Ultrastructural Analysis of the Role of Voltage-Gated Calcium Channel Intracellular Domains in Synaptic Vesicle Tethering at Presynaptic Terminals

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Physiology
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Abstract

Chemical synaptic transmission is the main basis by which neurons convey information. This transmission is mediated by the fusion of synaptic vesicles (SVs) and the release of their neurotransmitter payloads across the synapse from a presynaptic neuron onto the receptors of a postsynaptic cell. In response to a depolarizing action potential, voltage-gated calcium channels (CaVs) open and allow the influx of calcium ions (Ca$^{2+}$) into the presynaptic terminal which triggers the fusion of SVs with the active zone membrane (AZ). Evidence that fusion can be gated by the Ca$^{2+}$ influx from a single CaV suggests a close coupling of SVs and CaVs likely connected by molecular tethers (Stanley, 1993). We developed a method to easily visualize tethers under electron microscopy (EM) through the osmotic rupture of isolated presynaptic terminals (SSM ghosts) which flushes out obscuring organelles and structures. We observed two classes of SV-to-AZ filaments: SVs within 45 nm of the AZ were often observed to be tethered by multiple, short filaments while SVs beyond 45 nm were tethered invariably by single, long filaments of <200 nm in length. Based on previously published studies identifying a distal region of CaV2.2 C-terminals that binds SVs, we tested whether the long filaments were these C-terminals. Using a novel immuno-nanogold labeling technique, L45 antibody against the C-terminal tip was found to label SVs in the lumen of SSM ghosts. NmidC2 and C2Nt antibodies
against the middle and proximal C-terminal, respectively, were found to label the middle and peri-membrane portions of SV tethers. NmidC2 also labeled SVs, which suggests a second, more proximal SV binding site on the CaV C-terminal. Our results provide strong evidence that CaV C-terminals tether cytoplasmic SVs to the AZ and suggest that these long tethers may reel SVs into range of Ca$^{2+}$ nanodomains.
Acknowledgments

This thesis is the culmination of 7 years of my life in the Stanley laboratory. However, it owes its existence to the great many people I have met along the way. I would like to take the time and space to acknowledge and deeply thank some of them here:

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List of Abbreviations

Ab Buffer = Antibody buffer
AZ = Active zone
C1-2strep = Strep-tagged fusion protein replicating proximal 2/3rd of CaV2.2 C-terminal
C2Nt = C2 region N-terminal
C3strep = Strep-tagged fusion protein replicating distal 3rd of CaV2.2 C-terminal
Ca2+ = Calcium ions
CaV = Voltage-gated calcium channel
CCG = Chick ciliary ganglion
Cyto = Cytoplasm
DMSO = Dimethyl sulfoxide
EDTA = Ethylenediaminetetraacetic acid
EGTA = Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EM = Electron microscopy or Electron micrograph
Endo = Any non-SV membrane-bound organelles observed inside SSMs
EPSC = Evoked excitatory postsynaptic potential
E-PTA = Ethanolic phosphotungstic acid
Fab = Fragment, antigen-binding
HB = Homogenization buffer
HBK = Hepes-buffered Krebs
HEPES = (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IgG = Immunoglobulin G
IP = Immunoprecipitation
KLH = Keyhole limpet hemocyanin
MAGUK = Membrane-associated guanylate kinase
MCN = Magnocellular nucleus
NmidC2 = N-type middle of C2 region
ORB = Osmotic-Rupture Buffer
PBS = Phosphate buffered saline
PmidC2 = P-type middle of C2 region
PMSF = phenylmethylsulfonyl fluoride
PS = Postsynaptic scab
PVDF = Polyvinylidene fluoride
RIM = Rab3-interacting molecule
RIM-BP = RIM-binding protein
RIPA = Radioimmunoprecipitation assay
RRP = Readily-releasable pool
SET = Sucrose/EDTA/Tris
SNARE = Soluble, N-ethylmaleimide sensitive factor attachment receptors
SSM = Synaptosomes (Isolated presynaptic membranes)
SV = Synaptic vesicle
SV-PD = Synaptic vesicle pull down
SVTP = Synaptic vesicle tethering protein
Synprint = Synaptic protein interaction
TBST = Tris-buffered saline with Tween
TEM = Transmission electron microscope
TRS = Transmitter release site
Author’s Note

The bulk of the data presented in this thesis has been published in two articles. Data regarding the lengths of tethers and the comparison of single, long tethers to multiple, short tethers as observed under electron microscopy was published in an article titled “Synaptic vesicle tethering and the CaV2.2 distal C-terminal” (Wong et al., 2014) in which I am one of the supporting authors. Data involving the immuno-nanogold labeling of presynaptic terminals, as well as the experiments characterizing the NmidC2 and C2Nt antibodies, was published in an article titled “The calcium channel C-terminal and synaptic vesicle tethering: Analysis by immuno-nanogold localization” (Chen et al., 2017) in which I am the first author.

The figures used in this thesis that have been published in these papers include the relevant references in their figure legends.
1 Introduction

1.1 A Brief Overview of Chemical Synaptic Transmission

The ability to rapidly relay information from one anatomical region to another is critical for multi-cellular organisms to respond to their environment in a coordinated manner. In higher organisms, this process is mainly mediated through neurons that communicate with each other and with other cell types across synapses. The majority of these synapses are chemical in nature, which operates upon the release of neurotransmitters from a presynaptic neuron onto a postsynaptic cell. Chemical synaptic transmission allows for modulation of the transmitted signal that gives biological nervous systems greater plasticity than a purely digital, electrical transmission system (Nicholls and Purves, 1972).

Synaptic transmission is initiated by the membrane depolarization of the presynaptic neuron that propagates as an action potential along the presynaptic axon through the influx of sodium ions and efflux of potassium ions (Hodgkin and Huxley, 1952). This depolarization is made possible through the maintenance of an ion gradient between the intracellular and extracellular spaces of the neuron where there is a much lower concentration of sodium ions inside the cell than outside the cell, and a higher concentration of potassium inside the cell than outside. Because the ATPase (Skou, 1998) that maintains these gradients pumps 3 sodium ions out of the cell for every 2 potassium ions into the cell, an overall negative voltage potential is generated within the cell. Upon the depolarization of the membrane and the resulting activation of sodium channels, sodium ions flow along the concentration gradient into the neuron, which further depolarizes the segment of membrane. While not immediately involved with the depolarization of the neuron, the maintenance of a higher concentration of potassium ions inside the cell versus outside the cell and then the subsequent equilibrating of this ion gradient contributes to the rapid repolarization of the presynaptic neuron and the sharp “spike” of an action potential. Both sodium and potassium ion movement across the membrane during an action potential is through voltage-gated sodium and potassium channels, respectively, that open when the membrane in which they are embedded becomes depolarized by the ion movement in adjacent membrane regions, thus propagating the signal along an axon. Once the action potential reaches the presynaptic terminal, the membrane depolarization opens voltage-gated calcium channels (CaVs) and the greater concentration of calcium outside the cell drives the influx of calcium ions (Ca^{2+})
into the terminal (Fatt and Katz, 1953; Katz and Miledi, 1967; Reuter, 1967). These CaVs are embedded in active zones (AZs) of the presynaptic terminal plasmalemma that appose the postsynaptic plasmalemma across the synapse (Pumplin et al., 1981; Robitaille et al., 1990). The influx of Ca$^{2+}$ at these AZs lead to the fusion of synaptic vesicles (SVs) to the AZ membrane at transmitter release sites (TRSs). These SVs contain neurotransmitters (Whittaker et al., 1964) that are released upon SV fusion, and these neurotransmitters act on specific receptors of the postsynaptic cell.

1.2 Overview of Calcium Channel Organization and Classification

1.2.1 General Organization of Voltage-Gated Calcium Channels

CaVs comprise up to 5 distinct subunits depending on the type of CaV (Figure 1). The $\alpha_{1}$ subunit is the largest and forms the transmembrane pore that conducts Ca$^{2+}$. The $\gamma$ subunit is the only other transmembrane subunit (Jay et al., 1990). The $\beta$ subunit is the wholly intracellular component of the CaV (Ruth et al., 1989), while the dimerized $\alpha_{2}$ and $\delta$ subunits are the extracellular components attached to the membrane through a glycosyl-phosphatidylinositol anchor (Davies et al., 2010). While the $\beta$, $\alpha_{2}\delta$, and $\gamma$ subunits are important in the trafficking and modulation of the CaV as a whole (reviewed in Dolphin, 2016), it is the pore-forming $\alpha_{1}$ subunit that is the focus of this thesis. Indeed, the importance of the $\alpha_{1}$ subunit is underlined by the fact that the classification of CaV is based solely on its $\alpha_{1}$ subunit. The structure of the $\alpha_{1}$ subunit is based off of its similarities to the $\alpha$ subunit of voltage-gated sodium channels (Tanabe et al., 1987). The subunit is made of 4 “domains” (numbered I-IV) each with 6 transmembrane “segments” (numbered S1-6) (reviewed in Catterall, 2011). The 4 domains are clustered together so that the S5 and S6 segments from each domain are oriented inwards to form the channel pore. The N-terminal, C-terminal, and the “loops” connecting each of the domains all protrude from the intracellular face of the protein. Each of these intracellular domains are of varying sizes in terms of number of amino acids, with the C-terminal being the largest followed by II-III loop, the N-terminal, the I-II loop, and the III-IV loop in descending order.
Figure 1. Organization of a Voltage-Gated Calcium Channel.
An “exploded” schematic of the domains of the pore-forming α₁ subunit of the long-splice CaV2.2 and the relative location of other CaV subunits. The area above the plasmalemma (black horizontal lines) is the intracellular side of the cell. The synprint and C-terminal tail regions of the α₁ subunit are highlighted in yellow and green respectively. The secondary structure of the C-terminal tail is uncharacterized but thought to be unstructured, and is drawn with creative license here in a serpentine conformation for the sake of space due to its long length. Intracellular loops of the α₁ subunit are drawn roughly to scale, but the other subunits are not.

1.2.2 Voltage-Gated Calcium Channel Classification

There are currently 5 types of CaVs that are distinguishable through their pharmacology and the gene encoding their α₁ subunit (reviewed in Catterall, 2011). L-type channels are centered around CaV1.1-1.4 α₁ subunits and found mostly in muscle, heart tissue, as well as some endocrine cells and sensory neurons (see Section 1.6 for more on sensory neurons). They can be selectively blocked by dihydropines such as nimodipine and activated by agonists such as Bay K 8644 (Hess et al.) and are characterized by a non-inactivating current. They are also the only CaV type that is generally accepted to possess γ-subunits. CaV2.1 is the α₁ subunit for P- and Q-type channels, which were first discovered in cerebellar Purkinje (Llinas et al., 1989) and granule cells (Randall and Tsien, 1995), respectively, and eventually found to be different splice variants
of the same gene that alters the inactivation characteristics of the channel. CaV2.1 (both the P- and Q-type variants) can be selectively blocked with ω-agatoxin IVA. The α1 subunit of N-type channels is CaV2.2 and are mostly found in neurons. CaV2.2 is selectively blocked by ω-conotoxin GVIA (Kerr and Yoshikami, 1984; Olivera et al., 1984). However, it should be noted that in zebrafish, evidence has been presented that ω-conotoxin GVIA also blocks P/Q-type channels (Wen et al., 2013). R- and T-type channels are both largely resistant to specific pharmacological agents, although there is evidence that a subtype of R-type channels is blocked by SNX 482 (Arroyo et al., 2003; Newcomb et al., 1998). The α1 subunit for R-type channels is CaV2.3 while for T-type channels it is CaV3.1-3.3. T-type channels are activated at a lower voltage than the other channel types (Carbone and Lux, 1984a, 1984b) and have generally transient currents (Nowycky et al., 1985). They are selectively blocked by the drug mibefradil only when it is used at low concentrations (Bezprozvanny and Tsien, 1995) while NNC 55-0396 has been described as being more specific (Huang et al., 2004). CaVs are often referred to by their α1 subunit (i.e. CaV2.2 for N-type channels) and the remainder of this thesis will refer to CaVs in this fashion.

<table>
<thead>
<tr>
<th>Type</th>
<th>Family</th>
<th>α1 subunit</th>
<th>Specific Pharmacological Blocker</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>CaV1</td>
<td>CaV1.1-1.4</td>
<td>Dihydropines</td>
</tr>
<tr>
<td>P/Q</td>
<td>CaV2</td>
<td>CaV2.1</td>
<td>ω-agatoxin IVA</td>
</tr>
<tr>
<td>N</td>
<td>CaV2</td>
<td>CaV2.2</td>
<td>ω-conotoxin GVIA</td>
</tr>
<tr>
<td>R</td>
<td>CaV2</td>
<td>CaV2.3</td>
<td>SNX 482 (partial)</td>
</tr>
<tr>
<td>T</td>
<td>CaV3</td>
<td>CaV3.1-3.3</td>
<td>NNC 55-0396</td>
</tr>
</tbody>
</table>

Table 1. Classification of Voltage-Gated Calcium Channels.
While there are many ways of organizing the classification of CaVs due to their discovery and naming by different research groups, this thesis will refer to CaVs by the name of their pore-forming α1 subunit (column highlighted in green).
1.2.3 Two Regions of Interest on the $\alpha_1$ Subunit of Voltage-Gated Calcium Channels

CaV2.2 has a long-splice variant that results in a $\alpha_1$ subunit with an extended C-terminal tail (Figure 1). This long-splice CaV2.2 is targeted to the presynaptic AZ (Maximov and Bezprozvanny, 2002), but the mechanism by which this targeting occurs is still not fully understood. It was demonstrated that the loss of the PDZ-binding domain or the SH3-binding domain (Maximov et al., 1999) found in the alternatively-spliced region of the C-terminal prevents the proper targeting of the channel which would suggest a role for the synaptic proteins MINT and CASK in this process (Maximov and Bezprozvanny, 2002). CASK belongs to a family of scaffolding proteins known as membrane-associated guanylate kinases (MAGUKs), but the precise function of CASK itself is still unknown (Atasoy et al., 2007). MINT proteins are adaptor proteins thought to interact with munc18-1 (Ho et al., 2006). The PDZ- and SH3-binding domains are also found in the C-terminal of CaV2.1 (Maximov et al., 1999). However, immunofluorescence experiments using antibodies against the long-spliced CaV2.2, CASK, and MINT show evidence that neither MINT nor CASK co-localize with the long-splice CaV2.2 at the AZ suggesting that these proteins do not serve as a scaffold for CaVs (Khanna et al., 2006).

Another region of interest for CaV2.2 is the synaptic protein interaction or “synprint” region of its II-III intracellular loop (Figure 1). Of the intracellular domains of CaV2.2 other than the C-terminal tail, this region possesses the most number of amino acids and is thought to be the longest of the loops connecting the transmembrane domains. There is biochemical evidence that this region can bind proteins involved in SV exocytosis (Sheng et al., 1998). This binding and its implications on SV tethering will be discussed in more detail in Section 1.7.1.

1.3 Synaptic Vesicles

SVs were first described in various synaptic tissues using electron microscopy (EM) in the mid-1950s as 10 to 30 nm diameter vesicles in presynaptic terminals that tend to cluster near the thickened synaptic membrane of the AZ (De Robertis and Bennett, 1955; Palay and Palade, 1955). The later finding that these relatively uniformly-sized vesicles contained neurotransmitters (acetylcholine in the study of Whittaker et al., 1964) provided strong evidence that SVs were the “quantal units” (del Castillo and Katz, 1954; Fatt and Katz, 1952) that are the basis of synaptic transmission. These quantal units were first described from observing
“spontaneous” synaptic events that occur in the absence of action potentials and are thought to result from the fusion of very limited number of SVs. It is thought that “evoked” synaptic events that are in response to action potentials involve the combined fusion of many SVs.

A later study that focused on characterizing the physical properties of SVs described them as having an average diameter of 40 nm with a range of 30 to 80 nm (Takamori et al., 2006). While SVs are membrane-bound organelle, the authors of this study found that, on average, only 50% of the surface area of an SV is actually phospholipids and 20% of the surface area is made of the transmembrane domains of various and numerous SV proteins. These extra-vesicular domains of these proteins cover a large amount of the SV surface. Using electron tomography, various appendages can be seen protruding from the SV surface, some of which are thought to be large proteins such as V-ATPase (Takamori et al., 2006).

1.3.1 Different Pools of Synaptic Vesicles

SV fusion to the AZ membrane, as far as the process has been elucidated thus far, is a very intricate and complicated process. In a given presynaptic terminal, SVs can be roughly separated into three functionally distinct populations or pools (reviewed in Denker and Rizzoli, 2010). A readily-releasable pool (RRP) of SVs was first described through their stimulation of postsynaptic cells by the application of hypertonic sucrose to cultured hippocampal neurons (Stevens and Tsujimoto, 1995). These SVs are thought to be “docked” and “primed” for immediate fusion at the AZ membrane. An actively recycling pool was differentiated from the reserve pool of SVs by tracking styryl dye uptake in the neuromuscular junction of fruit fly larvae (Kuromi and Kidokoro, 1998). In that and subsequent studies, it appears that the reserve pool only comes into play when the SVs belonging to the recycling pool has been exhausted.

Only the RRP is thought to be spatially identifiable as being the SVs that are visibly touching (Imig et al., 2014) or closely-linked (Fernández-Busnadiego et al., 2010) to the AZ membrane. However, other studies have shown that SVs that are morphologically docked may not be necessarily available for immediate fusion. For example, munc13-1 deficient hippocampal synapses still have a normal complement of SVs morphologically docked to the AZ, but have a significant reduction in high-sucrose-induced neurotransmitter release (Augustin et al., 1999). Thus, it appears that the functional and morphological definitions of the RRP still remain to be reconciled. The recycling and reserve pools of SVs do not appear to be distinguishable in terms
of the location of the pools within a presynaptic terminal (Rizzoli and Betz, 2012). It is still not well understood what factors, if any, on the SVs themselves determine whether an SV is destined for a certain SV pool. However, a recent study has suggested that the protein tomosyn, in conjunction with the GTPase rab3a, may play a role in sorting SVs to the reserve pool (Cazares et al., 2016).

### 1.3.2 Synaptic Vesicle Fusion

Fusion of RRP SVs to the AZ membrane is currently thought to be directly mediated by the SNARE complex (reviewed in Söllner, 2009; see also Söllner et al., 1993). The core of this complex at synapses is comprised of three proteins; VAMP2, SNAP-25, and syntaxin-1. While their locations may change after SV fusion, VAMP (classified as a v-SNARE) is generally localized to vesicular membrane prior to fusion while the latter two proteins (t-SNAREs) are generally found on the target AZ membrane. The SNARE hypothesis requires that the three proteins interact in a zipper-like fashion to overcome the incredibly strong energy barrier posed by the water molecules sandwiched between the SV and AZ membrane that would otherwise inhibit the fusion of the two membranes. The actions of the SNARE complex are regulated by a variety of proteins. Munc18-1 acts both as a chaperone for closed, inactive syntaxin-1 proteins (McEwen and Kaplan, 2008) and as a facilitator for SNARE complex zippering (Shen et al., 2015) once the complex is formed. The latter function has been described to be dependent on the interaction of munc18-1 with munc13 (Ma et al., 2013). There is also evidence that complexin plays a role in regulating SNARE complex activity, although the exact nature of this role is still under heavy debate (reviewed in Brose, 2008). Perhaps the most important regulator of SNARE complex activity is the calcium sensor protein that triggers SV fusion. At this moment in time, the generally accepted hypothesis is that a member of the synaptotagmin family of proteins serve as the calcium sensor for fast, evoked transmitter release at classical synapses (Brose et al., 1992). Mice that are homozygote for a deleterious mutation of synaptotagmin-1 are no longer capable of evoked synaptic transmission, although spontaneous activity remains largely unaffected (Geppert et al., 1994). While this SNARE hypothesis model is currently the most widely-accepted model of SV fusion, it should be noted that many steps in this model—especially those involving synaptotagmin—are still poorly understood and controversial (reviewed in Meriney et al., 2014).
1.3.3 Endocytosis

After fusion, SVs are reformed through a process termed endocytosis (reviewed in Smith et al., 2008). There appears to be two forms of endocytosis: “bulk” and “ultrafast” endocytosis. What is now termed bulk endocytosis was first described in the classical study of the frog neuromuscular junction by Heuser and Reese (1973) where it was first suggested that SVs eventually undergo a recycling process after fusing with the plasmalemma. At sites on the edge of AZs, “coated vesicles” are retrieved from the plasmalemma post-fusion and coalesce into large “cisternae” from which bud newly-formed SVs (purple pathway in Figure 2). It is thought that the coat observed on the endocytosing vesicles is made of the protein clathrin that physically assists in retrieving vesicles from the synaptic plasmalemma (reviewed in Kirchhausen, 2000). It has now been suggested that this mode of bulk endocytosis is far too slow to account for SV replenishment seen under physiological conditions and that the cisternae may only form in response to extreme levels of SV fusion events to compensate for the sudden increase in plasmalemma surface area. Ultrafast endocytosis (orange pathway in Figure 2) involves the retrieval of smaller endosomes from the edges of AZs (Watanabe et al., 2013) which then split into SVs (Watanabe et al., 2014). These smaller endosomes are roughly ~80 nm in diameter (Watanabe et al., 2014), larger than the average diameter of SVs which is ~40 nm (Takamori et al., 2006). Reformed SVs then re-join the recycling pool and the SV cycle (a term coined by Heuser and Reese, 1973) continues. Furthermore, clathrin is now believed to only be involved in the forming of SVs from endosomes (Schikorski, 2014; Watanabe et al., 2014).

