any defects in either one of these pathways could be linked to a host of neurodegenerative diseases such as Alzheimer's disease. Exocytosis is triggered by influx of extracellular Ca^{2+}, which binds to the two tandem C2 domains of synaptic vesicular protein synaptotagmin 1 (Syt1), the putative Ca^{2+} sensor for exocytosis (reviewed in Chapman, 2008). Ca^{2+}-bound Syt1 interacts with both SNARE protein complexes and anionic phospholipids, largely phosphatidylserine (PS), which drive fusion and subsequent exocytosis (reviewed in Chapman, 2008; Murthy and De Camilli, 2003).

While current observations reveal Ca^{2+} may be involved in endocytosis: its role in endocytosis remains diverse (Yamashita, et al*.,* 2010): either stimulatory (Beutner et al., 2001; Klingauf et al., 1998; Sankaranarayanan & Ryan, 2001) or inhibitory (Rouze & Schwartz, 1998; Cousin & Robinson, 2000). It remains as an open question as to how Ca^{2+} regulates endocytosis and what is the biochemical sensor. Studies have suggested a coupling mechanism for the calcium-dependence of exo-and endocytosis by identifying Syt1 as an ancillary component for endocytosis (Poskanzer et al., 2006; Nicholson-Tomishima & Ryan, 2004). Again, it remains unknown as to how Syt1 regulates endocytosis and what is the involved mechanism? In this work, I attempt to characterize how Ca^{2+} is involved in the endocytic process, and its specific

biochemical interactions within the retrieval pathway of clathrin-mediated endocytosis (CME). By utilizing high time resolution cell-attached capacitance measurements to monitor CME of single vesicle in neuroendocrine chromaffin cells, we are capable of evaluating the dynamics of vesicle fission, the final stage of endocytosis, with fission-pore analysis. Here I establish that Syt1may serve as the biochemical Ca^{2+} sensor necessary for single endocytic CME. Additionally, I describe some of the key molecular interactions which are necessary to ensure vesicle fission including understanding how Syt1 and anionic phospholipids like phosphatidylserine (PS) cooperate in a Ca^{2+} dependent manner to enhance the fission dynamics. These results thus grant a novel perspective into how components, which contribute to the kinetics of endocytosis in our model system, may be occurring at the neuronal synapse.

1.2 Literature Review

1.2.1 Exocytosis and endocytosis of synaptic vesicles

Synaptic transmission is initiated when an action potential triggers the opening of voltage-gated Ca^{2+} channels (VGCC's) which results in the fusion of neurotransmitter-filled synaptic vesicles with the presynaptic membrane and subsequent neurotransmitter release into the synaptic cleft (Katz & Miledi, 1969). Because nerve terminals are secretory regions that are perpetually dedicated to consistent and repeated rounds of release at the synapse, there is a compensatory mechanism known as endocytosis, that allows synaptic vesicles to be recycled, refilled and primed for the next signaling event (Sudhof, 2004). Collectively known as the synaptic vesicle cycle, vesicles undergo this repeated sequence at the nerve terminal (Figure 1). First, neurotransmitters are actively transported into synaptic vesicles that are then docked near the active zone and primed for release. Once an action potential has generated the opening of VGCC's, synaptic vesicles fuse and release their content. Following exocytosis, vesicle retrieval is initiated and local recycling occurs through multiple endocytic pathways (Saheki & De Camilli, 2012)

Decades of research have been committed to understanding the processes and kinetic components behind the synaptic vesicle cycle with the majority of work contributing to our understanding of how the exocytic pathway is triggered. Recently, the field has shifted toward endocytosis due to a need to understand how the delicate balance between exo and endocytosis is maintained. Although blockade of endocytosis does not carry an immediate effect on neurotransmitter release (Yamashita et al., 2005; Hosi et al., 2009; Yao and Sakaba, 2012), it does lead to a severe depletion in the overall readily releasable pool after prolonged neuronal activity (Newton et al., 2005; Heerssen et al., 2008) suggesting that synaptic vesicle endocytosis

is essential for long term, continual release. The goal of my efforts here, is to contribute to the working knowledge in this area by defining the specifics behind one of the multiple endocytic forms. Here the kinetics during the last stage of vesicle internalization in the most classic form of endocytosis, clathrin-mediated endocytosis (CME) are observed and analyzed as a means towards a better understanding in this pathway. Due to the importance of this process, elucidating the mechanisms by which the synaptic vesicle cycle is maintained is critical in understanding the basic foundation of cellular communication within the brain.

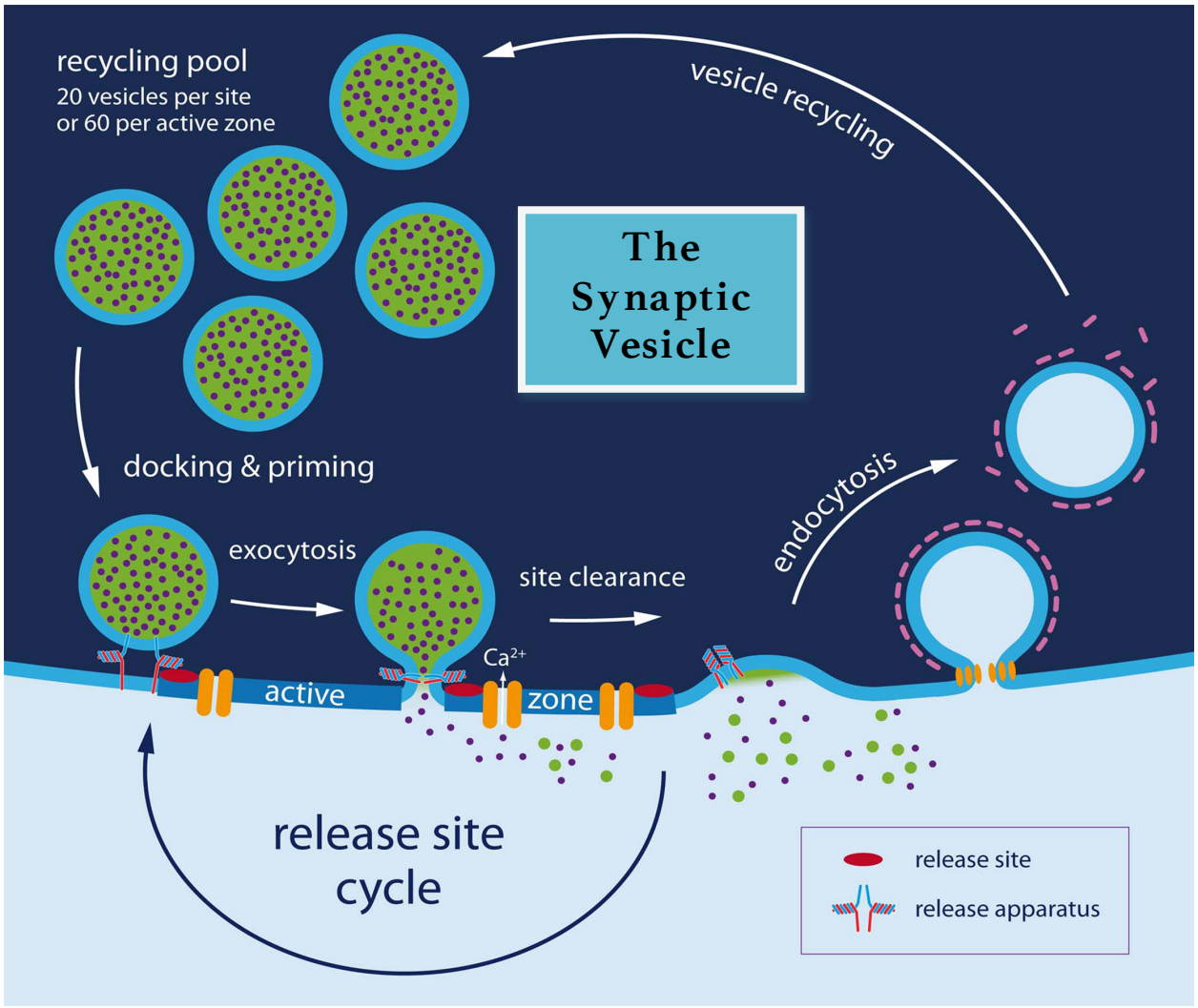


Figure 1. Neuronal communication is maintained by the synaptic vesicle cycle Synaptic vesicles undergo repetitive rounds of release through process known as exocytosis and endocytosis at the nerve terminal. Image was modified from original by Erwin Neher, 2010.

1.2.2 Calcium dependent regulation of release

The revelation of the action potential through Emil du Bois-Reymond (1818–1896) at the neuromuscular junction ignited the concept of neuronal signaling and in turn, the field of modern day neuroscience. Yet it was not until the work of Bernard Katz almost 100 years later, which defined how the action potential contributes to synaptic transmission. At the neuromuscular junction, Katz and Miledi (1969) described for the first time that an action potential travels to the end of the synaptic cleft where it triggers the opening of gated Ca^{2+} channels and allow for the influx of Ca^{2+}. This influx then triggers exocytosis of synaptic vesicles and this release then activates postsynaptic receptors, propagating cell signaling onward. It was from this groundbreaking work that multiple experimental approaches pursued an understanding of how Ca^{2+} and synaptic transmission are occurring at the synapse.

Several studies have contributed to the Ca^{2+} story, demonstrating that Ca^{2+} triggers release in a highly cooperative manner (Dodge and Rahamimoff, 1967) within a few hundred microseconds and this time scale is not much slower than the opening of a voltage-gated ion channel (Sabatini and Regehr, 1996). Due to the brief duration of an action potential, it is critical that the proper concentration of Ca^{2+} is achieved in order to trigger exocytosis. Because of this, it is predicted that high calcium concentrations are required for vesicle fusion and this is feasible due to the spatial constraint of primed vesicles within the vicinity of Ca^{2+} channels in the active zone (Neher and Sakaba, 2008). Some mathematical models predict that diffusion, around the mouth of open Ca^{2+} channels, yields an immediate accumulation of Ca^{2+} ions within an area covering only tens of nanometers (Simon & Linas 1985). Yet, other work claims that some components of neurotransmission are mediated by slower variations in Ca^{2+} of a much smaller concentration, which is thought to build up throughout the nerve terminal during episodes of

high synaptic activity and it is believed that this slow steady rise in internal Ca^{2+} is what accelerates the recruitment of vesicles, influencing the probability of release during action potentials (Dittman and Regehr 1998). Because of these competing theories, there remains a qualitative difference in how intracellular Ca^{2+} signals contribute to the kinetics of fast, synchronous release (Sudhof, 2004). Collectively it is well established that Ca^{2+} influx initiates exocytosis.

1.2.3 Synaptotagmin 1: The Ca^{2+} Sensor for Exocytosis

With the determination of Ca^{2+} as the signaling molecule which initiates release (Neher and Zucker 1993),the question then became: How is it that primed vesicles are coordinated with Ca^{2+}-triggered release? Close proximity to VGCC's at release sites enables precise coupling of an action potential to release but how is Ca^{2+} coordinated with synaptic vesicles, which are docked at presynaptic release sites for release? These questions created a need in understanding what kind of "sensor" of Ca^{2+} could be coordinating this ions signaling property.

While there are several protein families believed to play a broad function in regulating exocytosis, including soluble NSF attachment protein's (SNAREs), synaptotagmins (syts), and complexins (Sudhof & Rothman, 2009; Jahn & Scheller, 2006; Jahn & Fasshauer , 2012; Winkler & Westhead, 1980), syts have moved to the forefront as the Ca^{2+} dependent proteins which serve as the Ca^{2+} sensors of release. Syts are evolutionarily conserved vesiclular proteins that contain a transmembrane region with two tandem cytoplasmic C2 domains (Perin et al., 1990, 1991). C2 domains display a universal property of binding different ligands and substrates, phospholipids, inositol polyphosphates, and other proteins. However the

tandem C2 domains of most Syt isoforms bind Ca^{2+} ion (Nalefski & Falke,1996). Syts constitute a large family of abundant conserved proteins that are composed of a short short juxtalumenal domain, a single membrane spanning domain, and a large cytoplasmic domain consisting of tandem C2 domains, C2A and C2B, connected by a linker (Figure 2) (Perin et al.,1990, 1990a). Although multiple Syt isoforms have been revealed (Perin et al., 1990b; Geppert et a., 1991; Nonet et al., 1993; Mizuta et al., 1994), it was the identification of synaptotagmin 1 (Syt1), which led to the emergence of a putative Ca^{2+} sensor for exocytosis (Perin, Fried et al. 1990). Syt1's C2A and C2B domains respectively bind three and two Ca^{2+} ions (Brose et al., 1992; Augstine, 2001; Perin et al, 1991a) and the Ca^{2+} binding affinity of Syt1 is coordinated by negatively charged aspartate residues in two loops at the top of each C2 domain (Shao et al., 1996; (Ubach, Zhang et al. 1998); (Fernandez, Arac et al. 2001). Furthermore, it has been demonstrated that Syt1 forms homomultimers and binds Ca^{2+} and phospholipids through a ternary complex (Perin et al., 1990; Davletov and Sudhof, 1993; Sutton et al., 1995)which would be highly suitable to ensure close vesicle-membrane to plasma membrane proximity necessary for release.

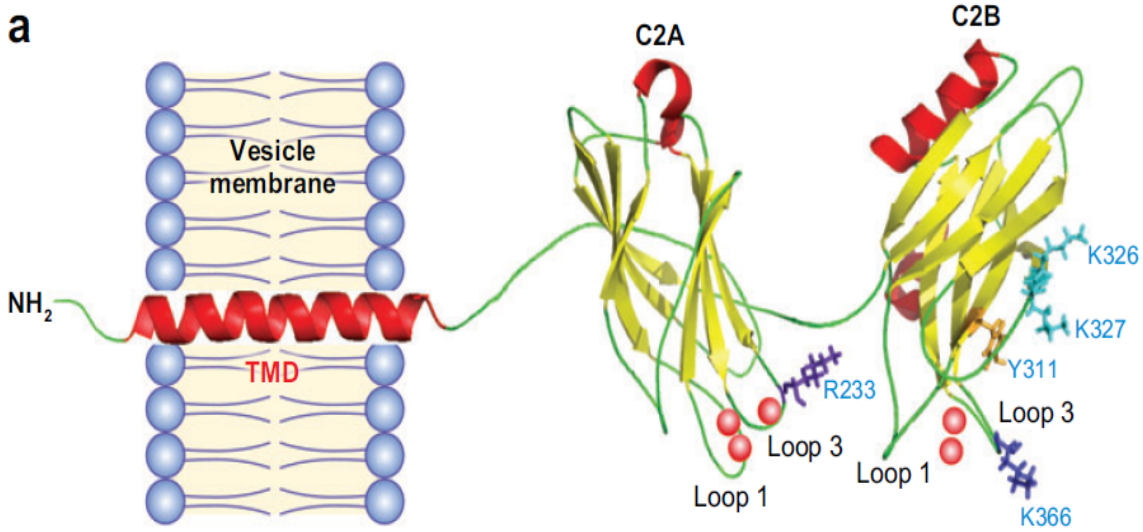
Initial experiments in C. elegans and Drosophila model systems proposed that Syt1 may be important in positioning vesicles next to voltage-gated Ca^{2+} channels rather than serving as the Ca^{2+} sensor of release (Nonet et al., 1993; DiAntonio et al., 1993; Littleton et al., 1993), and Neher and Penner (1994). However, subsequent work on transgenetic mouse model has revealed that Syt1 may directly serve as the Ca^{2+} sensor for synaptic vesicle exocytosis (Geppert et al., 1994; Fernandez-Chacon et al., 2001; Sørensen et al., 2003; Pang et al., 2006a). The following experiments further support the role of Syt1 as the Ca^{2+} sensor for exocytosis: 1) The Ca^{2+} dependence of vesicle release was completely disrupted by a Syt1-C2A^{3DA}C2B^{3DA} mutation in

which aspartate (D) residues were substituted for asparagine (N), that block Ca^{2+} binding (Sutton, Davletov et al. 1995, Ubach, Zhang et al. 1998, Sutton, Ernst et al. 1999, Fernandez, Arac et al. 2001); Shin et al., 2009); 2) A point mutation in a positively charged residue within the C2A domain, Arginine (R) 233, to glutamine (Q) (R233Q), decreases both the Ca^{2+} binding affinity of Syt1 and release by a factor of ~ 2 (Fernandez-Chacon et al., 2001); 3) Lastly, a gain-of- function mutation, $Syt1^{C2A3W\,,\,C2B3W}$, which replaces hydrophobic residues in the C2A and C2B Ca^{2+}-binding loops 1 and 3 for tryptophan (W) residues, augments neurotransmitter release by enhancing the Ca^{2+} binding affinity (Rhee et al., 2005).

The function of Syt1 as the Ca^{2+} sensor in vesicle release intrinsically depends on its interactions between SNARE proteins such as synaptobrevin, syntaxin-1, and SNAP (Sollner, Bennett et al. 1993); Chapman, 2008; Pang et al., 2006). Additionally, Syt1 interacts with anionic phospholipids, preferentially phosphatidylserine (PS), due to the generated electrostatic potential switch of Syt1 from positively charged Ca^{2+} ions binding to the negatively charged aspartaes in each of the C2 domains. This positively charged Syt1 in turn be inserted into the negative headgroups of PS in a Ca^{2+} dependent manner (Fernandez-Chacon et al., 2001; Earles et al., 2001; Rhee et al., 2005). While studies have attempted to genetically disrupt the interactions of Syt1 to either SNARE or PS (Pang et al., 2006), it turns out to be a challenge to independently disrupt one of these interaction as it appears that syt1 interactions with SNARE proteins and PS may be mutually inclusive (Chapman, 2008).

While Syt1 is well established as the Ca^{2+} sensor of release in neurons (Chapman, 2008) there exist a total of sixteen synaptotagmin isoforms within the brain and at least eight of these isoforms retain the Ca^{2+} binding affinity (Xu et al., 2007). Therefore, it is suggested that different Syt isoforms may serve as Ca^{2+} sensors in different cell types such as Syt1 in hippocampal

neurons and Syt2 in cylax of Held and even for different types of vesicles such as catecholamine and peptide hormone secretion (Syt1 & Syt7) (Schonn et al., 2008; Gustavsson et al., 2008).


a
C2A
C2B
Vesicle membrane
NH2
TMD
K326
K327
Y311
R233
Loop 3
Loop 1
Loop 3
Loop 1
K366

b

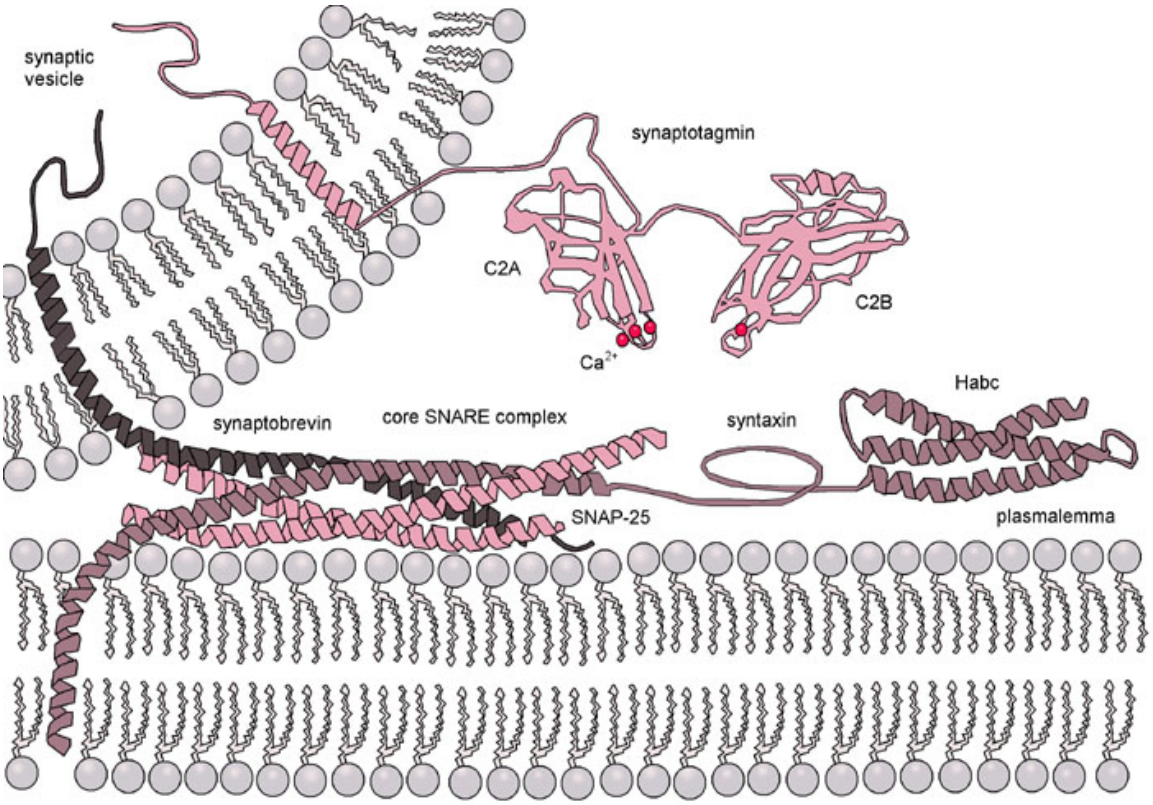

synaptic vesicle
synaptotagmin
C2A
C2B
Ca2+
Habc
synaptobrevin
core SNARE complex
syntaxin
SNAP-25
plasmalemma

Figure 2. Vesicle protein synaptotagmin 1 is the Ca^{2+} sensor for release. A. A schematic representation of Syt1 with a short interlumenal domain, a transmembrane domain and two tandem C2 domains, C2A and C2B, with bound Ca^{2+} ions (red). Three Ca^{2+} ions bind to the C2A domain and two Ca^{2+} ions bind to the C2B domain to ensure vesicle release. **B**. Cartoon representation of vesicle bound Syt1 and the key players of the SNARE complex: vesicle bound synaptobrevin and membrane bound SNAP-25 and syntaxin. Original image modified from Georgiev, Danko D & James F . Glazebrook from *Nano and Molecular Electronics Handbook,* 2007

1.2.4 Calcium dependent regulation of endocytosis

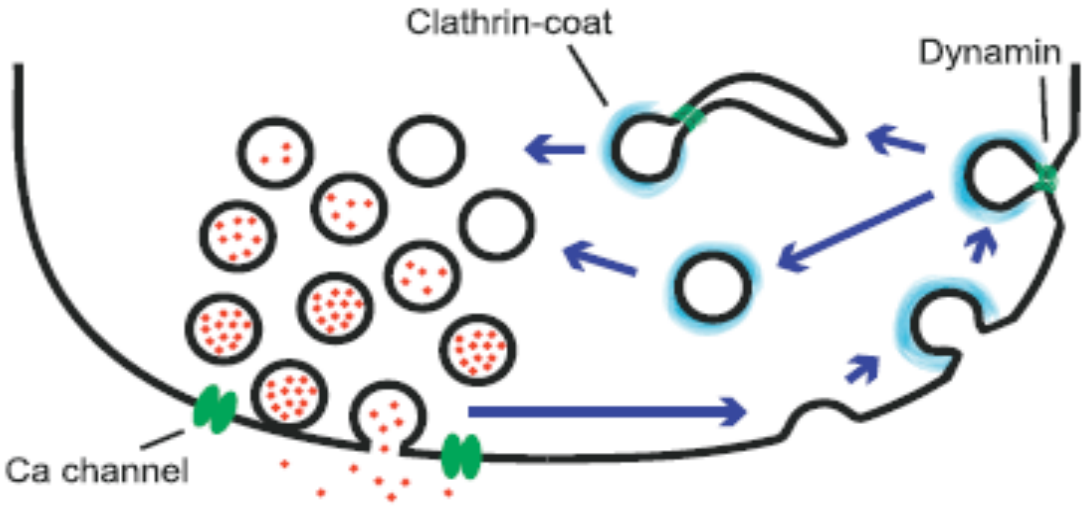
In an effort to understand how vesicles were recapitulated for the next round of release at the synapse, pioneering work in the frog neuromuscular junction by Heuser and Reese (1973) and Bruno Ceccarelli (1973) demonstrated that after vesicle exocytosis, vesicles can be retrieved by endocytosis locally at the axon terminal. It was initially demonstrated that the endocytic process could take up to tens of seconds (Miller and Heuser, 1984). However, endocytosis can occur within a few milliseconds after action potential firings.

