CRYSTALLOGRAPHIC INVESTIGATION AND CHARACTERIZATION OF THE
INTERACTION BETWEEN PRESYNAPTIC VOLTAGE-GATED CALCIUM CHANNELS
AND SNARE PROTEINS

by

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B.Sc., The University of British Columbia, 2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

January 2010

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Abstract

Voltage-gated calcium channels (Ca\textsubscript{v}) have functions ranging from regulating release of hormones and neurotransmitters, generating cardiac action potentials, and excitation-contraction coupling. At nerve terminals, N- and P/Q-type Ca\textsubscript{v}s convert the action potential into a Ca\textsuperscript{2+} signal that in turn triggers neurotransmitter release. Neurotransmitter release requires several components, such as SNARE proteins. SNAREs, as well as many other presynaptic proteins, can interact with Ca\textsubscript{v}s and inhibit them by increasing their inactivation. The interaction is localized in the intracellular loop between domains II and III of the \( \alpha_1 \) subunit, in a domain termed 'synprint' (synaptic protein interaction site). In this study, we tried to solve the structure of the synprint site by crystallography. To date, long needle-shape crystals were obtained; however, the quality of these crystals was not good enough for X-ray diffraction. In addition, isothermal titration calorimetry (ITC) was used to determine the interaction between SNARE protein syntaxin1A and the synprint site. It turned out that not any binding was detected, suggesting that the interaction between SNARE proteins and the presynaptic Ca\textsubscript{v}s, if at all present, is weak.
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List of Abbreviations

ΔH  Change in Enthalpy
ΔS  Change in Entropy
βME Beta-mercaptoethanol
Bov Bovine
CaM Calmodulin
CaMKII Ca2+/calmodulin-dependent protein kinase II
Ca, Voltage-gated calcium channel
CD Circular dichroism
CDI Ca2+ -dependent inactivation
Co-IP Co-immunoprecipitation
DMSO Dimethyl sulfoxide
E. coli Escherichia coli
ESI Electrospray ionization
GST Glutathione-S-Transferase
GPCR G-Protein-Coupled Receptor
IPTG Isopropyl β-D-1 Thiogalactopyranoside
ITC Isothermal Titration Calorimetry
Kd Dissociation Constant
kDa kilo-Dolton
LIC Ligation independent cloning
MBP Maltose Binding Protein
### List of Abbreviations (Continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MOLDI-TOF</td>
<td>Matrix-Assisted Laser Desorption/Ionization-Time of Flight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Rab</td>
<td>Rabbit</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophorosis</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive factor attachment protein receptors</td>
</tr>
<tr>
<td>Stx</td>
<td>Syntaxin 1A</td>
</tr>
<tr>
<td>Synprint</td>
<td>Synaptic protein interaction site</td>
</tr>
<tr>
<td>synA8</td>
<td>BovalBsynA8C784A/E794A/E795A</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>VDI</td>
<td>Voltage-dependent inactivation</td>
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</table>
Acknowledgements

I would like to thank my supervisor, Dr. Filip Van Petegem, for giving me the opportunity to work on this project and all his help during the two and half years. I would also like to thank all the previous and present lab members for help.

Finally, I would like to thank friends from Molday lab and Strynadka lab for kindly helping me with enzymes and antibodies, and friends from Accili lab and Ahern lab for allowing me to use some chemicals and instruments.
1. Introduction

1.1 Voltage-gated calcium channels and synaptic transmission

1.1.1 Overview of voltage-gated calcium channels

Virtually all excitable cells express plasma membrane voltage-gated calcium channels (CaVs) that convert the electrical signals into the cellular activities. Rapid entry of Ca$^{2+}$ through CaVs upon membrane depolarization regulates a wide range of physiological processes such as release of neurotransmitters, excitation-contraction coupling, hormone secretion, gene expression, and activation of calcium dependent enzymes (Sutton et al. 1999, Dolmetsch et al. 2001, Reid et al. 2003).