There is completely different form of SV cycling known as “kiss-and-run” where SVs do not completely fuse with the presynaptic plasmalemma (reviewed in Harata et al., 2006). Instead, the SV membrane maintain their general spherical shape with only a membrane “neck” connecting the SV to the AZ plasmalemma where the SV neurotransmitter content can escape. With the kiss-and-run model, SVs are described as retaining their identity throughout the entire SV cycling process.
Figure 2. The Synaptic Vesicle Cycle.
Cartoon of the SV cycle at a presynaptic terminal with the SVs (blue circles) fusing at the AZ (green membrane) and being recycled at sites adjacent to the AZ. SVs are shown held at the peri-AZ through filamentous tethers as discussed later in this thesis. While the concept of SV recycling dates back to (Heuser and Reese, 1973), this figure reflects the bulk (purple) and ultrafast (orange) modes (on the left AZ) of endocytosis, as well as the kiss-and-run model (grey, on the right AZ) of SV recycling. Note that all 3 modes of SV recycling are unlikely to occur simultaneously in the same synapse and are only shown together here for illustrative purposes. The fusion necks of SVs undergoing kiss-and-run have been exaggerated in this diagram to differentiate this process from the others. “T” shaped objects represent clathrin-coating of vesicles.

1.4 Calcium Microdomain Versus Nanodomain Gating of Synaptic Vesicle Fusion

The proximity between CaVs and SV during exocytosis—known as the Ca$_2^+$ microdomain versus nanodomain debate—is of great importance to this project. More specifically, this is the debate of whether the exocytosis of SVs can be triggered by the Ca$_2^+$ influx through a single CaV, which forms a Ca$_2^+$ nanodomain, or whether exocytosis requires a Ca$_2^+$ microdomain generated by multiple CaVs opening in conjunction (Figure 3). Early studies modeling the influx of Ca$_2^+$ suggested that multiple CaVs must be open simultaneously to trigger SV release (Simon and Llinás, 1985; Zucker and Fogelson, 1986) which would thus form a Ca$_2^+$ microdomain. A series of subsequent studies then suggested that SV fusion was gated by a Ca$_2^+$
nanodomain. Dose-response experiments using low doses of ω-conotoxin GVIA to block portions of the total CaV2.2 population and then measuring the effects on end-plate potentials did not fit a cooperative model of CaV gating as would be expected in a microdomain system (Yoshikami et al., 1989). Single-channel recordings of presynaptic calyces from the chick ciliary ganglion while simultaneously monitoring for acetylcholine release showed that the Ca$^{2+}$ influx from a single CaV is sufficient to gate the fusion of SVs, consistent with nanodomain gating of SV fusion (Stanley, 1993). Further studies using the neonatal rodent calyx of Held (beginning with Borst and Sakmann, 1996) found evidence that microdomain gating is predominant since the Ca$^{2+}$ influx could be “intercepted” by the Ca$^{2+}$-chelator EGTA before it triggered fusion. They concluded that there must be a significant distance between SVs and CaVs for such a relatively “slow” chelator to block fusion. However, it was eventually found that the mature, transmitting calyx of Held transitioned from a microdomain-gated system to a predominantly nanodomain-gated system during development (Fedchyshyn and Wang, 2005). While there are still relatively recent reports of synapses that are based on Ca$^{2+}$ microdomains based on EM immunogold labeling (Nakamura et al., 2015), a review of the literature suggests that SV fusion in the majority of synapse types are triggered by Ca$^{2+}$ nanodomains (reviewed in Stanley, 2016). Nanodomain gating is also supported by recent modeling studies (Dittrich et al., 2013; Stanley, 2015), the latter of which suggests that SV fusion is triggered by nanodomains even when SVs are adjacent to groups of CaVs.
Figure 3. Ca$^{2+}$ Microdomain versus Nanodomain Gating of Synaptic Vesicle Fusion.

Ca$^{2+}$ (green) enters the presynaptic terminal through CaVs with the greatest concentration of Ca$^{2+}$ near the CaV pores. In microdomain-gated fusion (top), the accumulated Ca$^{2+}$ influx from multiple CaVs is necessary to trigger the calcium sensor (orange) on the SV due to its distance away from the CaVs. In nanodomain-gated fusion (bottom), the SV is sufficiently close to the CaV for the Ca$^{2+}$ influx from this single CaV to trigger the calcium sensor. Note that distances drawn in this diagram are not to scale.

1.5 Synaptic Vesicle Tethering

The sequence of events before a SV docks and fuses with the AZ is still poorly understood. If SV fusion is gated by Ca$^{2+}$ nanodomains as it appears to be the case for most synapses, then it is insufficient for SVs to freely float in the presynaptic lumen if it is to remain part of the recycling and readily-releasable pools since the distances between CaVs and SVs would be unpredictable and, more importantly, too long. One of the early studies of nanodomain gating proposed that a
molecular anchor or tether must maintain SVs sufficiently close to individual CaVs so that the Ca\textsuperscript{2+} influx from an individual CaV will reach the Ca\textsuperscript{2+} sensor on the SV in sufficient concentration to trigger fusion (Figure 4; Stanley, 1993, 1997). It was estimated that the maximal distance that such a tether would maintain between the CaV and the SV would be \(~25\) nm (Stanley, 1993; Weber et al., 2010).

![Diagram of synaptic vesicle tethering](image)

**Figure 4.** A Molecular Anchor/Tether Holds Voltage-Gated Calcium Channels and Synaptic Vesicles Close Enough for Nanodomain Gating.

The diagram of a model for release site organization in Ca\textsuperscript{2+} nanodomain gating of SV fusion proposed and published in Stanley (1993). The “Anchor” labeled in this diagram is analogous to SV tethers. This tether holds the SV sufficiently close (~25 nm) to the CaV so that the diffused Ca\textsuperscript{2+} influx from this CaV is of sufficiently high concentration (~10 uM) to trigger the Ca\textsuperscript{2+} sensor (not shown here) on the SV. *Image reproduced with the permission of Elsevier (License No. 4057120801000).*

### 1.5.1 Imaging Synaptic Vesicle Tethers

Much of the direct evidence for SV tethering comes from electron microscopy as this technique is currently the only one with sufficient resolution to observe structures at the nanometer scale.
In early freeze-fracture studies of the frog neuromuscular junction, the AZ is observed to be laid out in a very distinctive pattern with two double-rows of particles that are flanked by SV fusion sites (Dreyer et al., 1973; Heuser et al., 1974). The freeze-fracture procedure involves an extremely rapid (<2 ms) freezing of the tissue sample and then the subsequent fracturing of this sample along fault lines that typically run between lipid bilayers. The newly-revealed faces of the sample are then shadowed and cast into a replica using platinum and carbon. These casts, or replicas, are what is imaged using EM. Subsequent studies were made of these freeze-fractured casts using 3D electron tomography, where the casts are progressively tilted relative to the electron beam so that a high-resolution 3D virtual reconstruction of the AZs can be made. Analysis of these reconstruction showed that an intricate structure of interconnecting ribs, beams, and pegs link SVs to the AZ membrane with the previously-described rows of AZ particles being the pegs (Harlow et al., 2001). These pegs, suggested to be composed in part by CaVs (Fukunaga et al., 1983; Harlow et al., 2001), were found to contract towards the centreline of the AZ during exocytosis (Stanley et al., 2003). The structured organization of molecules at the AZ is not limited to the frog neuromuscular junction. Electron tomography of the neuromuscular junction of mice also show an intricate array of beams, ribs, and pegs interacting with SVs at the AZ (Nagwaney et al., 2009). However, in the contrast to the frog neuromuscular junction where SVs flank the AZ array on either side, the murine AZ arrays flank a single row of SVs (Figure 5).
Figure 5. Active Zone Macromolecules at the mouse and frog neuromuscular junctions. Organization of the beams, ribs, and pegs at the AZs of mouse (top) and frog (bottom) neuromuscular junctions. The exposure of neuromuscular junctions to antibodies against CaVs resulted in the disruption of these structures (Fukunaga et al., 1983), and it is thought that the pegs are associated with CaVs. Image reproduced with the permissions of John Wiley and Sons (License No. 4060280161022), and Elsevier (License No. 4060280031741).

Freeze-etch studies of other presynaptic terminal types have suggested a complex organization of proteins at the AZ as well. In these studies, tissue samples were also rapidly frozen and a <50 nm outer layer of the frozen sample is sublimated, revealing non-aqueous structures near the surface. This surface is then coated with platinum and the replica is imaged using EM. While a great number of SVs and various filaments interacting with them were observed using the freeze-etching of presynaptic terminals, of particular significance to this thesis were the observations of filaments connecting SVs to the AZ (Landis et al., 1988). Landis and colleagues suggested that these filaments may be a series of spectrin and synapsin protein molecules, but did not pursue that hypothesis further in this study. A later study using immunogold labeling found evidence that synapsins may be involved in the filaments connecting SVs to each other rather than the filaments tethering SVs to the AZ (Siksou et al., 2007). Some of these SV-to-AZ filaments were
~100 nm long (Figure 6; Hirokawa et al., 1989). While the identities of these long filaments were not established in their study, Hirokawa and colleagues noted that these filaments did not have the periodicity that would be expected with actin filaments, which were observed to form a network interconnecting the SVs and other presynaptic structures.

Figure 6. Long SV Tethers from Hirokawa study.
A freeze-etch EM image of a presynaptic terminal from rat cerebellar mossy fiber synapses originally published in Hirokawa et al. (1989) with colours added to the arrow labeling. A long filament (green arrow) can be seen connecting a SV with the AZ. Filaments can also be seen connecting SVs to each other (yellow arrows) and actin filaments (purple arrow) are also present.
Conventional transmission EM of synaptic tissue, where the electron beam must penetrate aldehyde-fixed tissue has thus far provided limited information on TRS organization. This technique has many drawbacks when used for this purpose due to the crowding of presynaptic organelles and the condensation of cytoplasmic proteins from aldehyde fixation causing an electron-translucent haze through which it is difficult to make out fine, nanometer-wide structures. However, there were a few instances, whether inadvertently or not, where hints of structures at AZ membranes could be seen. One of the earliest studies of purified presynaptic membrane, or “ghosts,” separated using sucrose gradients from intact isolated presynaptic terminals (SSMs) show that these ghosts are largely devoid of organelles and cytoplasm (Whittaker, 1968). While the author does not directly comment on them, some of the published figures show residual SVs and string-like cytoplasm connecting these SVs to each other and to the AZ membrane. Another study used ethanolic phosphotungstic acid (E-PTA) to stain various synapses from a variety of species to visualize the proteinaceous material at the AZ (Bloom and Aghajanian, 1968). Since E-PTA does not stain phospholipid membranes, hazy “synaptic material” projections could be seen at the AZs, as determined by apposition to postsynaptic densities, of the various synapses. The proteinaceous nature of these projections was established by their dissolution upon application of trypsin. This “particle web” of synaptic material could also be dissolved with high pH and even reconstituted by subsequently lowering of the pH (Phillips et al., 2001). Mass spectroscopy of the reconstituted particles yielded a wide array of proteins which the authors then fit into a model based on the differential pH solubility of each protein. However, more recent studies have argued that the aldehyde fixation of the presynaptic material artificially collapses the cytoplasmic proteins near the AZ into these presynaptic particles (Rostaing et al., 2006).

More recently, filaments connecting SVs to the AZ membrane were observed with the electron tomography of high-pressure frozen samples (termed cryo-electron tomography) which allows for greater penetration into presynaptic terminals and overcomes the electron-dense condensation of cytoplasm during aldehyde fixation (Rostaing et al., 2006). In a study looking at mouse hippocampal neurons in slices, filaments connecting SVs to the AZ membrane were observed with lengths up to 200 nm long (Siksou et al., 2007). The authors then used EM immunogold
(on aldehyde-fixed samples) and knockout animals to investigate synaptophysin, synapsin, bassoon, and CAST as candidates. However, their results were largely inconclusive in terms of identifying what proteins make up the SV-to-AZ tethers. A recent study from the Reese group (Cole et al., 2016) examined dissociated rat hippocampal neurons using cryo-electron tomography and characterized two different types of filaments connecting SVs to the AZ membrane. Long, regularly kinked, “cluster” filaments ranging from 47-102 nm long were observed to connect multiple SVs to the AZ membrane. Curiously, filaments with similar kinked characteristics also existed in the cytoplasm without any observable AZ membrane interaction. These ranged from 30-169 nm in length, and the authors classified these long cytoplasmic filaments with the cluster filaments that contact the AZ membrane. A second class of shorter filaments, termed by the authors as “docking” filaments, ranged from 10-47 nm in length. What is striking about these docking filaments is that SVs within 16 nm of the AZ membrane could be tethered by up to 6 of these filaments. These multiple tethers echo the findings of earlier studies from the Lucic group (Fernández-Busnadiego et al., 2010, 2013) that examined SV tethering in rodent SSMs using cryo-electron tomography. The tethers observed in these studies only extend up to ~40 nm in length and are likely the docking filaments observed in the Cole study and, like Cole’s docking filaments, can tether individual SVs with multiple filaments. The presence of these multi-tethers can be reduced by using tetanus toxins (Fernández-Busnadiego et al., 2010) or by using SSMs from RIM1α mice (Fernández-Busnadiego et al., 2013). In SSMs from mice lacking all 3 forms of synuclein, a family of proteins associated with various neurodegenerative diseases, there is a significant increase of multi-tethers (Vargas et al., 2017). This last finding is particularly intriguing, as α-synuclein has been shown to interact with the small GTPase rab3a (Chen et al., 2013) which in turn has been described to be one of the many proteins that interact with RIM (see 1.5.2.4). While these three studies from the Lucic group provide strong candidates for the modulation of SV tethering, the authors acknowledge that they do not provide direct evidence for the identity of the actual tether itself. All the cryo-electron tomography studies described here have also identified filaments connecting SVs to each other in the cytoplasm, but these “interconnectors” are outside the scope of this thesis and will not be discussed here.
1.5.2 Some Candidates for the Identity of the Synaptic Vesicle Tether

1.5.2.1 Cytoskeletal Proteins

As of yet, there has been no direct evidence for the identity of the SV-to-AZ filaments seen under EM. One candidate for this molecular tether is actin, a major component of the cytoskeleton. At the calyx of Held, the application of latrunculin A, an actin depolymerization agent, reduced the recruitment of SVs to the RRP as measured electrophysiologically (Sakaba and Neher, 2003). Another group found similar effects using latrunculin B and also blebbistatin, an inhibitor of the cytoskeletal component myosin II (Lee et al., 2012). While the actin cytoskeleton is a critical part of organizing organelles such as mitochondria and SVs in the cytoplasm (Perkins et al., 2010), there is no direct evidence showing that actin specifically tethers SVs to the AZ. In fact, early freeze-etch studies of presynaptic terminals have observed filaments connecting SVs to the AZ that are morphologically distinct from actin (Figure 6; Hirokawa et al., 1989). More recent work has suggested that the cytoskeleton plays a role in trafficking SVs between synapses (Gramlich and Klyachko, 2017).

1.5.2.2 Bassoon and Piccolo

Some of the larger proteins localized to the AZ have also been implicated as SV tethers (Phillips et al., 2001). Bassoon and piccolo are two large (420 and 530 kDa, respectively) proteins with homological domains that are thought to act as scaffold proteins at the AZ (reviewed in Gundelfinger et al., 2016). Loss of bassoon at certain presynaptic terminals such as those of cerebellar mossy fibers (Hallermann et al., 2010) and the endbulb of Held (Mendoza Schulz et al., 2014) leads to the slowing of SV recruitment to the RRP. This role in SV recruitment and their large size makes them attractive candidates as SV tethers. However, neither bassoon nor piccolo are found in certain stereotypical synapses such as the neuromuscular junction (Fenster et al., 2000). Indeed, the structures connecting SVs to the AZ have been well described at the neuromuscular junction of frogs and mice (Figure 5; Section 1.5.1). Furthermore, the loss of both proteins does not seem to affect the apparent function of hippocampal synapses despite reducing the clustering of SVs in the cytoplasm (Mukherjee et al., 2010). Because of those series of observations, it is thought that bassoon and piccolo may play a role similar to that of another presynaptic protein, synapsins (Siksou et al., 2007), in regulating the interconnection of
cytoplasmic SVs and not that of tethering SVs to the AZ membrane in the majority of classical synapses.

1.5.2.3 Septins

Septins are a family of filamentous GTPase proteins that are most well-known for their role in cytokinesis (reviewed in Sanders and Field, 1994). Later work on septins have functionally and biochemically linked them to components of the “exocyst” complex (Hsu et al., 1998) that mediate pre-fusion steps of non-synaptic exocytosis in most cell types. The septin filaments described in this study using EM are of 8.25 nm in diameter and are of lengths that are multiples of 25 nm. Septins have been found at the presynaptic terminals of hippocampal neurons using immunofluorescent labeling (Tsang et al., 2011) and have also been described to bind to the SNARE protein syntaxin in biochemical assays (Beites et al., 1999). While these characteristics would make septins an attractive candidate as SV tethers, the filaments described to connect SVs to the AZ in early freeze-etch studies (Hirokawa et al., 1989) do not have the periodicity that would be expected with septin-based filaments. Furthermore, while exocyst-mediated exocytosis does occur in neurons, it does not appear to be involved in the exocytosis of SVs (Murthy et al., 2003). Septins have more recently been shown to play a role in the developmental shift in the calyx of Held from an immature Ca\(^{2+}\) microdomain-gated system to a nanodomain-gated system (Yang et al., 2010). In this study, the loss of septin5 at the immature calyx of Held renders the transmission at this synapse insensitive to the effects of EGTA that should only affect microdomain-gated systems (Adler et al., 1991), leading the authors to suggest that septin5 is responsible for maintaining the distance between SV and CaV (Yang et al., 2010). Thus, instead of a CaV-SV tether, septins would more likely act in an inverse fashion to separate CaVs from SVs. However, there have been few published studies that have continued pursuing the hypothesis of septins as a synaptic tethering molecule.

1.5.2.4 RIM

RIM (rab3-interacting molecule) was originally described as a putative effector protein for the small synaptic GTPase switch rab3a (Wang et al., 1997). Loss of RIM leads to an increase of paired-pulse facilitation at excitatory synapses and an increase of paired-pulse depression at inhibitory synapses (Schoch et al., 2002). The phenotype at inhibitory synapses is different than what is observed with rab3a knockout animals, leading the authors of that study to question
whether RIM interacts with rab3a at all synapses. RIM is also thought to play a scaffolding role at the AZ since it has such a wide array of binding partner proteins. Using biochemical and molecular biology assays, RIM has been shown to interact with the AZ proteins munc13-1, RIM-BP1/2 (RIM-binding protein 1/2), and multiple isoforms of liprins (Schoch et al., 2002).

Multiple reports have also suggested that RIM interacts with CaVs and may anchor the channels to the AZ. Using biochemical assays, RIM has been described to bind the synprint region (further described above and below) of CaV2.2 in a similar fashion to the interaction of synaptotagmin to CaV2.2 (Coppola et al., 2001). RIM interaction with the β subunits of CaVs has also been shown using biochemical and immunofluorescent measures (Kiyonaka et al., 2007). However, a follow up study also using biochemical and immunofluorescence assays could not find evidence of RIM interaction with CaV2.2 (Wong and Stanley, 2010).

RIM has also been described to bind SV-associated proteins. There is the eponymous connection with rab3a, although it should be noted that rab3a associates and dissociates with SV membranes in response to calcium (Fischer von Mollard et al., 1991) and thus a RIM-rab3a-SV tethering would be transient. The same study that found RIM binding to the CaV2.2 synprint site also found that the putative SV calcium sensor synaptotagmin will bind to RIM in a calcium-dependent manner (Coppola et al., 2001). The evidence for RIM tethering of SVs culminated with the work leading to the Kaeser model (Kaeser et al., 2011), which is described in more detail later in a later section (1.7.2) along with the lines of argument against this model.