The first line of evidence supporting the importance of in endocytosis came from the use of α-Latrotoxin, a neurotoxin isolated from black widow spider venom that triggers Ca^{2+} independent exocytosis (Sudhof, 2001). Application of α-Latrotoxin in a Ca^{2+}-free solution led to severe depletion of synaptic vesicles at the nerve terminal (Ceccarelli & Hurlburt, 1980), which likely due to arrests in the endocytic mechanisms in the absence of extracellular Ca^{2+}. Further evidence in support of this notion came from the lamprey reticulospinal synapse in which Ca^{2+} removal immediately after exocytosis inhibited endocytosis (Gad et al., 1998). Moreover, it has been suggested that Ca^{2+} may initiate and switch between different forms of endocytosis at nerve terminals, such as clathrin-mediated endocytosis, kiss-and-run and bulk endocytosis (Fig. 3) (Wu et al., 2009 Hosoi et al., 2009). Three forms of endocytosis are hypothesized at synapses (Royal & Langnado, 2003). In the best-characterized form, full collapse of the synaptic vesicle occurs after exocytosis, which is then followed by clathrin-mediated invagination and fission. "Kiss-and-run" exocytosis and endocytosis involve a transient fusion-pore followed rapidly by fission without full collapse of vesicle membrane and full retained identity of the vesicle and the third form is by forming an intermediate membrane compartment, the endosome-like structure, from which vesicles bud off; this form is identified as bulk endocytosis (Wu et al., 2013). These three

distinct endocytic forms may operate under different constraints with Ca^{2+} proximity playing a major role in contributing to the kinetic dispositions of the various modes. It has been demonstrated that Ca^{2+} may regulate these various individual forms by: 1) immediate vicinity of Ca^{2+} channels initially triggering endocytic forms and 2) by how far intracellular Ca^{2+} travels from the active zone to the periactive zone based upon stimulation intensity (Yamashita, 2012). Bulk endocytosis has been established to associate with large increases in Ca^{2+} influx during high and intense stimulation (Wu et al., 2009) while Kiss-and-Run kinetics of slow release and rapid retrieval, have been demonstrated to be favored in neuroendocrine cells at higher Ca^{2+} concentrations (Ales et al., 1999). Finally, CME has been identified to be Ca^{2+} dependent mechanism by witnessed effects of its kinetic components tailored to different Ca^{2+} concentrations (Wu et al., 2009; Hosoi et al., 2009; Yao et al., 2012). While most work has demonstrated a stimulatory role of Ca^{2+} in endocytosis, some preparations have shown that Ca^{2+} may not play any obvious roles (Ryan et al., 1996; Wu & Betz, 1996) or even an inhibitory role in endocytosis (von Gersdorff & Matthews, 1994b; Leitz & Kavalali, 2011).

Clathrin-Mediated Endocytosis

A



B

Kiss-and-Run

C

Bulk Endocytosis

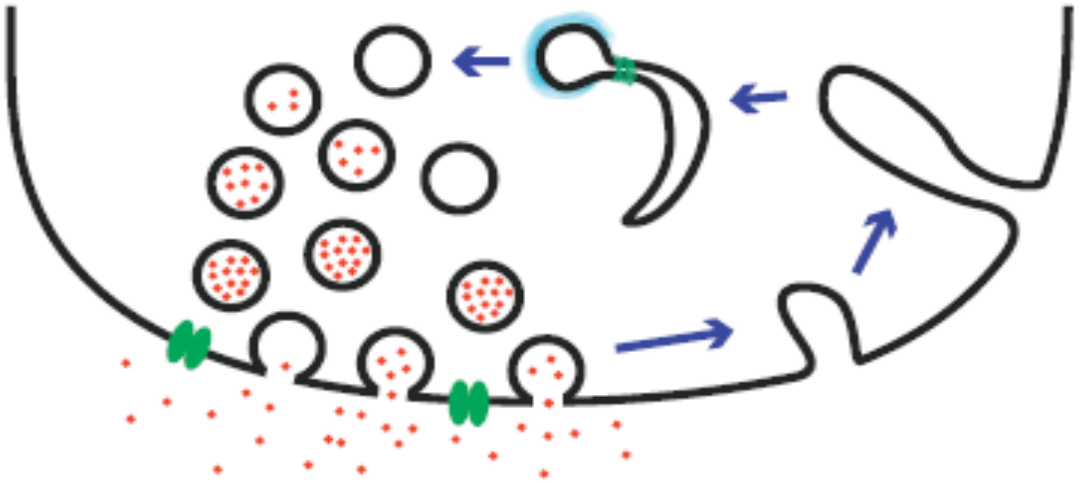


Figure 3. The three modes of vesicle retrieval at the synapse. A Clathrin-Mediated-Endocytosis: synaptic vesicle membrane is retrieved via a clathrin coat and pinched-off by GTPase Dynamin. **B**. Kiss-and-Run endocytosis is determined by a transient fusion pore with the plasma membrane with retrieval resulting in consistent identity of the synaptic vesicle. **C**. Bulk endocytosis: is believed to be the result of very high frequency, intense stimulation that generates large exocytosis. This massive addition to the plasma membrane requires an equally large generation of infolding membrane to maintain the presynaptic identity. It is believed that synaptic vesicles are then generated from this large endosome-like structure through clathrin-mediated budding. The original cartoon was from Yamashita et al., 2012, modified through Kelly Varga.

1.2.5 Syt1 in vesicle endocytosis

With the identification that calcium influx triggers and accelerates endocytosis in nerve terminals and nonneuronal secretory cells (Wu et al., 2014), the next question is what is the Ca^{2+} sensor for endocytosis? Some studies imply that calcium/calmodulin-activated calcineurin, which dephosphorylates endocytic proteins, may serve as the Ca^{2+} sensor for endocytosis (Wu, XS et al., 2009), since inhibitions in calmodulin kinase activities results in a blockage of rapid, slow, and bulk endocytosis and endocytosis overshoot at both calyces (Wu et al., 2009) and rapid endocytosis in chromaffin cells (Artalejo et al., 1996). However, this notion is still questionable, since there exist controversial results in different cell types, developmental stages, and endocytic forms (Wu et al., 2014).

Another protein that may serve as the Ca^{2+} sensor for endocytosis is Syt1. It has been demonstrated that synaptic vesicles are depleted at synaptic terminals in Syt1 null mutants in the nematode *Caenorhabditis elegans*, indicating a role of Syt1 in synaptic vesicle endocytosis (Jorgenssen et al., 1995). Additionally, timely Syt1 photoinactivation right after exoctyosis, inhibits endocytosis of synaptic vesicles at the *Drosophila* synapse (Proskanzer et al, 2003,). Nicholson-Tomishima and Ryan (2004) further demonstrate that Syt1 is also required for synaptic vesicle endocytosis in hippocampal neurons through the use of synapto-pHluorin. Finally, Proskanzer et al., (2006) proposes that Syt1 may directly control Ca^{2+} sensitivity of synaptic vesicle endocytosis. This concept is reiterated by a recent work demonstrating Syt1 as the Ca^{2+} sensor for synaptic vesicle endocytosis as measured by the whole-cell patch configuration (Yao et al., 2011). However, due to the existence of multiple forms of synaptic vesicle endocytosis, the proposal that Syt1 is the Ca^{2+} sensor in synaptic

vesicle endocytosis opens new questions: Is Syt1 a Ca^{2+} sensor that "switches" forms of retrieval? or Is Syt1 the universal regulator underlying all modes of endocytosis?

1.2.6 Clathrin-Mediated Endocytosis: a snapshot of Syt1 and adaptor proteins

Clathirn-mediated endocytosis (CME) is one of the most well studied endocytic pathway for membrane proteins, extracelluar ligands and material that is to be taken up from the cell surface (McMahon & Boucrot, 2011). Clathrin- coated vesicles proceed through a series of steps which include: initiation, cargo selection, coat assembly, scission and uncoating. Proteomic analysis of clathrin-coated vesicles from neurons reveals that up to 200 different gene products appear to be associated with proper function of this retrieval pathway (Blondeau, et al., 2004).

CME is believed to be initiated by the recruitment of the clathrin adaptor protein AP-2 (Schmid, 1997). AP-2 is a heterotetramer complex composed of α, β2, μ2, and σ2 subunits, which interact with both lipids in the membrane as well as tyrosine or dileucine-based motifs of endocytic proteins (Heilker et al., 1996). Biochemically, AP2 has widely been demonstrated to interact with Syt1 (Chapman et al., 1998; Zhang et al., 1994) and it has been shown that this Syt1- AP-2 interaction is possible due to Syt1 C2B domain binding the AP-2μ-related protein stonin-2 (Stn 2) which subsequently binds to AP-2 (Hauke & De Camilli, 1999; Kononenko et al., 2013). Thus, it is not surprising that synapses lacking functional Syt1 protein show a defect in endocytosis (Jorgensen et al., 1995; Nicholson-Tomishima & Ryan, 2004; Proskanzer et al., 2003). However it is not well defined if this is the only necessary contribution that Syt1 provides for the endocytic pathway and if this is in fact where the Ca^{2+}- dependent effects attributed to Syt1 is at play. It appears that Syt1 is a necessary component involved in recruiting molecular players like AP-2 and clathrin to endocytic sites for CME (Zhang et al., 1994). Additionally it

has been shown that Syt1 may be involved in the Ca^{2+} sensitivity of synaptic vesicle endocytosis (Poskanser et al., 2006; Yao et al., 2011). Therefore it is possible that Syt1 is involved in multiple steps in the CME pathway including serving as a Ca^{2+} sensor in this mode of vesicle retrieval. To better understand the role of Syt1 in CME, our lab has developed a novel technique to understand how this protein is regulating the kinetics associated with single endocytic events; the cell-attached capacitance method. Thus this technique allows a high-resolution examination of single endocytic events and can be used to study whether CME depends on Syt1and how single event kinetics are regulated by this protein.

CHAPTER II: METHODOLOGY

2.1 Solutions and culture media preparations

All solutions can be kept at -20 °C for up to six months and the culture media kept at 4°C for up to 3 months.

Culture media: 100ml**:** DMEM (1X) + GlutaMAX^{TM}-1 Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Frederick, MD), 1ml of Pen-Strep solution (Life Technologies), 1ml of ITSX (Insulin-Transferrin-Selenium-Supplementation,).

Enzyme solution: 250 ml of DMEM, 2.5 ml of 100 mM $CaCl_2$, 2.5 ml of 50 mM EDTA.

Inactivation solution: 225 ml of DMEM, 25 ml of FBS, 625 mg of Albumin, 625 mg trypsin inhibitor.

Locke's Solution: 154 mM NaCl, 5.6 mM KCl, 5.0 mM HEPES, 3.6 mM NaHCO3, 5.6mM Glucose, pH 7.3 with NaOH.

Poly-d-Lysine (PDL): (Sigma, St. Louis, MO) solution at 50 mg/ml and the final concentration to coat coverslips is 1 mg/ml.

Extercellular solution: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES-\ NaOH, and 10 mM glucose; pH is adjusted to 7.3 with NaOH. Osmolarity is ~ 310 nmol/kg.

Pipette solution:

50 mM NaCl, 100 mM TEACl, 5 mM KCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, and 10 mM HEPES; pH is adjusted to 7.3 with NaOH. Osmolarity is ~ 290 nmol/kg.

2.2 Chromaffin Cell Culture

Among cell model systems, the adrenal chromaffin cell has provided some of the most definitive insight into the molecular machinery underlying synaptic vesicle recycling. Adrenal glands of newborn pups of either sex (postnatal day 0) from wildtype (WT) or Syt1 heterozygous (HT) mouse matings were isolated in accordance with the guidelines of the National Institutes of

Health, as approved by the Animal Care and Use Committee of the University of Illinois at Chicago. Tails were kept for genotyping for newborn pups from Syt1 HT matings, and electrophysiological recordings on chromaffin cell cultures from littermate WT and Syt1 knockout (KO) were compared. WT chromaffin cells were typically from WT matings.

Since the whole adrenal glands were used for cell preparation, chromaffin cells in culture were mixed with cortical cells; mice taken between Day 0-Day 3 reduce the coincidence of mixed fibroblast among chromaffin cells. Mice were euthanized via cervical decapitation due to their resistance to CO_2 administration. Once the head was removed, a longitudinal cut down the back of the pup was made following the spine from cervical to the caudal region cutting superficially under the skin. Relieved skin was pinned away from spine and a transectional cut at the cervical spinal cord followed by two parallel cuts along the sides of the spine allows for access to the spinal column which was then pinned backwards towards the caudal region of the mouse. The resulting exposure of the kidneys allowed for easy access to the adrenal glands located atop each of the kidneys. Each gland was placed into an eppendorf tube filled with Locke's solution (for Locke's solution, see section 2.1); Glands were kept in this condition for up to 2 hrs. Enzyme solution (for enzyme solution, see section 2.1) was bubbled with 5% CO_2 + 95% O_2 for 15 min prior to use and equilibrated to turn from a bright pink color into pinkish/orange color. Papain (Worthington, Lakewood NJ), is added to the freshly bubbled enzyme solution at a final concentration of 20-25 unit/ml. Each pair of glands from each animal was incubated in their own individual tube for 40-60 min at 37° C. 75% of inactivation (Inactivation solution, see section 2.1) solution was then added to each tube and incubated at 37°C for another 10 min. Glands were then transferred into a newly labeled tube and then washed 3 times with culture media (Culture media, see section 2.1). After washing, glands were

placed in the culture media at 200 µl and titrated through a 200 µl pipette tip until tissue was dissolved in to the solution. An additional 250 µl of culture media was added to bring the total volume to 450 µl and cells were plated at 60 µl onto seven 12-mm PDL-precoated coverslips, which were placed in a 60x15mm culture dish and incubated in a incubator at 37 °C maintained with a steady flow/supply of 5% CO_2 for 30 min. After incubation, 5 ml of pre-warmed culture media was added into the culture dish and incubated for 24 hrs prior to cell-attached recordings. Cells can typically be used for electrophysiological recordings for up to 4 days. Chromaffin cells are typically very bright and near-perfectly circular with a brownish/beige coloring, while mixed cortical cells appear much smaller and dimmer.

Phospholipids such as PS, phoshatidylethanolamine (PE), phosphotidylcholine (PC), at 10 µM were added to the culture media for 24 hrs prior to cell-attached electrophysiological recordings (see section 2.6) or immunocytochemisty (described in section 2.5). PS, PE and PC were purchased from Sigma (St. Louis, MO).

2.3 PCR Assay for Syt1

The current standard for genotyping is the polymerase-chain reaction (PCR). 2mm of tails kept at the time of andrenal gland isolation (see section 2.2) had 200 µl of 50mM NaOH added and heated at 95 °C in a heat block for 30-60 min, vortexed before adding 20 µl of 1M Tris (Sigma) and vortexed again. A 5 min spin in a microcenterfuge was done at 15000g to separate out the aqueous top layer of DNA and lower solid layer of protein. DNA could be utilized immediately in PCR reactions or stored in a 4° C for later analysis.

Primer sets were designed according to Jackson Laboratories (Ben Harbor, ME) website in accordance to the Syt1 protocol for the Syt1 mice obtained from Jackson Laboratories.

WT Forward: 5' – GTATTCAGTGCGTCTCAGAGACAGTC -3'

WT Reverse: 5' – AACTATAATTTGTCACAGGCATTGCCTTTCA -3'

KO Forward: 5' – GAGCGCGCGCGCCGGAGTTGTTGAC -3'

KO Reverse: 5'- AACTATAATTTGTCACAGGCATTGCCTTTCA -3'

A Master mix solution was created for the number of tails that were to be genotyped with a 1X reaction as follows : 5.5 µl of ddH_2O, 10X buffer (Kappa PCR Kit, Kappa Biosystems, Boston, MA), 1.2 µl of 2.5 mM dNTPs, 1 µl of 10 mM WT Forward primer (sequence listed above), 1 µl of 10mM WT Reverse (sequence listed above), 1 µl of KO Forward primer (sequence listed above), 1 µl of KO Reverse (sequence listed above), 0.1 µl of Taq (Kappa PCR Kit). 2µl of extracted DNA was added to each tube bringing the total reaction volume per PCR tube to 10 µl. The PCR program run was as follows:

Step 1: 93 °C for 10 min

Step 2: 93 °C for 30 sec

Step 3: 60 °C for 45 sec

Step 4: 65 °C for 2 min

Step 5: Go to step 2, 40 cycles

Step 6: 65 °C for 10 min

Step 7: 4 °C forever

Each primer pair was shown to be gene specific by producing only a single amplicon of the expected size through PCR amplification and subsequent gel electrophoresis. The expected size

of WT gene was 700 bp and KO gene was expected at 400 bp based against a 1Kb ladder used to determine gene amplification sizing

2.4 Lenti-virus System in mouse adrenal chromaffin cell

Please see Chapter IV for an in depth description.

2.5 Immunocytochemistry

Chromaffin cells were cultured as described previously (see section 2.2) and fixed for 15 min in 4% paraformaldehyde (65% ddH_2O, 10% 10X PBS (800 ml of ultrapure H2O, 80g NaCl, 2g KCl, 7.62g Na2HPO4, 0.77g KH2PO4; pH of 7.4, final volume to 1000 ml), 25% of 16% paraformaldehyde). After fixation cells were rinsed three times at 5 min each in 1X PBS (see aforementioned recipe for 10X PBS and brought to a 1X concentration; 1:10). Cells were then blocked, covered for 40 min in blocking buffer (IF buffer: 3% BSA in 1X PBS, 0.1% Trition X-100 and 2% Normal Donkey Serum). Cells were then washed three times with IF buffer and incubated with primary antibody for 1 h. Following 1 h incubation, cells were washed three times in IF buffer and incubated in secondary antibody for 30 min and washed again for three times in IF buffer. Cells were then mounted using Vectasheild mounting medium for fluorescence imaging (Vector Laboratories, Burlingame, CA).

Primary anti-GFP was derived in chicken (Abcam, Cambridge, MA) and anti-tyrosine hydroxylase, a marker for catecholaminergic cells, was derived in rabbit (Abcam). Both were utilized at a 1:2000 dilution. Goat anti-chicken 488 (Abcam) and Goat anti-rabbit 555 (Abcam) were utilized as secondary antibodies at a 1: 2000 dilution. Additionally, PS insertion capability into cells was tested with a fluorescent 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine-N-(7-nitro-2-

1,3-benzoxadiazol-4-yl (NBD) labeled PS (McIntyre and Sleight 1991). The incorporation of 10-μM NBD-PS (Avanti Polar Lipids, Alabaster, AL) into chromaffin cells for 24 hrs prior to fixation and visualization was confirmed by the detection of fluorescence. NBD-PS is a pre-fluorescently labeled marker for PS with an excitation wavelength of 488nm. Cells were then fixed and stained for TH as a chromaffin marker.

To stain for intracellular PS by Annexin-V-FITC, chromaffin cells pre-incubated with an additional 10 μM cellular PS in culture media were stained and compared to WT cells. Cells were fixed with a 4% paraformaldehyde for 15 min and then washed three times in 1x PBS. The plasma membrane was permeabilized using 0.3% Triton X-100 and washed three more times in PBS. Cells were incubated with Annexin V-FITC (SouthernBiotech, Birmingham, AL) in the Annexin-V Binding Buffer (SouthernBiotech, Birmingham, AL) with 3mM $CaCl_2$ for 30 min, washed with 1X PBS, and fixed for 15 minutes in 4% paraformaldehyde in PBS and stained for TH as described. FITC (488 nm) was utilized to visualize Annexin V-FITC and TRITC (555 nm) was used to visualize TH.

All images were captured using an Olympus Fluoview (FV10i) confocal microscope (Center Valley, PA) using a 60x oil objective. Image visualization and quantitative analysis was done through the use of ImageJ (National Institute of Health, Bethesda, MD) software. Cells were only considered if they passed the following criteria: 1) Cells must first be visualized under the TRITC filter for TH in order to appropriately determine that the cell was in fact a chromaffin cell and not a mixed cortical cell, and 2) those cells that were TRITC positive were then examined under the FITC filter the filter for Syt1-GFP or GFP alone. Control cells were fixed and stained side by side to experimental cells and imaged in parallel. Images were then cropped for figure presentation using Adobe Illustrator or Adobe Photoshop (San Jose, CA).

2.6 Use of Botulinum Neurotoxin A (BoNT/A)

To determine the importance in CME of the interaction between soluble N-ethylmaleimide-sensitive factor receptor (SNARE) and Syt1, we utilized botulinum neurotoxins, which cleaves SNARE proteins (Baldwin & Barbieri, 2009). Specifically, we utilized botulinum neurotoxin A (BoNT/A) (METABIOLOGIS, INC, Madison, WI), which cleaves the last 9 amino

acid residues from the c-terminal of SNAP-25, the binding site of Syt1 to SNAP-25 (Fang et al., 2008). BoNT/A was incorporated into the cell via a 45 min incubation period as described in the literature (Fang et al., 2008). To determine the proper BoNT/A concentration, several different concentrations were utilized ranging from 5 to 25 ng/ml. 10 ng/ml was determined to be the optimized amount as concentrations above 10 ng/ml completely inhibited the number of exocytic events and thus severely reduce the number of endocytic events we were able to record per patch. Cells were utilized for 1 h after the 45 min incubation period. Cells were recorded for the standard 5 min recording length (Yao et al., 2012). The $[Ca^{2+}]_e$ was at the standard working concentration of 2 mM (Yao et al., 2012).

2.7 Cell-Attached recordings

Changes in membrane capacitance and conductance were recorded using the cell-attached patch clamp capacitance technique (Debus and Lindau 2000, Dernick, Gong et al. 2005). Patch pipettes were pulled in four stages with a programmable P-97 puller (Sutter Instruments, Novato CA) and coated with a sticky wax (Kerr, Romulus MI). Pipettes were fire-polished and had a typical resistance of approximately 2 MΩ in the bath solution. For cell-attached capacitance measurements, an EPC-7 plus patch clamp amplifier (HEKA-Elektronik, Lambrecht/Pfalz, Germany) was used. Command voltage was applied to the bath solution. Changes of patch

admittance were measured, as described previously (Debus and Lindau 2000), with a SR830 lock-in amplifier (Stanford Research Systems, Sunnyvale CA) using a sine wave amplitude of 50 mV (root mean square) at a frequency of 20 kHz. The output filter of the lock-in amplifier was set to a 1 ms time constant, 24 db. The phase of the lock-in amplifier was set such that transient capacitance changes produced by gentle suction pulses appeared only in the Im trace with no

projection into the Re trace (Debus and Lindau 2000). Since the phase may change during the recording, the phase for sections with endocytic events of interest was readjusted such that the baseline of the Re trace was at the same level before and after completion of the capacitance step.

All endocytic events were recorded by the cell-attached patch configuration unless mentioned otherwise. Capacitance steps and fission-pore durations were reliably detected for step sizes > 0.2 fF, and smaller steps were not included in the analysis. The number of endocytic events per patch detected in the cell-attached recordings was counted as the total number of downward capacitance steps within the first 5 min of recordings.

2.8 Double (cell-attached/whole-cell) patch recordings

* This work was done by Li-Hua Yao for our 2012 publication (Yao et al., 2012)

The endocytic events were also recorded by the double (cell-attached/whole-cell) patch configuration in some experiments. In this double patch configuration, the cells held at -65 mV were stimulated by a train of 100-ms depolarization to 0 mV at 0.2 Hz through the whole-cell pipette to induce endocytosis of single vesicles that were recorded by the other cell-attached patch pipette. The number of endocytic events recorded per patch in the double (cell-attached/whole-cell) patch configuration was counted as the total number of downward capacitance steps > 0.2 fF within 5 min after the first depolarization.

2.9 Fission-pore analysis

Fission-pore closures of endocytic events were analyzed in the same way as exocytotic fusion-pore openings as described previously (Debus and Lindau 2000, Dernick, Gong et al.

2005, Gong, de Toledo et al. 2007). During fission-pore closure of endocytic events in which a transient increase in the

Re signal was associated with a decline in the Im step, the vesicle capacitance (Cv) was determined as the total change in the Im trace for a particular event and the fission-pore conductance (Gp) was calculated using the formula $Gp = \frac{\omega \bullet Cv}{\sqrt{(\frac{\omega \bullet Cv}{\mathrm{Im}}) - 1}}$ (Breckenridge and Almers 1987). The fission-pore duration was defined as the time interval from the first point where Gp decreased below 2 nS and the final drop in Gp to zero. This final drop reflects the step response of the low pass filter setting of the lock-in amplifier (1 ms, 24 dB). At this setting, 90% of the final value is reached within ~7 ms, so the last point of the fission-pore was taken as the time at 7–10 ms before the final drop to zero in the Gp trace. The fission-pore conductance Gp was taken as the average Gp value during the fission-pore duration time interval. Analysis of the fission-pore kinetics were restricted to fission-pores with durations >15 ms, since shorter events were distorted by the lock-in amplifier low-pass filter (set to 1 ms, 24 dB).