To date, five different types of CaVs have been identified based on their specific physiological functions, electrophysiological and pharmacological properties, and subcellular distributions (Table 1). They can also be grouped into the high-voltage activated channels (HVA) such as L-, P/Q-, N-, R-type channels which require large membrane depolarization to open and the low-voltage activated channels (LVA) such as the T-type channels that activate in response to smaller membrane depolarization (Catterall 2000).

Channels can also undergo different states. Besides the simple 'open' and 'closed' states, the channels can reside in an 'inactivated' state, a state that prevents passage of ions even when a depolarizing signal is present. When the plasma membrane is depolarized, CaVs open to allow the entry of Ca$^{2+}$, while they are closed as the plasma membrane is hyperpolarized. Channel inactivation typically occurs during membrane
<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+} current type</th>
<th>Primary localizations</th>
<th>Specific blocker</th>
<th>Previous name of ( \alpha ) subunits</th>
<th>Specific functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca\textsubscript{v}1.1</td>
<td>Skeletal muscle</td>
<td>( \alpha _1S )</td>
<td>DHPs</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2</td>
<td>Cardiac muscle</td>
<td>( \alpha _1C )</td>
<td>DHPs</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.3</td>
<td>Endocrine cells</td>
<td>( \alpha _1D )</td>
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<td>Ca\textsubscript{v}1.4</td>
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<td>Ca\textsubscript{v}2.1</td>
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<td>( \alpha _1A )</td>
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</tr>
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<td>Ca\textsubscript{v}2.2</td>
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<td>( \alpha _1B )</td>
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<tr>
<td>Ca\textsubscript{v}2.3</td>
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<td>None</td>
<td>DHPs</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>Ca\textsubscript{v}3.1</td>
<td>Cardiac muscle</td>
<td>( \alpha _1G )</td>
<td>None</td>
<td>Neurotransmitter release</td>
</tr>
<tr>
<td>Ca\textsubscript{v}3.2</td>
<td>Skeletal muscle</td>
<td>( \alpha _1H )</td>
<td>None</td>
<td>Neurotransmitter release</td>
</tr>
<tr>
<td>Ca\textsubscript{v}3.3</td>
<td>Neurons</td>
<td>( \alpha _1I )</td>
<td>None</td>
<td>Neurotransmitter release</td>
</tr>
</tbody>
</table>

(Catterall 2000)
depolarization, thus reducing channel availability. Inactivation is an important process for channels to prevent excess Ca\textsuperscript{2+} to enter the cells when the channels are open. Ca\textsubscript{s} experience two types of inactivation: the intrinsic voltage-dependent inactivation (VDI) and the influx of calcium–triggered Ca\textsuperscript{2+}-dependent inactivation (CDI). Both inactivation processes are regulated by different modulators (such as auxiliary Ca\textsubscript{r}\textsubscript{b} subunits and calmodulin) and different signaling pathways (such as GPCR-mediated pathways and cAMP-dependent PKA) (Catterall 2000, Budde \textit{et al} 2002, Halling \textit{et al} 2006, Findeisen \textit{et al} 2009).

1.1.1.1 Molecular structure of voltage-gated calcium channel

HVA channels consist of the principle pore-forming \(\alpha_1\) subunit (Ca\textsubscript{r}\(\alpha_1\)) in association with two auxiliary subunits: a membrane-anchored, predominantly extracellular, disulfide-linked \(\alpha_2\delta\) subunit (Ca\textsubscript{r}\(\alpha_2\delta\)) and a cytoplasmic \(\beta\) subunit (Ca\textsubscript{r}\(\beta\)). In addition, though it is still controversial, some HVA channels (e.g. Ca\textsubscript{1.1}) are found to have another auxiliary transmembrane \(\gamma\) subunit (Ca\textsubscript{r}\(\gamma\)) (Kang and Campbell 2003; Moss \textit{et al} 2002) (Figure 1). LVA channels also have an \(\alpha_1\) subunit, but whether other auxiliary subunits are also present is still uncertain. However, recent studies show the possibility of \(\beta\) subunit and \(\alpha_2\delta\) subunit existing in T-type channels (Walsh \textit{et al} 2009).