### 1.6 Ribbon Synapses in Sensory Neurons

The AZ organization at ribbon synapses in sensory neurons is extremely specialized and presents a fascinating example of SV tethering. Photoreceptor cells and hair cells, among others, fall under this classification and are characterized by the presence of “ribbon” structures at the AZ (reviewed in Sterling and Matthews, 2005). These structures extend 200 nm into the presynaptic lumen and are held to the presynaptic plasmalemma by an “arciform density” that includes proteins such as bassoon (Dick et al., 2003). Using either the freeze-etch or high-pressure freezing techniques described earlier (Section 1.5.1), SVs were observed to be tethered to ribbons by multiple filaments of 30-50 nm in length (Usukura and Yamada, 1987). The freeze-etch images of these tethers are remarkably similar to the 3D reconstructions of the multiple tethers observed decades later (Fernández-Busnadiego et al., 2010) at more typical synapses.
using cryo-TEM. The protein identity of these filaments is currently unknown. The tethering of numerous SVs to ribbons allows for a greater density of SVs to be kept near the AZ, which is thought to allow these sensory neurons to maintain relatively high rates of neurotransmitter release over extended periods of time.

Apart from the visually striking ribbons, these sensory neurons are also interesting in terms of calcium domains. The CaVs in sensory neurons belong to the L-type family, with CaV1.3 found in hair cells while CaV1.4 is found in photoreceptor neurons. The extreme sensitivity of these photoreceptor neurons to light and hair cells to stereocilia movement suggests that Ca2+ nanodomain gating could be prevalent in these cell types (reviewed in Kim et al., 2013). However, a recent study on gerbil hair cells has found that SV fusion in hair cells that detect high-frequency sounds are gated by Ca2+ microdomains while those that detect low-frequency sounds are gated by Ca2+ nanodomains (Johnson et al., 2017). Interestingly, biochemical studies from the Stanley group did not find a SV-binding motif on the C-terminal tail of CaV1.3 and CaV1.4 (Gardezi et al., 2016), which could suggest that the longer grab-tethering function of CaV C-terminals (Wong et al., 2014) may be supplanted by the ribbon structure in these specialized synapses (Gardezi et al., 2016).

1.7 Voltage-Gated Calcium Channel Tethering of Synaptic Vesicles

The simplest, and most-readily testable, model for a molecular tether between the SV and the CaV is for the direct binding of the CaV with some protein or element of the SV. Early studies from various groups found that CaV2.2 co-immunoprecipitates with syntaxin (Bennett et al., 1992; Yoshida et al., 1992), synaptotagmin (Leveque et al., 1992), and VAMP (el Far et al., 1995). As discussed above, syntaxin and VAMP are both SNARE proteins implicated in the machinery that facilitates the fusion of SVs to the AZ membrane. While VAMP is the SV-associated element and syntaxin is the plasmalemma-associated element in classical descriptions of SNARE complex function, both proteins have been found on SVs (Takamori et al., 2006). Synaptotagmin is the putative calcium sensor for SV exocytosis (Brose et al., 1992) and is found on the SV membrane (Matthew et al., 1981).
1.7.1 The Synprint Region

A series of studies published by the Catterall group found evidence of a binding site for syntaxin, synaptotagmin, and also SNAP-25 on the synprint region of the II-III intracellular loop of the CaV2 α1 subunit (reviewed in Sheng et al., 1998). His-tagged fusion proteins replicating the synprint region of CaV2.2 were found to bind GST-tagged syntaxin fusion proteins (Sheng et al., 1994). In the same study, peptides mimicking the CaV2.2 synprint region were found to prevent the binding of native CaV2.2 to GST-tagged syntaxin. SNAP-25, another SNARE protein traditionally thought to be on the synaptic plasmalemma but also found on SVs (Takamori et al., 2006), was also found to bind directly to CaV2.2, while VAMP’s interaction with CaV2.2 appeared to be mediated through syntaxin and SNAP-25 (Sheng et al., 1996). Evidence for the interaction between the CaV2.2 synprint site with synaptotagmin was established using affinity chromatography and solid-phase immunoassay (Sheng et al., 1997). The authors of this study also found that the interaction between syntaxin and synaptotagmin can be disrupted by the presence of the synprint peptide, which led them to propose a model by which the binding partner of CaV2.2 would change depending on the priming stage of the SV. This model is further supported by the finding that the CaV interaction with syntaxin and SNAP-25 was strongest at the Ca\(^{2+}\) concentration of 18 μM while the interaction with synaptotagmin did not appear to be Ca\(^{2+}\)-dependent (Sheng et al., 1997). On the physiological and function level, mimetic synprint peptides were reported to block evoked excitatory postsynaptic potentials (EPSPs) in superior cervical ganglion neurons (Mochida et al., 1996). Asynchronous release was not affected, however, leading to the conclusion that the interaction between the CaV synprint site and its binding partners are a critical part in synchronous SV fusion. The interaction between CaV2.1 and synaptic proteins were shown to only occur with certain CaV2.1 splice variants (Rettig et al., 1996). The II-III intracellular loop of CaV1 is not known to bind any of these proteins (Sheng et al., 1994).

There are a few findings that argue against the CaV II-III loop as being an SV tether. First, there is the relative weakness of the interaction with syntaxin and SNAP-25 at physiologically baseline concentrations of Ca\(^{2+}\) (<0.1 μM) when compared to the interaction at ~20 μM Ca\(^{2+}\). The higher concentration is more comparable to the concentration found at the Ca\(^{2+}\) nanodomain of an open CaV, and would suggest that the binding of the synprint region to syntaxin and SNAP-25 is more relevant at a late stage of SV exocytosis rather than at rest in a tethering role. However, the
interaction between the synprint region to synaptotagmin does not appear to be calcium-dependent and thus could be the CaV-to-SV tether during resting conditions. Second, a study using mimetic synprint peptides at the calyx of Held was found to only affect SV endocytosis and not SV exocytosis as measured through changes in synaptic membrane conductance (Watanabe et al., 2010). This finding is in direct opposition to the finding in superior cervical ganglion neurons (Mochida et al., 1996), and the reason for the discrepancy between the two studies is unknown. However, SV fusion in the calyx of Held in younger animals, as used by the Watanabe study, is dependent upon Ca\(^{2+}\) microdomains that do not necessarily require the tethering of SVs close to CaVs. Thus, the role of the CaV synprint region in SV tethering is promising but still unclear.

1.7.2 The C-terminal Tail

More recently, another region of CaV2 that has been associated with SV-binding is the C-terminal tail of the \(\alpha_1\) subunit. This tail is defined as the portion of the subunit that extends from the transmembrane domain IV into the cellular lumen. Two of the conserved regions (the PDZ- and SH3-binding domains) found in the C-terminal tail have been associated with the synaptic proteins CASK and MINT (Maximov et al., 1999) and are thought to be involved in the trafficking of CaVs to the presynaptic plasmalemma (Maximov and Bezprozvanny, 2002; discussed above). More recently, the C-terminal tail has also been found to be associated with SVs. One study used yeast two-hybrid screening to show that the same PDZ- and SH3-binding domains near the distal end of the CaV2.1 C-terminal that bind MINT and CASK (Maximov et al., 1999) also bind RIM and RIM binding protein (RIM-BP) (Kaeser et al., 2011). The same study also used EM to show that the synapses of cultured hippocampal cells from RIM knockout mice have a significantly reduced number of docked SVs, which was interpreted to suggest that RIM is a molecular tether linking SVs to CaVs. RIM (Wang et al., 1997) and RIM-BP (Wang et al., 2000) are both associated with rab3a, a small GTPase that binds to SV membrane (Fischer von Mollard et al., 1991). It is thought that RIM acts as a scaffold for the various proteins that it binds at the AZ and the loss of RIM decreases the release probability of SVs (Schoch et al., 2002; see above section on RIM). It should be noted that RIM KO synapses are still capable of neurotransmission despite being “less efficient.” It requires a loss of both RIM and RIM-BP to truly cripple SV exocytosis (Acuna et al., 2016). Acuna and colleagues interpreted their results as suggesting that RIM and RIM-BP provide functional redundancy for SV tethering. However,
the structure of and binding motifs in both proteins are grossly dissimilar (Kaeser et al., 2011; Wang et al., 1997, 2000). It is important to note that none of these studies investigating the RIM and RIM-BP have demonstrated any direct, biochemical binding of the native forms of these proteins with native CaVs \textit{in situ}.

Because of its many reported binding partners at the AZ, it is difficult to discern whether RIM is the SV tether or whether phenotypes arising from the loss of RIM is due to a general disruption of the AZ. The yeast two-hybrid assays conducted by Kaeser and colleagues (2011) cannot fully establish that the RIM and RIM-BP binding to CaVs resulted in a complex with actual SVs. If RIM tethers SVs through its interaction with rab3a as suggested in the Kaeser model, then how is this tether occurring in inhibitory synapses where RIM may not be interacting with rab3a at all (Schoch et al., 2002)? In fact, the role of RIM as the predominant SV-to-CaV tether has been recently called into doubt. A prodigious number (>600) of amino acids make up the CaV C-terminal tail and it has been estimated that the tail of the long-splice variant of CaV2.2 could presumably extend up to 200 nm long, especially since the tail has been predicted to be largely without secondary structures to shorten its length (Wong et al., 2014). While EMs of RIM KO synapses show a reduction of SVs docked on the AZ membrane (Kaeser et al., 2011), there does not appear to be a reduction of tethered SVs that are more than 5 nm away from the AZ membrane (Fernández-Busnadiego et al., 2013). If RIM is the tether that connects SVs to the CaV C-terminal, these EMs beg the question of why there is no disruption of the more-distal tethered SVs that would still be within range of a 200 nm CaV C-terminal tail?

A recent series of publications from the Stanley laboratory has provided compelling biochemical and functional evidence that the CaV C-terminal tail binds to some element on SVs that is neither RIM nor RIM-BP. They (that is, Dr. Stanley and members of the laboratory other than me) developed a novel pull-down assay (SV-PD) designed to test which proteins are capable of directly interacting with intact SVs (Wong et al., 2013). SV-PD involves the conjugation of native proteins or fusion proteins to precipitation beads through antibodies or tag-specific interactions, respectively. These conjugated beads are then incubated with SVs harvested from synaptosomes in the absence of detergents, which means these SVs should be intact and in possession of their normal complement of proteins. The pull-down of intact SVs is assessed through western blotting for various integral SV proteins such as VAMP, SV2, and/or synaptotagmin. This system allows the testing of the interaction between SVs and the protein
conjugated to the beads in a more physiological state than what can be accomplished using traditional techniques such as yeast two-hybrids or detergent-based immunoprecipitations. SV-PD was observed to pull-down entire SVs using native CaV2.2 harvested from synaptic membrane (Wong et al., 2013). A fusion protein replicating the distal third of the CaV2.2 C-terminal tail was also able to pull down intact SVs. However, a fusion protein replicating a distal region of the tail containing the SH3-binding domain failed to pull-down intact SVs (Wong et al., 2014). Furthermore, two separate fusion proteins replicating the distal end of the CaV C-terminal tail without the PDZ-binding domain were observed to pull-down intact SVs (Wong et al., 2014). These results argue against critical roles for RIM and RIM-BP in SV tethering as was previously suggested by Kaeser and colleagues (Kaeser et al., 2011) and narrowed the SV-binding site on the CaV C-terminal tail to the distal 49 amino acids that are proximal to the PDZ-binding domain at the C-terminus (Wong et al., 2014). This conclusion was further supported by the finding that mimetic blocking peptides replicating the PDZ-binding domain had no effect on CaV C-terminal tail fusion protein pull-down of intact SVs. These peptides were incubated with the intact SVs prior to incubating the SVs with fusion protein-conjugated beads, and one would expect a loss of SV binding to the beads if RIM or any protein with a similar PDZ domain such as MINT was necessary for the CaV-SV interaction.

Further painstaking work using other mimetic blocking peptides mimicking sequential sections of the SV-binding region on the CaV2.2 C-terminal has found a novel motif that binds SVs (Gardezi et al., 2016). A “HxxRR” motif, and variations on this motif, is conserved for both CaV2.2 and CaV2.1 in many organisms and somewhat conserved in CaV2.3. These channel types are present in the majority of neural synapses (Catterall, 2011). In CaV2.2, this binding site is 34 amino acids away from the C-terminus. Mimetic blocking peptides containing this motif significantly reduced the pull-down of SVs in SV-PD using fusion proteins replicating the distal third of the CaV2.2 C-terminal tail.

1.8 Cryoloading

The blocking peptides used with SV-PD have also been used by the Stanley laboratory in SV-recycling assays using a novel technique called cryoloading. Cryoloading is a process (Figure 7) by which compounds can be introduced into SSMs while maintaining the SSMs’ ability to exocytose and endocytose SVs (Nath et al., 2014). These otherwise impermeant compounds
pass through the plasmalemma through a freeze-thaw cycle (Nichols et al., 1989), and the health of the SSMs is maintained by a cryopreservative buffer (Daniel et al., 2012). A wide variety of compounds have been shown to be successfully cryoloaded into SSMs, such as 10 nm-wide colloidal gold particles, BAPTA, fluorescently-labeled dextran, and the light chain of botulinum A (Nath et al., 2014). SSMs cryoloaded with ~0.476 kDa-large BAPTA molecules have impaired SV cycling, likely due to the chelation of calcium by BAPTA. Botulinum light chain is ~53 kDa large (Syuto and Kubo, 1977) and cryoloading this compound, known to cleave SNAP-25, into SSMs also impairs SV recycling (Nath et al., 2014). Both 3 kDa- and 10 kDa-large dextran molecules that were fluorescently-tagged were observed to be retained inside SSMs after cryoloading. While cryoloading does not deliver its payload into every single SSM in a given population, co-cryoloading with the fluorescently-tagged dextran molecules allows the end-user to identify which SSM has been successfully cryoloaded when observing the SSMs under a fluorescent microscope (Nath et al., 2014). Cryoloaded SSMs can be then be used in styryl dye SV recycling assays to test the effect of cryoloaded compounds on the SV cycle. Styryl dyes, also known as FM dyes, are only fluorescent when associated with lipid membranes but are easily washed off of these membranes (Betz et al., 1996). However, once the SV cycle of a SSM is stimulated, the FM dyes trapped in the endocytosed endosome and SVs are retained (Betz and Bewick, 1992). Thus, compounds cryoloaded into SSMs that are unable to take up FM dyes can be inferred to interfere with the SV cycle, as was observed with cryoloaded botulinum A light chain (Nath et al., 2014).
Figure 7. Overview of Cryoloading.
(A) Graphical representation of cryoloading process. (1) SSMs (represented by the single, large blue circle) is incubated in a cryopreservative buffer along with the payload of interest (orange *s). (2) During freeze-thaw of the sample, the SSM plasmalemma becomes compromised which allows the diffusion of the payload into the SSM. (3) After thawing, the SSM plasmalemma regains its integrity which traps the payload inside the SSM even after subsequent washes. (B) Evidence of cryoloading using EM. 10 nm colloidal gold particles conjugated to avidin (denoted by black *s) were observed inside an SSM after cryoloading. Image is from an experiment conducted with Mr. Arup Nath and reproduced from Nath et al., 2014.

The mimetic blocking peptides used in SV-PD to test CaV-SV interactions (Gardezi et al., 2016; Wong et al., 2014) were cryoloaded into SSMs. Peptides replicating the PDZ-binding domain at the tip of the CaV2 C-terminal tail, which was originally thought to bind to RIM and thus to SVs (Kaeser et al., 2011), were found to have no effect on SV recycling as assayed by the FM recycling assay, which would argue that the RIM interaction with the CaV2 C-terminal tail does
not play a critical role in SV exocytosis and endocytosis (Wong et al., 2014). The mimetic peptide HQARRVPNGY containing the later-discovered HxxRR SV-binding motif was found to significantly reduce SV recycling when cryo-loaded into SSMs (Gardezi et al., 2016). These results, along with those from SV-PD experiments, strongly suggest that CaV C-terminal tails can bind SVs in a RIM-independent fashion.
2 Rationale and Hypothesis

A multitude of EM studies investigating the AZ and the peri-AZ have described the fibrous filaments connecting SVs to the AZ membrane (Cole et al., 2016; Fernández-Busnadiego et al., 2010; Hirokawa et al., 1989; Landis et al., 1988; Siksou et al., 2007). While there lacks a strong consensus in the literature as to the precise dimensions of these filaments, it is generally accepted that they function as some sort of SV tethers. The identity of these tethers is still quite unclear, largely due to the difficulty of immunolabeling proteins and interpreting this immunolabeling at the nanometer scale. However, until fluorescent super-resolution techniques advance much further into the nanoscale, EM is the only technique with sufficient resolution to reliably observe these filaments and better EM immunolabeling techniques must be developed towards this aim.

Biochemical and functional studies have provided a variety of putative SV tether proteins. As of right now, the strongest such evidence points towards CaVs binding to some yet-unknown element of SVs as the tether (Wong et al., 2013). The finding that a specific site on the CaV C-terminal tail binds SVs suggests that this tail makes up the critical core of this tether (Gardezi et al., 2016; Wong et al., 2014). However, these biochemical studies cannot study this relationship in situ, and it is difficult for functional studies to discern whether disruptions in either SV recruitment to the RRP or SV recycling is specifically due to SV tether malfunctions or another step during these processes. Once again, observation of these tethers using EM is the most direct way to study them.

This study pursues the hypothesis that the CaV C-terminal tail tethers SVs to the AZ membrane in an in situ setting. Its general aims are to:

1. Develop a simple and effective method to visualize these tethers in situ at presynaptic AZs.
2. Measure the lengths of these tethers. If these tethers are CaV C-terminal tails, we hypothesize that the lengths of the tethers will not exceed 200 nm which is the estimated maximal length of the C-terminal tail.
3. Develop a simple and effective method to immunogold label structures in presynaptic terminals.
4. Develop a quantitative measure to quantify this immunogold labeling. We hypothesize that immunogold labeling using antibodies against the CaV C-terminal tail will significantly label tethers and/or associated SVs.

5. Generate more antibodies against different regions of the CaV C-terminal tail for immunogold labeling. If CaVs extend from the AZ membrane to tether SVs, we hypothesize that antibodies against regions of the C-terminal tail closer to the transmembrane domain will immunogold label parts of the SV tethers closer to the AZ membrane.
3 Methods and Materials

3.1 Synaptosome (SSM) Ghost Tissue Preparation

Synaptosome ghost preparation (Figure 8) was based on the protocol established for isolating SVs from SSMs (Whittaker et al., 1964) with some modifications to optimize the yield of ghosts without causing undue kinetic damage to the tissue. For each individual EM sample, the whole brains of 4 to 5 E15-E17 chicks were dissected out (i.e. I would use 20 chick brains for 4 samples). All subsequent steps were either done on-ice or at 4 °C. Up to 15 brains were homogenized together at a time in Homogenization Buffer (“HB”: 320 mM sucrose, 10 mM HEPES at pH 7.4, 1 mM EGTA, 1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)) with 10 strokes of a Dounce homogenizer (Thomas Scientific, Swedesboro, NJ). The resulting supernatant was centrifuged for 15 min at 1000 xg on a fixed tabletop centrifuge. The supernatant was further centrifuged twice for 35 min at 200 000 xg in a fixed 70Ti rotor (Beckman Coulter, Brea, California) while keeping the pellet and resuspending it in ~25 mL of HB after each centrifugation. The resuspended pellet was passed through a 22.5-gauge needle six times and then loaded onto a 0.8 M / 1.2 M discontinuous sucrose gradient and centrifuged for 1.5 h at 100 000 xg in a SW41 swing bucket rotor (Beckman Coulter, Brea, California) with the brakes turned off. The SSMs found in the brown-coloured layer of the 0.8 M / 1.2 M sucrose interface was taken, diluted in ~25 mL of HB, and centrifuged at 2000 xg for 30 min in the 70Ti rotor. For experiments involving intact SSMs, I used the pellet obtained at this step.

The synaptosomes in the resulting pellet were osmotically ruptured using an Osmotic-Rupture Buffer (“ORB”: 50 mM Na HEPES at pH 7.4, 10 mM free CaCl buffered using 1 mM EGTA). I made sure to add at least 10x the volume of ORB as the volume of the pellet to maximize the osmotic shock to the SSMs, and our typical preparations used 25 mL of ORB to a ~1 mL SSM pellet. Ruptured SSMs were centrifuged for 30 min at 2000 xg in the 70Ti rotor. The subsequent pellet was resuspended in HB diluted to 0.2 M of its sucrose content, passed through a 22.5-gauge needle six times, and then loaded onto a 0.4 M / 0.6 M / 0.8 M / 1.0 M discontinuous sucrose gradient. This gradient was centrifuged at 100 000 xg in the SW41 swing bucket rotor with the brakes disabled for 1.5 h and left overnight in the centrifuge. The purified SSM ghosts at the 0.8 M / 1.0 M interface were taken the next morning, diluted in ORB, divided into the desired number of EM samples, and centrifuged into pellets at 20 000 xg for 30 min on a
fixed tabletop centrifuge. The dilution in ORB is intended to re-rupture SSM ghosts to further ensure that their free-floating contents are released as well as lower the density of the solution to facilitate the pelleting of the ghosts.