2.10 Amperometry

* This work was done by Li-Hua Yao for our 2012 publication (Yao et al., 2012)

Conventional carbon fiber amperometry for catecholamine detection used 5 μm carbon fibers (ALA Scientific Instruments, Farmingdale NY) as described previously (Gong, Di Paolo et al. 2005, Gong, de Toledo et al. 2007). The freshly cut tip of the carbon fiber electrode was positioned closely against the cell surface to minimize the diffusion distance from release sites. The amperometric current, generated by catecholamine oxidation at the exposed tip of the carbon fiber electrode, was measured using an EPC-7 plus amplifier, operated in the voltage-clamp

mode at a holding potential of +700 mV. Amperometric signals were low-pass filtered at 1 kHz and digitized at 4 kHz. Similar to the stimulation pattern in double patch configuration, secretion was induced by a train of 100-ms depolarization to 0 mV at 0.2 Hz through a whole-cell patch pipette. Amperometric recordings were collected, and then analyzed with a customized macro for IGOR software (WaveMetrics, Lake Oswego, OR) to extract spike information according to the criteria of Chow and Von Ruden (Chow, von Ruden et al. 1992). The number of amperometrical spikes was counted as the total number of spikes with the amplitude > 10 pA within 120 s after the first depolarization.

2.11 Reagents

* This work was done by Li-Hua Yao for our 2012 publication (Yao et al., 2012)

Anti-clathrin heavy chain antibody (X22) was obtained from Affinity BioReagents (Golden CO). The KR-peptide, a Syt1 C2B domain derived peptide with the sequence of KRLKKKKTTIKK, was utilized to block the interaction of Syt1 and AP-2 (Grass, Thiel et al. 2004). The control experiments were performed using the scrambled version of the KR-peptide (Scr-peptide, KKKRTLTKKKKI) and the mutant version of the KR-peptide (AA-peptide, KRLKKAATTIKK) with two of the conserved lysines (KK) replaced by alanines (AA), which displays no interaction with AP-2 as shown by previous study (Kastning, Kukhtina et al. 2007). These three peptides were synthesized through Biomatik (Wilmington DE) and NeoBioscience (Cambridge MA). The intracellular application of anti-clathrin heavy chain antibody, KR-peptide, Scr-peptide or AA-peptide was through a whole-cell patch pipette for at least 15 min in the double (cell-attached/whole-cell) patch configuration while endocytosis of single vesicles was recorded by the other cell-attached patch pipette.

2.12 Statistical Analysis

All the experiments were performed at room temperature. Data is expressed as Mean ± s.e.m., and Newman of one-way ANOVA tests was used for statistical analysis. Since typically 1 or 2 analyzable endocytic events can be obtained from each individual cell, every endocytic event was treated as statistically independent and the number of events was used for statistical testing of the fission-pore kinetics.

CHAPTER III:

SYNAPTOTAGMIN 1 IS NECESSARY FOR THE CA^{2+} DEPENDENCE OF CLATHRIN-MEDIATED ENDOCYTOSIS

Yao LH, Rao Y, Varga K, Wang CY, Xiao P, Lindau M, Gong LW
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3.1 Abstract

The role of Ca^{2+} in synaptic vesicle endocytosis remains uncertain due to the diversity in various preparations where several forms of endocytosis may contribute variably in different conditions. While recent studies have demonstrated that Ca^{2+} is important for clathrin-mediated endocytosis (CME), the mechanistic role of Ca^{2+} in CME remains to be elucidated. By monitoring CME of single vesicles in mouse chromaffin cells with cell-attached capacitance measurements that offer millisecond time resolution, we demonstrate that the dynamics of vesicle fission during CME is Ca^{2+} dependent but becomes Ca^{2+} independent in synaptotagmin 1 (Syt1) knockout cells. Our results thus suggest that Syt1 is necessary for the Ca^{2+} dependence of CME.

3.2 Introduction

Neurotransmitter release is mediated by exocytosis of synaptic vesicles and subsequent endocytosis is essential for supporting rapid and repeated rounds of release (Stevens 2003). While the roles of Ca^{2+} in exocytosis are well established (Zucker and Regehr 2002, Neher and Sakaba 2008), its role in synaptic vesicle endocytosis remains uncertain (Ceccarelli and Hurlbut 1980, Ramaswami, Krishnan et al. 1994, von Gersdorff and Matthews 1994, Cousin and Robinson 1998, Gad, Low et al. 1998, Marks and McMahon 1998, Beutner, Voets et al. 2001, Neves, Gomis et al. 2001, Sankaranarayanan and Ryan 2001, Wu, Xu et al. 2005), largely because the results are diverse among various preparations where several forms of endocytosis may contribute variably in different conditions (Wu, Ryan et al. 2007). It has been recently demonstrated that Ca^{2+} is critical in clathrin-mediated endocytosis (CME), the slow form of endocytosis, in the Calyx of Held (Hosoi, Holt et al. 2009, Wu, McNeil et al. 2009). However, it remains to be elucidated how Ca^{2+} regulates the process of CME and which molecules determine the Ca^{2+} dependence of CME. In the present study, CME of single vesicles has been monitored using cell-attached capacitance measurements, in which a sinusoidal voltage is superimposed to the holding potential and a two-phase lock-in amplifier is used to analyze the current output signal. When the phase of the lock-in amplifier is properly adjusted, the two outputs of the amplifier directly provide the changes of membrane conductance in one channel and the changes of membrane capacitance in the other channel (Neher and Marty 1982, Debus and Lindau 2000). When the tubular membrane neck that connects the endocytic vesicle to the plasma membrane prior to vesicle pinch-off has a low conductance, the vesicle capacitance is not fully charged and the values in the capacitance trace are reduced while a transient increase in the conductance trace appears. As for exocytotic fusion pores (Debus and Lindau 2000), the analysis of these changes

can be utilized to estimate the dynamic behavior of endocytic fission-pore conductance (Rosenboom and Lindau 1994, Dernick, Alvarez de Toledo et al. 2003, MacDonald, Eliasson et al. 2005, Zhao, Fang et al. 2010). Our results demonstrate that the dynamics of vesicle fission during CME is Ca^{2+} dependent but becomes Ca^{2+} independent in Syt1 KO cells, indicating that Syt1 is required for the Ca^{2+} dependence of CME.

3.3 Results

3.3.1 Characterization of CME, single vesicles, and the fission pore-analysis

Fig. 4A shows a recording with both upward and downward capacitance steps. In this recording, the upward capacitance steps, which are associated with exocytosis as reported previously (Gong, Hafez et al. 2003, Gong, de Toledo et al. 2007), occurred at earlier times than the downward capacitance steps associated with endocytosis (Fig. 4A). The plot of endocytic events as a function of time shows that the frequency of endocytic events increased after the first depolarization (Fig. 4B), suggesting that the stimulation was effective to increase the rate of endocytotic events. The diameter of individual vesicles associated with endocytosis was calculated assuming a specific capacitance of 9 fF/μm^2 (Albillos, Dernick et al. 1997, Gong, Hafez et al. 2003) and spherical geometry. The resulting frequency distribution of vesicle diameters for these endocytic events did not show evidence for a multimodal distribution (Fig. 4C), suggesting that the downward capacitance steps reflect a single type of endocytic events. Fig. 4D exemplifies a cell-attached recording with patch conductance (Re, top) and capacitance (Im, bottom) in a control chromaffin cell. This trace displays five downward capacitance steps, indicating sequential endocytosis of five individual vesicles. When an anti-clathrin heavy chain antibody was delivered into the cells through the whole-cell pipette in double (cell-attached/whole-cell) patch configuration, the number of endocytic events detected by the cell-attached pipette was significantly decreased in a concentration dependent manner ($p < 0.01$) while control IgG had no significant effect (Fig. 4E and F), demonstrating that these endocytic events represent CME. For a subset of individual endocytic events, the decrease in Im was associated with a detectable transient change in the Re trace, reflecting detection of a narrow fission-pore (Fig. 4G), which was characterized by the fission-pore conductance (Gp) and

duration (Fig. 4H). We then went on to examine the fission-pore kinetics of endocytic events in cells treated with 60 μg/ml anti-clathrin heavy chain antibody, which inhibited the number of endocytic events by ~ 65% (Fig. 4F), since treatments with this antibody at concentrations of 120 or 240 μg/ml reduced the occurrence of endocytic events to such a low level that a statistical analysis was impossible. Our results showed that cells pre-treated with 60 μg/ml antibody displayed no changes in the capacitance step size of the endocytic vesicles (Fig. 4I), the fission-pore Gp (Fig. 4J) and the fission-pore duration (Fig. 4K). It has been shown by electron microscopy that AP-2 and associated endocytic accessory proteins assemble into coated membrane sub-domains that lack any significant curvature in the absence of clathrin, demonstrating that clathrin is important for invagination during CME (Nossal 2001, Hinrichsen, Meyerholz et al. 2006). Our results thus indicate that the fission-pore kinetics cannot be affected by defects in endocytic invagination.

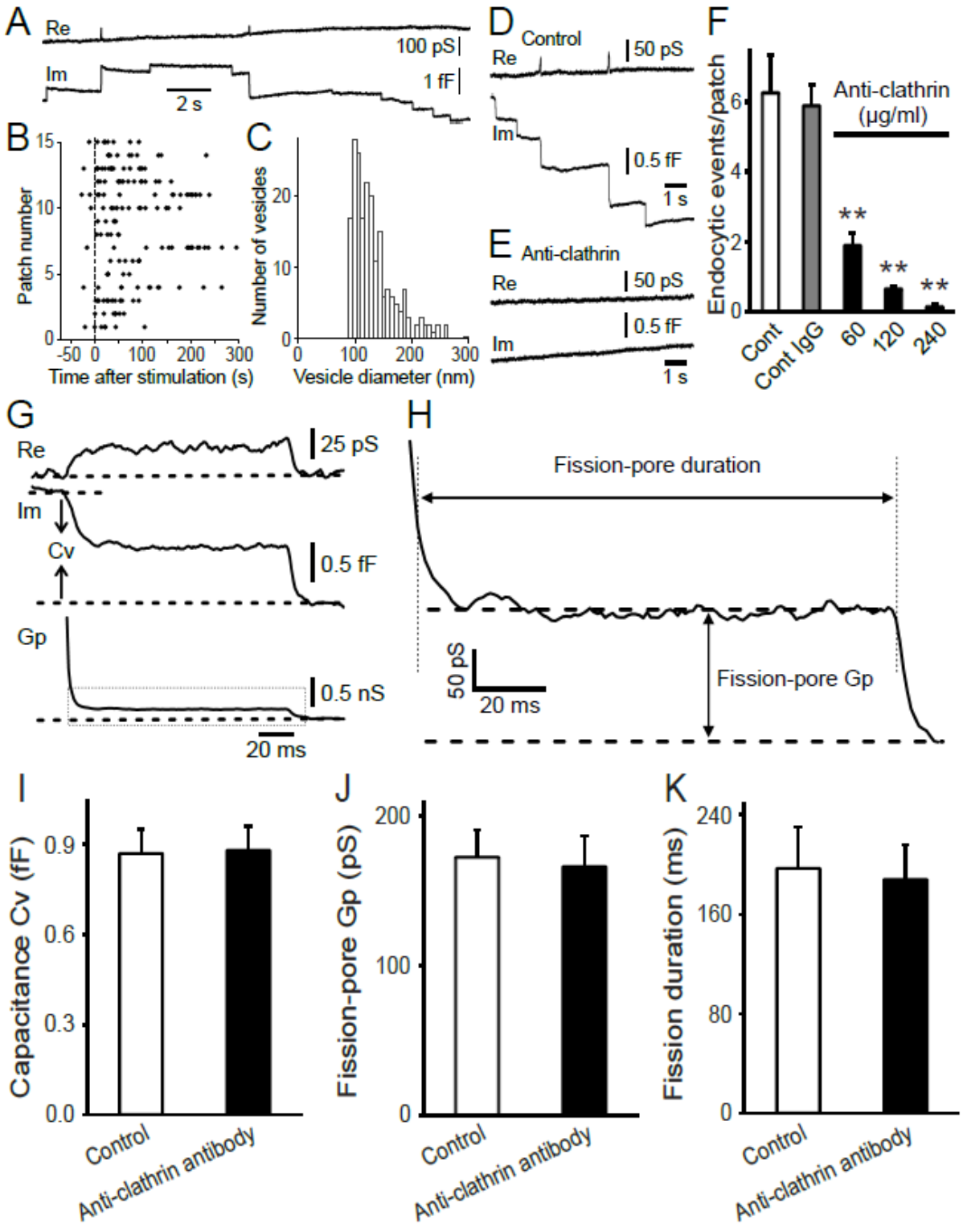

A
Re
100 pS
Im
1 fF
2 s
B
Patch number
Time after stimulation (s)
C
Number of vesicles
Vesicle diameter (nm)
D
Control
Re
50 pS
Im
0.5 fF
1 s
E
Anti-clathrin
Re
50 pS
Im
0.5 fF
1 s
F
Endocytic events/patch
Anti-clathrin (μg/ml)
Cont
Cont IgG
60
120
240
G
Re
25 pS
Im
Cv
0.5 fF
Gp
0.5 nS
20 ms
H
Fission-pore duration
50 pS
20 ms
Fission-pore Gp
I
Capacitance Cv (fF)
Control
Anti-clathrin antibody
J
Fission-pore Gp (pS)
Control
Anti-clathrin antibody
K
Fission duration (ms)
Control
Anti-clathrin antibody

Figure 4. CME of single vesicles and fission pore analysis. **A,** Simultaneous recordings of Re and Im by a cell-attached patch pipette in the double (cell-attached/whole-cell) patch configuration showed upward capacitance steps associated with exocytosis and downward capacitance steps associated with endocytosis. **B,** The occurance of individual endocytic events as a function of time for each of 15 patches with at least six endocytic events. Each dot represents an individual endocytic event and the vertical dotted line indicates the onset of stimulation. **C,** Distribution of vesicle diameters derived from endocytic capacitance step sizes. The mean vesicle diameter is 122 ±2.7nm (*n* = 238 events). **D,E,** Downward steps in Im associated with single vesicle endocytosis in a control cell **(D)** were largely abolished in a cell treated with 240 μM anti-clathrin antibody **(E)**. **F,** Statistical analysis demonstrating that applications of anti-clathrin heavy chain antibody through the whole-cell patch pipette significantly decreased the number of endocytic events recorded within 5 min by the cell—attached patch pipette (Control: n = 48 patches; 240 μg/ml Control IgG: n = 40 patches, $p > 0.05$; 60 μg/ml anti-clathrin antibody: n = 75 patches, $p < 0.01$; 120 μg/ml anti-clathrin antibody: n = 28 patches, $p < 0.01$; 240 μg/ml anti-clathrin antibody: n = 30 patches, $p < 0.01$), demonstrating that these endocytic events likely represent CME of single vesicles. **G,**. Detection in the fission-pore of an individual event. The traces from top to bottom represented the time course of: Re, Im, the fission-pore Gp, and capacitance of the endocytic vesicle (Cv). The horizontal dashed lines indicated the baselines of the respective signals. The box in **G** is shown on an expanded time scale in **H** for the demonstration of the fission-pore kinetics. **H,** Fission-pore kinetics was characterized by the fission-pore Gp and duration as described in the section of Material and Methods. **I,** Application of anti-clathrin antibody at 60 μg/ml through a whole-cell patch pipette did not alter the capacitance size of the endocytic vesicles (Control: 0.87 ± 0.08 fF, n = 47 events; Anti-clathrin antibody: 0.88 ± 0.08 fF, n = 45 events) ($p > 0.05$) (**J**), the fission-pore Gp (Control: 407 ± 48 pS; Anti-clathrin antibody: 433 ± 61 pS) ($p > 0.05$) (**K**), and the fission-pore duration (Control: 197 ± 33 ms; Anti-clathrin antibody: 188 ± 28 ms) ($p > 0.05$). The double asterisk indicates $p < 0.01$ in F. *Electrophysiology preformed by Lihua-Yao.

3.3.2 Ca^{2+} dependent properties of endocytic events

The endocytic events observed in the cell-attached recordings are Ca^{2+}-dependent and triggered by Ca^{2+} influx into the cell during patching, since removal of external Ca^{2+} substantially reduced the occurrence of these events ($p < 0.01$) (Fig. 5A). The number of endocytic events was decreased by ~ 70% at 0.5 mM $[Ca^{2+}]_e$ as compared to 2 mM $[Ca^{2+}]_e$ ($p < 0.01$) (Fig. 5A). Meanwhile, exocytosis, as measured by carbon fiber amperometry was also inhibited to a similar extent at 0.5 mM $[Ca^{2+}]_e$ ($p < 0.01$) (Fig. 5B). As a result, the endocytosis/exocytosis ratio was indistinguishable at 2 and 0.5 mM $[Ca^{2+}]_e$ ($p > 0.05$) (Fig. 5C), suggesting that the inhibition in endocytosis at lower $[Ca^{2+}]_e$ may be a consequence of the reduction in exocytosis. The capacitance step size (Fig. 5D and E) and the fission-pore Gp (Fig. 5D and F) of these endocytic events were not statistically different in these two groups ($p > 0.05$). However, the fission-pore duration was prolonged by ~ 250% at 0.5 mM $[Ca^{2+}]_e$ as compared to 2 mM $[Ca^{2+}]_e$ ($p < 0.01$) (Fig. 5D and G), indicating a direct role of Ca^{2+} in vesicle CME fission kinetics.

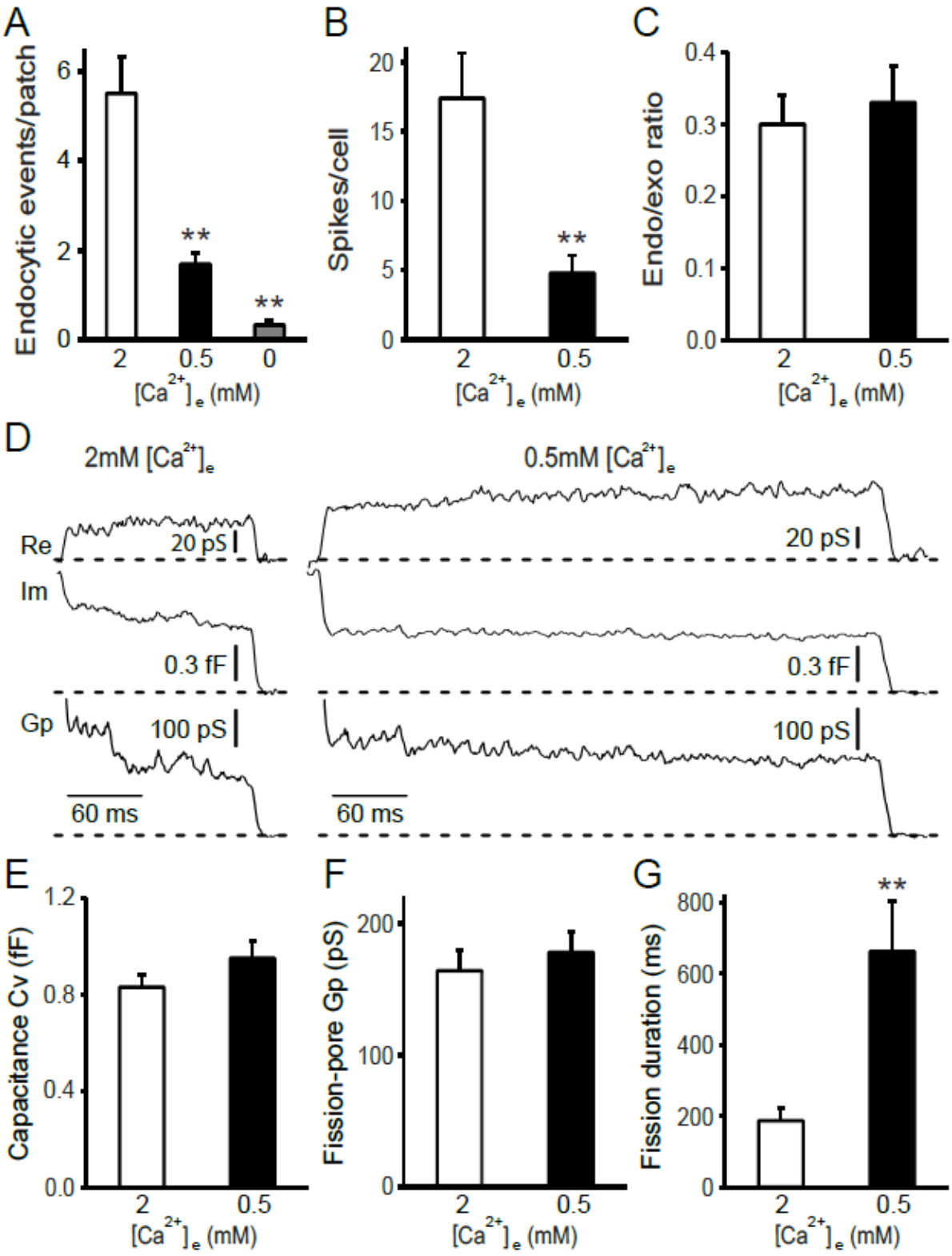

A
Endocytic events/patch
6
4
2
0
**
**
2
0.5
0
[Ca2+]e (mM)
B
Spikes/cell
20
15
10
5
0
**
2
0.5
[Ca2+]e (mM)
C
Endo/exo ratio
0.4
0.3
0.2
0.1
0.0
2
0.5
[Ca2+]e (mM)
D
2mM [Ca2+]e
0.5mM [Ca2+]e
Re
20 pS
20 pS
Im
0.3 fF
0.3 fF
Gp
100 pS
100 pS
60 ms
60 ms
E
Capacitance Cv (fF)
1.2
0.8
0.4
0.0
2
0.5
[Ca2+]e (mM)
F
Fission-pore Gp (pS)
200
100
0
2
0.5
[Ca2+]e (mM)
G
Fission duration (ms)
800
600
400
200
0
**
2
0.5
[Ca2+]e (mM)

Fig. 5. Dependence of endocytic event properties on $[Ca^{2+}]_e$. **A**. The number of endocytic events within 5 min of cell-attached recordings was reduced by ~ 70% at 0.5 mM $[Ca^{2+}]_e$ (n = 102 patches) ($p < 0.01$) and by ~ 95% at zero $[Ca^{2+}]_e$ (n = 46 patches) ($p < 0.01$) as compared to 2 mM $[Ca^{2+}]_e$ (n = 95 patches). Zero $[Ca^{2+}]_e$ was achieved through a combination of removal of Ca^{2+} and addition of 5 mM EGTA. **B**. The number of amperometrical spikes at 0.5 mM $[Ca^{2+}]_e$ (n = 18 cells) was reduced as compared to 2 mM $[Ca^{2+}]_e$ (n = 18 cells) ($p < 0.01$). The experiments in A and B were performed on different cells from the same preparations. **C.** The endocytosis/exocytosis ratio was not statistically different between 2 and 0.5 mM $[Ca^{2+}]_e$ ($p > 0.05$). **D**. Representative fission-pore events at 2 mM (left) and 0.5 mM $[Ca^{2+}]_e$ (right) in the cell-attached patch configuration. **E**. The mean capacitance step size of endocytic vesicles was indistinguishable between these two groups (2 mM $[Ca^{2+}]_e$: n = 43 events; 0.5 mM $[Ca^{2+}]_e$: n = 42 events) ($p > 0.05$). **F.** The fission-pore Gp was independent of $[Ca^{2+}]_e$ ($p > 0.05$). **G**. The fission-pore duration was significantly increased ($p < 0.01$) at 0.5 mM $[Ca^{2+}]_e$. The double asterisk indicates $p < 0.01$ in A, B and G. *Electrophysiology preformed by Lihua-Yao.

3.3.3 Ca^{2+} dependence is lost in Syt1 KO cells

Given the Ca^{2+} binding capability of Syt1 and its putative role as the Ca^{2+} sensor in exocytosis (Chapman 2008, Pang and Sudhof 2010), we tested whether Syt1, the predominant synaptotagmin isoform in chromaffin cells (Marqueze, Boudier et al. 1995), may be responsible for the Ca^{2+} sensitivity of fission-pore dynamics during CME by examining the fission-pore kinetics of endocytic events in Syt1 KO cells (Fig. 6A). There was a significant decrease in the number of endocytic events in KO cells as compared to wild-type (WT) cells ($p < 0.01$) (Fig. 6B). Although exocytosis, as measured by carbon fiber amperometry, was also inhibited ($p < 0.01$) (Fig. 6C), the endocytosis/exocytosis ratio was decreased in KO cells ($p < 0.01$) (Fig. 6D), pointing to a direct role of Syt1 in CME beyond exocytosis. Both, the capacitance step size of endocytic vesicles (Fig. 6A and E) and the fission-pore Gp (Fig. 6A and F), were indistinguishable between WT and KO cells ($p > 0.05$). However, the fission-pore duration was increased by ~ 150% in KO cells as compared to WT cells ($p < 0.01$) (Fig. 6A and G). Comparison of the fission-pore durations at 2, 1 and 0.5 mM $[Ca^{2+}]_e$ between WT and KO cells revealed that the Ca^{2+} dependence of the fission-pore duration observed in WT cells was abolished in Syt1 KO cells (Fig. 6H), indicating that the Ca^{2+} dependence of fission dynamics during CME requires Syt1.

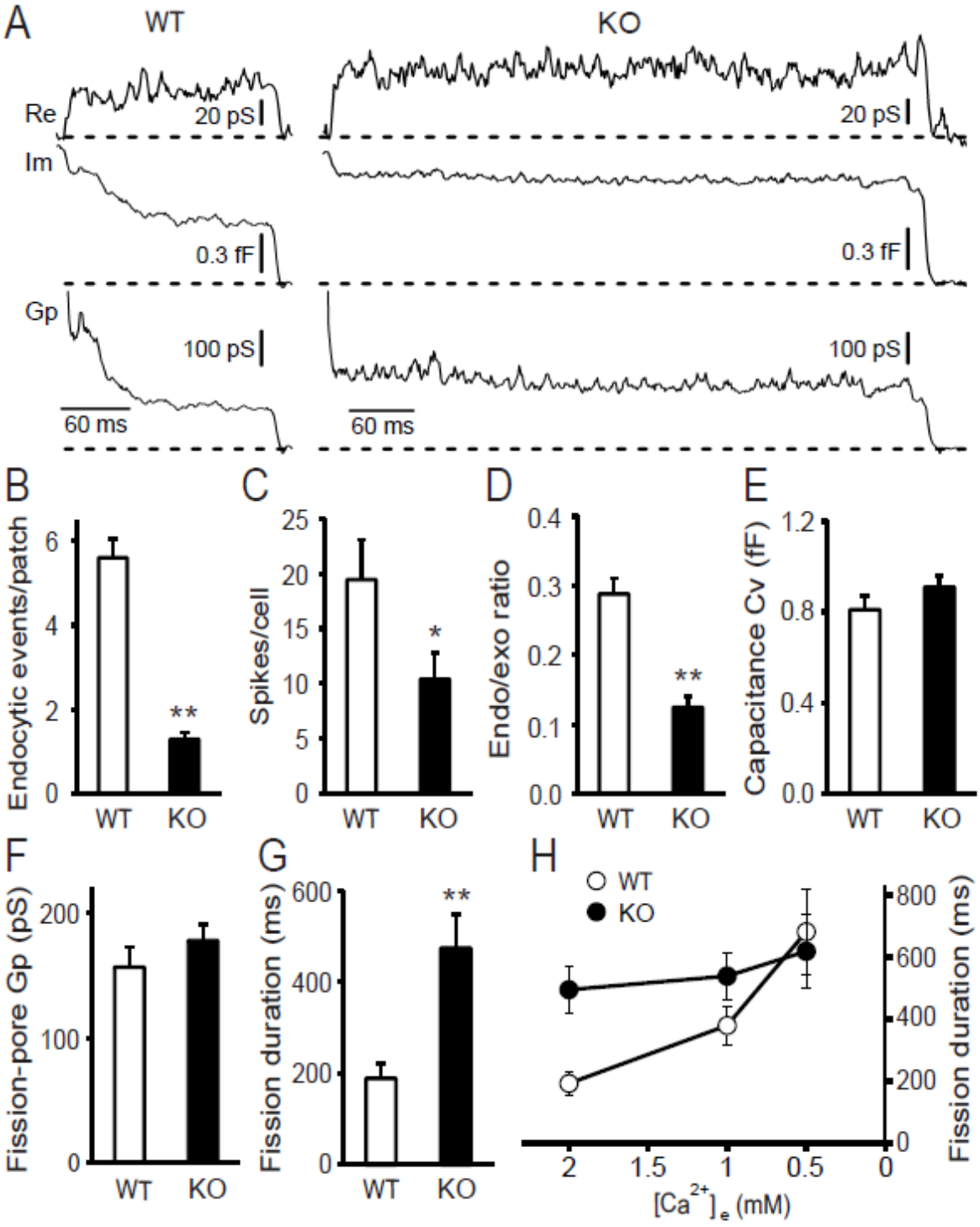

A
WT
KO
Re
20 pS
Im
0.3 fF
Gp
100 pS
60 ms
B
Endocytic events/patch
C
Spikes/cell
D
Endo/exo ratio
E
Capacitance Cv (fF)
F
Fission-pore Gp (pS)
G
Fission duration (ms)
H
WT
KO
[Ca2+]e (mM)

Fig. 6. The fission-pore duration is prolonged and its Ca^{2+} dependence is reduced in Syt1 KO cells. A. Representative events at 2 mM $[Ca^{2+}]_e$ in WT (left) and Syt1 KO cells (right) recorded in the cell-attached patch configuration. **B.** The number of endocytic events within 5 min of recordings was inhibited in Syt1 KO cells (n = 281 patches) as compared to WT cells (n = 89 patches) ($p < 0.01$). **C**. There was a decrease in the number of amperometrical spikes in Syt1 KO cells (WT: n = 21 cells; KO: n = 21 cells) ($p < 0.05$). The experiments in B and C were performed on different cells from the same preparations. **D**. The endocytosis/exocytosis ratio was also reduced in Syt1 KO cells ($p < 0.01$), indicating a defect of the capacity of CME in the absence of Syt1. **E**. The capacitance size of the endocytic vesicles was indistinguishable between WT (n = 52 events) and KO cells (n = 43 events) ($p > 0.05$). While the fission-pore Gp was not statistically different ($p > 0.05$) **(F),** the fission-pore duration was significantly increased in Syt1 KO cells ($p < 0.01$) **(G). H**. The fission-pore duration was significantly increased upon reductions in $[Ca^{2+}]_e$ in WT cells (2 mM: n = 44, 1 mM: n = 49, $p < 0.01$; 0.5 mM: n = 46, $p < 0.01$) but not in Syt1 KO cells (2 mM: n = 42, 1 mM: n = 50, $p > 0.05$; 0.5 mM: n = 39, $p > 0.05$), suggesting that the Ca^{2+} dependence of fission-pore dynamics is abolished in Syt1 KO cells. The single asterisk indicates $p < 0.05$ in C and the double asterisk indicates $p < 0.01$ in B, D and G. *Electrophysiology preformed by Lihua Yao. PCR optimization and PCR product to ensure correct genotype utilized in aforementioned experiments preformed by Kelly Varga. All mouse colony maintenance, matting and correct collection times of the mice utilized in these experiments were done by Kelly Varga and Lihua Yao.

3.4 Discussion

In the present study, we have monitored CME of single vesicles by two different configurations: cell-attached patch configuration, where endocytic events may be spontaneous and/or triggered by mechanical stimulation during patching, and the double (cell-attached/whole-cell) patch configuration, where endocytic events are stimulated by a train of depolarizations applied through the whole-cell patch pipette. The number of exocytotic events, which co-exist with these endocytic events as shown in Fig. 1A, was increased in the double (cell-attached/whole-cell) patch configuration as compared to cell-attached patch configuration (data not shown), indicating that the depolarizations were effective in stimulating exocytosis. Our results showed that the frequency of endocytic events was also increased after stimulation (Fig. 1B). It may, therefore, be expected that the number of endocytic events recorded in the double patch configuration would be higher than that in the cell-attached patch configuration. However, contrary to this expectation, our results showed that the numbers of endocytic events recorded in the two configurations were comparable (Fig. 1F, 2A, 3B and 4A). The number of endocytic events in the double patch configuration could possibly be lowered due to a loss of some factors necessary for CME when the cells are infused with intracellular solution in the whole-cell pipette. Consistent with this implication, a previous study has demonstrated that compensatory endocytosis, which occurs consistently in perforated patch configuration, shows rapid wash-out in the whole-cell configuration (Smith and Neher 1997).

We have shown here that the dynamics of fission-pore closure in chromaffin cells is Ca^{2+} dependent, which is consistent with Ca^{2+}-dependent fission-pore kinetics in insulin secreting INS-1 cells (MacDonald, Eliasson et al. 2005). In insulin secreting cells, fission-pore kinetics exhibit two distinct kinetic components (Zhao, Fang et al. 2010). Further experiments will be

necessary to determine if this is also the case in chromaffin cells and if the different kinetic components display different Ca^{2+} sensitivity.

The central finding of the present study is that the Ca^{2+} dependence of fission-pore kinetics requires Syt1. In the absence of Syt1 fission-pore dynamics is Ca^{2+} independent. The complete inhibition of endocytosis by the KR peptide at 100 μM concentration (Fig. 4A) indicates that endocytosis is mediated via an AP2-Syt interaction. Similarly, a peptide derived from synaptotagmin 2 (Syt2) blocks CME in the calyx of Held where Syt2 is the predominant synaptotagmin isoform (Hosoi, Holt et al. 2009). Like Syt1 (Zhang, Davletov et al. 1994), all synaptotagmin isoforms including synaptotagmin 4 (Syt4) bind AP2 (Ullrich, Li et al. 1994). In contrast to Syt1, the C2 domain of Syt4 does not display Ca^{2+} binding activity (Ullrich, Li et al. 1994, Li, Ullrich et al. 1995, Dai, Shin et al. 2004). It therefore appears possible that the endocytic events with Ca^{2+}-independent fission-pore dynamics in Syt1 KO cells are mediated by Syt4. Interestingly, a recent study showed that over-expression of Syt4 increased the endocytic fission-pore duration in PC12 cells (Zhang and Jackson 2010), consistent with our result that at physiological $[Ca^{2+}]_e$ the fission-pore duration is increased in Syt1 KO cells (Fig. 3G and H).

It has been proposed that Syt4 may play a role in regulating neurotransmitter release (Moore-Dotson, Papke et al. 2010), fusion pore dynamics during exocytosis (Wang, Grishanin et al. 2001, Wang, Lu et al. 2003) and granule maturation (Ahras, Otto et al. 2006) in PC12 cells, a chromaffin cell-derived cell line. However, in a recent immunocytochemical analysis confirming that Syt4 is also expressed in primary rat chromaffin cells, it was found that Syt4 was not present on dense core vesicles (Matsuoka, Harada et al. 2011). Our results suggest that in the absence of Syt1, Syt4 may support endocytosis.

Although NMR studies indicate that purified Syt1 has relatively low intrinsic Ca^{2+} affinity (Fernandez, Arac et al. 2001), recent evidence suggests that, when anchored to membrane with physiological lipid composition, Syt1 displays Ca^{2+}-dependent enhancement in exocytosis even at sub-μM Ca^{2+} levels (Lee, Yang et al. 2010). Such Ca^{2+} levels are achievable in most nerve terminals following a single-action potential or low-frequency stimulation, suggesting that Syt1 may serve as the Ca^{2+} sensor in endocytosis as well. Previous studies have noted that the rate of synaptic vesicle endocytosis is slower in Syt1 KO animals (Poskanzer, Marek et al. 2003, Nicholson-Tomishima and Ryan 2004, Poskanzer, Fetter et al. 2006) but an elevation in $[Ca^{2+}]_e$ rescues the endocytic defect caused by a Syt1 mutant with low Ca^{2+} affinity (Poskanzer, Fetter et al. 2006). However, due to the evidence that there exist multiple forms of endocytosis such as CME, kiss-and-run and bulk endocytosis in synapses (Wu, Ryan et al. 2007) and that the contribution of each form of endocytosis in synaptic vesicle endocytosis varies markedly in different conditions such as when Ca^{2+} level is changed (Wu, McNeil et al. 2009), the mechanism for the defect in synaptic vesicle endocytosis from Syt1 KO animals (Poskanzer, Marek et al. 2003, Nicholson-Tomishima and Ryan 2004, Poskanzer, Fetter et al. 2006) remains uncertain. Our results identify that, in contrast to WT cells, the Ca^{2+} dependence of fission dynamics during CME is abolished in Syt1 KO cells (Fig. 3H), suggesting that Syt1 is a necessary component for the Ca^{2+} dependence of CME.

CHAPTER IV:

INFECTION OF MOUSE CHROMAFFIN CELLS USING LENTIVRUS

4.1 Abstract

The lentiviral system has been demonstrated to have the unique ability amongst retroviruses of being able to infect non-cycling cells. Vectors that are derived from lentiviruses have provided a huge advancement in the scientific field by offering a technologically sound methodology to observe different genetic contributions at varied levels of gene transfer *in vivo*. These lentiviral vectors can serve to introduce genes into a broad range of tissues and it has been demonstrated that *in vivo* expression can be sustained for significant timeframes without detectable pathology. Due to our recent finding that Syt1 is necessary for the Ca^{2+} dependence of CME, we wanted to determine how specifically Syt1 is contributing to the Ca^{2+} dependence by mutating the Ca^{2+} binding pockets within each of the C2 domains. While there has been a host of varied findings, which demonstrate the use of the lentiviral systems to address this question in several cell types, it remains to be determined whether this method can be utilized in adrenal chromaffin cells. Here we describe that the lentiviral system can be applied to mouse chromaffin cell, providing an additional tool for genetic manipulation in chromaffin cells.

4.2 Introduction

The adrenal chromaffin cell has been widely used as a model system to understand the basic mechanisms for synaptic vesicle exocytosis and endocytosis, although the recycling of granules/vesicles is considerably more complex than synaptic vesicle recycling at synaptic terminals. These studies include examinations of: (1) exocytic kinetics of distinct vesicle pools (Stevens, Schirra et al. , Rettig and Neher 2002) and rate of endocytosis (Smith and Neher 1997, Artalejo, Elhamdani et al. 2002) using whole-cell capacitance measurements; (2) exocytosis at the single vesicle level using cell-attached capacitance measurement (Gong, Hafez et al. 2003, Lindau and Alvarez de Toledo 2003, Gong, de Toledo et al. 2007) and amperometry (Chow, von Ruden et al. 1992); and (3) endocytosis of single vesicles using celled-attached capacitance measurements (Yao et al., 2012). Additionally, primary cultures of adrenal chromaffin cells are utilized to understand the synthesis and storage of catecholamine (Livett, Boksa et al. 1983, Stachowiak, Jiang et al. 1990).

Introduction of genetic material into post mitotic mammalian cells such as chromaffin cells has often been a challenge. Traditional transfection methods for mammalian cell, such as calcium phosphate precipitation, lipid-mediated transfection and electroporation have been met with limited success and have variable efficiencies (Ma, Holz et al. 1992, Wick, Senter et al. 1993, Wilson, Liu et al. 1995, Liu, Housley et al. 1996). Efficiencies of 20-40% (Ashery, Betz et al. 1999, Duncan, Don-Wauchope et al. 1999) or 90-100% (Duncan, Greaves et al. 2002) in bovine chromaffin cells have been reported using the Semliki Forest viral expression system, however this method is typically associated with significant cytotoxicity as this virus shuts off protein synthesis within the host cells (van Steeg, Thomas et al. 1981). More recently, it has been claimed that nearly 100% efficiency can be obtained using a recombinant adenoviral expression

system (Li, Drakulich et al. 2002). However, since protocols involved in developing recombinant adenovirus are complicated, this adenoviral-based infection of chromaffin cells is limited (Gong, Di Paolo et al. 2005).

The lentivirus system is unique in that it has the ability to infect non-dividing cells, while delivering a significant amount of the DNA construct of interest into a host cell without prevalent cytotoxicity (Naldini et al., 1996). The original lentivirus vectors described by Naldini et al (1996a,b) was a retroviral vector system based on the human immunodeficiency virus (HIV) that was developed to transduce heterologous sequences into HeLa cells and rat fibroblasts to block the cell cycle. Moreover, the HIV vector could mediate stable *in vivo* gene transfer into terminally differentiated neurons thus demonstrating that the ability of viral vectors to deliver genes *in vivo* into nondividing cells. Since this pioneering work, numerous studies and scientific approaches have been performed in treating, analyzing and delineating gene therapies and targets through its application. Hui et al., 2009 effectively demonstrated that the lentivirus application could be utilized to address synaptotagmin-mediated bending of target membranes by transfecting constructs with different binding capabilities in Syt1 C2 domains in Syt KO neurons. Furthermore, because Syt1 C2 domains harbor the Ca^{2+}binding domains necessary to serve as the Ca^{2+} sensor for exocytosis (Chapman, 2008), studies have extensively used lentiviral applications to determine the biophysical properties of these domains in Syt1 and how they ensure fast synchronous neurotransmitter release (Rhee et al, 2005; Shin et al., 2009, Hui et al., 2009; Yao et al, 2012). We, therefore obtained known lentiviral constructs for GFP and $Syt1^{WT}$ (a generous gift from Dr. Scott Shippy's Lab) and through the help and guidance of Dr. Hua Jin, were able to effectively demonstrate for the first time that the lentiviral system could be applied and utilized in mouse adrenal chromaffin cell.

4.3 Materials and methods

4.3.1. Chromaffin cell culture

See methodology section 2.2

4.3.2. Infection of chromaffin cells with lentivirus

Chromaffin cell were transduced with lentivirus containing either CMV- GFP or synaptotagmin 1 (Syt1) vector inserts at 1.2ul/2mL of culture media. Lentiviral particles were sustained in the culture media for 12 hrs to ensure full transduction before virus removal. After washing with fresh media (1X), cells were incubated in fresh media with no virus for another 12 hrs at 37° C with 5% CO2 until electrophysiological recordings or fixation for immunostaining unless mentioned otherwise.

4.3.3. Immunochemistry

See methodology section 2.5

4.4.4. Cell-attached capacitance recordings and fission-pore analysis

See methodology section 2.6 & 2.8

4.4.5. Statistical Analysis

See methodology section 2.10

4.5 Results

Chromaffin cells were identified with an antibody against tyrosine hydroxylase (TH), a marker for chromaffin cells, which were mixed with other types of cells such as cortical cells and fibroblasts as the whole adrenal glands were used for cell preparation. Determination of GFP-positive infected cells was achieved using an anti-GFP antibody, since the intensity of the GFP signal was too weak to be evident under direct fluorescence after cells were fixed. Fig. 8A shows a representative live cell image with 5 cells, which are TH-positive (left) and GFP-positive (right), suggesting these cells are GFP-transfected chromaffin cells. Our results indicated that more than 95% of chromaffin cells were infected with variable infection durations (Fig. 7B) or when the infection application was applied at different days after culture (Fig. 7C). This indicates that the extent of infection is independent of infection duration (beyond the one day minimum) and the age of cell (days in culture).

We then analyzed the effects of lentivirus-based GFP expression on CME kinetics via our established cell-attached capacitance measurements (Yao, Rao et al. 2012). Our results revealed that lentivirus-based GFP expression had no obvious effects on the capacitance step size of endocytic vesicles (Fig. 8A), the fission-pore Gp (Fig. 8C) or the fission-pore duration (Fig. 8D) of these endocytic events ($p > 0.05$). Likewise, the number of endocytic events (Fig. 8D) displayed no difference between these two groups ($p > 0.05$). Therefore, our results indicate a minimal cytotoxicity of the recombinant lentivirus to chromaffin cells.

Due to our previous work which demonstrated that the fission-pore duration is prolonged in synaptotagmin 1 (Syt1) knockout (KO) cells, thus indicating a Ca^{2+}-dependent role of Syt1 in vesicle fission during CME (Yao, Rao et al. 2012), we then asked whether lentivirus-based expression of Syt1 could rescue the endocytic defects in Syt1 KO cells (Yao, Rao et al. 2012).

While the Cv of endocytic vesicles (Fig. 9A) and the fission-pore Gp (Fig. 9B) were not affected by Syt1 expression ($p > 0.05$), the fission-pore duration was significantly rescued by Syt1 expression ($p < 0.01$) (Fig. 9C) to levels that is comparable to WT cells. Furthermore, there was a significant increase in the number of endocytic events in KO cells with Syt1 expression ($p < 0.01$) (Fig. 9D), suggesting that lentivirus-based Syt1 expression also rescues defects in the endocytic event number in Syt1 KO cells.

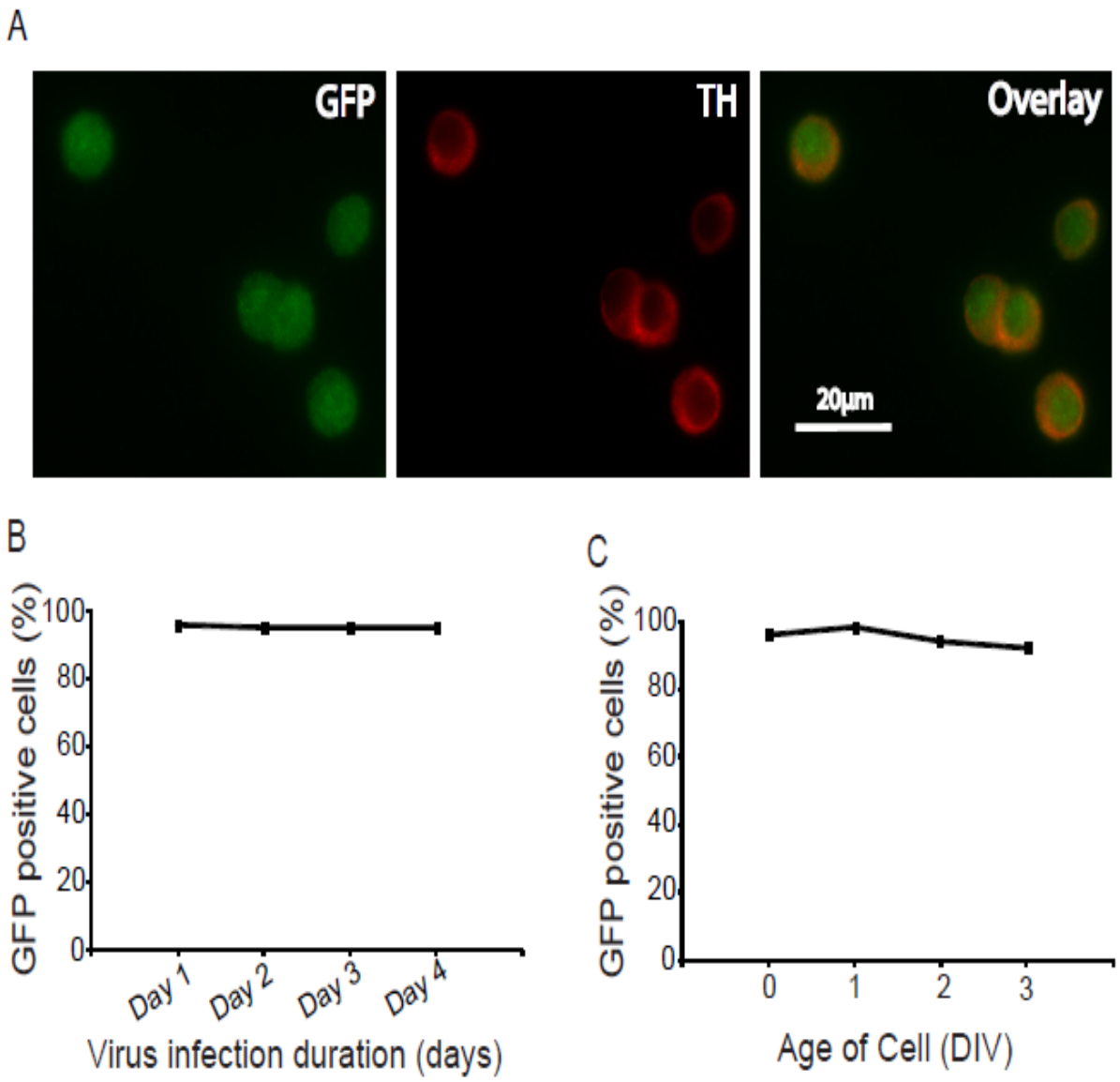


Figure 7. Lentivirus application can effectively incorporate into mouse chromaffin cells in culture. **A.** Confocal microscopy displaying chromaffin cells expressing lentiviral-GFP (left); tyrosine hydroxylase (TH) is a chromaffin cell marking antibody (middle) and overlay of co-expression in the same five cells (right). **B.** Duration of incubation of lentivirus reveals no change in positively transfected cells (Day 1: 96/100 cells, n=3; Day 2: 95/100 cells, n=3; Day 3: 95/100 cells, n=3; Day 4: 95/100 cells, n=3)($p > 0.05$). **C.** The age of the cell at time of lentiviral application demonstrates no change in transfection efficiency (Day 1: 96/100 cells, n=3; Day 2: 98/100 cells, n=3; Day 3: 94/100 cells, n=3; Day 4: 92/100 cells, n=3) ($p>0.05$).