![Image of synaptosome ghost preparation](image)

Figure 8. Synaptosome Ghost Preparation.
An outline of the major steps in preparing synaptosome (SSM) ghosts from chick brains. Whole brains are homogenized and SSMs are isolated from the homogenate using differential centrifugation. SSMs are then osmotically ruptured and a crude fraction including SSM ghosts is pelleted using low-speed centrifugation. An enriched fraction of SSM ghosts is achieved through another round of differential centrifugation (not shown here), and the resulting ghosts can be observed under electron microscopy (left inset). More details for each step can be found in the text.

3.2 SSM Ghost Passive Diffusion Antibody Labeling

SSM ghost pellets were kept undisturbed while being fixed for 1 h at room temperature with 50 uL of Fix Solution #1 (4% paraformaldehyde, 0.1% glutaraldehyde, 0.1 M cacodylate buffer pH
followed by two gentle rinses without dislodging the pellet with 100 uL of 150 mM Tris-HCl (pH 7.2) for 15 min each to saturate residual aldehydes. The pellets were resuspended with 50 uL of a non-selective antibody binding site blocking solution (1.2 mg/mL of goat serum in 20 mM Tris-HCl pH 7.2) for 30 min on ice. Primary antibody (L4569 from Khanna et al., 2006; or 1 mg/mL non-specific rabbit IgG from Jackson ImmunoResearch, West Grove, PA) was added at 1:100 dilution and the mixture was left on a rocker overnight at 4C. The following morning, samples were centrifuged at 20 000 xg for 1 h in a tabletop centrifuge and the resulting pellet was gently rinsed twice with 100 uL of 20 mM Tris-HCl. The pellet was then resuspended in 100 uL of 20 mM Tris-HCl and centrifuged again at 20 000 xg for 30 min. Pellets were then resuspended in 50 uL of 20 mM Tris-HCl with 1:100 6 nm colloidal gold goat anti-rabbit secondary antibody (Electron Microscopy Sciences, Hatfield, PA). Samples were then incubated for 2 h at room temperature before being centrifuged for 30 min at 20 000 xg. Pellets were then rinsed gently twice with 1 mL of 20 mM Tris-HCl and then resuspended. After centrifugation at 20 000 xg for 30 min, pellets were rinsed twice with 100 uL of 0.1 M cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA). Samples were then fixed again as described below.

### 3.3 Immuno-Nanogold Cryoloading of SSM Ghosts

Cryoloading is described in detail in a previous publication (Nath et al., 2014). SSM ghost pellets were resuspended in sucrose/EDTA/Tris buffer (“SET buffer”: 320 mM sucrose, 1 mM EDTA, 5 mM Tris at pH 7.4) with 5% DMSO at room temperature. Primary antibodies were added such that the final antibody concentration was 1:50. Samples were then frozen slowly by enclosing them in a parafilm-wrapped Styrofoam freezer box stuffed with lab diapers prior to being put into a -80 °C freezer. Samples were left in the freezer either overnight or over a series of days (no changes in labeling efficacy were observed in relation to the number of days left frozen), and then were thawed quickly in a 37 °C incubator for 2.5 min. Ice-cold ORB was added at 10 times the volume of each sample to rupture any resealed SSM ghosts and to flush out unbound primary antibody. Ghosts were then re-cryoloaded with Nanogold® goat anti-rabbit secondary Fab (Nanoprobes, Yaphank, NY) at a 1:50 concentration in SET buffer and 5% DMSO in the same fashion as was done for cryoloading of the primary antibodies. After cryoloading with the secondary Fab, ghosts were again thawed and ruptured with ice-cold ORB
to flush out unbound secondary Fab prior to being centrifuged at 20 000 xg for 1h at 4 °C. Ghost pellets at this point were generally ~1 mm³ in size and were then fixed as described below.

Figure 9. Immuno-Nanogold Cryoloading of SSM Ghosts.
(1) SSM ghosts (represented by a single, large blue circle) are incubated in SET buffer containing 5% DMSO and 1:50 concentration of primary antibody (green “Y”s). (2) The freeze-thaw process of cryoloading allows entry of the primary antibody into the SSM ghosts. (3) Cryoloaded SSM
ghosts are ruptured using ORB and unbound antibodies are flushed out of the SSM ghosts. (4) SSM ghosts are then incubated in SET buffer containing 5% DMSO and 1:50 concentration of secondary, nanogold-conjugated Fab (orange “i”s, which are (5) cryoloaded into the SSM ghost. (6) SSM ghosts are again ruptured using ORB and unbound Fab are flushed out of the SSM ghosts.

### 3.4 SSM Ghost Fixation

All subsequent steps were done at room temperature and samples were kept in the dark during incubations to minimize any disturbance of the cross-linking process. SSM ghosts were fixed as pellets using 200 uL of Fix Solution #2 (2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2) for 1 h. Without disturbing the pellets, the supernatant was exchanged for 500 uL of a 150 mM pH 7.4 Tris buffer to quench the fixatives and allowed to sit for 30 min so that the Tris buffer can fully saturate the pellet. The supernatant was then exchanged for 500 uL of 0.1M cacodylate buffer (pH 7.4) twice and allowed to sit for 30 min after each exchange. The samples were then left overnight to maximize the diffusion of Tris out of the pellets as Tris has the potential to react with and precipitate uranyl acetate.

### 3.5 Electron Microscopy (EM) Preparation and Imaging

The sample pellets were stained with 2% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M cacodylate buffer for 1 h. The pellets were washed twice for 10 min each with 0.1 M cacodylate buffer and then twice for 10 min each with distilled water. The pellets were then stained en bloc with 1% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) for 1 h. After three 10 min washes in distilled water, the pellets were put through a series of 30%, 50%, 70%, 90%, and 100% ethanol washes of 10 min each. The pellets were then put through a series of 1:3, 1:1, and 3:1 dilutions of Spurr’s resin (Electron Microscopy Sciences, Hatfield, PA) in ethanol, and left in undiluted Spurr’s resin overnight. The next day, the resin was replaced with fresh, undiluted resin twice in ~5 h intervals before the samples were placed into a 60 °C oven overnight to cure. 100 nm sections of each sample were cut using a Leica EM UC6 ultramicrotome (Concord, Canada) and placed onto copper grids (Electron Microscopy Sciences, Hatfield, PA).

Sections were examined using a Hitachi (Tokyo, Japan) H7000 (for passive diffusion labeling experiments) or HT7700 transmission electron microscope (all subsequent experiments) at 75 kV or 80 kV, respectively (imaging done at Cell & Systems Biology Imaging Facility, University of
Toronto, Toronto, Canada). The top portions of pellets (where the density of sample material was low enough to easily differentiate synapses from structures randomly abutting each other from centrifugation) were scanned at low magnification (50 Kx magnification) for ghosts with visibly attached postsynaptic scabs. Higher magnification images were then taken of these ghosts to be used for quantification purposes. Ghosts labeled with 6 nm colloidal gold secondary antibodies using passive diffusion were imaged at 300 Kx magnification. Ghosts labeled with L45 or Ab571 antibodies were imaged at 500 Kx magnification. Ghosts labeled with NmidC2 antibodies prior to December 2015 were imaged at 500Kx, while those afterwards as well as C2Nt labeled ghosts were imaged at 530Kx. The change in high magnification imaging was due to an upgrade to the TEM software beyond our control.

3.6 Compartment Analysis

Images at 500Kx or 530Kx magnification (where nanogold particles are readily visible) were subdivided by hand using ImageJ (NIH, Bethesda, MD) into separate compartments (Figure 17A). These compartments are AZ membrane, non-AZ membrane, filaments, SVs, endosomes, dense cytoplasm, and empty cytosol. Any membrane-bound organelle that was not readily identifiable as SVs were deemed as endosomes. Dense cytoplasm was any electron-dense portion of the SSM ghost that was not associated with the AZ membrane. The combined area for each compartment from each image for each condition were quantified using ImageJ, and the total number of gold particle clusters within these compartments were tallied. The compartment area vs number of gold clusters were plotted on a scatterplot and 95% prediction bands were calculated using statistical software. Compartment analysis was devised by Dr. Stanley, and the compartment analysis of the L45 immunolabeling experiments were originally conducted blind by her using Origin (OriginLab, Northampton, MA). I re-plotted her data using Prism 6 (GraphPad, La Jolla, CA) so that the compartment analysis graphs looked consistent throughout this thesis. Compartment analysis of subsequent experiments were done by me using Prism 6.

3.7 Binomial Tests

Binomial tests were done using the online QuickCalcs program available on the GraphPad website. “Successes” were defined by the total number of nanogold clusters observed in experiments using either L45, Ab571, NmidC2, or C2Nt antibody while the hypothetical probability of success was defined by the total number of nanogold clusters observed in
respective negative control experiments divided by the number of negative control trials. For binomial tests comparing individual tethered SVs, the number of trials was the number of individual tethered SVs analyzed. For binomial tests comparing the extent of immuno-nanogold labeling, the number of trials was the number of ghosts analyzed.

3.8 TAT-Conjugated Blocking Peptides

Intact SSMs were incubated with 5 uM of TAT-conjugated peptides containing the HQARRVPNGY sequence shown to interfere with SV binding to the distal C-terminal of CaV2.2 (Gardezi et al., 2010) or containing a AHRVPQGYNR sequence as a scrambled control in a HEPES-Buffered Krebs solution (HBK; Daniel et al., 2012) for 1 h at room temperature. Higher concentrations of TAT-conjugated peptides were also tested, but were found to sufficiently alter the density of the SSMs that I was unable to recover them from subsequent sucrose gradients.

Treated SSMs were then depolarized using 40 mM K+ in HBK for 2 min in a 30 °C water bath. These SSMs were then osmotically ruptured into SSM ghosts and then prepared for EM as described above.

3.9 Antibodies

The presynaptic marker SV2 antibody (DSHB, Iowa City, Iowa) and Purkinje neuron marker calbindin (clone D28K from Synaptic Systems, Gottingen, Germany) are commercially available antibodies.

Rabbit polyclonal antibodies that were generated in house and that have been characterized previously included L4569 and L4570 (Khanna et al., 2006). These two antibodies were generated in two separate rabbits immunized with the same CaV2.2 C-terminal tip antigenic peptide GTGRSYYHEADDDWC. An equal volume combination of L4569 and L4570 was termed L45 and was used for labeling in this study. Ab571 was raised against a GAEAPRKHHHRHDKEKL peptide replicating part of the synprint region of the chick CaV2.2 II-III loop and has been characterized extensively (Li et al., 2004).

Three new antibodies were generated for this study. A RPHPMHLYEYSLER peptide, replicating a sequence in the middle region of the chick CaV2.2 C-terminal (Figure 22) tail was used to generate NmidC2. A second antibody, PmidC2, was generated against a LGTDLSVTQSGDLPS peptide replicating a sequence in the middle region of the chick CaV2.1 C-terminal (Figure 22;
Chick CaV2.1 full length sequence submitted to Genbank by Ms. Brittany Elliott, Dr. Qi Li, and Dr. Elise Stanley). A \textit{LSPKNLDLLVTPHK} peptide, replicating a sequence within the proximal third of the chick CaV2.1 C-terminal tail (Figure 22; Chick CaV2.1 full length sequence submitted to Genbank) was used to generate C2Nt. This region shares strong homology with chick CaV2.2.

KLH-conjugated immunogenic peptides (NmidC2 peptide made by SPARC BioCentre, Hospital for Sick Children, Toronto, Canada; C2Nt peptide made by Biomatik, Cambridge, Canada) were used to immunize white rabbits (immunization and bleeding done by the animal facility of the Division of Comparative Medicine, University of Toronto, Toronto, Canada based on protocols established in Harlow and Lane, 1988). 500 ug of immunogenic peptides (diluted to 1 mg/mL in PBS) were injected into rabbits with Freund’s Complete Adjuvant as the primary immunization while 250 ug of peptides along with Freund’s Incomplete Adjuvant was injected as subsequent boosts. Rabbits were bled 2 weeks after an immunization and rabbits were re-inoculated with boosters a week after being bled.

Serum with the NmidC2 antibody was affinity-purified using a SulfoLink Immobilization Kit (#44999 from ThermoFisher, Waltham, MA). The affinity-purified NmidC2 was used only for EM experiments that did not involve antibody-blocking. NmidC2 serum was used directly for all other experiments. C2Nt experiments were all carried out using serum.

### 3.10 Quantification of Immuno-Nanogold Distance from Active Zone Membrane

Images of individual tethered SVs from SSM ghosts were used for this analysis. Using ImageJ software, I manually traced the distance from each nanogold cluster along the tether to where the tether met the AZ membrane. In the rare occasions where the nanogold cluster was located on the SV away from the tether itself, I traced directly from the nanogold cluster to the nearest part of the tether and then continued tracing along the tether until it met the AZ membrane. I then used ImageJ to quantify the length of each trace. These lengths were manually sorted into bins of 25 nm ranging from 0 nm to 225 nm for a total of 9 bins. To compensate for non-specific labeling for each of the L45, Ab571, NmidC2, or C2Nt experiments, Dr. Stanley suggested that I count the number of nanogold clusters on individual tethered SVs in the respective control experiments for each antibody. The number of nanogold clusters observed in each control
experiment was then normalized to the number of bins (9), and this value was subtracted from the frequency of experimental nanogold clusters observed for each bin. Finally, I normalized these compensated frequencies to the number of individual tethered SVs used in the analysis for each antibody and plotted these final values as line graphs in Prism 6.

3.11 Immunoprecipitation

70 uL of protein A agarose bead slurry (Sigma-Aldrich, St. Louis, Missouri) for each individual immunoprecipitation (IP) were washed four times in phosphate buffered saline (PBS; ThermoFisher, Waltham, Massachusetts). Either 0.8 uL of Ab571 or 0.5 uL of non-specific control rabbit IgG (Jackson Immunoresearch, West Grove, Pennsylvania) were added to the beads and more PBS was added until the total volume of each tube was 500 uL. This mixture was incubated at 4 °C on a rotator for 2 h. Beads were then washed three times with 1 mL of PBS. Chick brain ghost lysate from 10 E14-17 embryos were solubilized in 500 uL of RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 1mM EDTA, pH 7.4) for 20 min on ice. The lysate was then centrifuged for 10 min at 20 000 xg on a tabletop centrifuge, and the supernatant was added to the beads. The beads with the lysate were left overnight at 4 °C on a rotator. The next morning, the beads were washed three times with 1 mL of RIPA buffer. 50 uL of 2X Laemmlie sample buffer (Bio-Rad, Hercules, CA) with 5% β-mercaptoethanol was added to each tube of beads and samples were then boiled for 5 min at 100 °C prior to western blotting.

3.12 Western Blotting

Western blotting was done using conventional methods (Wong et al., 2013). Samples were mixed with 2X Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% β-mercaptoethanol at a 1:1 ratio and boiled for 5 min at 100 °C prior to being loaded onto 10% SDS-PAGE gels and run at 120 V. Gels were then transferred onto PVDF membrane (Bioshop, Burlington, Canada) and blocked for 1 h with 5% milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20; Bioshop, Burlington, Canada). Blots were incubated overnight at 4 °C with primary antibody (at 1:200) in 5% powdered milk in TBST. Blots were then washed 3x 10 min with TBST prior to incubation for 1 h at room temperature with secondary goat anti-rabbit antibodies (at 1:3000; Jackson Immunoresearch, West Grove, PA) conjugated with horseradish peroxidase in 5% milk in TBST. Blots were washed again 3x 10 min with TBST and treated with ECL (GE
Healthcare, Chicago, IL). Chemiluminescence was visualized using a ChemiDoc XRS System (Bio-Rad, Hercules, CA). Western blotting of SSM ghost lysate probed with NmidC2 or C2Nt antibodies were conducted by me, while those of fusion proteins probed with NmidC2 or C2Nt were conducted by Dr. Sabiha Gardezi or Ms. Christine Snidal, who generously shared their data for this thesis.

### 3.13 Dot Blotting

Nitrocellulose membrane was cut into squares and each square was marked with a circle near each corner (i.e. 4 circles in total) using pencil. 1 uL of fusion protein was applied within each circle and allowed to air dry for ~15 min. Two different fusion proteins were used at different concentrations. The C3strep fusion protein (Wong et al., 2013) replicates a distal region of the CaV2.2 C-terminal and contains the antigenic region for the L45 antibodies but not the antigenic region for the NmidC2 antibodies. 5 ng of C3strep was applied to the top left hand circle of a square while 50 ng was applied to the top right hand circle of a square. The other fusion protein used was C1-2strep which replicates a more proximal region of the CaV2.2 C-terminal that contains the antigenic region for the NmidC2 antibodies but not the antigenic region for the L45 antibodies. Because the C1-2strep fusion protein was eluted from purification beads by heat alone because we were concerned that the use of SDS would affect the dot blotting process, the amount of C1-2strep fusion protein applied to the bottom two circles of the dot blots is unknown. However, like the circles for C3strep, the left hand circle contains C1-2strep at a 10x lower amount than the right hand circle. Blots were blocked for 1 h at room temperature in 5% milk in TBST. Blots were then incubated overnight at 4 °C with either L45 or NmidC2 antibody sham-blocked or blocked with various concentrations of their respective antigenic peptides (see below for antibody blocking). The next day, the blots were washed 3 times for 10 min each with TBST. Blots were then incubated for 1 h at room temperature with goat-anti-rabbit-HRP secondary antibodies (same as above for western blotting) at 1:3000 dilution in TBST with 5% milk. After another three rounds of 10 min washes in TBST, blots were incubated with ECL for 5 min and then imaged on the ChemiDoc XRS System.

### 3.14 Fluorescent Immunostaining

Fluorescent immunostaining was done either on chick cerebellar slices from E19 embryonic chicks, magnocellular nucleus cells from E15 embryonic chicks, or chick ciliary ganglion (CCG;
Stanley and Goping, 1991) cells from E15 embryonic chicks. Immunostaining was conducted as described (Li et al., 2004) and at room temperature, unless otherwise stated.

Chick cerebella were dissected and fixed in 2% paraformaldehyde and a series of 30% (2 h), 20% (2 h), and 10% (overnight) sucrose in PBS (1x phosphate buffered saline pH 7.4; ThermoFisher, Waltham, MA). The cerebella were then embedded in M1 Freezing Medium (ThermoFisher, Waltham, MA) and sectioned into 15 μm thick slices using a Leica CM3050 S cryostat. Slices were then permeabilized with 0.1% Triton-X100 (Sigma-Aldrich, St. Louis, Missouri) in PBS and then washed repeatedly with Ab Buffer (500 mM NaCl, 10 mM MgCl2, 10 mM NaN3, 20 mM Tris, 0.1% BSA, pH 7.2).

Immunohistochemistry of magnocellular nucleus cells used chick brainstems isolated according to a previously published protocol (Sanchez et al., 2011). These slices were then fixed and sectioned in the same way as chick cerebellar slices.

CCGs were fixed (150 mM cyclohexylamine, 20 mM EGTA, 20 mM MgCl2, 20 mM PIPES, and 2% paraformaldehyde, pH 6.5) on glass coverslips for 45 min. Fixatives were then quenched (150 mM Tris, 20 mM MgCl2, 20 mM NaN3, pH 7.4) for 30 min before being washed for with Ab Buffer for 30 min.

Both CCGs and slices were blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, Pennsylvania) in Ab Buffer for 45 min. Incubation with primary antibody (diluted in pH 6.8 Ab Buffer) was at 4 °C overnight. Tissue was then washed 3x 5 min with pH 7.2 Ab Buffer prior to being blocked again with 5% donkey serum in Ab Buffer for 45 min. Tissue was then incubated with 1:100 secondary (Jackson ImmunoResearch, West Grove, Pennsylvania) donkey anti-mouse antibody conjugated to AlexaFluor 594 and/or donkey anti-rabbit conjugated to FITC for 1 h. After another 3x 5 min wash with Ab Buffer, tissue was mounted using Vectashield (Vector Laboratories, Burlingame, CA) and imaged using an Axioplan 2 microscope (Zeiss, Oberkochen, Germany). Cerebellum slice immunohistochemistry was prepared and imaged by Mr. Henry Mah and generously shared with me for this thesis, while CCG immunocytochemistry and magnocellular nucleus immunohistochemistry were prepared and imaged by both me and Dr. Qi Li.
3.15 Antibody Blocking

Nm1dC2 antibody was incubated with an equal volume of 40 \( \mu \text{M} \) (unless otherwise stated) of peptide corresponding to the NmidC2 antigenic site (RPHPMHLYEYSLER) for 2 h on ice (Nm1dC2 peptide made by SPARC BioCentre, Hospital for Sick Children, Toronto, Canada; other peptides made by Biomatik, Cambridge, Canada). A combination of two peptides (GTGTRGRSYY and HEADEDWDC) corresponding to the L45 antigenic site at 40 uM concentration (unless otherwise stated) was used as a control. This solution containing both antibody and peptides was used at twice the concentration as normally used for antibody alone.
4 Results

4.1 Visualizing Synaptic Vesicle Tethers in Situ

To pursue our goal of testing whether synaptic vesicles (SVs) are tethered by voltage-gated calcium channels (CaVs), we first had to establish a system by which we could visualize tethers in situ. We knew that at the nanometer scale necessary to resolve tethers, we would have to use transmission electron microscopy (EM). Thus, we began looking at isolated presynaptic terminals (synaptosomes; SSMs) made from whole chick brain prepared using various standard EM preparative protocols. The interior of intact synaptosomes (Figure 10) as fixed overnight at 4 °C and then stained with 1% osmium tetroxide were a compacted mass of SVs, other membrane-bound organelles (which we collectively termed endosomes), and hazy clouds of cytoplasm. It was impossible to reliably identify any links between the SVs and the plasmalemma. Furthermore, there often appeared to be a scattering of particularly electron-dense particles across the synaptosomes that—although we did not know it at the time—would have been indistinguishable from the nanogold particles I would eventually use for immunolabeling. However, these first attempts at visualizing synaptosomes were greatly useful in learning how to identify active zones (AZs). In many instances, we could observe the remains of postsynaptic processes (termed postsynaptic scab from here on) still attached to the synaptosome. These scabs could be identified by their close apposition to synaptosomal membrane and electron-dense post-synaptic densities sometimes still be attached to the scabs. The plasmalemma at these synapses were noticeably darker and thicker than the plasmalemma at other points of the synaptosome, and we took the presynaptic side of this darkening/thickening to be the AZ.
Figure 10. Electron Micrograph of an Intact Synaptosome. 
An EM of an intact SSM with an active zone (AZ) apposed to the attached postsynaptic scab (PS). Under conventional transmission EM, intact SSMs fixed with aldehydes are typically observed to contain a multitude of synaptic vesicles (SVs), other membrane-bound organelles (Endo), and have a characteristically condensed, electron-dense cytoplasm (Cyto).