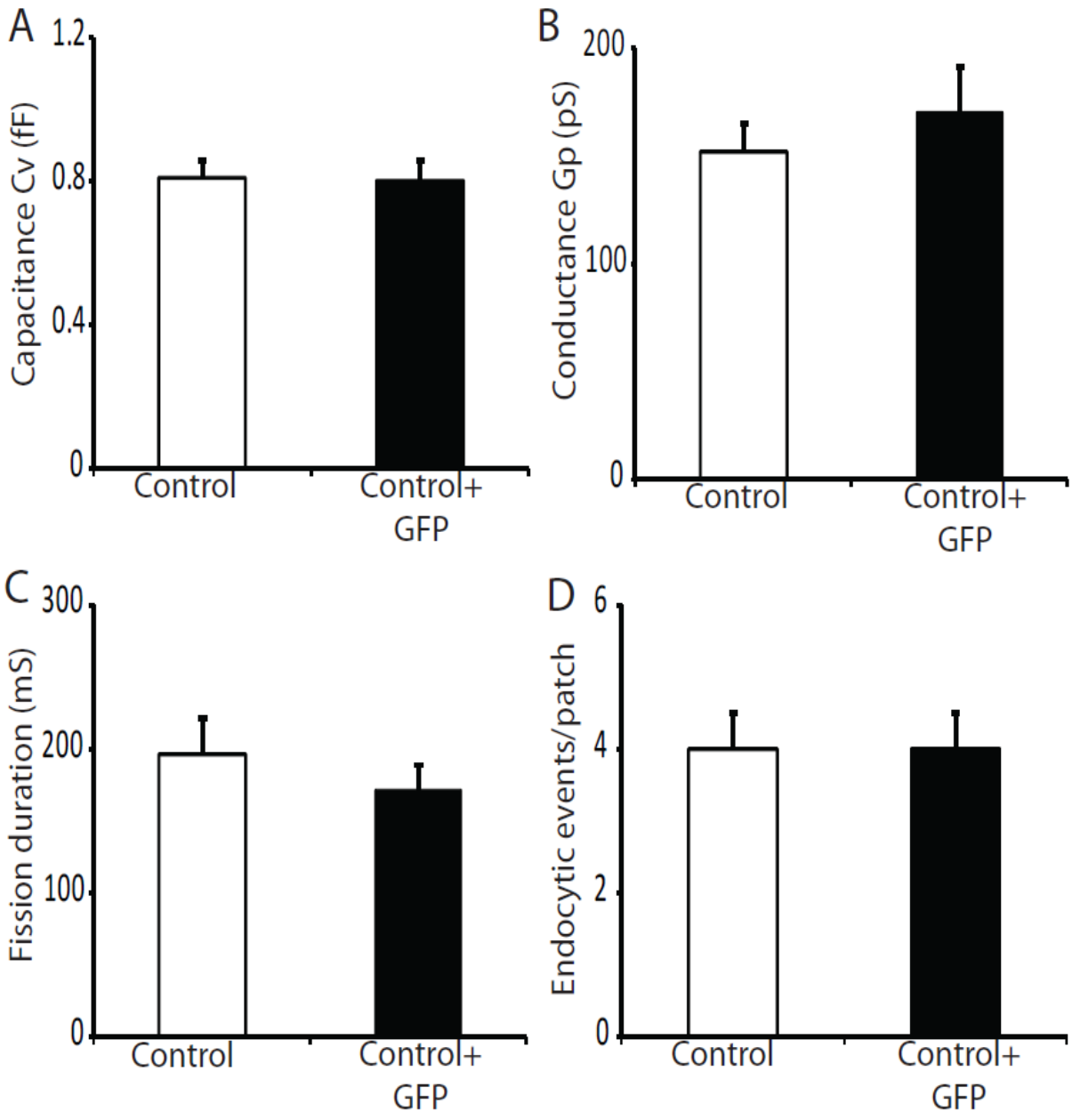


Figure 8. Lentiviral application demonstrates no alteration in CME fission kinetics. **A.** Cells transfected with GFP (n=35 events) reveal no change in the size of the endocytic vesicle (Cv) when compared to control (n=36 events)($p>0.05$). **B-C.** Both the fission-pore conductance **(C)** and the fission-pore duration were indistinguishable between control cells and cells transfected with GFP (control n=36 events; GFP n=35 events) ($p>0.05$). **D.** The number of endocytic events per patch also showed no difference when comparing control cells to GFP treated cells (Control n= 123 cells; GFP n= 105 cells) ($p>0.05$).

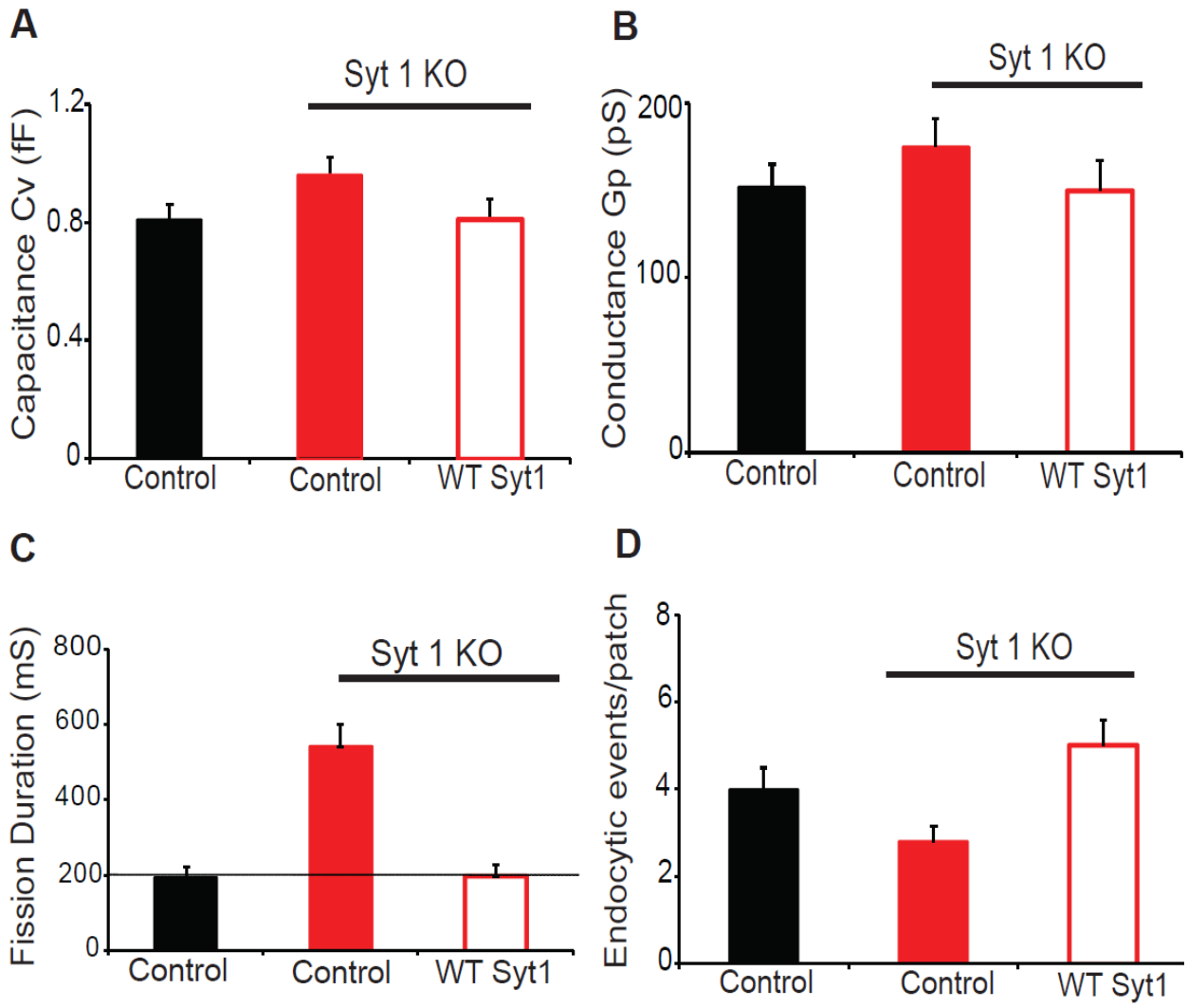


Figure 9. Transfection with full-length Syt1 rescues kinetic defects in Syt1 KO cells. **A-B.** Lentiviral application of $Syt1^{WT}$ into KO cells reveals no change in vesicle size **(A)** (Control= 0.81 ± 0.05fF, n= 36 events; $Syt1^{WT}$=0.81±0.07fF, n=40 events; KO=0.96±0.06fF, n=44 events ($p>0.05$) or vesicle conductance **(B)** when compared to control cells or Syt1 KO (Control= 152 ±13pS, n=36 events; $Syt1^{WT}$=149±20pS, n=40 events; KO=175±16pS, n=44events)($p>0.05$). **C.** Insertion of $Syt1^{WT}$ rescued a defect of a prolonged fission-pore duration in Syt1 KO cells back to control duration (Control= 196 ± 26mS, n=36 events; $Syt1^{WT}$=197±33mS, n= 40 events, KO=543±58mS, n=44 events)($p<0.01$). **D.** Additionally, cells expressing the $Syt1^{WT}$, insertion, recovered the reduced endocytic event number per patch to control levels when compared to Syt1 KO cells alone (Control= 4 ±0.5 events, n= 123 cells; $Syt1^{WT}$=5±0.6events, n=61 cells; KO=2.8±0.36events, n=106 cells)($p<0.01$).

4.6 Discussion

Since genetic manipulations are essential to understand the mechanisms of Syt1 in CME, here we examined the possibility of using recombinant lentivirus to infect primary neuroendocrine mouse chromaffin cells. Identification of infected cells was performed by co-transfecting GFP, along with Syt1 in the viral plasmid. Our results demonstrate that the susceptibility of chromaffin cells to the lentivirus application is independent of the virus infection time (beyond 1 day) and the age of the cells up to 4 days.

Our electrophysiological data showed that the number of endocytic events, the capacitance size of the endocytic vesicle and the fission-pore kinetics such as the fission-pore conductance Gp and duration are identical in both control cells and infected cells with GFP expression, indicating minimal cytotoxicity of chromaffin cells associated with the lentiviral infection protocol. Moreover, we showed that expression of exogenous Syt1 using the recombinant lentivirus reduced the fission-pore duration in the Syt1 KO cells to a level comparable to that in the WT cells as previously reported (Yao, Rao et al. 2012). Collectively, this work indicates that the lentivirus application is efficient to insert exogenous genes of interest into adrenal chromaffin cells.

Initial approaches reported a 0.1-5% transfection efficiency in bovine chromaffin cells using calcium phosphate transfection (Ross, Evinger et al. 1990, Wick, Senter et al. 1993); even under optimal conditions, only 35% of chromaffin cells proved to be transfected with same technique (Wilson, Liu et al. 1995). Along this line, electroporation, which requires a vast amount of cells in the protocol, demonstrated low transfection efficiency when applied to bovine chromaffin cells (Criado, Gil et al. 1999). Hence, the desire to achieve efficient transfection of eukaryotic cells has driven the development of several viral transduction systems. Both the Semliki Forest

virus (Ashery, Betz et al. 1999, Duncan, Don-Wauchope et al. 1999, Duncan, Greaves et al. 2002) and adenovirus (Li, Drakulich et al. 2002) have been utilized in bovine chromaffin cells for the investigation of mechanisms in exocytosis. Here, our study establishes that the lentivirus system can be utilized to transfect constructs of interest into the mouse adrenal chromaffin cells with nearly 100% efficiency, indicating the lentivirus system is a useful tool to deliver genetic material of interest effectively and efficiently into our model cells. Compared to Semilki Forest virus and adenovirus, lentivirus has many advantages such as lower cytotoxicity and long-term stable expression (Dreyer 2010), which make it suitable for gene rescue as well as gene silencing in chromaffin cells. Moreover, compared to knockout genetics, the use of RNAi-based gene silencing through lentivirus application is rapid, cost effective, and can be easily adapted to study homologous gene function in a wide variety of organisms.

Therefore, this work reveals an additional approach of genetic manipulation in the mouse adrenal chromaffin cell model system. Because of this work, we can now confidently use this system to address questions of how Syt1 is involved in the Ca^{2+} dependence of CME.

CHAPTER V:

SYT1 IS THE BIOCHEMICAL CA^{2+} SENSOR OF CME KINETICS

5.1 Abstract

Neurotransmitter release is triggered by a cooperative interaction between the two tandem C2 domains (C2A/B) of the synaptic vesicle protein synaptotagmin-1 (Syt1) and Ca^{2+}, triggering exocytosis. While the two C2 domains of Syt1 serve different but convergent effects when binding Ca^{2+} during exocytosis, it is well established that this protein serves as the Ca^{2+} sensor for fast, synchronous vesicle release. Although, there also appears to be Ca^{2+}-dependent regulation of synaptic vesicle endocytosis, multiple endocytic forms continue to generate uncertainty about the mechanisms involved. Although there has been a general consensus that Ca^{2+} is important for the classic mode of retrieval known as clathrin-mediated endocytosis (CME) and that Syt1 KO cells exhibit a loss in the Ca^{2+} dependence of endocytosis overall, it has yet to be determined if Syt1 is the biochemical Ca^{2+} sensor for CME. Recent work in our lab established that Syt1 is necessary for the Ca^{2+} dependence of CME kinetics. Here we wanted to expand upon this finding through the use of several Syt1 mutations ($Syt1^{6DA}$, $Syt1\ C2A^{3DA}$, $Syt1\ C2B^{3DA}$) which variably disrupt the Ca^{2+}binding pockets of each of the two Syt1 C2 domains. We demonstrate for the first time that biochemical alteration in the Ca^{2+} binding pockets of C2A, C2B or both C2 domains collectively fail to rescue the prolonged fission pore duration observed in Syt1 KO cells alone. This work establishes Syt1 as the Ca^{2+} sensor of individual CME event kinetics.

5.2 Introduction

It is widely accepted that Ca^{2+} influx triggers fast, synchronous neurotransmitter release, and that Syt1 serves as the major Ca^{2+}sensor during exocytosis (Koh & Bellen, 2003; Chapman, 2008). How Syt1 senses Ca^{2+} is through its two tandem C2 domains, C2A and C2B, which bind two and three Ca^{2+} ions respectively through the five conserved aspartate residues found on the top loops of conserved β-sandwich structures (Sutton et al., 1995; Fernandez et al., 2001; Shao et al., 1996; Ubach et al., 1998). These negatively charged aspartate residues coordinate Ca^{2+} binding and thus, determine the Ca^{2+} binding affinity of Syt1 (Shao et al., 1996; Ubach et al., 1998; Fernandez et al, 2001). Currently there exists a substantial amount of evidence relating the Ca^{2+} binding activity of Syt1 to exocytosis including: 1) The Ca^{2+} dependence of vesicle release is completely disrupted by a Syt1C2AB^{6DA} mutation in which aspartate (D) residues are substituted for alanine (A) changes that block Syt1 Ca^{2+} binding affinity (Xu et al., 2009); 2) A point mutation in a positively charged residue within the C2A domain, Arginine (R) 233, to glutamine (Q) (R233Q), decreases the Ca^{2+} binding affinity of Syt1 and simultaneously decreases release by a factor of ~ 2 (Fernandez-Chacon et al., 2001); 3) Lastly, a gain-of-function mutation, Syt1$^{C2A3W\ ,\ C2B3W}$, which replaces hydrophobic residues in the C2A and C2B Ca^{2+}-binding loops 1 and 3 for tryptophan (W) residues, augments neurotransmitter release by enhancing the Ca^{2+} binding affinity (Rhee et al., 2005; Chapman, 2008) while driving Syt1 deeper into anionic phospholipids in the membrane. This last point is consistent with evidence that Ca^{2+} binding to Syt1 C2 domains induces a rapid interaction with both SNARE proteins as well as phospholipids in the plasma membrane to ensure close vesicle-membrane proximity for fusion and subsequent neurotransmitter release (Nishiki and Augustine, 2004; Chapman, 2008).

Recent work has demonstrated that Syt1 is also required for normal rates of endocytosis (Nicholson-Tomishima & Ryan, 2004; Poskanzer et al., 2003); a major constituent being the C2B domain of Syt1 binding to endocytic adaptor proteins like AP-2 and stonin-2 (Stn 2) which are necessary for clathrin-mediated endocytosis (CME) (Haucke & De Camilli, 1999; Zhang et al, 1994; Diril et al., 2006). However, it has also been shown that the C2 domains of Syt1 can independently or (Poskanzer et al., 2006) interchangeably (Yao et al., 2011) regulate endocytic rates of synaptic vesicles. Yet other reports suggest that mutations disrupting the Syt1 C2B domain, specifically perturbing the binding of Stn 2/AP2, do not affect the kinetics of synaptic vesicle endocytosis (Drill et al., 2006). Because of these conflicting results, a definitive understanding of the independent or collective function of Syt1 C2 domains in endocytosis remains elusive.

Understanding how Ca^{2+} regulates the overall exocytic/endocytic rates at the synapse remains an important focus of investigation. Proskanzer et al., (2006) has suggested that Syt1 may be involved in the Ca^{2+} sensitivity of synaptic vesicle endocytosis by showing that mutations which disrupt the Ca^{2+} binding sites of the C2B domain demonstrate defects in the overall endocytic rate at *Drosophilla* NMJ. Additionally, it has been shown that Ca^{2+} is critical for endocytosis at the Calyx of Held (Hosoi, Holt et al. 2009, Wu, McNeil et al. 2009) and that Syt1 is necessary for the Ca^{2+} dependence of CME (Yao et al., 2012)

Although Syt1 and Ca^{2+} have an interaction in endocytosis, definitive answers to the specifics of how Ca^{2+} binding to Syt1 may be regulating CME remains to be determined. While it is well established that both C2 domains can bind Ca^{2+}, the C2B domain appears play a prominent role in triggering fast, synchronous release during exocytosis with the C2A domain serving a more ancillary function (Chapman, 2008). Yet in CME, it appears that both Syt1 C2

domains interact with endocytic adaptor proteins (Yao et al., 2011) and thus may both contribute to the Ca^{2+} dependence of CME (Yao et al., 2012). Here we attempt to address the relative importance of Ca^{2+} binding to the two C2 domains of Syt1 during CME. Through the use of high-resolution cell-attached capacitance measurements, we were able to resolve how Syt1 C2 domains collectively and independently contribute to the fission dynamics during the final stages of CME. We confirm that not only does Syt1 contribute to the Ca^{2+} dependence of CME, but that both C2 domains of Syt1 are required to maintain the proper kinetics of single CME events.

5.3 Results

5.3.1 The expression level of Syt1 does not determine the kinetics of CME events

To assess the physiological relevance of Syt1 C2 domains of mediating the Ca^{2+} dependence in CME events, we utilized the lentiviral vector system (See Chapter IV). First of all, we determined whether potential variation in Syt1 expression levels could possibly affect the kinetics of single CME events. Therefore, we analyzed varied expression levels of Syt1 to test if copy number versus exogenously added copies would be incorporated differently into the cells and thus alter CME kinetics. WT cells with two endogenous copies of the Syt1 gene were compared to heterozygous Syt1 (HT) cells expressing only one endogenous copy of Syt1. Additionally, to determine if overexpression of Syt1 could pose a potential problem, we transfected WT cells with additional copies of Syt1 (Cntl + Syt1) (1.2µl/2mL culture media) via the same full-length construct we designed for our rescue experiments (Figure 10 & 11). Syt1 HT cells revealed no change in the capacitance, conductance Gp, or the fission-pore duration when compared to control cells alone (Figure 10) ($p>0.05$). Furthermore, Cntl + Syt1 cells demonstrated no alteration in the capacitance (Cv), conductance (Gp) or fission-pore duration when compared to control cells (Figure 10 A,B,& C) suggesting that the number of endogenous Syt1 copies present, aside from the complete Syt1 KO, did not affect CME event kinetics. In addition, there was no observable difference in the number of endocytic events per patch when each of these three groups were compared (control, HT, Cntl +Syt1) (Figure 10D) ($p > 0.05$), further confirming that the exogenous addition of Syt1 into chromaffin cells, does not play a role in determining the kinetics of single CME events.

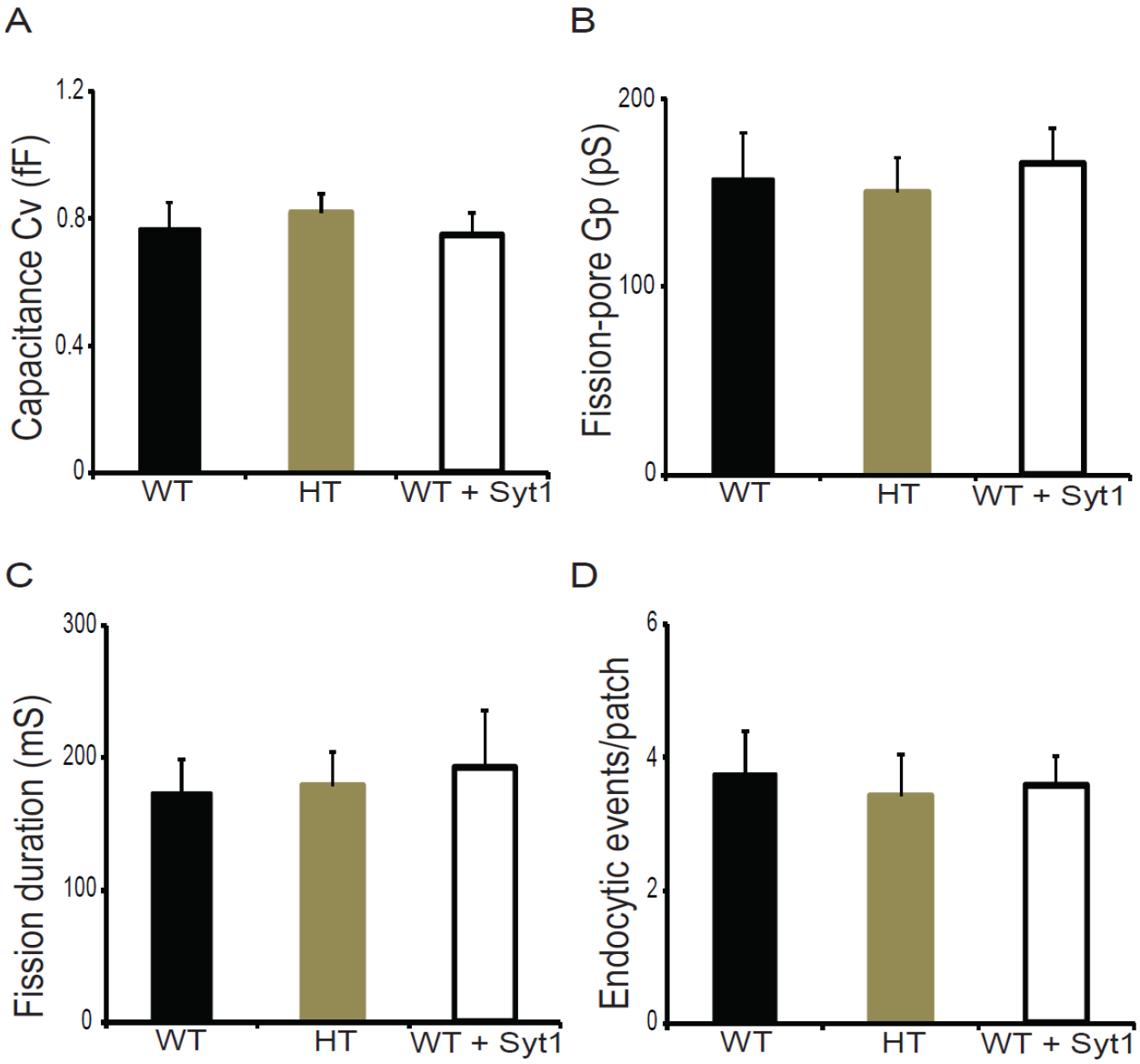


Figure 10. Expression levels of Syt1 do not determine the endocytic fission-pore kinetics. **A.** Both heterozygous Syt1 cells (HT) and control cells overexpressing Syt1 (control + Syt1) reveal no change in the size of the endocytic vesicle when compared to control cells alone (control= 0.77fF ±0.08, n= 41 events; HT= 0.82±0.06fF, n=43 events; Control + Syt1= 0.75±0.07fF, n=33 events) ($p>0.05$). **B-C.** Neither single gene expression of Syt1 (HT) or overexpression of Syt1 (control + Syt1) demonstrate an alteration in the fission pore Gp **(B)** nor in the amount of time necessary for vesicle pinch off **(C)** when compared to control cells alone (Control, n= 41 events; HT, n= 43 events; Control + Syt1, n= 33 events)($p>0.05$). **D.** The number of endocytic events per patch was comparable among all three groups (Control, n=37 cells; HT, n= 49 cells; Control + Syt1, n=24 cells)($p>0.05$).

5.3.2 Blocking Ca^{2+} binding to both C2 domains disrupts CME kinetics

After verifying that the Syt1 expression level has no obvious effects on CME event kinetics, we next examined the effects of the Ca^{2+} binding activity of individual Syt1 C2A, C2B domain or both C2 domains on the CME kinetics. A previous study has shown that a Syt1-$C2A^{3DA}$ mutant (mutation of Syt1 C2A domain aspartate residues (D) to alanine (A) : D178A, D230A, D232A) plus a Syt1-$C2B^{3DA}$ mutant (mutation of Syt1 C2B domain aspartate (D) residues to alanine (A): D309A, D363A and D365A),collectively known as the $Syt1^{6DA}$ mutant, eliminates the Ca^{2+}-binding affinities of both C2A/B domains (Xu et al., 2009). All these mutant constructs were obtained from Dr. Zhiping Pang's Lab. We first investigated whether blockade of all Ca^{2+} binding affinity in *both* C2 domains affects CME kinetics. Syt1 KO cells were transfected with either $Syt1^{6DA}$ mutation or wild-type Syt1 ($Syt1^{WT}$) and compared side by side to Syt1 KO without infection. The capacitance Cv and conductance Gp remained unaltered in all three groups ($p>0.05$)(Figure 11 B &C) demonstrating that the Ca^{2+} binding pockets in each of Syt1 C2 domains do not play a major role in determining the synaptic vesicle size or the amount of current flowing through the fission-pore before vesicle pinch-off. In contrast expression of the $Syt1^{WT}$ insert fully restored the fission duration of Syt1 KO cells ($p < 0.01$)(Figure 11 D), expression of the $Syt1^{6DA}$ mutant in Syt1 KO cells failed to rescue the kinetic defect of the prolonged fission pore duration observed in the Syt1 KO cells (Figure 11C) ($p >0.05$), suggesting that Ca^{2+} binding affinity of both Syt1 C2 domains participate in determining the final rate of vesicle pinch-off during CME. Therefore, it appears that the ability of Ca^{2+} to bind efficiently to Syt1 C2 domains is necessary for single vesicle fission in CME, indicating that Syt1 may serve as the biochemical Ca^{2+} sensor for CME kinetics.

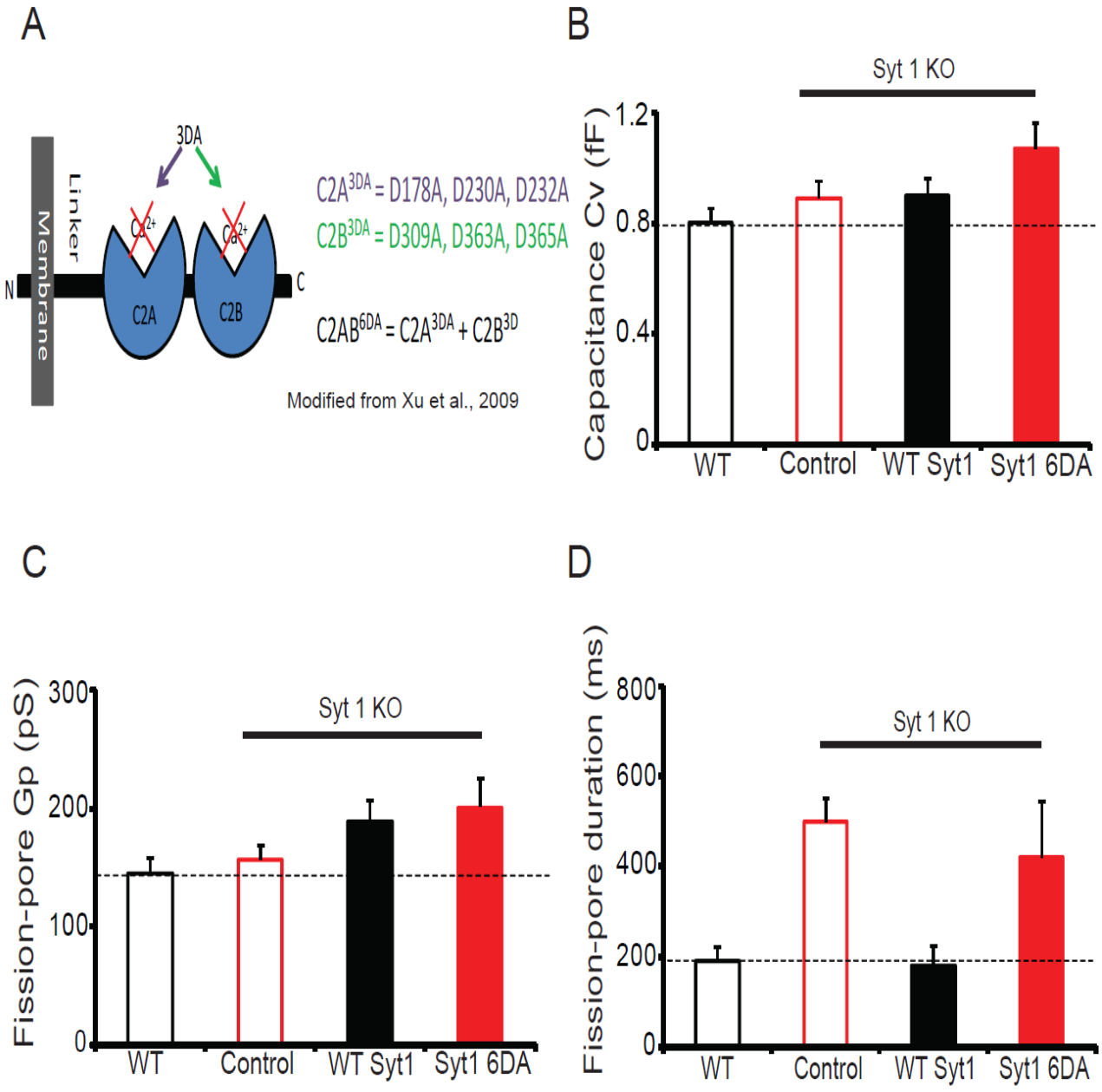

A
3DA
Linker
Membrane
N
C
C2A
C2B
Ca2+
Ca2+
C2A3DA = D178A, D230A, D232A
C2B3DA = D309A, D363A, D365A
C2AB6DA = C2A3DA + C2B3D
Modified from Xu et al., 2009
B
Syt 1 KO
Capacitance Cv (fF)
1.2
0.8
0.4
0
WT
Control
WT Syt1
Syt1 6DA
C
Syt 1 KO
Fission-pore Gp (pS)
300
200
100
0
WT
Control
WT Syt1
Syt1 6DA
D
Syt 1 KO
Fission-pore duration (ms)
800
600
400
200
0
WT
Control
WT Syt1
Syt1 6DA

Figure 11. $Syt1^{6DA}$ mutant fails to rescue the prolonged duration in Syt1 KO cells. **A.** Modified schematic drawing demonstrating the $Syt1^{6DA}$ mutation from Xu et al., 2009 ("3DA," corresponding to three aspartate (D) to alanine (A) mutations); mutations generously provided by Dr. Zhiping Pang's Lab. **B.** Fission-pore capacitance was unaltered between Syt1 KO cells and cells transfected with either $Syt1^{6DA}$ or $Syt1^{WT}$ when compared to control cells (Control= 0.80± 0.05fF, n=37 events; KO=0.89±0.06fF, n=42 events; $Syt1^{6DA}$=1.07±0.09fF, n=31events; $Syt1^{WT}$ = 0.90±0.06fF, n=40 events)($p>0.05$). **C.** The absence of Ca^{2+} binding in the $Syt1^{6DA}$ mutation revealed no change in overall conductance when compared to cells transfected with $Syt1^{WT}$ or control cells alone (Control= 145 ±13 pS, n= 37 events; Syt1 KO=156±12pS, n=42 events; $Syt1^{WT}$ = 188±18pS, n=40 events; $Syt1^{6DA}$ = 201±23pS, n=31 events;)($p>0.05$). **D.** The fission pore duration was significantly reduced in Syt1 KO cells expressing $Syt1^{WT}$ (179±45ms, n=40 events) ($p<0.01$) compared to Syt1 KO cells expressing $Syt1^{6DA}$ (421±124ms, n=40 events) or Syt1 KO cells alone (500±52mS, n= 42 events)($p>0.05$)

5.3.3 Both C2 Domains of Syt1 are necessary for vesicle fission of CME

It is well established that Ca^{2+} binding to the C2B domain of Syt1 is required to trigger fast synchronous exocytosis (Mackler et al., 2002; Nikishi & Augustine, 2004) and that mutations in the C2B domain are more severe than those in the C2A domain (Paddock et al., 2011; Chapman, 2008, review; Nikishi & Augustine, 2004). Moreover, the C2B domain of Syt1 binds to AP-2 (Zhang et al., 1994; Haucke and De Camilli, 1999) during the initiating stages of CME suggesting that the C2B domain of Syt1 is intrinsically more important than C2A for exo and endocytosis. However, while some reports suggest that this interaction occurs through the adaptor protein complex Stonin-2 (Walther et al., 2001), others suggest that both C2 domains of Syt1 can interact with Stonin-2 (Diril et al., 2006; Jung et al., 2007) implying that possibly *both* C2 domains are necessary for inducing AP2-dependent CME. Since C2 domains contribute differentially to the Ca^{2+} dependence of vesicular release and that we have revealed that mutations in to all Ca^{2+} binding sites in both C2 domains have defects in CME fission-pore kinetics (Figure 11), we next explored whether C2A or C2B domain differentially regulates CME by examining the effects of Syt1-$C2A^{3DA}$ mutant or Syt1-$C2B^{3DA}$ mutant on the fission-pore kinetics using the cell-attached capacitance measurements. While the endocytic vesicle size (Figure 12A) as well as the amount of current flowing through the neck of the fission pore (Gp) (Figure 12B) were comparable between all four groups (Syt1 KO, $Syt1^{WT}$, Syt $C2A^{3DA}$ and Syt $C2B^{3DA}$)($p>0.05$), neither the Syt $C2A^{3DA}$ mutant nor the Syt $C2B^{3DA}$ mutant could rescue the prolonged fission-pore duration (Figure 12C) in Syt1 KO cells (Syt1 KO: 559 ± 89 mS; $C2A^{3DA}$ mutant: 581 ± 78 mS; Syt $C2B^{3DA}$ mutant: 588 ± 96 mS) ($p > 0.05$). However, expression of $Syt1^{WT}$ into Syt1 KO cells exhibited rescue in the extended duration back to WT levels ($Syt1^{WT}$:

196 ± 33 mS) ($p < 0.01$), suggesting that Ca^{2+} binding affinity to both C2B *and* C2A domains is necessary for CME fission kinetics.

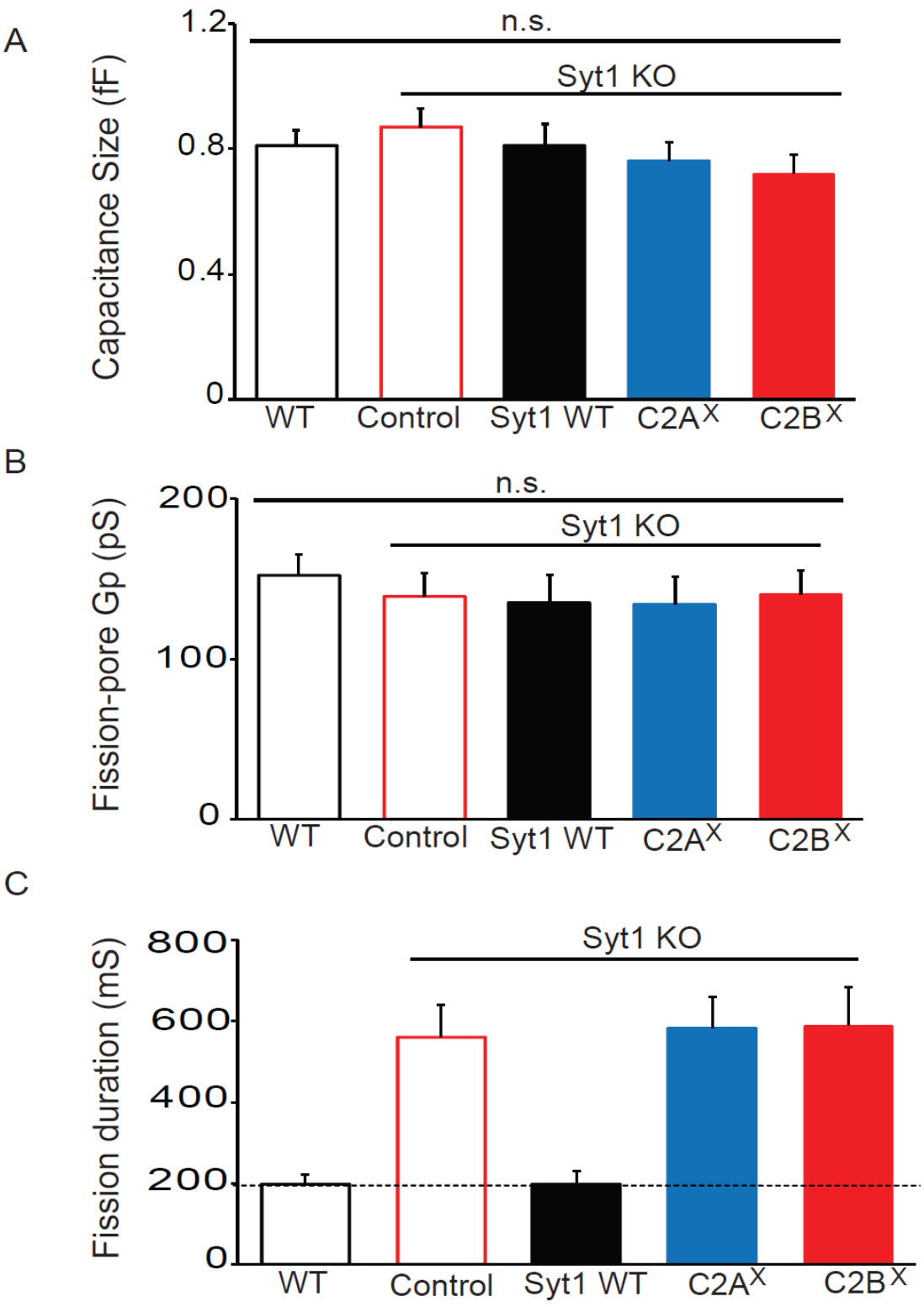

A
1.2
0.8
0.4
0
Capacitance Size (fF)
n.s.
Syt1 KO
WT
Control
Syt1 WT
C2A^X
C2B^X
B
200
100
0
Fission-pore Gp (pS)
n.s.
Syt1 KO
WT
Control
Syt1 WT
C2A^X
C2B^X
C
800
600
400
200
0
Fission duration (mS)
Syt1 KO
WT
Control
Syt1 WT
C2A^X
C2B^X

Figure 12. Altered endocytic fission properties in Syt1 C2A and C2B Ca^{2+}binding mutants. **A.** The endocytic vesicle size was unaltered in all four groups (KO =0.87±0.06fF, n=40 events; Syt1 $C2A^{3DA}$ = 0.81±0.07fF, n=35 events; Syt1 $C2B^{3DA}$ =0.76±0.06fF, n= 33 events; $Syt1^{WT}$=0.72±0.06fF, n=39 events) when compared to control (control=0.81fF, n=36 events) ($p>0.05$). **B.** Total current flowing through the endocytic fission-pore (Gp) revealed no significant change between control (132±14pS, n=36 events), Syt1 KO (139±14pS, n=40 events, Syt1 $C2A^{3DA}$ (135±17pS, n=35 events), Syt1 $C2B^{3DA}$ (134±17pS, n=33 events) or $Syt1^{WT}$ (140±15pS, n=39 events) ($p>0.05$). **C.** The prolonged fission-pore duration is not rescued by Syt1 $C2A^{3DA}$ or Syt1 $C2B^{3DA}$ (Syt1 $C2A^{3DA}$ = 581±78mS, n=35 events; Syt1 $C2B^{3DA}$=588±96mS, n=33 events) in Syt1 KO cells, however $Syt1^{WT}$ significantly rescued the prolonged duration back to control levels in Syt1 KO cells ($Syt1^{WT}$=196±33mS, n=39 events; control 196±26mS, n=36 events) ($p<0.01$)

Discussion

It has been proposed that any defects in exocytosis will inevitably alter the driving forces that initiate and maintain endocytosis (Wu et al., 2009). Consequently, determining if defects observed in endocytosis are merely secondary outcomes due to biochemical alterations in exocytosis have been difficult to resolve. Therefore, establishing whether or not alterations in endocytosis in Syt1 mutants are directly attributing to Syt1's role in endocytosis have remained a challenge. Recent attempts have tried to uncouple the function of Syt1 in exo vs endocytosis and have pointed to a role of Syt1 in endocytosis (Poskanzer et al., 2003; Marek & Davis, 2002; Yao et al., 2012). Here we demonstrate that we can effectively delineate the effects of Syt1 on single-vesicle CME kinetics without affecting exocytosis.

It is well established that Syt1 interacts with Ca^{2+} while simultaneously interacting with SNARE complexes driving Syt1/SNARE proteins towards anionic phospholipids in the plasma membrane to promote exocytosis (Chapman, 2008). Thus, examining whether Syt1 requires these interactions during endocytosis furthers our understanding of the CME process. Neale et al., (1999) was one of the first groups to demonstrate that botulinum neurotoxin A can uncouple vesicle exo- from endocytosis by cleaving the interaction between Syt1 and the SNARE complex protein SNAP-25, and provided evidence that Ca^{2+} is required for synaptic vesicle membrane retrieval in the absence of Syt1/SNARE interactions. Due to the recent findings in our lab that determined that Syt1 is necessary for the Ca^{2+}dependence of single CME events (Yao et al., 2011), we utilized BoNT/A to help us further explore the molecular mechanisms by which Syt1 acts in endocytosis (Data shown in Chapter VI, Figure 16).

While it is known that Syt1 plays an ancillary role in recruiting clathrin to endocytic sites, and that Syt1 appears to be involved in the Ca^{2+} sensitivity of synaptic vesicle endocytosis

(Poskanser et al., 2006; Yao et a., 2011) it remains to be understood how Syt1 specifically contributes to the kinetics of endocytosis: Does Syt1 determine the number of endocytic events? Or is its role to determine the Ca^{2+}dependence? Alternatively, is it serving both functions? Limitations with techniques such as whole-cell capacitance measurements do not allow for the single vesicle CME resolution needed to identify and separate out the kinetic components of CME events. While it is understood that the Syt1- Stonin-2- AP-2 interaction is critical for CME, we have demonstrated that although inhibition of this interaction alters the number of endocytic events (Yao et al., 2012), it does not impact single event kinetics. Building upon this observation, here we reveal here that Ca^{2+} binding to both the C2A and C2B domains is necessary to ensure the kinetics of vesicle fission of CME. Furthermore, our preliminary results demonstrate that mutations which disrupt the Ca^{2+} binding capability of Syt1 C2 domains do not appear to disrupt number of endocytic events of CME (data not shown). This preliminary work in conjunction with our previous findings that Syt1 KO cells display a reduction in the number of endocytic events (Figure 6B) suggests for the first time, that Syt1 has two separate functions in the endocytic pathway of CME. Supporting this, is work that was done in both the whole-cell configuration and through the use of quantitative live imaging with synapto-pHluorin that showed mutations in the AP-2/stonin-2 binding motif of C2B or in the Syt1 binding domain of stonin-2, do not disrupt the kinetics of synaptic vesicle endocytosis (Diril et al., 2006).

In addition to suggesting that Syt1 appears to serve dual functions during CME, we have also demonstrated, for the first time, that the C2A domain of Syt1 has a novel function in assisting in the Ca^{2+} dependence of CME event kinetics. It has been widely proposed that the C2A and C2B domains of Syt1 have different roles in that mutations in the Ca^{2+} binding sites of the C2B domain reveal a more severe defect than mutations of Ca^{2+} binding pockets of C2A

during vesicular release (Earles et al., 2001). This suggests that Ca^{2+} binding to syt1 C2B domain plays a more dominant role in synaptic transmission compared to its C2B domain. Unlike exocytosis, and contrary to recent work demonstrating that either C2A or C2B can rescue kinetic defects of endocytosis (Yao et al., 2012), we have unveiled an original finding that the C2A domain is *equally* required to ensure the last stages of vesicle pinch-off kinetics (Figure 12). While this finding further confirms Syt1 as the biochemical Ca^{2+} sensor of single vesicle CME events, it also reveals a novel understanding of the Ca^{2+} sensing capability of Syt1 during synaptic vesicle retrieval.

It has often been stated that endocytosis occurs on timescales which are much slower than that of exocytosis, and that the rate at which Syt1 binds and unbinds Ca^{2+} and or membranes is within the millisecond time scale (Hui et al., 2005). This suggests that a time frame of seconds for endocytosis is unreasonable for a Syt1 function alone. In lieu of this, proposed models have suggested that Syt1 may be acting in concert with another protein or complex to ensure endocytosis (Yao et al., 2011). Some studies have proposed calcineurin (Marks & McMahon, 1998; Cousin & Robinson, 2001) and calmodulin (Wu et al., 2009; Artalejo et al., 1996) contribute to the affects of Syt1 in CME. Moreover, it has been proposed that calmodulin serves as the Ca^{2+} sensor specifically in chromaffin cells (Artalejo et al., 1995; Artalejo et al., 1996) yet the form of endocytosis examined was clathrin independent. In the present study, and through the use of the high-resolution cell-attached configuration, we for the first time demonstrate that Syt1 serves as the biochemical Ca^{2+} sensor of CME event kinetics.

CHAPTER VI:

PHOSPHATIDYLSERINE IS CRITICAL FOR VESICLE FISSION DURING CLATHRIN-MEDIATED ENDOCYTOSIS

6.1 Abstract

It is well established that Syt1 interacts with the plasma membrane anionic phospholipid phosphatidylserine (PS) in a Ca^{2+} dependent manner, and this interaction is critical for the Ca2+ sensing role of Syt1 in exocytosis. This interaction may influence exocytosis in two ways: 1) by enhancing the early stages of vesicle priming leading to fusion pore opening and 2) an additional subsequent step which aids in the stabilization of dilating fusion pores. We have previously established that Ca^{2+} binding to Syt1 is also required for CME. Here we investigated whether this process involves interactions between Syt1 and PS. Our results demonstrate that PS, but not neutrally charged phoshpatidylcholine (PC) or phosphatidylethanolamine (PE), plays an essential role in defining CME kinetics and furthermore that Syt1-PS interaction is critical in regulating CME kinetics.

6.2 Introduction

Phosphatidylserine (PS) is an abundant negatively charged glycerophospholipid found predominantly at the inner leaflet of the plasma membrane which contributes to the recruitment and concentration of proteins that are critical to various physiological functions. (Leventis and Grinstein, 2010; Yeung et al., 2008; Fairn et al., 2011). Although PS has been widely implicated in many membrane trafficking processes (Vance and Tasseva, 2013), one of its main physiolgical functions is to facilitate synaptic vesicle fusion by binding the synaptic vesicle protein Syt1 in a Ca^{2+}-dependent manner (Zhang et al., 2009, 2010; Chapman, 2008; Pang and Sudhof, 2010). It has been demonstrated that Syt1 binds to PS- containing liposomes and that this interaction in conjunction with Ca^{2+} dependent binding of Syt1 to soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) are necessary for exocytosis (Brose et al., 1992, Chapman, 2002). Carbon fiber amperometry suggests that PS may modulate both the open pore of fusing vesicles as well as the frequency of exocytic events (Zhang et al., 2009). Furthermore, studies of mutations that prevent Syt1 C2 domains from interaction with PS indicate that this interaction is required for release (Paddock et al., 2011).