To look for SV tethers that could potentially be hidden by the cytoplasmic haze, we looked for ways to remove parts of the cytoplasm that were not essential to this study. The simple but effective solution we arrived at was to osmotically rupture the synaptosomes, which “flushed out” anything not physically linked to the synaptosomal plasmalemma. I took these synaptosomal “ghosts” and prepared them with the same preparative protocol as I had done with the intact synaptosomes (Figure 11). As predicted, the interiors of these ghosts were much less cluttered. In a few exciting instances, filaments could be seen to connect SVs to the AZ.
membrane. However, these were very faint and difficult to reliably visualize. After consulting with the personnel at the Cell System Biology Imaging Facility (University of Toronto, Toronto, Canada) where the imaging was done, we made some changes to the EM preparative protocol by fixing for 1h at room temperature, doubling the concentration of osmium tetroxide, and by using *en bloc* uranyl acetate staining (see Methods). It was dramatically easier to see the filaments connecting SVs to the AZ (and on occasion, to each other) in ghosts prepared using this modified protocol (Figure 12). Furthermore, the electron-dense particles scattered throughout our previous specimens were either gone or no longer visible. Months into this project, the Imaging Facility upgraded to a Hitachi HT7700 TEM system which is easier to calibrate on a per-use basis and has a more sensitive camera as compared to the previous H7000 TEM system. Coupled with our greater skill at operating TEMs in general, this system allowed us to image SSM ghosts at higher magnification without the loss of contrast (see subsequent EM figures). We now had a viable method of visualizing SV tethers *in situ*, and this EM preparative protocol would be at the heart of all future EM experiments in this project.

Figure 11. Early Electron Micrograph of Synaptosome Ghost.
An EM of a SSM ghost prepared using an early EM preparative protocol using 1% osmium tetroxide and without *en bloc* uranyl acetate staining. Note the significant decrease of electron-dense cytoplasm and the retention of active zone (AZ) membrane apposition to postsynaptic scabs (PS). Synaptic vesicles (SVs) and other membrane-bound organelles (Endo) are still present, but in far lower numbers. More importantly, faint filaments (FM) can now be seen.

**Figure 12.** Electron Micrograph of Synaptosome Ghost Prepared Using a Modified EM Preparative Protocol. An EM of a SSM ghost prepared using a modified EM preparative protocol with 2% osmium tetroxide and 1% *en bloc* uranyl acetate staining. Unattached filaments (FM) and those tethering (T) synaptic vesicles (SVs) are now easily discernable. Endo: non-SV membrane-enclosed organelle; AZ: active zone; PS: postsynaptic scab.
4.2 Characterizing SV Tether Lengths

Having established a reliable method for visualizing the filaments connecting SVs to the AZ, we then proceeded to characterize their lengths. Using 72 images of ghosts, I measured the lengths of the filaments connecting SVs to the AZ membrane by tracing them out by hand in ImageJ. We soon realized that multiple (up to 4) filaments could be seen connecting some SVs to the AZ, but SVs that were farther from the AZ membrane appeared to possess only a single tether. After plotting the distance between SVs and AZ membranes against the number of tethers (Figure 13). While I measured the lengths of all tethers connecting the SVs to AZs, we decided to take only the shortest member of any multi-tethers we encountered in our analysis of tether lengths along with all single, long tethers. This analysis showed that the longest of the SV tethers observed was 186 nm long and 95% of the tethers were less than 98 nm long (Figure 14).

![Image showing SVs and AZs connected by tethers](image)

**Figure 13. Single Long Tethers versus Multiple Short Tethers.**
Two general classes of tethers (red arrows) were observed in SSM ghosts. Synaptic vesicles (SV) were observed to be connected to the active zone membrane (AZ) by single long tethers (example on top left panel) or multiple short tethers (example on top right panel). Multiple
short tethers were only observed when the SV was within 45 nm of the AZ (bottom graph; n=52 SVs from 71 SSM ghosts prepared over 2 separate experiments). Figures reproduced from Wong et al., 2014 with minor changes to the labeling.

![Frequency Histograms of Tether Lengths](image)

**Figure 14. Frequency Histograms of Tether Lengths.**
Frequency histogram of the lengths of SV tethers as traced along the tethers themselves. A cumulative frequency histogram of the same data is provided in the inset of the figure. The 95% (dashed line) and 99% (dotted line) confidence limits correspond to 98 nm and 176 nm, respectively. n=72 tethers from 71 SSM ghosts prepared over 2 separate experiments. Figure reproduced from Wong et al., 2014.

4.3 Development of a Robust EM Immunogold Labeling Technique

I immunogold labeled ghosts with CaV antibodies to directly test whether the filaments we observed tethering SVs to the AZ are intracellular components of these channels. Based on the length of the amino acid backbone, the only intracellular domain of CaV2 (the family containing CaV2.1 and CaV2.2, which account for the majority of CaVs in the central nervous system) that could account for all the long single-tethers we have observed was the C-terminal tail. Thus, we began this segment of the project using an in-house and well-characterized L4569 antibody
against the very tip of the CaV2.2 C-terminal tail (Khanna et al., 2006). Our initial attempts to immunogold label ghosts using this antibody sought to take advantage of the compromised plasmalemma of the ghosts resulting from their osmotic rupture to passively diffuse antibodies into the interior of the ghosts (See Methods). While there were a few instances of immunogold labeling of filaments extending from the AZ and of SVs using this method with the L4569 antibody (Figure 15A), these were rare and did not appear to occur more frequently than in control experiments using non-specific IgG.

We thus developed a new immunogold labeling technique incorporating new technologies such as cryoloading and nanogold particles to improve labeling efficacy (See Methods for protocol). In this “immuno-nanogold labeling” technique, I introduced our primary antibody into SSM ghosts using a cryoloading process (Nath et al., 2014) that was developed by Dr. Stanley and Arup Nath, a previous graduate student in the laboratory. For this set of experiments, the primary antibody used was an equal-volume mixture (see 5.4 for reasoning) of the L4569 and L4570 antibodies against the same antigenic region of the CaV2.2 C-terminal tail tip (Khanna et al., 2006) which we termed L45. After cryoloading, I osmotically ruptured the SSM ghosts to release any unbound L45. I then re-cryoloaded these SSM ghosts with Fab covalently conjugated with nanogold. These SSM ghosts were osmotically ruptured again to release unbound Fab and then prepared for EM.

When I used this new technique for L45 labeling on ghosts, we found much greater labeling efficacy than with our previous attempts using passive diffusion (Figure 15B). In contrast to the 6 nm and perfectly spherical colloidal gold particles previously used, gold labeling with the 1.4 nm nanogold secondary Fab mainly appear as clusters of electron-dense material. While there are presumably single nanogold particle labeling present in our images, these are difficult to distinguish from the staining from other metal (i.e. osmium and uranium) used in our preparation (see the “X” in Figure 16) and we defined positive labeling as a cluster of two or more gold particles. This decision has the added advantage of ensuring that we were observing actual antigenic sites for our antibodies and secondary Fab, which are all polyclonal and would bind in multiple copies onto a stretch of antigens.
Figure 15. Development of a Robust EM Immunogold Labeling Technique.

(A) Early attempts at immunogold labeling SSM ghosts involved incubating fixed SSM ghosts with the L4569 antibody against the CaV2.2 C-terminus. After washing, SSM ghosts were subsequently incubated with 6 nm colloidal gold-conjugated secondary antibody. SSM ghosts
were then imaged at 300 Kx magnification. (i) Gold particle (*) associated with filaments (FM) extending from the active zone membrane (AZ) that is in apposition to the postsynaptic scab (PS). (ii) Two gold particles associated with synaptic vesicles (SV) in the peri-AZ. (iii) A trio of gold particles associated with FMs extending from the AZ. (B) The novel immuno-nanogold labeling technique used on SSM ghosts involved the cryoloading of L45 antibody (a combination of L4569 and L4570; see Methods) and then the subsequent cryoloading of nanogold particles covalently bound to secondary Fab. SSM ghosts were re-ruptured after each round of cryoloading to flush out unbound antibody or Fab. SSM ghosts were then imaged at 500 Kx magnification, where nanogold particles could be visualized as clusters (circles and arrows) associated with tethers (T), synaptic vesicles (SV; in crimson), or the active zone membrane (AZ; in blue). Figure reproduced from Chen et al., 2017.

4.4 L45 Labeling in Ghosts

L45 antibody immunogold labeling of ghosts yielded quite striking images (Figure 16). We observed gold clusters mainly on AZ and non-AZ membrane, as well as on SVs. There were also significantly more gold particles in ghosts labeled with L45 when compared to those labeled with control non-specific rabbit IgG (p<0.005, b=binomial test). Many of the gold particles on SVs were located near the junction of SVs and their tethers. To objectively test this exciting, but qualitative observation, we developed two novel statistical analyses.
Figure 16. L45 Immuno-Nanogold Labeling of SSM Ghosts.
SSM ghosts were immuno-nanogold with either L45 antibody (upper panel) or non-specific control rabbit IgG (lower panel) as described in the Methods and Figure 15B. Nanogold clusters (circles and arrows) in L45-cryoloadeed SSM ghosts were generally found on tethers, synaptic vesicles (T; SVs; in crimson), or the active zone membrane (AZ; in blue). Gold clusters were also sometimes observed to be associated with other presynaptic structures (black). Nanogold
clusters in IgG controls were fewer and were not associated with specific presynaptic structures.

PS: postsynaptic scab; AZ?: possible but unconfirmed active zone; X: region of membrane with osmium tetroxide that is similar to nanogold particles, but are likely to be false-negatives. Figure reproduced from Chen et al., 2017.

Dr. Stanley devised a novel “compartment analysis” to evaluate which structures within the ghosts were targeted by the L45 antibodies. This analysis involved dividing the images of ghosts into separate compartments by hand according to common presynaptic structures using ImageJ (Figure 17a). Dr. Stanley amalgamated the “tether” compartment, defined as filaments that connected SVs to the AZ, into the overarching “filament” compartment (abbreviated to “FM”) under the assumption that a portion of filaments extending from the AZ but not terminating in an SV were unoccupied tethers. She used the ImageJ software to measure the area in each compartment and counted the number of gold clusters in each compartment by eye. The gold cluster counting for this set of experiments was done blind by Dr. Stanley to prevent observer bias and she did not consider compartments devoid of structures, which she termed “empty cytosol,” for quantification due to the lack of clusters in that compartment (as we had expected since there is nothing in those compartments for antibodies to bind). Dr. Stanley plotted the cumulative area of each compartment and their respective number of gold clusters on a scatterplot and found that the plot for control IgG followed a relatively linear relationship. We expected such a relationship since the amount of non-specific binding should relate to the amount of area for this binding to occur. Using regression analysis, Dr. Stanley plotted out 95% prediction bands based from these points which she took as being the boundaries for non-specific labeling. When she took the data corresponding to L45 labeled ghosts, she found that our eyes had been correct in identifying AZ membrane, non-AZ membrane, and SVs as structures that were specifically labeled with gold (Figure 17b). While labeling for filaments were on the border of the 95% prediction zone, it should be noted that we deemed any gold clusters at the junction between SVs and their tethers to be associated with the SV in order to simplify the quantification.
Figure 17. Compartment Analysis of L45 Immuno-Nanogold Labeling in SSM Ghosts. 

(A) Example of SSM ghost image (same as the one in Figure 16A but image contrast was lowered for this figure to better see the drawn yellow lines) partitioned into compartments (yellow lines) corresponding to active zone membrane (AZ), non-AZ plasmalemma (Memb), synaptic vesicles (SV), e-cytoplasm (e-Cyto), and d-cytoplasm (d-Cyto).
(SV), non-SV membrane-bound organelles (Endo; none in this particular example), filaments extending from the AZ (FM), electron-dense cytoplasm (d-Cyto), empty cytosol (e-Cyto). Compartments were drawn using ImageJ (see Methods) and drawn only of structures within 200 nm of the AZ. (B) Scatterplot of the cumulative number of nanogold clusters in each compartment plotted against the cumulative area of each compartment from images of SSM ghosts cryo-loaded with either L45 antibody (green circles) or non-specific control IgG (black squares). e-Cyto data points were not plotted since, understandably, no nanogold clusters were found in that compartment. Regression line (black line) and 95% prediction bands (dotted lines) were plotted on this graph based on the control IgG data points. n=12 each of L45 or control IgG immuno-nanogold labeled SSM ghosts from 3 paired experiments. Figure reproduced from Chen et al., 2017.

An alternative, simpler analysis that we also used was to pick out all instances of SVs with a visible tether to the AZ (Figure 18A). We then looked for gold clusters along the entire tethered-SV structure and noted when a gold cluster was within 10 nm of the SV, the AZ membrane, or whether it was associated with portions of the tether away from either the SV or the AZ membrane which I quantified as being on the tether itself. When I used this analysis on images of L45-labeled ghosts as compared to control IgG-labeled ghosts, we found was that the number of gold clusters near the SV or on the tether (when normalized to the number of tethered SVs analyzed) was significantly higher in L45 labeled ghosts (Figure 18B).
Figure 18. Analysis of Individually Tethered SVs in L45 Immuno-Nanogold Labeled SSM Ghosts. 

(A) Cropped images of individual tethered SVs from images of SSM ghosts cryo-LOADED with L45 antibody and then cryo-LOADED with nanogold-conjugated Fab. Nanogold clusters (circles and arrows) can be seen associated with individual tethered SVs within 10 nm of the SV (orange), of the AZ membrane (green), or of the tether only (blue). 

(B) Analysis of how many nanogold clusters are associated with the SV, AZ membrane, or tether only of individual tethered SVs from images of control IgG versus L45 immuno-nanogold labeled SSM ghosts. The number of nanogold clusters was normalized to the number of individual tethered SVs analyzed. n=15 or 10 individual tethered SVs of control IgG or L45 immuno-nanogold labeled SSM ghosts, respectively. pb values on bars are a comparison of L45 vs control IgG conditions using the binomial test. The lack of observed nanogold clusters associated with the tether only in control IgG condition resulted in the approximation of pb→0. Figure reproduced from Chen et al., 2017.

While the L45 immunogold labeling was consistent with the hypothesis that CaVs tether SVs, there is the alternative interpretation that the SV-labeling with L45 was labeling CaVs that had
their transmembrane pore located on SVs rather than on the AZ (See Discussion). The remainder of this project was directed towards testing this alternative hypothesis and further exploring our original hypothesis.

4.5 Ab571 Labeling of Ghosts

To test the alternative hypothesis that L45 was labeling CaVs whose transmembrane pores were on SVs, I began by labeling ghosts with a previously-characterized Ab571 antibody against the II-III intracellular loop of CaV2.2 (Li et al., 2004). If this alternative hypothesis was correct, we would expect to see Ab571 labeling SVs in much the same fashion as L45 did. Using compartment analysis, I found that ghosts labeled with Ab571 had gold clusters specifically associated with AZ membranes and—to our surprise—SVs (Figure 19). To further understand these results, I took these images as well as images of L45-labeled ghosts, and measured the distances along the tether that SV- or tether-associated gold clusters were from the AZ membrane. The frequency of gold clusters was binned by 25 nm interval distances from the AZ membrane. To compensate for non-specific labeling for each condition, we normalized the number of gold clusters in their respective control IgG experiments to the number of ghosts analyzed and subtracted that value from the frequencies of the L45- or Ab571-associated gold clusters (see Methods for more detail). When these compensated values were plotted in a frequency histogram (Figure 20), it became readily apparent that the gold clusters on SVs or their tethers in Ab571-labeled ghosts were generally closer to the AZ membrane than those in L45-labeled ghosts. In fact, 50% of the gold clusters in the Ab571-labeled ghosts appeared to be within 45 nm of the AZ while 50% of gold clusters in L45-labeled ghosts were roughly within 75 nm of the AZ. It should be noted that there was greater Ab571 labeling than L45 labeling at the 125 nm data point. We currently do not have an explanation for this deviation from the overall trend.
Figure 19. Compartment Analysis of Ab571 Immuno-Nanogold Labeling of SSM Ghosts. 
(A) Example image of a SSM ghost cryoloadoed with the Ab571 antibody against the CaV2.2 II-III intracellular loop and then cryoloadoed again with nanogold-conjugated secondary Fab. A nanogold cluster (crimson circle and arrow) can be seen on associated with a synaptic vesicle (SV) with a tether (T) to the active zone membrane (AZ) apposing the postsynaptic scab (PS). Note that this nanogold cluster is ~65 nm away from the AZ membrane. Another nanogold
cluster (blue circle and arrow) can be seen associated with the AZ membrane. **(B)** Compartment analysis of Ab571 immuno-nanogold labeled SSM ghosts as described for L45 immuno-nanogold labeled SSM ghosts in Figure 17B. n=19 for each of Ab571 (green circles) or control IgG (black squares) immuno-nanogold labeled SSM ghosts over 2 paired experiments. Figure reproduced from Chen et al., 2017.

![Gold Cluster Cum Freq](image)

**Figure 20. Comparison of the Distance of Nanogold Clusters from the AZ Membrane Along Tethered SVs.**
The distance between nanogold clusters to the AZ membrane was measured by tracing along SVs and tethers in images of Ab571 (green) and L45 (blue) immune-nanogold labeled SSM ghosts. This data was compensated for non-specific labeling as measured with respective IgG controls and normalized to the number of ghosts analyzed for each condition. Data was then plotted as an inverse cumulative frequency histogram with dashed lines denoting the distance for 50% nanogold cluster frequency for each condition. n=19 and 12 for Ab571 and L45 immuno-nanogold labeled tethered SVs, respectively. Figure reproduced from Chen et al., 2017.

4.6 TAT-Peptide Blocking of SV Tethering

Our lab has successfully used blocking peptides in biochemical and functional assays to disrupt SV tethering (*HQARRVPNGY* from Gardezi et al., 2016). The difficulty in applying this approach to electron microscopy was to find a method by which we would know whether the peptide blocker had entered a particular ghost or not (**See Discussion**). We decided to obtain
peptides that were conjugated with TAT tags with the assumption these peptides would gain access into most if not all the ghosts in a sample. We predicted that exposing synaptosomes to TAT-HQARRVPNGY peptides, stimulating them with 40 mM KCl, osmotically rupturing them into ghosts, and then labeling them with L45 antibody would lower the population of tethered SVs by a quantifiable extent and thus the number of L45-labeled SVs in comparison to control TAT-scrambled peptides. However, I immediately observed that the higher the concentration of TAT-conjugated peptides used, the faster the SSMs appeared to sink in solution. These SSMs also tended to adhere to the side of tubes more than untreated SSMs after centrifugation. Despite this first anomaly, I continued with our experimental plan. When visualized under EM, we found large, presumably uranyl acetate crystals scattered all throughout the ghost sample which made imaging very difficult as the gain for the camera would have to be altered to a point where gold clusters were hard to identify (Figure 21A). The presence of larger crystals in a field of view under EM also often seemed to distort the image. In the few occasions when I could get interpretable images despite the uranyl acetate crystals, we still occasionally observed SVs with gold cluster labeling (Figure 21B). However, the paucity of usable images for TAT-HQARRVPNGY and control TAT-scrambled peptide treated ghosts, we were unable to distinguish any significant difference in labeling between the two conditions. We were also unable to ascertain whether the number of tethered SVs were different.
Figure 21. L45 Immuno-Nanogold Labeling of SSM Ghosts Pre-Treated with TAT-Conjugated SV-Tether Blocking Peptides.

SSMs were exposed to TAT-conjugated peptides containing the HQARRVPNGY sequence shown to block the SV interaction with the distal third of the CaV2.2 C-terminal and stimulated with a high-potassium solution prior to being osmotically ruptured into SSM ghosts. SSM ghosts were then cryoloadeed with L45 antibody and then cryoloadeed again with nanogold-conjugated Fab. Large crystals (presumably of uranyl acetate; yellow arrows) were observed scattered across various structures (top panel). In the occasional occurrence where there were synapses...
unobstructed by these crystals, nanogold clusters (crimson circle and arrow) could sometimes still be seen (bottom panel).

4.7 Antibodies Against More Central Regions of the CaV C-Terminal Tail

In order to further test whether the L45 antibody was labeling CaVs whose C-terminals spanned from the transmembrane pore on the AZ to SVs in the cytoplasm, we made two more polyclonal antibodies against different regions of the CaV C-terminal tail (Figure 22). The NmidC2 antibody is targeted against a 14 amino acid sequence located approximately halfway between the tip of the CaV2.2 C-terminal tail and where the tail becomes Domain IV’s S6 transmembrane helix. The C2Nt antibody is targeted against another 14 amino acid sequence in the C-terminal tail that is ~130 amino acids distal to the S6 helix of CaV2.1 that shares high homology (11/14 amino acids) with CaV2.2 (see Methods for polyclonal antibody generation).
Figure 22. Overview of Antibodies. 
(A) A table of the antibodies against the CaV C-terminal used in this study as well as the corresponding antigenic sequence, whether the antibody was intended to be specific for CaV2.1 or CaV2.2, and the estimated maximal distance the antigenic target could be from the membrane. (B) Sequence alignment of the C-terminal of CaV2.1 and CaV2.2 along with the antigenic sites for each antibody corresponding to the colour code in the table in (A). (C) A cartoon of a CaV2.1 embedded in the membrane with the target locations of each antibody.
indicated. The intracellular loops have been drawn roughly to scale. Figure C reproduced from Chen et al., 2017.

We characterized both antibodies using biochemistry and fluorescent immunocytochemistry prior to using them for EM immunogold labeling. Western blots of chick ghost lysate and a C1-2strep fusion protein emulating the distal half of the CaV2.2 C-terminal tail (Wong et al., 2013, 2014) that contains the NmidC2 antigenic site were probed with the NmidC2 antibody at various stages after the inoculation of the rabbits (Figure 23A). In lanes loaded with chick ghost lysate, we observed a series of bands including a band at a higher molecular weight than 250 kDa that is where we would expect CaV2.2 (Khanna et al., 2006; Li et al., 2004) and that was not seen when I probed with pre-immune serum. There were also a pair of darker bands at ~60 kDa and a prominent band under 37 kDa. NmidC2 also identified a ~47 kDa band in the C1-2strep lane that corresponded to the molecular weight of the fusion protein.

To further biochemically characterize the NmidC2 antibody, I immunoprecipitated CaV2.2 from chick ghost lysate using the Ab571 antibody. When then probed the immunoprecipitated CaV2.2 on western blots using NmidC2 antibodies that were either blocked with the NmidC2 immunogen or sham-blocked with control peptides (Figure 23C). We found that the sham-blocked NmidC2 antibody picked up the >250 kDa band corresponding to the immunoprecipitated CaV2.2 as well as a band at 50 kDa which is likely IgG heavy chain from the Ab571 immunoprecipitation. The antigen-blocked NmidC2 antibody did not identify the >250 kDa band at all while there was no change with the 50 kDa band.

We further tested the blocked and sham-blocked NmidC2 antibodies on bacterial fusion proteins replicating different regions of the CaV2.2 C-terminal. I made dot blots of fusion proteins containing only the NmidC2 antigenic site or only the L45 antigenic site (C1-2 and C3, respectively) which I then probed with NmidC2 or L45 antibodies that were blocked- or sham-blocked with varying concentrations of their respective antigenic peptides (Figure 23D). We found that the NmidC2 and L45 antibodies only identified fusion proteins that contained their antigenic sites and that blocking these antibodies using their respective antigenic peptides prevented this identification.
Figure 23. Biochemical Characterization of the NmidC2 Antibody.

(A) Western blots of chick brain SSM ghost lysate and the C1,2Strep fusion protein containing the NmidC2 antigenic site. These blots were probed with serum from a rabbit before immunization with the NmidC2 immunogen (Pre-Immune) and the 3rd and 4th post-immunization bleeds (Bleed 3 and Bleed 4).
The expected molecular weight for CaV2.2 in samples boiled prior to western blotting is above 250 kDa (indicated with red arrow and ‘Native CaV2.2’) as identified by the previously characterized Ab571 (see C) while the estimated molecular weight of the C1,2Strep (replicating the proximal 2/3rd of the CaV2.2 C-terminal) fusion protein is 47 kDa. Red arrows denote the expected location for these bands according to molecular weight. (B) Western blot of chick brain SSM ghost lysate probed with affinity purified NmidC2 antibody (see Methods). The red arrow indicates the expected molecular weight for CaV2.2 in boiled samples. Note that the contrast of this blot had to be severely adjusted to visualize the bands, which is why the blot is so pixelated. (C) Immunoprecipitate from Ab571 IPs and control IgG IPs of chick brain SSM ghost lysate were run on western blots along with whole chick brain SSM ghost lysate. Western blots were then probed using NmidC2 serum pre-blocked using 40 uM of the peptide used to generate NmidC2 (NmidC2-blocked NmidC2), NmidC2 serum pre-blocked using 40 uM of peptides corresponding to the L45 antigenic region (L45-blocked NmidC2) as an antibody-blocking control, or unadulterated Ab571 as a IP positive control. (D) A scan of 10 dot blots (each denoted by black squares) of the C3 fusion protein (green circles) containing the L45 antigenic site but not the NmidC2 antigenic site and of the C1-2 fusion protein (blue circles) containing the NmidC2 antigenic site but not the L45 antigenic site. Dots on the right contain 10x the amount of protein as compared to dots on the left in each dot blot square. Blots were then probed with either L45 or NmidC2 antibody sham-blocked with the peptides corresponding to the other antibody, or actual-blocked with the indicated concentrations of their own antigenic peptide. Figures A and D reproduced from Chen et al., 2017.

We also tested the NmidC2 antibody using fluorescent immunocytochemistry on chick ciliary ganglion (CCGs; Li et al., 2004). The presynaptic calyces of CCGs (as denoted by SV2 counter-staining) showed puncta-like staining near the release face when stained with NmidC2 (Figure 24). Staining CCGs with pre-immune serum did not show the same staining pattern while SV2 staining was still nominal.
Figure 24. Immunocytochemistry of Chick Ciliary Ganglion Cells using NmidC2 Antibody. Dissociated chick ciliary ganglion (CCG) neurons synapse with thick, calyx-shaped presynaptic terminals. These cells were stained with the SV protein SV2 (magenta) to identify these calyces, and were also stained with either NmidC2 antibody (bottom panels) or control pre-immune serum (top panels). Note the punctate-like staining along the release face of the calyx with NmidC2 that is absent with the pre-immune serum. The green bloom where the postsynaptic neuron is with the pre-immune serum staining is an imaging artifact. These experiments were done in conjunction with Dr. Qi Li. Figures in lower panels reproduced from Chen et al., 2017.

We also characterized the C2Nt antibody by western blotting (Figure 25A). We observed a series of bands ranging from 150 kDa to below 37 kDa, but also observed one weak band above 250 kDa and another just under 250 kDa. A colleague (Christine Snidal) used C2Nt on western blots of CaV2.2 fusion proteins (C1-2gst and CproxA) and a CaV2.1 fusion protein (C5) containing the C2Nt antigenic region and observed bands corresponding to each fusion protein (~73 kDa, ~52 kDa, and ~69 kDa, respectively) but bands were not observed with a CaV2.2 fusion protein (CmidB at ~52 kDa) lacking the C2Nt antigenic site (Figure 25B).
Figure 25. Biochemical Characterization of the C2Nt Antibody.

(A) Western blots of chick brain SSM ghost lysate were probed with the pre-immune serum (Pre-immune) from a rabbit immunized with the C2Nt immunogen or subsequent bleeds (Bleed 4, Bleed 5) from the same rabbit. Red arrows and text denote the expected molecular weight for CaV2.2 (as previously identified with Ab571) and CaV2.1 (See Discussion).

(B) Western blots of various CaV C-terminal GST-tagged fusion proteins probed with C2Nt antibody. C1-2 and CproxA fusion proteins replicate proximal regions of the CaV2.2 C-terminal containing the C2Nt antigenic site. CmidB replicates a middle region of the CaV2.2 C-terminal distal to the C2Nt antigenic site. C5 replicates a portion of the CaV2.1 C-terminal containing the C2Nt antigenic site. Black arrows denote the expected molecular weights of each fusion protein as determined by probing blots.
with anti-GST antibody. C1-2 fusion protein in this blot is GST-tagged and thus has a higher molecular weight than the C1,2strep in Figure 23. These fusion protein experiments were done by Ms. Christine Snidal. Figure reproduced from Chen et al., 2017.

We also tested C2Nt antibody on CCGs with fluorescent immunocytochemistry. We observed puncta-like staining of the CCG calyces using the C2Nt, but not a control pre-immune, antibody (Figure 26). Henry Mah, a fellow graduate student in the Stanley laboratory, used this antibody on chick cerebellar slices to test whether it would also immunostain CaV2.1 (See Discussion). Using an anti-calbindin antibody identify Purkinje cell bodies, we observed that the C2Nt antibody stained the somas and the axons of calbindin-positive cells (Figure 27). The C2Nt staining of the axons was much stronger than the staining of the calbindin-positive somas. The C2Nt staining of calbindin-positive somas and axons is similar to the staining using a CaV2.1-specific antibody, PmidC2. However, PmidC2 also appeared to stain cells in the same cerebellar layer as the Purkinje dendritic trees that are smaller than Purkinje cells and are not calbindin-positive.

Figure 26. **Immunocytochemistry of Chick Ciliary Ganglion Cells using C2Nt Antibody.** Dissociated chick ciliary ganglion (CCG) neurons synapse with thick, calyx-shaped presynaptic terminals. These cells were stained with the SV protein SV2 (magenta) to identify these calyces, and were also stained with either C2Nt antibody (bottom panels) or control pre-immune serum
(top panels). Note the punctate-like staining along the release face of the calyx with C2Nt that is absent with the pre-immune serum. Lower panel figures reproduced from Chen et al., 2017.

Figure 27. Immunohistochemistry of Chick Cerebellar Slices Using C2Nt Antibody. Chick cerebellar slices from E19 chicks were stained using antibody against the Purkinje cell marker calbindin (magenta) and either C2Nt (top panels) or PmidC2 (bottom panels) antibody (green). The Purkinje layer of the slices were then imaged at (A) 16x magnification and (B) 63x.
magnification. These experiments were carried out by Mr. Henry Mah (Mah and Stanley, Unpublished).

The PmidC2 antibody that we used as a comparison to the C2Nt antibody was originally made in support of another study characterizing chick CaV2.1. The antibody is against a region near the NmidC2 antibody (Figure 22), but the antigenic region shares little to no homology with chick CaV2.2 (4 out of 14 amino acids). On western blots of chick ghost lysate, the PmidC2 antibody identifies a strong band just above 250 kDa, but lower than what would be expected for CaV2.2 (Figure 28A). Fluorescent immunocytochemistry of CCGs was negative when stained using PmidC2 antibodies (Figure 28B). PmidC2 antibodies did not stain the characteristic presynaptic calyces of magnocellular nucleus (MCN) slices (Parks and Rubel, 1978), but instead appeared to stain some kind of structure adjacent to the postsynaptic cell where the presynaptic calyx (as defined with SV2 staining) was absent (Figure 28C). However, PmidC2 antibodies did stain the calbindin-positive neurons in chick cerebellar slices that are presumably Purkinje neurons (Figure 27).
Figure 28. Characterization of PmidC2 Antibody.

(A) Western blot of chick brain SSM ghost lysate probed with pre-immune serum (Pre-) from a rabbit that was immunized with the PmidC2 immunogen and with a subsequent bleed (Bleed 4) of the same rabbit. The black arrow marks the band near the expected molecular weight for CaV2.1 (See Discussion).

(B) Immunocytochemistry of dissociated chick ciliary ganglion cells stained with the SV marker SV2 (magenta) to visualize the calyx-shaped presynaptic terminal and PmidC2 (green). Note the lack of specific green staining on the calyx structure. These experiments were done in conjunction with Dr. Qi Li.

(C) Immunohistochemistry of chick magnocellular nucleus (MCN) from a E15 embryonic chick brainstem stained with the SV marker SV2 to visualize the presynaptic calyces in this region and PmidC2 (top panels) or Ab571 (bottom panels) antibody (in green). Note that while PmidC2 staining is evident in the MCN, it does not
seem to stain the SV2-positive calyx while Ab571 co-stains with SV2. These immunohistochemistry experiments were done entirely by Dr. Qi Li.

4.8 NmidC2 EM Immunogold Labeling

Prior to using the NmidC2 for EM immunogold labeling, we decided to affinity-purify the antibody using the immunogen used to generate the antibody. When I used this purified antibody to probe chick ghost lysate in western blots, we observed a faint band above 250 kDa and a stronger band above 50 kDa (Figure 23B). The other bands that we had observed with non-purified NmidC2 antibody were no longer present, although the bands in general much weaker using the purified NmidC2 and the western blots had to be exposed for a greater amount of time (a 1 h exposure and the contrast on the blot had to be severely adjusted, as opposed to the usual ~30 min exposure for the other antibodies) in order to visualize any bands at all.

While EM immunogold labeling of ghosts using the purified NmidC2 antibody resulted in some very provocative images (Figure 29A), we did not observe a significantly greater density of gold clusters overall when compared to immunolabeling with control IgG (p>0.1; n=38 and 39 for NmidC2 and control IgG, respectively). It is possible that there was a significant loss of antibody during the affinity purification process, which would also explain why the western blot probed with purified NmidC2 had to be exposed for so long before bands were visible, and thus the amount of control IgG used may not have been strictly equivalent with the amount of IgG in the purified NmidC2. Nevertheless, I decided to use compartment quantification on this data set, which showed that the filaments extending from the AZ were significantly (albeit at the edge of significance) labeled with gold clusters while SVs were not significantly labeled (Figure 29B). This result with the affinity-purified NmidC2 antibody contrasts with the predominately SV and AZ membrane labeling using the L45 antibody.
Figure 29. Immuno-Nanogold Labeling and Compartment Analysis with NmidC2 Antibody. 
(A) Sample EM image of SSM ghost cryo loaded with affinity-purified NmidC2 antibody and then 
cryo loaded with nanogold-conjugated secondary Fab. Nanogold clusters can be seen associated 
with the AZ membrane (blue circle and arrow) and with filaments extending from the AZ (red 
circles and arrows). (B) Compartment analysis of images of SSM ghosts cryo loaded either with
affinity-purified NmidC2 (green circles) or non-specific control rabbit IgG (black squares) as per Figure 17. n=39 and 38 for control and NmidC2 conditions, respectively, in 2 paired experiments. Figure reproduced from Chen et al., 2017.

To independently test whether the NmidC2 immunogold labeling was real or not, I used the same blocked and sham-blocked NmidC2 antibodies used previously on western and dot blotting (Figure 23C and 23D) for EM immunogold labeling of ghosts. I analyzed where gold particle clusters were located on individual tethered SVs much like how I had analyzed L45-labeled individual tethered SVs, except we agreed to limit the data sets to SVs tethered by filaments longer than 80nm to make the determination of whether the nanogold cluster is associated with the SV, tether, or AZ membrane less ambiguous. We found that there were significantly less gold clusters on tethered SVs, their tethers, and their tethers near the AZ membrane in ghosts labeled with blocked NmidC2 versus sham-blocked NmidC2 (Figure 30).

![Figure 30](image)

**Figure 30. Analysis of Blocked Versus Sham-Blocked NmidC2 Immuno-Nanogold Labeling of SSM Ghosts.**

SSM ghost were cryoloadered with NmidC2 antibody either sham-blocked with 40 uM of peptides against the L45 antigenic region or actually blocked with 40 uM of peptides against the NmidC2 antigenic region (as per Figure 23C). SSM ghosts were then cryoloadered with nanogold-conjugated secondary Fab. EMs of individual tethered SVs from these SSM ghosts with tethers longer than 80 nm were analyzed (as per Figure 18) and the reduction of nanogold clusters in the actual-blocked condition compared to the sham-blocked condition using the Binomial Test. n=16 individual tethered SVs for each condition.
4.9 C2Nt EM Immunogold Labeling in Comparison with L45 and NmidC2

I used the C2Nt antibody as an unpurified serum to immunolabel ghosts. Using compartment analysis, the C2Nt-immunolabeled ghosts had a significant amount of gold clusters associated with filaments extending from the AZ (Figure 31). This result was similar to that for NmidC2 immunogold labeling of ghosts, albeit to a greater extent, and different to that of L45 immunogold labeling. However, we were curious to see whether there were any quantifiable differences between the NmidC2 and C2Nt immunolabeling as would be predicted by the location of their respective antigenic sites on the CaV C-terminal tails.
Figure 31. Compartment Analysis of C2Nt Immuno-Nanogold Labeling of SSM Ghosts.

(A) Sample EM image of a SSM ghost that was cryoloaded with C2Nt antibody and then cryoloaded with nanogold-conjugated secondary Fab. Nanogold clusters were seen associated with the AZ membrane (blue circle and arrow) and filaments extending from the AZ (red circles and arrows).

(B) Compartment quantification of images of C2Nt (green circles) and control pre-immune serum (black squares) immuno-nanogold labeled SSM ghosts. n=38 and 41 for control
and C2Nt labeled SSM ghosts, respectively, in 2 paired experiments. Figure reproduced from Chen et al., 2017.

I first compared the location of gold particle clusters on individual tethered SVs for ghosts labeled with L45, NmidC2, and C2Nt (Figure 32). We found that while L45- and NmidC2-labeled ghosts had significantly greater gold labeling on SVs and tethers when compared to cumulated controls, C2Nt-labeled ghosts had only labeling on tethers, the AZ membrane, and no labeling on SVs at all. When compared to the cumulated controls, L45 labeling of the tethers near the AZ membrane were also significant. To more precisely investigate the distribution patterns of the antibodies, I measured the distances along the tethers between gold clusters and the AZ membrane as I had done previously for Ab571 immuno-labeled individual tethered SVs (also see Methods), we found that gold clusters were generally closer to the AZ membrane for the C2Nt-labeled ghosts than in the NmidC2-labeled ghosts, which were themselves closer to the AZ membrane than those of the L45-labeled ghosts (Figure 33). Half of the gold clusters on individual tethered SVs from C2Nt-, NmidC2-, and L45-labeled ghosts were within 45 nm, 60 nm, and 80 nm away from the AZ membrane, respectively. 75% of the gold clusters for the same conditions were within 65 nm, 80 nm, and 100 nm of the AZ membrane, respectively.

Figure 32. Comparison of Nanogold Location on Individual Tethered SVs Labeled with L45, NmidC2, or C2Nt Antibody.
Individual tethered SVs from SSM ghosts cryoloadeed with either L45, NmidC2, or C2Nt antibody and then cryoloadeed again with nanogold-conjugated Fab were analyzed for whether nanogold clusters were associated with the SV, the tether itself, or the AZ membrane (as per Figure 18). These values were statistically tested against the amalgamated values of the controls (pooled from the respective controls of each of the 3 antibodies) using the Binomial Test ($p_b$). All $p_b$ values shown on the graph are statistically significant except for those of nanogold clusters associated with the AZ membrane in NmidC2 labeled ghosts and with SVs in C2Nt labeled ghosts (values not shown) and are almost statistically significance for nanogold clusters associated with tethers in NmidC2 labeled ghosts. Note the lack of labeling on SVs in C2Nt labeled ghosts. $n=55$, 18, 12, and 10 for controls, C2Nt, NmidC2, and L45, respectively. Figure reproduced from Chen et al., 2017.

Figure 33. Comparison of the Distances of Nanogold Clusters from the AZ Membrane as Measured Along Tethered SVs in SSM Ghosts Immuno-Nanogold Labeled with C2Nt, NmidC2, or L45 Antibody.