Interestingly, PS has recently been suggested to be important for endocytosis as well (Ory et al., 2013), although the mechanism by which PS regulates endocytic events is not fully understood. While Syt1-PS and Syt1-SNARE interactions are essential for exocytosis (Sollner et al., 1993, Jahn & Scheller, 2006), uncoupling Syt1-PS interaction while determining if Syt1-SNARE complex binding is necessary during endocytosis appears difficult to resolve.

By applying the cell-attached capacitance technique in mouse adrenal chromaffin cells, we can resolve the kinetics of individual CME vesicular events (Yao et al., 2012, 2013). In the

present study we applied this technique to address the role of PS on CME dynamics through a series of different manipulations: 1) determining the contribution of different phospholipids PC or PE to PS, to the kinetics of CME 2) attempting to dissect out Syt1-SNARE complex interaction separate from Syt1-PS interaction through the use of botulinum neurotoxin A and 3) determining whether the Syt1 PS-mediated effect on the fission-pore duration is Ca^{2+} dependent.

6.3 Results

6.3.1 PS addition to chromaffin cells

Before determining any potential effects of PS on CME, we explored whether exogenous PS could be incorporated into chromaffin cells. PS insertion into cells was first tested with a fluorescent NBD labeled PS (McIntyre and Sleight 1991). The incorporation of NBD-PS into chromaffin cells was assesed by the detection of NBD fluorescence in cells treated with 10-μM NBD-PS (Avanti Polar Lipids, Alabaster, AL) for 24 hrs prior to fixation and visualization (Fig. 1A). We next quantified the cellular PS level using FITC tagged Annexin V (Southernbiotech, Birmingham, AL), a PS binding protein. This detection method, commonly used to monitor apoptosis (Janko, Jeremic et al. 2013), can be used to label intracellular PS (Montaville, Neumann et al. 2002, Zhang, Hui et al. 2009). When 10 μM exogenous PS (Sigma, St. Louis, MO) (or vehicle alone) was added to culture media and cells were fixed and visualized 24 hrs later, the intensity of Annexin-V-FITC signals was significantly increased in PS-treated cells as compared to control cells ($p < 0.01$) (Fig. 13 B and C), indicating an increase in the cellular PS level. In contrast, cells treated with PC or PE at the same concentration of 10 μM displayed no obvious change in Annexin-V-FITC intensity ($p > 0.05$) (Fig. 13 C). Collectively, our results indicate that exogenous PS addition can be effectively incorporated into chromaffin cells and thus cause an increase in cellular PS levels, consistent with previous studies (Zhang, Hui et al. 2009)Uchiyama et al (2007).

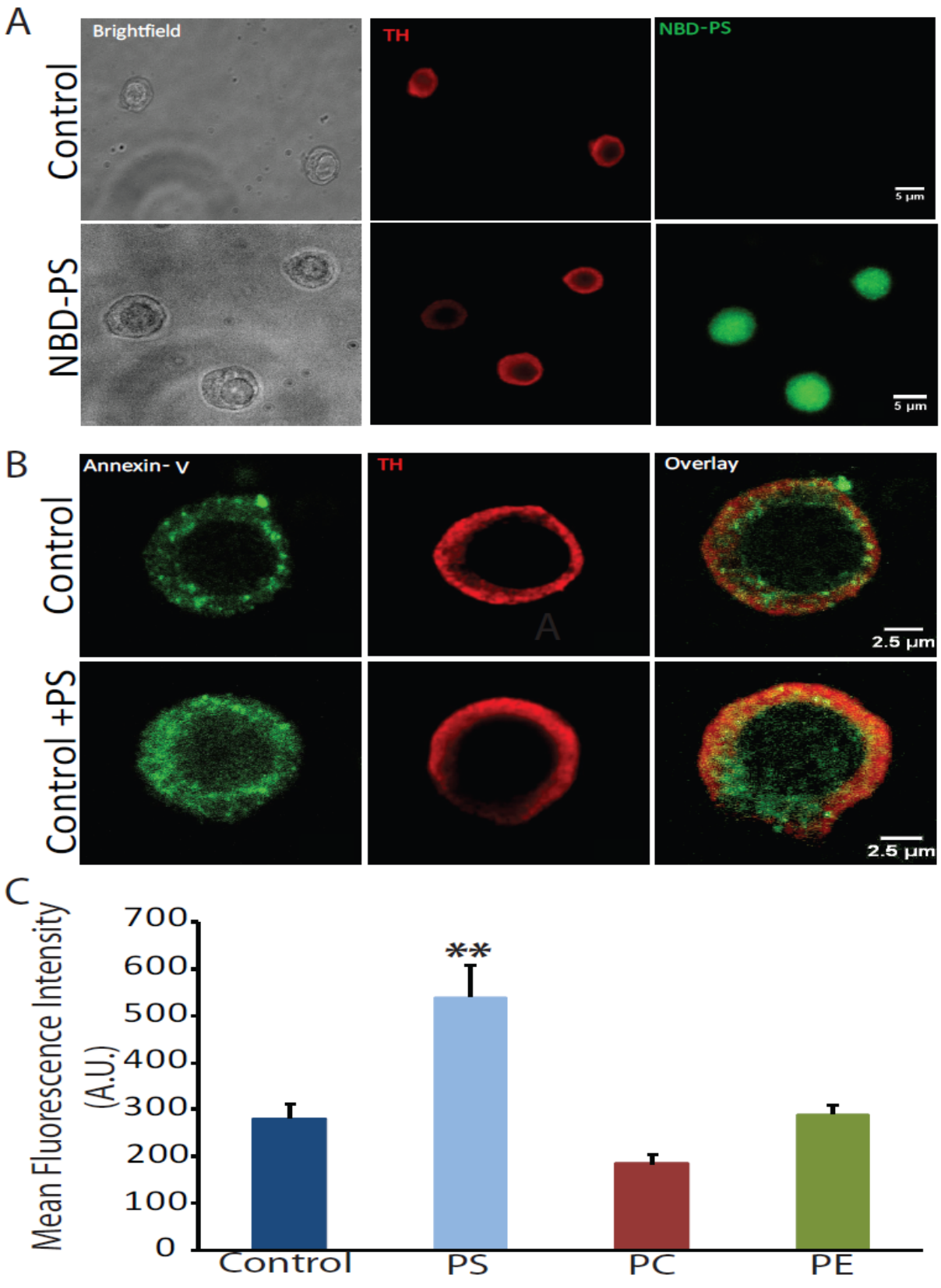

A
Brightfield
TH
NBD-PS
Control
NBD-PS
5 µm
5 µm
B
Annexin- V
TH
Overlay
Control
Control +PS
2.5 µm
2.5 µm
C
Mean Fluorescence Intensity (A.U.)
700
600
500
400
300
200
100
0
**
Control
PS
PC
PE

Fig. 13 The characterization of exogenous PS in Chromaffin Cells**. A**. Confocal images demonstrating the efficiency of fluorescent probe NBD-PS. Control cells without NBD-PS supplementation express the chromaffin cell marker TH (upper middle) but reveal no NBD-PS staining compared to cells supplemented for 24 h with 10 μM NBD-PS (bottom right). Brightfield images of both control and NBD-PS treated cells are shown in left. **B.** Endogenous PS levels in control cells (upper panel) were compared to the incorporation of exogenous PS by Annexin-V-FITC. PS-treated cells revealed a ~70% increase in PS levels (lower left) when compared to endogenous PS levels in control cells (upper left). TH marks chromaffin cells (middle panels) and overlays are shown at right. **C.** Cellular PS level, estimated by Annexin-V-FITC, is increased by addition of PS but not PC or PE. (control: n= 40 cells, PS: n= 32 cells; PC: n=36 cells; PE: n= 38 cells) ($p<0.01$).

6.3.2. PS addition reduces the fission-pore duration

After verifying that exogenously supplemented PS can be effectively incorporated into cells, we used the cell-attached capacitance technique to explore the potential influence of PS on the kinetics of individual CME events. PS addition did not alter the capacitance Cv of endocytic vesicles (Fig. 14B) or the fission-pore conductance Gp (Fig. 15C) ($p > 0.05$), indicating that PS may not be critical in determining the size of endocytic vesicles or the geometry of the tubular membrane neck. However, PS addition caused a ~ 50% reduction in the fission-pore duration ($p < 0.01$) (Fig. 14 A, D), suggesting a role for PS in regulating the kinetics of individual CME events. In contrast to anionic PS, neutral PC or PE had no obvious effect on the fission-pore duration ($p > 0.05$) (Fig. 14D). Comparison of the fission-pore durations at 2, 1 and 0.5 mM $[Ca^{2+}]_e$ between control cells and cells treated with PS revealed that the Ca^{2+} dependence of the fission-pore duration observed in control cells was downwardly shifted by PS addition (Fig. 16E), implying that PS may enhance the Ca^{2+} dependence of the fission-pore duration of individual CME events. Therefore, these results indicate that PS is critical for the kinetics of unitary CME events.

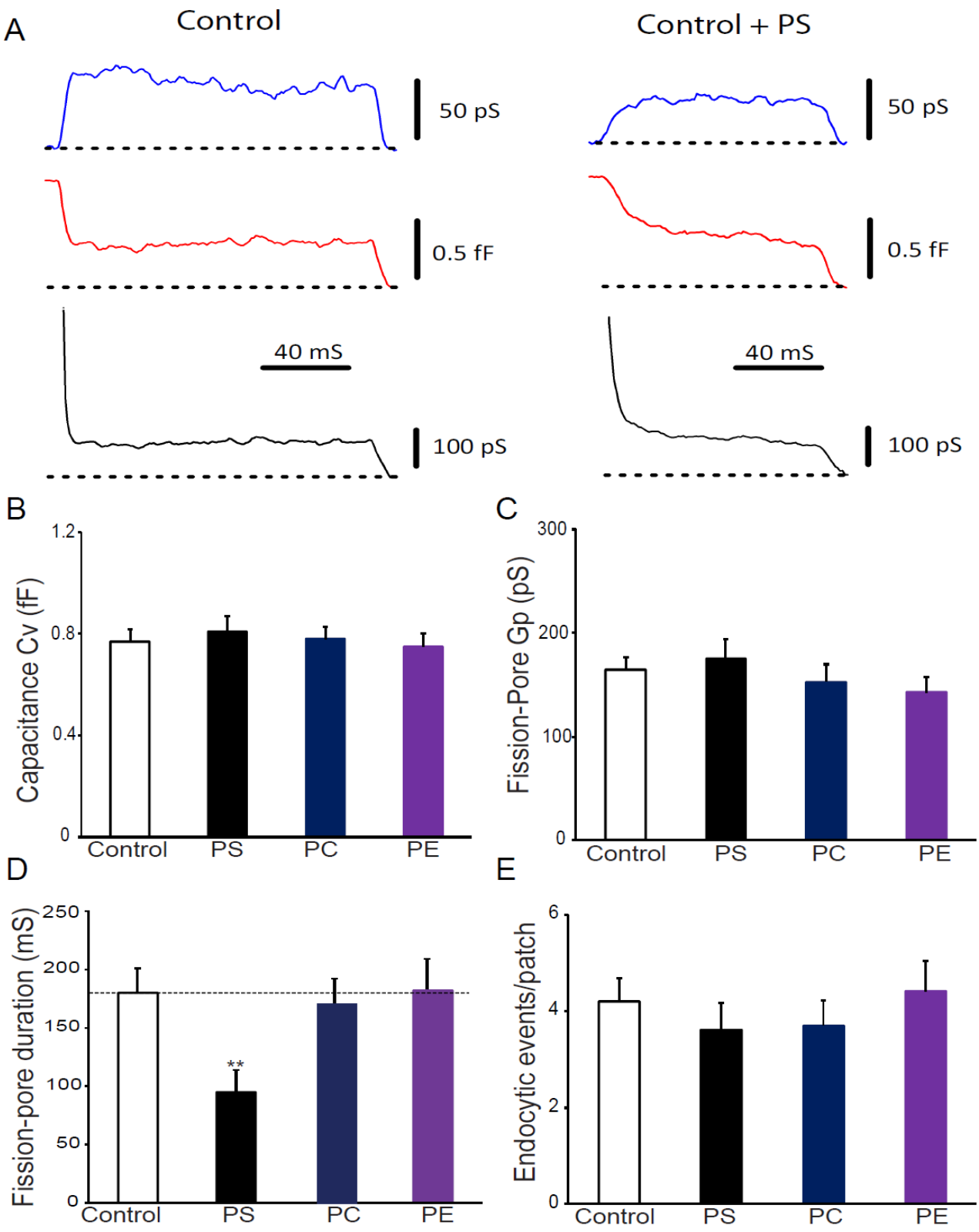
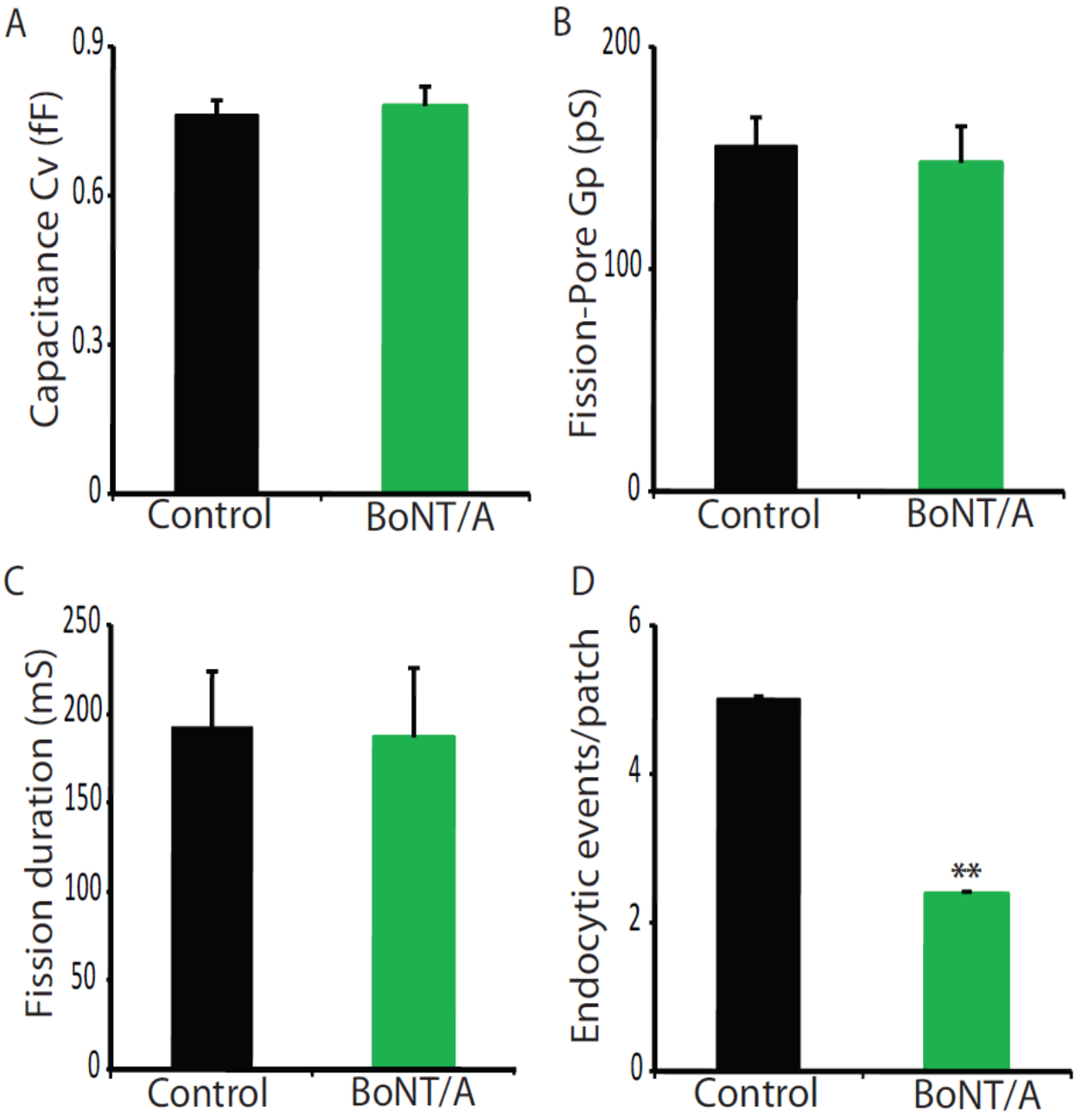

A
Control
Control + PS
50 pS
0.5 fF
40 mS
100 pS
B
Capacitance Cv (fF)
1.2
0.8
0.4
0
Control
PS
PC
PE
C
Fission-Pore Gp (pS)
300
200
100
0
D
Fission-pore duration (mS)
250
150
50
**
E
Endocytic events/patch
6
4
2

Fig. 14. The fission kinetics are accelerated by the addition of PS. **A.** Representative fission-pore event at 2mM $[Ca^{2+}]_e$ in a control cell (left) and a cell supplemented with 10 μM PS (right) recorded in the cell-attached configuration. **B-C.**The Cv of endocytic vesicles **(B)** was unchanged and the fission pore conductance Gp **(C)** was indistinguishable between control cells (n= 61 events) and cells incubated with each PS (n=46events), PC (n=56 events), or PE (n=52 events) ($p>0.05$). **D.** Fission-pore analysis revealed a ~ 50% reduction in the fission-pore duration when cells were treated with 10 μM PS ($p<0.01$). **E.** The number of endocytic events recorded within a 5min time frame, was not altered in groups supplemented with either PS (n= 118 cells) , PC (n=129 cells) or PE (n= 91 cells) when compared to control cells (n=152 cells) ($p>0.05$).

6.3.3 Use of BoNT/A toxin: indicates that a defect in SNARE complex formation has no affect on fission-pore kinetics of CME.

Syt1 binds to the target t-soluble N-ethylmaleimide sensitive factor receptor (t-SNARE) SNAP-25, to ensure vesicle-membrane proximity during exocytosis (Dai et al, 2007; Bhalla et al, 2006) Syt1 also interacts with PS in the membrane. Although mutations exist in Syt1 that were thought to selectively affect either Syt1 interaction with SNARE proteins or phospholipids (Pang et al., 2006), it was later shown that these mutations disrupt both simultaneously (Chapman, 2008). Thus, Syt1 interactions with SNARE proteins and phospholipids are not mutually exclusive (Chapman, 2008) and genetic ablation of either Syt1-SNARE or Syt1-phospholipid is experimentally challenging. We attempted to circumvent this problem using a separate approach, through the use of botulinum neurotoxin A (BoNT/A) (METABIOLOGIS, INC, Madison, WI), a neurotoxin which cleaves the last 9 amino acid residues from the c-terminal of SNAP-25, thus inhibiting Syt1-SNAP-25 binding (Fang et al., 2008). This allowed us to examine the importance of Syt1-t-SNARE interaction in CME. BoNT/A at 10 ng/ml did not alter the size of the endocytic vesicle (Cv) (Fig. 15 A) ($p>0.05$), conductance (Gp) flowing through the fission-pore neck (Fig. 15 B) ($p>0.05$) or the fission-pore duration (Fig. 15 C) ($p>0.05$), suggesting that the Syt1-t-SNARE interaction may not be critical in regulating endocytic fission kinetics. Furthermore, our results show that an exocytic defect does not alter the reliability of the endocytic machinery in forming the fission pore during CME. Although, the number of endocytic events per patch was decreased ~ 57% (Fig 15 D) ($p<0.01$) we concluded that the BoNT/A-induced endocytic inhibition was attributable to the exocytic slow-down (Gerona et al., 2000; Zhang et al., 2002).


A
Capacitance Cv (fF)
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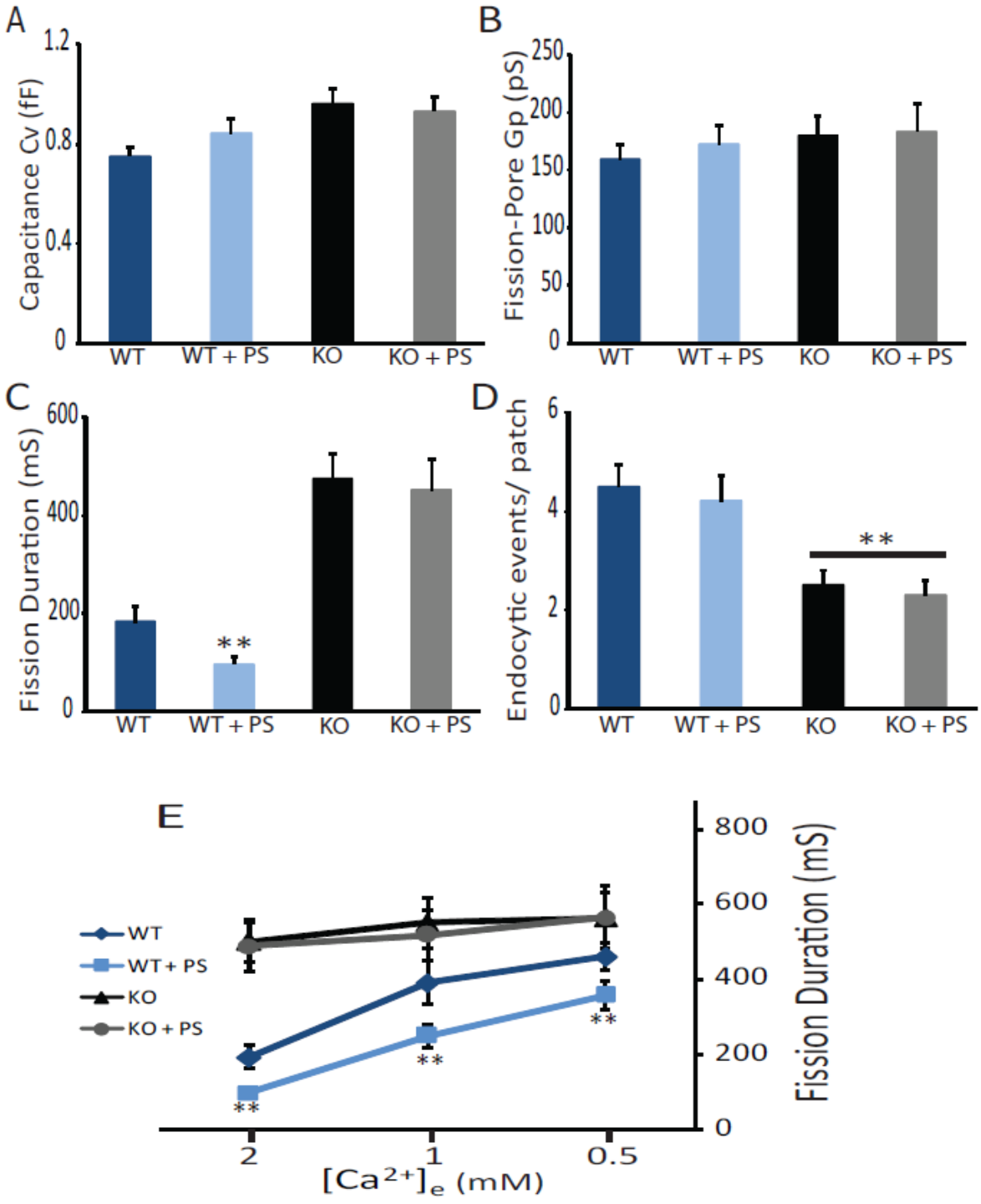
0.6
0.3
0
Control
BoNT/A
B
Fission-Pore Gp (pS)
200
100
0
Control
BoNT/A
C
Fission duration (mS)
250
200
150
100
50
0
Control
BoNT/A
D
Endocytic events/patch
6
4
2
0
**
Control
BoNT/A

Fig. 15 Disruption in Syt1- SNARE complex binding by BoNT/A does not affect CME fission kinetics. **A.** Cleavage of the last 9 amino acid residues in the c-terminal of SNAP-25 with BoNT/A (10ng/ml, 45 min incubation) thus inhibiting Syt1 binding interaction, revealed no difference in the size of the endocytic vesicle (Cv) when compared to control (control: n=43 events, BoNT/A: n=42 events)($p>0.05$)**. B-C.** Both the fission pore conductance Gp **(B)** and the fission pore duration **(C)** were indistinguishable between control cells (n= 43 events) and cells incubated with BoNT/A (n=42events)($p>0.05$). **D.** The number of endocytic events recorded within a 5min time frame, however was altered in groups supplemented with BoNT/A (n=108 cells) when compared to control cells (n=117 cells) ($p<0.01$) suggesting that exocytic slow down contributes to the number endocytic events per patch observed.