The distance of each nanogold clusters on individually tethered SVs for each condition were measured by tracing along the tether to the AZ membrane. These distances were compensated for non-specific labeling as measured with their respective controls and normalized to the
number of ghosts analyzed (n=40, 38, and 12 for C2Nt, NmidC2, and L45, respectively). These values were then plotted on an inverse cumulative frequency histogram and vertical dashed and dotted lines were drawn to denote the 50% and 75% nanogold cluster frequency distances, respectively, for each antibody condition. Figure reproduced from Chen et al., 2017.
5 Discussion

Our intention in this study was to investigate whether we could observe SV-tethering by the C-terminals of CaVs in situ. To accomplish this goal, we created a new paradigm for examining structures in presynaptic terminals under EM by using osmotically ruptured synaptosomes, termed ghosts. By combining this model system with our newly developed immunogold technique, I was able to use antibodies against different regions of CaVs to label various presynaptic structures, including SVs and their tethers. The most striking of these experiments was the labeling of SVs that were retained within ghosts, but not directly docked to the plasmalemma, with the L45 antibody against the tip of the CaV2.2 C-terminal tail. We interpreted this result as suggesting that the CaV C-terminal tail extends from the AZ membrane to tether SVs in the presynaptic lumen. This key result using the L45 antibody was the springboard upon which we proceeded to further test and explore our hypothesis.

5.1 Development of a Suitable EM Preparative Technique

We chose to use isolated presynaptic terminals (synaptosomes; SSMs) and their derivatives as the model system for this study because they provide a concentrated and purified sample of active zones for us to image. Because the nanometer scales that we must work at necessitates the use of electron microscopy (EM), the less time spent searching in nano- or micrometer increments for suitable presynaptic terminals in intact tissue sections meant more time spent gathering useful data. SSMs were created and described by Gray and Whittaker (1962), and are still functionally capable of exo- and endocytosis (Cousin and Robinson, 1998; Nicholls and Sihra, 1986). Much of our published biochemical and functional studies investigating SV-tethering has also been done either on SSMs or using components derived from them (Gardezi et al., 2016; Wong et al., 2013, 2014), and EM-based studies of tethers have also used this model system (Fernandez-Busnadiego et al., 2013).

Previous EM studies investigating SV tethers or other structures at the peri-AZ have always had to overcome the overwhelming concentration of particles at presynaptic terminals. It was quickly found that aldehyde fixation of samples caused many of these particles to coalesce into a morass of electron-dense “soup.” Some of the earliest studies used freeze-etching to overcome this issue (Hirokawa et al., 1989; Landis et al., 1988). However, freeze-etching is limited in how deep into the sample you could observe (20 nm according to Landis et al., 1988) and is thus of
limited value for looking at tethers despite Hirokawa’s observation of a >100nm SV tether. Later studies used electron tomography and computer reconstruction (while still fixing the sample using high pressure freezing) to image and make sense of all the particles present near the AZ (Cole et al., 2016; Fernández-Busnadiego et al., 2013; Siksou et al., 2007). For our study, we adapted a relatively common and simple protocol for isolating SVs (Whittaker et al., 1964) to visualize tethers without the complex and specialized setup necessary for high-pressure freezing and electron tomography. By osmotically rupturing SSMs into “ghosts,” (Whittaker, 1968), I not only flush out the cytoplasm that could obscure tethers after aldehyde fixation, but I also retain only the structures associated with the presynaptic plasmalemma (see Figure 10 as compared to Figures 11 and 12). These structures would logically include tethered SVs and the structures tethering them, and thus the osmotic rupture serves a secondary purpose of “filtering out” non-membrane-bound structures such as untethered SVs that are outside the scope of this study.

It should be noted that while examining ghosts facilitates the observation of tethers in situ, a common criticism that arises is that the kinetic force of the osmotic rupturing could potentially have affected some of the characteristics of the tethers. Because I cannot precisely control the force of the osmotic rupture and thus how much of the internal structures of the ghosts I am evacuating, this could explain why certain ghosts have many tethered SVs remaining and some have very little. It is possible that the kinetic force applied by the osmotic shock could also account for the discrepancy in tether lengths observed by us (up to 190 nm; Figure 14) and those seen in one previous study (only up to ~40 nm in Fernandez-Busnadiego et al., 2013) by somehow stretching or unraveling any secondary or tertiary structures. However, most other EM-based studies have found SV tether lengths that are ~100 nm or above (Cole et al., 2016; Hirokawa et al., 1989; Siksou et al., 2009), which are closer to—although not quite at—our observed lengths. Furthermore, if the long tethers are indeed CaV C-terminals as the results of this study and others from the Stanley laboratory (Gardezi et al., 2016; Wong et al., 2013, 2014) would suggest, then there should be very little secondary structure inherent in the tether (Wong et al., 2014). If fact, ghosts may provide a better model for observing long tethers as compared to intact presynaptic terminals since there is less electron-dense material present to obscure their observation. However, it is important to keep in mind that this study can only provide limited information on what the tethers look like in a truly physiological state. Regardless of whether their secondary or tertiary structure have been somehow altered by the osmotic rupture, the
length of our observed tethers do not exceed 200nm which is consistent with the length of the CaV C-terminal tail if it were fully stretched out. The immunogold labeling experiments that identify some of the tethers as the CaV C-terminal tail are likewise not dependent on whether or not the tether is in its natural conformation.

We spent many months of trial and error to develop a satisfactory protocol of preparing and visualizing these SSM ghosts under EM. I started with a standard protocol (see Methods) provided by the Imaging Facility at the Cell & Systems Biology department (University of Toronto, Toronto, Canada) that resulted in images that were encouraging, but difficult to interpret (Figure 11). By increasing the concentration of osmium tetroxide and including an en bloc uranyl acetate staining step, I eventually obtained images of SSM ghosts where we could consistently identify structures such as active zones, tethers, and SVs (Figure 12).

Osmium tetroxide is a popular fixative and lipid stain due its reactivity with the double bonds, especially those found on phospholipids (Porter and Kallman, 1953). Thus, when I doubled the concentration of osmium tetroxide used in our sample preparation, I enhanced the deposition of this heavy metal on both the plasmalemma and the membranes of intra-synaptosomal organelles such as SVs. Uranyl acetate is also quite commonly used for EM sample preparation. It is thought to preferentially bind phosphate groups such as those found on phospholipid membranes, it was originally described to also stain many different proteins that do not possess phosphate groups (Watson, 1958). It has been suggested that uranyl acetate will also react with sialic groups (Pandithage, 2013). By using the uranyl acetate en bloc (i.e. before the tissue is embedded in plastic), I am likely increasing the penetration and efficacy of the uranyl acetate staining.

5.2 Tether Lengths and CaV Intracellular Domains

Our observation that SVs could be tethered by multiple tethers only if the SV is within 45 nm of the AZ membrane (Figure 13) suggested to us that there are multiple classes of SV tethers of different lengths. It should be noted that because I am imaging 100 nm-thick sections using a conventional 2D transmission EM system (see Methods), I have no way of measuring any deviations that these tethers may have in the z-axis. Thus, it is possible that we are consistently underestimating the true lengths of our observed tethers. The extent of this underestimation is difficult to assess since it could vary depending on whether the deviation undulates along the z-
axis or merely travels at a constant angle to the viewing plane. However, our overall observation of multiple tethers when the SV is closer to the membrane is still valid and is consistent with those made by other EM studies (Fernandez-Busnadiego et al., 2013; Cole et al., 2016). This led us to hypothesize that there are separate “Grab-” and “Lock-” tethers (Figure 34A); the former of which brings SVs close enough to the membrane that the latter can maintain the SV at a suitable range that the calcium influx through CaVs will be of sufficient concentration to trigger the calcium sensor on the SV (Fogelson and Zucker, 1985; Simon and Llinás, 1985; Weber et al., 2010).

Figure 34. Working Model of CaV Tethering of SVs.
(A) **Working model of the G- and L-tethering system** where a long Grab-tether (G-tether) binds a SV tethering protein (SVTP) on the SV in the cytoplasm and brings the SV to the docking site on the AZ membrane. Once at or near the docking site, shorter Lock-tether(s) (L-tether) bind a protein on the SV (L-tether binding site) that can either be the SVTP or a different SV protein altogether to ensure that the SV is sufficiently close to the CaV for Ca\(^{2+}\) nanodomain-gated fusion. Figure reproduced from Wong et al., 2014. (B) **A cartoon summary of the findings of this study** investigating whether the G-tethers are CaV C-terminals. Three separate antibodies (L45, NmidC2, and C2Nt) against the C-terminal tails of CaV2.1 and/or CaV2.2 have been shown to immuno-nanogold label different regions of SV tethers as observed under EM in relative accordance to where their antigens are located on the tail.

Because previous biochemical studies from the Stanley laboratory have suggested the CaV C-terminal, an intracellular domain that could potentially span 200 nm, as a SV tether candidate (Gardezi et al., 2016; Wong et al., 2014), we decided to simplify the project by identifying the longer, single tethers. If the hypothesis made preceding this study (Wong et al., 2013) that the CaV itself is a tether of SVs is correct, then one or more of the 5 CaV intracellular domains is likely involved. Based on the predicted secondary structures (as per the Phyre\(^2\) bioinformatics server), and the number of amino acids, Dr. Stanley estimated the “reach” into the presynaptic lumen of the CaV N-terminal head, I-II loop, II-III loop, III-IV loop, and C-terminal tail to be ~20 nm, ~13 nm, ~65 nm, ~6 nm, and ~200 nm respectively. These estimations were based off the assumptions that the approximate length of an individual amino acid in a disordered strand is 0.36 nm per amino acid, that the length in an alpha-helix is 0.54 nm per 3.6 amino acids, and that the length in beta-sheets (which are predicted to be unlikely) is 0.1 nm per amino acid. Of these intracellular domains, the C-terminal tail has the greatest likelihood to be the >100 nm SV tethers. 95% of the 72 tethers I measured in this portion of the study were within 98 nm in length while none of them exceeded the 200 nm that the CaV C-terminal could theoretically reach. Beyond the EM data, Dr. Stanley and other members of her laboratory have also published biochemical and functional studies showing that the C-terminal can bind to SVs (Gardezi et al., 2016; Wong et al., 2013, 2014). We thus proceeded to further test whether the CaV C-terminal tail was a tether of SVs in situ.

### 5.3 Development of a Novel EM Immunogold Labeling Technique

We decided to use EM immunogold labeling to directly test whether any of the AZ-to-SV filaments we have observed under EM were CaV C-terminal tails. Our lab has previously
generated and characterized multiple polyclonal antibodies against different regions of chick CaVs. These include two antibodies (L4569 and L4570) from two different rabbits against the very tip of the C-terminal tail (Khanna et al., 2006). The antigenic region for these antibodies does not include the C-terminal SV-binding site as was identified using biochemical means (Gardezi et al., 2016). We based our first attempts at immunogold labeling ghosts on a classic immunogold labeling study (De Camilli et al., 1983) where intact SSMs were fixed while osmotically ruptured and then embedded in agarose. The compromised plasmalemma of the SSMs from the osmotic shock allowed greater antibody access to epitopes inside the SSMs. From a study that successfully introduced trypsin into previously osmotically ruptured SSMs (Mehta et al., 1996), we assumed the same would apply to SSM ghosts. According to the study, the agarose embedding allows the SSMs to be more easily manipulated as a bulk sample. However, I found it surprisingly difficult to find the agarose blocks after plasticizing which made it difficult to section the samples for EM. Thus, I decided to maintain our samples as pellets that were more visible and could be counted on to be at the bottom of our sample tubes after centrifugation. We had limited success using our adaption (See Methods) of this method. While we observed a few instances where SVs or AZ filaments were labeled with the 6 nm colloidal gold particles conjugated to our secondary antibodies (Figure 15A), these were too infrequently encountered to quantify reliably. We thus developed our own immunogold labeling technique.

We had two major concerns that we wanted to address when developing our new technique. The first was how to improve the access of our antibodies into the SSM interior without resorting to methods that would severely disrupt the presynaptic ultrastructure such as the use of detergents. Dr. Stanley and Arup Nath, then a graduate student in the Stanley laboratory, had recently developed a new method of introducing foreign compounds into intact SSMs through freeze-thawing that they had christened “cryoloading” (Nath et al., 2014). Cryoloaded SSMs are still capable to recycling SVs and look intact under EM. While cryoloading had not been tested on SSM ghosts during its initial development, we were confident that the compromised plasmalemma (even after resealing) and lack of free cytoplasm of the ghosts would only serve to make the technique more effective. The only drawback of cryoloading is that only a percentage of SSMs would take up the payload, but this percentage turned out to still be greater than what I had when using only passive diffusion.
The second concern that we had revolved around our choice of secondary antibodies. Our initial attempts at immunogold labeling used 6 nm colloidal-gold secondary antibodies, where the gold particles were merely adsorbed to the antibodies by electrostatic forces (Faulk and Taylor, 1971). Thus, there was no way of knowing the ratio of antibodies and gold particles, and also no way of knowing whether the gold particles were still associated with the secondary antibodies after cryoloading. Upon the suggestion of Dr. Milton Charlton, I began using Nanogold®-conjugated secondary Fab (See Methods). These 1.4 nm-large nanogold particles are covalently-bound to the Fab at a near 1-to-1 ratio, which greatly reduces the likelihood that the nanogold particles will dissociate during sample preparation and deposit elsewhere. We also expected the smaller size of the nanogold-coupled Fab as compared to full sized antibodies conjugated with 6 nm colloidal gold to have improved access to antigenic targets. We had concerns that the nanogold particles would be too small to visualize or to differentiate from the osmium tetroxide and uranyl acetate staining. Fortunately, likely due to the polyclonal origin of the secondary Fab, nanogold labeling appear as multi-pixel clusters of gold particles (Figure 15B) which were generally more electron-dense than the heavy metal staining. These clusters are what we decided to quantify as positive labeling. The only regions where we had difficulty distinguishing between heavy metal staining and nanogold labeling were phospholipid bilayers (an example is noted in Figure 16). Thus, it is possible that our analyses underestimate the amount of labeling of particularly electron-dense membranous regions such as the AZ membrane. However, since antibodies are unlikely to be embedded within the phospholipid bilayer, this underestimation is likely minimal.

5.4 Immunogold Labeling of the CaV C-Terminus

Using our new immunogold labeling technique, I cryoloaded ghosts with a combination of L4569 and L4570 antibodies, which we termed L45. The idea, suggested by Dr. Stanley, behind combining the two polyclonal antibodies was to further maximize our labeling efficacy and does not introduce any complications since both L4569 and L4570 were raised against the same antigenic peptide. L45-labeled ghosts generally had more gold clusters than their paired rabbit IgG control-labeled ghosts, which suggested to us that the labeling was real. In fact, there were many exciting images where we could observe gold clusters near the junction of SVs and their tethers that were perfectly consistent with our hypothesis that the CaV C-terminal tethers SVs (Figure 16, 18). Our worries that these images were almost too perfect and that we were biasing our data were allayed by the knowledge that I was searching for ghost AZs at such a low
magnification (50 Kx) that I would be unable to tell if the ghost had gold clusters until I increased the magnification to 500 Kx or 530 Kx. To prevent myself from biasing the data, I made sure to take a high-magnification image of every ghost AZ I came across while scanning at low magnification.

We quantified the gold cluster number and localization in two different ways. Compartment analysis (See Methods and Figure 17A), which partitions every ghost analyzed into their component regions and then plots the total number of gold clusters in each compartment to the summed area enclosed by each compartment, gives us a broad overview of whether the L45 antibodies were labeling certain presynaptic structures beyond what we would expect for non-specific labeling as represented by the control IgG-labeled ghosts. The result of this analysis (Figure 17B) suggested that L45, and thus the CaV2.2 C-terminus, was specifically localized to the AZ membrane and SVs, as well as filaments extending from the AZ to a lesser extent. The second, more direct analysis method was to count the number of gold clusters associated with individual tethered SVs found in our images of L45-labeled ghosts. I further kept track of whether the gold labeling were within 10 nm (to account for the length of the antibody and the Fab fragment) of the AZ membrane or the SV, while the remainder were assigned to the intervening tether. When compared to the number and distribution in the IgG control-labeled ghosts, the gold clusters on individual tethered SVs in L45-labeled ghosts were significantly greater on SVs and their tethers, but not near the AZ membrane (Figure 18). The fact that compartment analysis identified CaV C-termini on the AZ membrane and SVs but the individual tethered SV analysis found insignificant amounts of L45 labeling near the AZ membrane region of tethers was excitingly consistent with our hypothesis that the CaV C-terminal extends from the AZ membrane to tether SVs in the presynaptic cytoplasm.

5.5 Determining whether the CaV pore is on the AZ or SVs

Although we were relatively content that the results of the L45 experiments were strongly supportive of our hypothesis, we sought alternative interpretations of the data in order to be thorough. One such interpretation is that there is a population of CaVs whose transmembrane domains were embedded in SV membrane instead of in the AZ membrane, and that the L45 antibodies were labeling this SV-residing population of CaVs that had nothing to do with tethering. While biochemical (Wong et al., 2013) and mass spectrometric (Takamori et al.,
studies have not detected the pore-forming portion of CaVs on SVs, a single EM immunogold study claims to have found such results (Zhang et al., 2000). Upon further examination, the anti-CaV antibody (commercially available from Alamone Labs) used in the EM immunogold study appears to be targeted against the synprint region of the CaV II-III intracellular loop. As discussed above in 5.2, but not taken into account by Zhang and colleagues, the II-III loop has the second most amino acids of any intracellular domain of CaV2.2 after the C-terminal and could potentially extend ~65 nm away from the plasmalemma. Intriguingly, the synprint region is also the original site suggested to bind to SVs (Sheng et al., 1998). As it turned out, we also have a well-characterized antibody, Ab571, that is targeted against the same region of CaV2.2 (Li et al., 2004) which allowed us to investigate the role of the II-III loop as well as test whether our L45 labeling was of SV-residing CaVs.

I carried out the Ab571 immunogold labeling in the same fashion as I had carried out the L45 immunogold labeling. Compartment analysis of Ab571 labeled ghosts suggested that the CaV synprint region could be localized to the AZ membrane and to SVs (Figure 19). This result is consistent with both the previous biochemical reports that the CaV synprint binds SVs (Sheng et al., 1998) and the immunogold labeling report suggesting that CaVs are entirely located on SVs (Zhang et al., 2000), but does not answer the question of whether the CaV C-terminals labeled by L45 originate from the AZ or from SVs. To properly investigate this issue, I measured the distances of the gold clusters on individual tethered SVs along their tethers. If the CaVs I was labeling with L45 and subsequently with Ab571 were completely on SVs instead of originating from the AZ membrane, we would expect the distances of the gold clusters from the AZ membrane to be the similar for the two antibodies. This turned out not to be the case, as gold clusters on Ab571-labeled individual tethered SVs were generally much closer to the AZ membrane than those on L45-labeled individual tethered SVs (Figure 20). Since the CaV2.2 C-terminal can theoretically extend much farther away from the AZ than the II-III loop, the difference in gold cluster distances from the AZ would suggest that the CaVs that I am labeling have their transmembrane pore domains in the AZ membrane and not SV membrane. It is likely that the immunogold labeling experiments using anti-CaV2.2 antibodies by Zhang and colleagues had similar results, but were misinterpreted as evidence that the CaV2.2 pore is on SVs since they never accounted for the location of the antibody’s epitope. In fact, 50% of the
Ab571 gold clusters on tethered SVs were within 45 nm of the AZ, which is well within the projected maximum of 65 nm estimated from the number of amino acids in the II-III loop.

The finding that the II-III synprint region can be localized to SVs according to our compartment analysis is exciting, however, in that it is consistent with previous biochemical studies suggesting that this region binds SVs (Sheng et al., 1998) and has great implications for a SV tethering system. While an up-to 200 nm C-terminal-based G-tether is an attractive option for tethering distant SVs, these SVs would be much too far beyond the 25 nm threshold to reliably detect the calcium influx from the CaV pore (Stanley, 1993, 1997, 2016; Weber et al., 2010). While a \( \leq 65 \) nm long tether based on the II-III loop can still be too far from the CaV for \( \text{Ca}^{2+} \) nanodomain gating, it may be part of a tethering system that progressively brings the SV closer to the CaV where it can dock and participate in fusion.

5.6 Disrupting SV tethering

One of the curiosities of the L45 immunogold labeling experiments was the paucity of gold clusters on filaments extending from the AZ but not terminating in a SV. While it is possible that the kinetic force caused by the osmotic rupturing of SSMs has artificially removed SVs from their tethers, it is easy to imagine that at least a population of these free-floating filaments are SV tethers that have not yet grabbed a SV. This concept has been proposed before (Landis et al., 1988; Phillips et al., 2001), although such real-time events are difficult to capture using EM. Compartment analysis of the L45-labeled ghosts had gold cluster labeling of filaments at the threshold of being more significant than control labeling, we would have expected to see much greater L45 labeling of filaments if they were CaV C-terminals. One explanation to explain this observation, or lack thereof, was that the tip of the CaV C-terminal spends most of its time either at the AZ membrane or bound to an SV. We thus wondered whether we could better catch the CaV C-terminal in its SV-less form by disrupting the tethering process.

Dr. Stanley and Dr. Sabiha Gardezi, then a graduate student in the laboratory, had recently developed blocking peptides that have been shown to disrupt SV-CaV binding in biochemical assays and SV-recycling in functional styryl-dye uptake assays (Gardezi et al., 2016). We predicted that if I was to introduce these blocking peptides into our SSMs prior to osmotic rupture into ghosts, I would be left with a larger population of CaV C-terminals that have not grabbed an SV and that I would get a correspondingly larger population of free AZ filaments.
labeled by L45 antibodies. While we had previously been able to identify using fluorescent microscopy which SSMs had been cryoloading with the blocking peptide by co-cryoloading with a fluorescent dextran marker, I was unable to employ the same strategy for my EM experiments as any marker would be flushed out in the osmotic rupture. We thus had a batch of our blocking peptides engineered with TAT-tags (Fawell et al., 1994) with the assumption that these TAT-conjugated peptides would have access to the entire population of SSMs in a sample. However, the use of these still-poorly-understood TAT-tags (Brooks et al., 2005) led to many complications with sample preparation and imaging (Figure 21). Thus, the reason behind why we had relatively little L45-labeling of free filaments remains a mystery.

5.7 Antibodies against central regions of the CaV C-terminal

If the CaV C-terminal extends from the AZ membrane to tether SVs, as suggested by the results of our L45 and Ab571 immunogold labeling experiments, it stands to reason that central regions of the C-terminal can be localized to parts of the filaments spanning between the AZ and the SVs. To test this hypothesis, we generated another two antibodies against the CaV C-terminal (NmidC2 and C2Nt; Figure 22). NmidC2 is targeted to a stretch of amino acids near the middle of the CaV2.2 C-terminal, while C2Nt is against a region almost a quarter of the way away from the AZ membrane in the CaV2.1 C-terminal that shares strong homology with CaV2.2. The reasoning behind making C2Nt against both CaV2.1 and CaV2.2 was to maximize our chances for immunolabeling CaVs at the AZ. The vast majority (94%) of synaptic transmission in chicks have traditionally been attributed to CaV2.2 activity that can be blocked by ω-conotoxin GVIA (Kasai et al., 1987). However, a recent discovery that zebrafish CaV2.1 activity is also sensitive to this toxin (Wen et al., 2013) led us to design the C2Nt antibody against both CaV2.1 and CaV2.2 in case the same sensitivity could be applied to chick CaV2.1. Based on the number of amino acids, I projected the NmidC2 antigenic region to be ~120 nm away from the AZ membrane if the C-terminal is fully extended, while the C2Nt antigenic region should be ~50 nm away (Figure 22A).

Both antibodies were characterized using biochemistry and fluorescent immunocytochemistry. In both cases, western blots of chick ghost lysate probed with either NmidC2 or C2Nt identified a band well above 250 kDa (Figures 23A, 25A). Such a band is consistent with what we have previously observed with Ab571 probing of boiled samples (Khanna et al., 2006), while non-
boiled samples typically yield bands at around 250 kDa (Li et al., 2004). It should be noted that when we probed samples with Ab571 alongside NmidC2, the >250 kDa band identified by NmidC2 were slightly higher than the Ab571-identified band. We have no explanation for this discrepancy at this time, but our concerns were attenuated by experiments where NmidC2 was able to identify the CaV2.2 immunoprecipitated by Ab571, and this identification was even sensitive to the blocking of the NmidC2 antibody using the NmidC2 antigenic peptide (Figure 23B). Apart from the band above 250 kDa, C2Nt also identified a lower band at ~250 kDa. While we no longer had a well-characterized antibody against chick CaV2.1, the Stanley laboratory had previously found that the CW65 antibody against CaV2.1 identified a band lower than the CaV2.2 band identified by Ab571 (Li et al., 2004). While both of these bands run at a higher molecular weight with boiled samples, we suspect that the second, lower band identified by C2Nt at ~250 kDa may be CaV2.1. Western blots of a specifically anti-CaV2.1 PmidC2 antibody that we were developing at around the same time for another project also identified a band at around 250 kDa (Figure 28A). One concern we had with our western blot experiments using our NmidC2 and C2Nt antibody was that there were other bands that were identified by these antibodies that were not at molecular weights normally attributed to CaVs. We understood that this would be the case when using non-affinity purified antibodies and assumed that the probability that L45, NmidC2, and C2Nt would all identify the same presynaptic non-CaV protein to be sufficiently low as to be absurd. I did affinity-purify the NmidC2 antibody for use in my EM experiments (Figure 29). However, while western blots using this purified NmidC2 antibody showed that the purification worked by identifying a band corresponding to where we would expect CaV2.2 to be and no other bands, the blots also showed that there was a loss of the specific NmidC2 antibody as the signal-to-noise ratio was not favourable (Figure 23B). Thus, I did not affinity purify any of the other antibodies for my other experiments.

We assume that fluorescent immunocytochemistry (ICC) would be the technique most similar to EM immunogold that did not require multiple weeks of sample preparation and data analysis, and thus would be the best test for the viability of our antibodies. The Stanley laboratory had previously characterized our anti-CaV2.2 Ab571, L4569, and L4570 antibodies using ICC on chick ciliary ganglion (CCG) preparations (Khanna et al., 2006; Li et al., 2004). The presynaptic calyx structures in this preparation (Stanley and Goping, 1991) are large and thick enough to allow transmitter release sites to be observed using light microscopy. Anti-CaV2.2 ICC of CCGs
typically show punctate-like staining patterns that are believed to be the transmitter release sites. NmidC2 and C2Nt ICC of CCGs show this pattern as well, while their respective pre-immune controls do not (Figures 24, 26), which we interpreted to be confirmation that these antibodies do indeed target CaV2.2. C2Nt was designed to also target CaV2.1 and we decided to test this on cerebellar slices. The Purkinje cells in the cerebellum were where CaV2.1 was first identified (Llinas et al., 1989) and could be identified using staining against calbindin. The C2Nt antibody did indeed stain the cerebellar slices (Figure 27A), especially in the region where Purkinje dendritic trees and previous anti-CaV2.1 staining have been described (Westenbroek et al., 1995). This staining co-localized well with the calbindin-staining (Figure 27B) and the overall staining shared many similarities with that of the CaV2.1-specific PmidC2 antibody. This PmidC2 antibody stained neither the chick CCG calyces nor the calyces in the chick magnocellular nucleus, which have been described to be mostly gated by CaV2.2 (Sivaramakrishnan and Laurent, 1995). Curiously, apart from the calbindin-positive Purkinje cells, the PmidC2 antibody also appeared to stain smaller cells intermixed with the Purkinje dendrites (Figure 27). These cells were not stained by the C2Nt antibody, and we were unable to establish the identity of these smaller cells and also unable to find an explanation for why PmidC2 stained them while C2Nt did not. It is possible that one of the non-CaV bands observed in the western blots probed with PmidC2 (Figure 28A) may correspond to a protein expressed in these smaller cells, but we currently do not have any evidence for such a hypothesis. Regardless, the staining of calbindin-positive Purkinje neurons in chick cerebellum slices strongly suggested that both the C2Nt and PmidC2 antibodies will target CaV2.1.

5.8 EM localization of central regions of the CaV C-terminal

EM immunogold experiments using either NmidC2 or C2Nt antibodies were consistent with our hypothesis that CaV C-terminals tether SVs (a summary of the immunogold labeling results for the various antibodies used in this thesis can be found in Table 2). Compartment analyses of ghosts treated with either antibody showed that the only structure specifically labeled in either set of experiments were filaments extending from the AZ (Figures 29, 31). This was in contrast to the AZ- and SV-specific labeling observed with L45, and further supports the hypothesis that the C-terminal extends from the AZ membrane to tether SVs. While not all filaments extending from the AZ that do not terminate in an SV are necessarily unoccupied SV tethers, it’s a reasonable assumption that at least some of them are tethers. The fact that enough of these
filaments have been labeled by antibodies against the CaV C-terminal to reach statistical significance lends credence to this assumption.

<table>
<thead>
<tr>
<th>Antibody Used for Immuno-Nanogold Labeling</th>
<th>Compartment Analysis</th>
<th>Individual Tethered SVs</th>
<th>Ranked Proximity of Nanogold Labeling (1st is closest to AZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L45</td>
<td>SVs, AZ, Memb, FM</td>
<td>SVs, tethers, AZ</td>
<td>4</td>
</tr>
<tr>
<td>Ab571</td>
<td>AZ, SV</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>NmidC2</td>
<td>FM</td>
<td>SVs, tethers, AZ</td>
<td>3</td>
</tr>
<tr>
<td>C2Nt</td>
<td>FM</td>
<td>Tethers, AZ</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. Summary of Results of Immunogold Labeling.

Antibodies used for immunogold labeling (see Figure 22 for antigenic targets) in this thesis are listed in this table along with structures that they label to a statistically significant extent as analyzed by either Compartment Analysis or Individual tethered SVs. Note: NmidC2 result for individual tethered SV analysis is based off the antibody-blocking experiment. Antibodies are also ranked by how close their associated nanogold clusters were from the AZ membrane according to the 50% nanogold cluster frequency distances in Figures 20 and 33. AZ: active zone, SV: synaptic vesicle, FM: filament.

The analysis of individual tethered SVs labeled with C2Nt or NmidC2 antibodies also yielded very interesting results. We began these experiments with the prediction that, assuming the CaV C-terminal follows a simple structure, antibodies against more proximal regions (i.e. C2Nt) would label points of SV tethers closer to the plasmalemma than antibodies against more distal regions (i.e. NmidC2, and then L45). C2Nt appears to exclusively label the middle and base of SV tethers, and we did not observe a single occurrence of C2Nt labeling the SV portion of individually tethered SVs (Figure 32). In contrast, NmidC2 and L45 both labeled SVs, the base of the tethers, and the tethers themselves (although NmidC2 labeling of the tether itself only reached a p value of <0.06 as compared to controls). When I measured the distances of the gold particle clusters along tethers associated with each antibody, it was readily apparent that C2Nt labeling was closer to the plasmalemma than NmidC2 labeling, which was itself closer to the plasmalemma than L45 labeling (Figure 33). All of these results are consistent with our
prediction that the antibody labeling patterns should roughly follow the regions from which their antigens were selected and the visibility of this pattern was an interesting suggestion that the CaV C-terminal is a relatively linear structure, at least when it is in the midst of tethering SVs. Such a claim will have to be further investigated in the future through a different system as it is entirely possible that the osmotic rupture necessary to generate ghosts has “unraveled” the C-terminals from their native configuration. However, the long, linear structures observed in other studies (Cole et al., 2016; Hirokawa et al., 1989) would support our observations as well.

The NmidC2 labeling of SVs when analyzed through individual tethered SVs was a further surprise (Figures 30, 32). Our biochemical study into the location of the SV-tethering region of the CaV C-terminal suggested that it was near the distal tip of the C-terminal (Gardezi et al., 2016), while the NmidC2 antigenic region is located roughly in the middle of the C-terminal. We were able to think of only two reasonable explanations for why both the middle and the distal tip—as suggested by the L45 experiments—of the C-terminal would also be in contact with the SV. First, it’s possible that the C-terminal tether folds or rolls upon itself with the tethered SV. This process could be one of many responsible for bringing the SV from deep in the cytoplasm to the transmitter release site in the AZ prior to docking and fusion. Second, NmidC2 labeling of SVs could suggest a second SV-binding site that is proximal to the one Dr. Stanley and Sabiha had previous located using biochemical means, which had focused on the distal third of the C-terminal or the whole channel until now (Gardezi et al., 2016). These two possibilities are not mutually exclusive, and it will be interesting to see whether future studies will confirm or refute them. A study published in 2016 by Cole and colleagues also suggested that long SV tethers have multiple SV-binding sites (Cole et al., 2016). However, we rarely observed tethers that have multiple SVs attached to them but are unsure of whether this is an artifact caused the osmotic rupturing process. It should be noted that while compartment analysis of NmidC2 labeled ghosts did not identify SVs as being statistically significantly labeled (Figure 29), the graph clearly shows that there was greater labeling with NmidC2 and control rabbit IgG. The discrepancy between the two analyses is likely because compartment analysis is inherently less sensitive but more broad than analyzing individual tethered SVs, as the controls for all compartments contribute to the 95% prediction zone.

Our confidence in our NmidC2 results were bolstered by the experiments where I blocked the NmidC2 antibody using its immunogen (Figure 30). First, these experiments were an
independent verification that the antibody labels SV tethers. Since blocked NmidC2 antibody failed to identify fusion proteins corresponding to the CaV C-terminal (Figure 23D), this was further evidence that these terminals must be SV tethers. Second, sham-blocked NmidC2 labeled tethered SVs to a significantly greater extent than actual-blocked NmidC2. This result is consistent with the one we observed in our previous use of NmidC2 for EM immunogold experiments and supports the argument that the NmidC2 antigenic site is close to an SV-binding site on the CaV C-terminal under certain yet-to-be-determined conditions.
6 Implications of the CaV C-terminal tethering SVs and Future Directions

The combination of L45, NmidC2, and C2Nt labeling of various components of tethers and SVs provides compelling evidence that the CaV C-terminal is an SV tether. From observations made by us and other groups, there appears to be multiple tethers of varying lengths (Cole et al., 2016; Fernández-Busnadiego et al., 2013; Wong et al., 2014). In our study, we have classified tethered SVs that are farther than 45 nm from the AZ membrane to be tethered by a longer G-tether (Figure 34). Since the CaV C-terminal possesses well over 600 amino acids in a mostly disordered conformation, we strongly suspect that the C-terminal is one of the, if not the only, candidate G-tethers. We also know the calcium sensor, presumably on the SV itself, that detects the rise in calcium and triggers SV fusion should theoretically be within 25 nm of the CaV pore (Fogelson and Zucker, 1985; Simon and Llinás, 1985; Stanley, 1993; Weber et al., 2010), which is much closer than the observed G-tethers. This begs the question of the purpose of such a long tether. Our proposal that G-tethers “grab” SVs that are far in the presynaptic lumen would suggest that some mechanism then draws or propels these G-tethered SVs to the transmitter release site. Unfortunately, this mostly EM-based study is limited in its inability to capture tethering and the subsequent pre-fusion steps in real-time. We have observed that our gold clusters corresponding to L45 and NmidC2 labeling still persist on SVs that are within 45 nm of the AZ membrane (Figure 33) which would suggest that the C-terminus “follows” the SV on its journey to the AZ membrane. Whether the CaV C-terminal has some intrinsic retraction properties or some other mechanism, perhaps itself calcium-triggered, is responsible for actually moving the SV will be an interesting future direction for SV-tether research. One intriguing piece of this puzzle came when we pooled all the tether length data from our subsequent immunogold labeling experiments with the initial tether length data (Figure 14). While not strictly part of our current study, the frequency histogram of this pooled data can be plotted with functions possessing 3 or 4 peaks depending on the binning of the data (Figure 35). The second of these peaks ranges from being 20 to 32 nm away from the AZ membrane, which is attractively consistent with the estimated 25 nm distance of the CaV pore from the SV-based calcium sensor. Whether this is coincidence or whether these peaks reflect certain distances that SVs are kept at in a physiological system will have to be determined by future studies.
Figure 35. Frequency Histograms of Tether Lengths with Larger Sample Size.
Frequency histograms (similar to those of Figure 14) of the lengths of filaments connecting SVs to the AZ. Data from experiments done since the publication of Figure 14 in Wong et al., 2014 have been added so that the sample size is now 119 tethered SVs from 24 separate experiments. These lengths were counted using (A) bins of 4 nm, (B) bins of 5 nm, or (C) bins of 6 nm. The frequencies were then plotted and curves with 3 to 4 peaks were fitted to the data. (D) The lengths (in nm) corresponding to each peak were tabulated according to the bins used in the frequency histograms, and the averaged length value of each peak is presented in bold, italic font at the bottom of the table.

It would also be very worthwhile to determine what governs this putative contraction of the C-terminal tail. The electron tomography study published by the Reese group suggested that the docking and fusion of closely-tethered SVs may pull farther-tethered SVs on the same tether towards the AZ membrane (Cole et al., 2016). Another attractive hypothesis is that either the influx of calcium through the CaV or even the pore conformation change in response to the depolarization of the AZ membrane may cause the C-terminal tail to contract. Both the CaV1 and CaV2 families contain an “IQ” motif on the C-terminal tail near the pore that interacts with
calmodulin and allows the CaV to “sense” rises in Ca$^{2+}$ (Liang et al., 2003). While this interaction is thought to regulate calcium-dependent inactivation of CaVs, it is not hard to imagine that a similar system may regulate the length of CaV C-terminal tail tethers. Using EM, it may be possible to test the calcium hypothesis by changing the Ca$^{2+}$ concentration and measuring the length of tethers either within SSM ghosts or intact SSMs. The voltage-dependent hypothesis can be tested in a calcium-free preparation of ghosts or SSMs that are depolarized using high-potassium and then rapidly slam-frozen prior to being prepared for EM (Watanabe et al., 2013). The hypothesis presented by the Reese group could be tested using pharmacological compounds such as alpha-latrotoxin (Henkel and Sankaranarayanan, 1999) that trigger SV release without an elevation in intracellular calcium.

The other, shorter L-tethers also remain to be identified by future studies. We have observed up to 4 concurrent SV tethers (Figure 13) which is consistent with what has been described in other studies (Cole et al., 2016; Fernández-Busnadiego et al., 2013). It is a reasonable assumption that at least one of these tethers is a retracted G-tether since L45 and NmidC2 labeling was observed with both short and long tethers (Figure 33). Our observation that Ab571 immunogold labels SVs that are close to the AZ membrane (Figures 19 and 20) would suggest that one of the other tethers is the CaV II-III loop, which is consistent with biochemical studies from other groups (Seagar and Takahashi, 1998; Sheng et al., 1998). However, it remains to be seen if the SV binding site on the II-III loop suggested by our findings is in fact the synprint site. It will also be very interesting to see exactly how calcium affects this relationship. It is possible, but unlikely, that the remaining 2 or so tethers are components of a second CaV since that would be inconsistent with single-CaV gating of SV fusion. Allowing for a second, distant CaV’s intracellular domains to bind the same components on SVs would lead to tug-of-war-like complications as the SV is brought closer to the AZ membrane and could result in the SV landing on a site that is remote from the calcium influx of either CaVs. While another layer of regulatory complexity could prevent such an event from occurring, there has been no indication yet as to the existence of such a molecular switch for tethering.

One of the largest remain pieces of the SV-tethering puzzle is the identity of the protein on the SV that binds to the tethers (SV Tethering Protein, or SVTP). In order to prevent tethering by multiple CaVs (see above), this SVTP would likely exist in relatively low-copy number or have some regulatory switch to prevent errant tether attachment. One previous study has suggested
that RIM and RIM-BP (RIM binding protein) as a binding partner for the CaV C-terminal (Kaeser et al., 2011), but our more recent work has strongly argued against these two candidates (Gardezi et al., 2016; Wong et al., 2014). Studies on the CaV II-III loop have suggested SNAP-25, VAMP, syntaxin, and synaptotagmin as binding partners (Charvin et al., 1997; el Far et al., 1995; Seagar and Takahashi, 1998; Sheng et al., 1998; Yoshida et al., 1992). The high copy number (an average of 69.8) of VAMP estimated to be on each SV (Takamori et al., 2006) makes it an unlikely candidate, while there are caveats for the binding of the other candidates (see Introduction). It is also completely unknown whether SVTPs are shared between the II-III loop and the C-terminal of CaVs. At one point in this study, we had the notion of using antibodies against a panel of known SV-associated proteins in EM immunogold experiments to identify the SVTP. However, now that we have a better appreciation of how long it takes to conduct such experiments as well as how difficult it is to account for the inherent length of antibodies when working at EM scales, we believe that other approaches such as biochemistry or mass spectroscopy will be more suitable as a first step in identifying SVTPs. The simplest of such biochemistry experiments, at least conceptually, would be to immobilize beads with peptide sequences mimicking the SV binding motif and then using these beads to “fish” for prey protein in solubilized SV lysate. The prey protein could then be identified through western blotting or mass spectrometry. These other approaches will have their own difficulties and limitation, but knowing that the CaV C-terminal is a binding partner to the SVTP provides a foundation for these future studies.
7 Conclusion

Until recently, SV-tethering has been somewhat of a “black box” in that SVs somehow went from being transported to the presynaptic terminal to being docked to the AZ membrane in preparation for fusion without a well-described mechanism or underlying structure. On one hand, EM structural studies have revealed that SV-tethering exists in situ (Cole et al., 2016; Fernández-Busnadiego et al., 2013; Hirokawa et al., 1989; Landis et al., 1988; Siksou et al., 2007), but without strong evidence for the identity of these tethers. On the other hand, biochemical studies have described the interaction between SVs and SV-associated proteins with CaVs (Gardezi et al., 2016; Seagar and Takahashi, 1998; Sheng et al., 1998; Wong et al., 2013, 2014) without being able to definitively place these interactions at the exact site of SV tethering. This study is an attempt to bridge these two halves of the SV-tether field and hopefully provide strong evidence that CaV C-terminals tether SVs in situ at the peri-AZ zone.
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