6.3.4 Syt1 is required for the PS-induced effect on the fission-pore duration

Anionic PS is implicated in direct binding to C2 domains (Powell et al., 2000; Verdaguer et al., 1999), suggesting the ability to modify catalytic activities of many C2 containingsignaling proteins. Syt1- a vesicular protein with two C2 domains- functions as the Ca^{2+} sensor in vesicle release. The positively charged surface of Ca^{2+}-bound Syt1 favors interactions with PS but not neutral charged PC or PE, in a Ca^{2+}-dependent manner (Sollner et al., 1993; Fernandez et al., 2001; Chapman 2008; Pang et al., 2006; Zhang et al., 2002). Given the PS-mediated effect on the fission-pore duration (Fig. 14C) and the defect in the fission-pore duration in Syt1 KO cells we previously reported (Yao, Rao et al. 2012), we next examined whether Syt1 is required for the PS-mediated effect on fission-pore duration, by measuring the effect of 10 μM PS on CME kinetics both WT and Syt1 KO cells (Fig. 16). While the capacitance (Cv) of endocytic vesicles (Fig. 16 A) and the fission-pore conductance (Gp) (Fig. 16B) were similar among all four groups ($p > 0.05$), PS induced a reduction in the fission-pore duration in WT cells ($p < 0.01$), but had no significant effect on the fission-pore duration in Syt1 KO cells ($p > 0.05$) (Fig. 16C). Furthermore, comparison of PS-mediated effects on the fission-pore durations at 2, 1 and 0.5 mM $[Ca^{2+}]_e$ between wildtype (WT) cells and Syt1 knockout (KO) cells revealed that PS addition downwardly shifted the Ca^{2+} dependence of the fission-pore duration in WT cells but had no obvious effect on the fission-pore duration in Syt1 KO cells at all the $[Ca^{2+}]_e$ tested (16E). Collectively, these results demonstrate that Syt1 is required for the PS-mediated effect on the fission-pore duration, suggesting that the Syt1-PS interaction may be critical for the kinetics of unitary CME events.


A
Capacitance Cv (fF)
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0.8
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WT
WT + PS
KO
KO + PS
B
Fission-Pore Gp (pS)
250
200
150
100
50
0
WT
WT + PS
KO
KO + PS
C
Fission Duration (mS)
600
400
200
0
**
WT
WT + PS
KO
KO + PS
D
Endocytic events/ patch
6
4
2
0
**
WT
WT + PS
KO
KO + PS
E
WT
WT + PS
KO
KO + PS
**
**
**
800
600
400
200
0
Fission Duration (mS)
2
1
0.5
[Ca2+]e (mM)

Fig. 16 The Ca^{2+} dependent, PS-mediated affect is abolished in Syt1 KO cells. **A-B** The Cv of endocytic vesicles (**A**) and the fission-pore conductance Gp (**B**) were comparable between all groups: control cells (n= 40 events), control + PS (n=39 events), Syt1 KO cells (n=38 events) and Syt1 KO +PS (n=41 events)($p>0.05$). **C.** The fission-pore duration was not altered in Syt1 KO cells treated with 10 μM PS when compared Syt1 KO cells alone ($p>0.05$). **D.** The number of endocytic events per patch in a 5 min recording is reduced in Syt1 KO cells, both treated with 10 μM PS and non-treated cells (KO: n= 89 cells, KO + PS: n= 95 cells) in comparison to both control cells and cells incubated with 10 μM PS (Control: n=96 cells; Control + PS: n= 85 cells) ($p<0.05$). **F.** A significant downward shift in the fission-pore duration was observed in control cells treated with 10 μM PS (2mM: n=39 events, 1mM: n=37 events, 0.5mM: n=37; $p<0.01$) at different $[Ca^{2+}]_e$ when compared to control cells alone (2mM: n=37 events, 1mM: n=36 events, 0.5mM: n=39 events) and this affect was abolished in both Syt1 KO (2mM: n=42 events, 1mM: n=33 events, 0.5mM: n=34 events) cells and Syt1 KO cells supplemented with10 μM PS (2mM: n=36 events, 1mM: n=38 events, 0.5mM: n=34 events; $p>0.05$) implying a PS enhanced affect on the fission-pore duration is achieved through Syt1 in a Ca^{2+} dependent manner.

Discussion

Using our established cell-attached capacitance measurement technique in mouse chromaffin cells (Yao, Rao et al. 2012, Yao, Rao et al. 2013), our results demonstrated that the number of single CME events was not altered by PS addition, indicating that PS may not be critical for endocytic initiation. In contrast, a recent study showed that a PS deficient mutant caused a reduction in the endocytic site in yeast (Sun and Drubin 2012), which predicts a likely reduction in 'effective' endocytic events. It is of note that another anionic phospholipid, phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$], is also critical for CME (Di Paolo and De Camilli 2006). While it has been shown that $PI(4,5)P_2$ is not crucial for the initiation of endocytic sites in budding yeast (Sun and Drubin 2012), $PI(4,5)P_2$ has been demonstrated to be essential for endocytic site initiation in mammalian cells, since $PI(4,5)P_2$ depletion results in the near absence of AP-2 from the plasma membrane (Zoncu, Perera et al. 2007, Abe, Inoue et al. 2008). Therefore, in contrast to $PI(4,5)P_2$, it appears that PS may be obligatory for the initiation of endocytic sites in yeast but not in mammalian cells.

Our data show that PS addition shortens the fission-pore duration in WT cells, indicating a stimulatory role of PS in vesicle fission during CME. It has been proposed that PS may also facilitate endocytic invagination by enhancing membrane bending (Zha, Genest et al. 2001). Consistently, Itoh et al. have shown that the presence of PS is required for the *in vitro* binding to phospholipid bilayers of F-BAR protein (Itoh, Erdmann et al. 2005), which is essential for endocytic invagination (Itoh and De Camilli 2006). Additionally, it is implied that PS may enhance membrane curvature in the endocytic pathway between the trans-Golgi network and early endosome (Xu, Baldridge et al. 2013). Taken together, it is reasonable to speculate that PS may function in multiple steps such as invagination and vesicle fission during endocytosis.

Dynamin is the key mechanochemical enzyme for vesicle fission during CME (Slepnev and De Camilli 2000). It has been shown that PS stimulates dynamin helix assembly (Sweitzer and Hinshaw 1998) as well as GTPase activity (Rasmussen, Rusak et al. 1998), leading to the possibility that dynamin could be the molecular mediator for the PS-induced effect on the fission-pore duration we have observed. However, this possibility is unlikely, since inhibition of dynamin GTPase activity results in increases in both the fission-pore conductance Gp and duration (Yao, Rao et al. 2013) while PS addition only alters the fission-pore duration (Figure 15C) and has no obvious effect on the fission-pore conductance (Figure 15B). More importantly, the PS-induced effect on the fission-pore duration was diminished in Syt1 KO cells, indicating Syt1 rather than dynamin is the mediator for the PS-induced effect.

When bound to Ca^{2+}, Syt1 binds PS in the plasma membrane to facilitate exocytosis (Chapman 2008, Pang and Sudhof 2010). Furthermore, the Syt1-PS interaction may be involved in the formation of a fusion pore, the last step of exocytosis. Our data found that PS decreases the endocytic fission-pore duration in WT cells but has no effect in Syt1 KO cells, implying that Syt1 may also be required for the PS-induced effect on the fission-pore duration. Therefore, it is possible that Syt1 plays a functionally similar role in exocytosis and endocytosis. In parallel, it is worth mentioning that Syt may serve as the Ca^{2+} sensor for synaptic vesicle endocytosis (Yao, Kwon et al. 2011), in addition to its putative Ca^{2+} sensing role in exocytosis (Chapman 2008, Pang and Sudhof 2010).

In conclusion, our results demonstrate that PS addition decreases the fission-pore duration in WT cells but not in Syt1 KO cells, arguing for a role of the Syt1-PS interaction in vesicle fission during CME.

CHAPTER VII: DISCUSSION

7.1 Summary and Important Findings

Neurotransmission requires a balance between exo and endocytosis in the synaptic vesicle cycle (Jarousse and Kelly 2001) and this balance is mediated by a distinct ensemble of proteins that are somehow coupled and maintained at sites of cellular communication. This equilibrium maintains a readily releasable pool of vesicles relatively constant throughout repetitive rounds of release. Thus, defining proteins and complexes that are involved throughout the vesicle cycle is critical towards a better understanding of how this process is maintained. Syt1 has been proposed as a possible link between the exo and endocytosis (Poskanzer et al., 2003; Nicholson-Tomishima & Ryan, 2004), however the mechanistic details have yet to be determined. Here our result adds to the emerging view that Syt1 is one of *the* critical components in controlling the kinetic efficiency of CME. Therefore, the work I have described here has provided new insights into the role of Syt1 in CME.

Ca^{2+} has been shown to regulate the endocytic process at the Calyx of Held, hippocampus, and retinal biopolar cell synapses (Neale et al., 1999; Neves et al., 2001; Wu et al., 2005; Cousin & Robinson, 1998, von Gersdorff & Matthews, 1994, Marks & McMhanon, 1998) although the role of Ca^{2+} in endocytosis is debated due to opposing effects among different preparations and experimental conditions (Wu et al., 2007; Hosi et al., 2009; Wu et al., 2009). More importantly, it remains uncertain how Ca^{2+} regulates endocytosis. Here we demonstrate, through the use of the cell-attached capacitance technique, the ability to define the Ca^{2+} dependence of CME kinetics by observing its contribution towards individual events (Figure 5) and more importantly, that Syt1 serves as the biochemical sensor in defining the Ca^{2+} dependence of individual CME events (Figure 6).

Syt1 contributes to two distinct kinetic components of CME

Currently, literature suggests that Syt1 could be contributing to the kinetics of CME at the whole cell level through interaction(s) with the endocytic adaptor proteins AP-2 and stonin-2 (Zhang et al., 1994; Haucke et al., 2000; Grass et al., 2004; Walther et al., 2004; Diril et al., 2006). Yet, there remains disconnect in the specifics of *how* CME kinetics are regulated by Syt1. This is due to the fact that the CME kinetics at the whole cell level is determined by a series of parameters: Is Syt1's only function to determine the number of endocytic events through recruitment of adaptor proteins AP-2/stonin-2? Is its role to act as a Ca^{2+} sensor for individual endocytic events? Or could it be that Syt1 is serving both platforms? Here our high-resolution technique allowed us to surpass the limitations of whole-cell capacitance measurements, allowing us to resolve precisely how Syt1 is contributing to the kinetics of CME. We have concluded that Syt1 contributes to two distinct functional roles during CME. First, Syt1 regulates the endocytic capacity by determining the number of endocytic events (Figure 6) (Yao et al., 2012). Secondly, we reveal here that Syt1 has a direct role in serving as the biochemical Ca^{2+} sensor for individual CME event kinetics during the last stages of vesicle fission (Figure 12 & 13). Therefore, we have for the first time separated and defined a piece of the kinetic puzzle associated with how Syt1 contributes to the endocytic kinetics of one mode in the vesicle retrieval pathway, CME.

The importance of Syt1-PS interaction in CME kinetics

An alternative approach to understanding the function of Syt1 in CME is to determine if the molecular interactions that are necessary for Syt1 function in exocytosis and endocytosis. Ca^{2+}-bound Syt1 binds to PS to facilitate vesicle fusion (Hui et al., 2009; Martens et al., 2007)

and it is understood that membrane bending and $Ca^{2+}$are important in both exo and endocytosis (Hosoi et al., 2009; Wu et al., 2009; Zhang et al., 2002). Due to the positively charged surface of Ca^{2+}-bound Syt1 this state promotes interactions with PS but not neutral charged PC or PE, in a Ca^{2+}-dependent manner (Sollner et al., 1993; Fernandez et al., 2001; Chapman 2008; Pang et al., 2006; Zhang et al., 2002). Here, I have established that PS enhances the fission kinetics of CME furthermore that Syt1-PS interaction is critical for the fission kinetics of CME (Figure 17).

Novel understanding in the Ca^{2+} sensing capability of Syt1

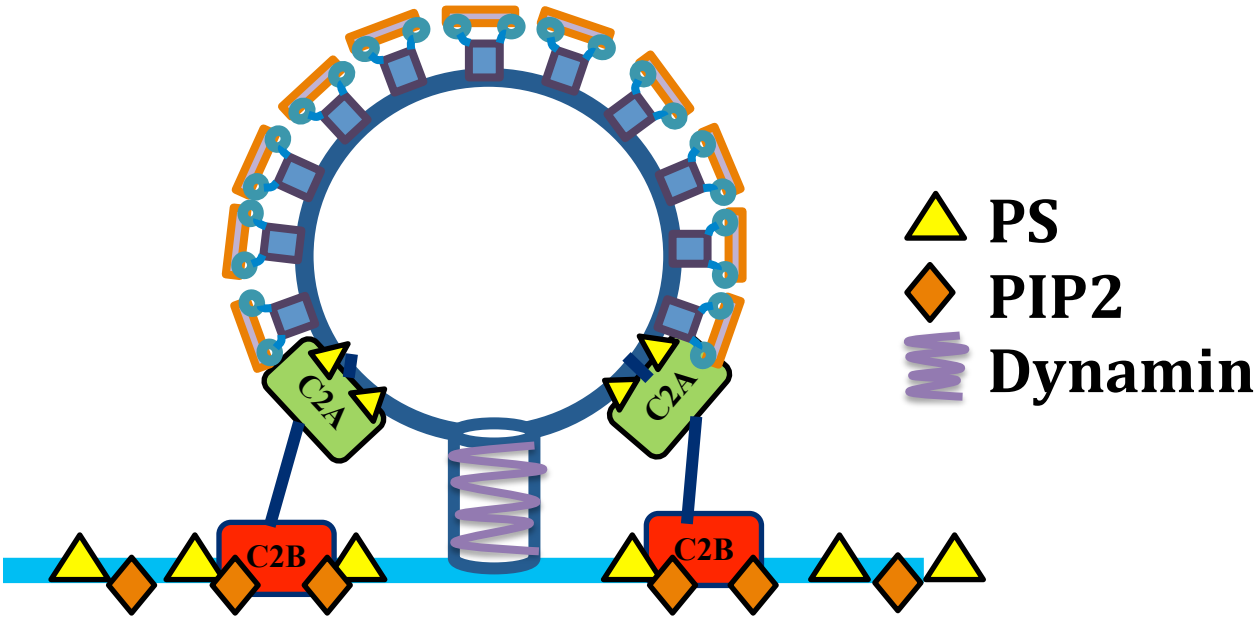
Syt1 C2 domains are known to bind and coordinate Ca^{2+} as well as integrate into lipid membranes. Thus, our goal is to determine how Syt1 C2 domains participate in the Ca^{2+} dependence of CME kinetics. Interestingly, we found that unlike exocytosis, the C2A domain is *equally* required to ensure the last stages of vesicle pinch-off during CME event kinetics. While this finding further supports Syt1 as the Ca^{2+} sensor of single vesicle CME events, it also reveals a novel understanding in the Ca^{2+} sensing capability of Syt1 during the synaptic vesicle cycle.

7.2 Working Model and Future Directions

This thesis identifies novel details regarding the role of Syt1 in CME. It is well established that Dynamin 1 serves as the key mechanochemical enzyme for vesicle fission during CME (Slepnev and De Camilli 2000). Moreover, it has been demonstrated that dynamin sustains a mechanoenzyme activity during endocytosis, however it has been implied that constriction of dynamin at the neck of the invaginating vesicle is not sufficient enough to induce vesicle fission (Roux et al., 2006). Thus, it has been proposed that at the neck of endocytic vesicles, other factors (Praefcke & McMahon, 2004;Slepnev & De Camili, 2000;Merrifield et al., 2005) may

provide the necessary tension which is cooperating with the constricting activity of dynamin in vesicle fission (Itoh et al,. 2005; Qualamann et al., 2000). It has been demonstrated that Ca^{2+} dependent membrane attachment of Syt1 C2A domain is mediated by electrostatic interactions with negatively charged membrane constituents like PS which occur when Ca^{2+} binds C2A domain (Murray & Honig, 2002). Likewise, this positive-to-negative interaction is important in the Ca^{2+} independent membrane access of C2B domain to membranes mediated by the highly charged polybasic face of C2B domain (Kuo et al., 2009). Moreover, PIP2, enriched in the plasma membrane, will accelerate the membrane binding of the C2B domain and alters the membrane-binding mode of C2B (Bai & Chapman, 2004; Rufener et al., 2005). Thus while the Ca^{2+}-binding of C2A penetrates more deeply into the lipid interface of the endocytic vesicle, the Ca^{2+}-binding loops of C2B may interact with the plasma membrane bilayer interface through PIP2, and the resulting change in orientation of C2B resulting in a shorter distance between the two opposing bilayers (Kuo et al., 2009) for exocytosis. Since PIP2 is well understood to play a role in CME (Wenk & De Camili, 2004; McLaughlin & Murray, 2005; Padron et al., 2003) and based on our findings here that show: 1) C2A has a novel function in CME by contributing equally to the Ca^{2+}dependence as C2B and 2) that PS has a facilitating role in fission-pore kinetics during CME through a Ca^{2+} dependent interaction with Syt1, we propose the following working model of how this work potentially fits into the literature of PIP2, dyanmin and CME fission-kinetics over all:

Fig. 17: Working model for the molecular interactions of Syt1 during CME



This model implies that dynamin could be stabilized through the cooperative interaction of: 1)the Syt1 C2B domain to PIP2+PS in the plasma membrane in a Ca^{2+} dependent manner and 2) Syt1 C2A domain to PS in the vesicular membrane in a Ca^{2+}dependent manner thus enhancing the tension on the membrane tubular neck of the endocytic vesicle which may consequently facilitate the constricting activity of dynamin in vesicle fission.

Given the similarities in exocytic mechanisms between chromaffin cells and neurons (Rettig & Neher, 2002), it is plausible that the fission mechanism in chromaffin cells may also apply to synaptic vesicle endocytosis in neurons, which we would like to test in the future. Because one of the main overall goals in neurobiology is to understand how *synaptic*

transmission is mediated, moving this work into neurons is our next priority. Moreover, based on the findings uncovered within this work, it would be interesting to determine *how* differently Syt1 is participating in exo and endocytosis in synaptic transmission. It remains unknown whether disruption of the juxtatransmembrane domain linker between the vesicle and the C2A domain could potentially affect exo-endocytosis. Finally, because the work here has found that Syt1 is also involved in determining event frequency in CME, we are interested to figure out the involved mechanisms for this Syt1-mediated effect. Along this line, we have obtained Stonin-2 KO mice, which is critical for Syt1-AP2 interaction (Kononenko et al., 2013).

In conclusion, given the essential role of synaptic transmission in the brain, the work described here provides a further understanding of the molecular machinery that controls the synaptic vesicle cycle and builds upon an essential foundation towards a better comprehension in synaptic transmission in the brain as a whole.

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Teaching Assistant 2009 to present University of Illinois at Chicago

- Taught junior and senior level undergraduates laboratory coursework.
- Duties involve lecturing and supervising the students in performing designated experiments.

- Administered tests, quizzes, assigned projects, and presentations.
- Graded and maintained grading records through Blackboard.
- Acted as a mentor and advisor.
- List of classes in which I have been a teaching assistant:
    - ✓ BIOS 489, Cellular Neurobiology Lab
    - ✓ BIOS 289, Biology of the Brain
    - ✓ BIOS 350, General Microbiology
    - ✓ BIOS 351, Microbiology Laboratory
    - ✓ BIOS 223, Cell Biology Lab
    - ✓ BIOS 272, Comparative Vertebrate Anatomy Lab

Laboratory Technician 2007-2009, Children's Memorial Research Center, Dr. Laura Herzing Lab

- Coordinated and assisted in research activities as they relate to the human genome research project on Autism.
- Duties included: acting lab manager, mouse colony maintenance, ordering and organizing general lab equipment.
- Received training in PCR genotyping, qPCR, sequencing, mouse surgeries, and helped to coordinate novel mouse phenotyping assays.
- Involved in data collection and interpretation.
- Responded to the needs of graduate students performing research in the laboratory.

Undergraduate Research Assistant 2005-2007, Dr. Randolph Krohmer, Department Chair Biological Sciences, St. Xavier University

- Investigated the neuroendocrine aspects of reproductive physiology and behavior in the brain of the male red-sided garter snake.
- Learned how to do snake brain slice and cryostat preparation in addition to immunocytochemistry.
- Met regularly with the Tri Beta Biological Sciences team to discuss and present new data and ideas.

AWARDS AND ACHEIVEMENTS

- **Graduate Student Travel Award**, 2014.
- **Department of Biological Sciences Travel Grant**, 2014
- **College of Liberal Arts and Sciences Travel Award**, 2013.
- Awarded **Best Teaching Assistant** by undergraduate colloquium in Biological Sciences, 2012- one award per academic year.
- **St. Xavier Research Grant Award***,* 2007
- **Excellence in Biological Research Award***,* acknowledges one student per year who has excelled in the conduct of original research projects, 2007.
- **Who's Who Among Students in American Universities And Colleges**. Recognized by faculty and staff as one of 39 others who demonstrate outstanding educational leadership, 2007.

MEMBERSHIPS

Chicago Brain Foundation	2012-2013
Society for Neuroscience	2013-2014
Chicago Society for Neuroscience	2012-2013
International Society for Autism Research	2007-2009

RESEARCH TECHNIQUES AND APPLICATIONS

- Patch-Clamp Electrophysiology, expertise in cell-attached capacitance measurements
- Immunohistochemistry, Immunocytochemistry
- Confocal Microscopy
- Mouse Chromaffin Cell/Neuron culture
- Lentivirus transfection
- PCR
- Basic experience in molecular biological applications: qPCR, DNA sequencing, Western Blots
- Mouse surgical techniques
- Mouse colony maintenance and breeding
- Mouse behavior assays
- Computer skills: both Windows and Mac platforms; Igor Pro, Adobe Illustrator CS, Adobe Photoshop CS, Word processing (Word,WordPerfect), Excel, PowerPoint.

RESEARCH ACTIVITIES

Participation on Animal Care and Use- Approved Animal Research Protocols

- IBC protocol for lentivirus application in chromaffin cell
  (Doctoral Student, University of Illinois at Chicago; 2012)

ABSTRACTS

- *Phosphatidylserine is Important for CME Kinetics*. (Chicago Brain Foundation) Chicago, IL. January, 2014.

- *Phosphatidylserine is Important for Clathrin-Mediated Endocytosis*. (Society for Neuroscience) San Francisco, CA. November, 2013.

- *Incidence and Density of Gonadotropin Releasing Hormone Immunoreactivity in the Forebrain of Adult Male Red-Sided Garter Snakes.* (Society for Behavioral Neuroendocrinology) Monterey, CA. April, 2007.

- *Role of the Hypothalamus-Pituitary Adrenal (HPA) Axis on the Incidence and Density of Gonadotropin Releasing Hormone Immunoreactive (GnRH-ir) Cells in Adult Male Red-Sided Garter Snakes*. (Society for Behavioral Neuroendocrinology) Pittsburg, PA. April, 2006.

PRESENTATIONS

- Understanding the dynamics of clathrin-mediated endocytosis. Departmental lecture, UIC. October, 2013.

- Reuse! Reduce! Recycle! The Synaptic Vesicle Cycle. Chicago Brain Bee, UIC Medical Campus, 2012.

- Phosphatidylserine is important in CME. Department Lecture, UIC. February, 2014.

- Reduce, Reuse, Synaptic Vesicle Recycling: a novel look at the kinetics of Clathrin-Mediated Endocytosis. Keynote Speaker, National Biological Honors Society Regional Research Conference, St. Xavier University, March, 2014.

PUBLICATIONS

1. Yao LH, Rao Y, Bang C, Kurilova S, **Varga K,** Wang CY, Weller BD, Cho W, Cheng J, Gong LW. *Actin polymerization does not provide direct mechanical forces for vesicle fission during clathrin-mediated endocytosis*. J. Neurosci 2013; 33(40): 15793-8.

2. Yao LH, Rao Y, **Varga K**, Wang CY, Xiao P, Lindau M and Gong LW. *Synaptotagmin 1 is necessary for the Ca2+ dependence of clathrin-mediated endocytosis.* J Neurosci 2012; 32: 3778-3785.

3. **Varga,** K, Jiang, X, Gong, L-W. *Cell-Attached Capacitance Measurements in Mouse Adrenal Chromaffin Cell*- JoVE Journal; in press, March, 2014.

PAPERS IN PROGRESS

1. Phosphatidylserine is important for the dynamics of vesicle fission in clathrin-mediated endocytosis- submitted, *Journal of Neuroscience* April, 2014- **Varga, K**, Yao, L-H, Wang, X, Jiang, X, Gong, L-W.