The largest Ca\textsubscript{r}\(\alpha_1\) subunit is approximately 190kDa. The Ca\textsubscript{r}\(\alpha_1\) subunit is the core of all functional Ca\textsubscript{s}, defines the channel subtypes and incorporates the ion conduction pore, the voltage sensor, the gating apparatus and several sites that allow channel modulation (Catterall 2000, Kisilevsky and Zamponi 2008). The Ca\textsubscript{r}\(\alpha_1\) subunit is composed of four
Figure 1 Architecture of the voltage-gated calcium channel. The pore-forming Ca,α1 subunit consists of four homologous domains (I - IV), connected by cytosolic loops and flanked by cytosolic N- and C-termini. The cytoplasmic Ca,β subunit associates with the Ca,α1 subunit through the I–II linker alpha interaction domain (AID). The Ca,α2δ subunit is composed of the transmembrane δ subunit and heavily glycosylated extracellular α2 subunit, which are linked by disulfide bonds. The Ca,γ subunit is another transmembrane protein with cytoplasmic N- and C-termini. (Kisilevsky and Zamponi 2008)
homologous domains I-IV, each of which consists of six transmembrane segments (S1-S6). S1 to S4 are considered to be the voltage-sensing domain, whereas S5, S6 and the P-loop between them are believed to form the conduction pore and confer Ca\textsuperscript{2+} selectivity (Figure 1). The S4 segment contains characteristic positively charged residues (Arg and Lys). Upon membrane depolarization, these residues sense the potential change, and the S4 segment undergoes rotation and translation. Via the S4-S5 linker helix, the S4 motion exerts force on the major pore-forming S6 segment to “pull” the channel open (Borjesson and Elinder 2008). The four pore-lining P loops between S5 and S6 contain an EEEEE locus, which is believed to form the selectivity filter for Ca\textsuperscript{2+} ions (Sather and McCleskey 2003). In addition, the cytoplasmic loops between domain I, II, III and IV (I-II loop, II-III loop and III-IV loop, respectively), and the N- and C-terminal regions provide crucial sites for regulatory and adaptor proteins, as discussed later. So far, ten different \( \alpha_1 \) subunits have been identified, divided into three families (\( \alpha_1 \), \( \alpha_2 \) and \( \alpha_3 \)) (Catterall 2000, Lipscombe et al 2002, Bell et al 2004).

The auxiliary intracellular \( \alpha_\beta \) subunit, approximately 55 kDa, has multiple effects on \( \alpha \) by increasing the channel expression, altering the membrane trafficking, and modulating the biophysical properties on the channel. This is also the only calcium channel subunit for which crystal structures are available (Chen et al 2004, Van Petegem et al 2004). To date, four different genes coding for \( \alpha_\beta \) subunits have been cloned (\( \alpha_\beta 1, \alpha_\beta 2, \alpha_\beta 3 \) and \( \alpha_\beta 4 \)). All the \( \alpha_\beta \) subunits possess a common-core structure, which consist of a Src homology 3 (SH3) domain and a guanylate kinase (GK) domain (Figure 2). The GK domain of \( \alpha_\beta \) associates with \( \alpha \alpha_1 \) through a high-affinity interaction with a region in the I-II loop of \( \alpha \alpha_1 \) (Figure 2) (Van Petegem et al 2004). \( \alpha_\beta \) has several
effects on the channel: increased trafficking to the plasma membrane, hyperpolarization of the G-V curve, and modulation of the inactivation. (He et al 2007).

To date, four distinct auxiliary α2δ subunits (α2δ-1, α2δ-2, α2δ-3 and α2δ-4) have been described (Klugbauer et al 1999, Qin et al 2002). The extracellular α2 domain and the transmembrane δ domain are the products of the same gene, and the precursor polypeptide is cleaved post-translationally into α2 and δ, which are linked by disulfide bonds (Jay et al 1991). The α2 domain is extensively glycosylated, which has been found to be essential for the stability of the interaction with the α1 subunit (Gurnett et al 1997). The Ca,α2δ subunit assists in the channel trafficking, (Arikkath and Campbell 2003, Klugbauer et al 2003), and alters activation and inactivation kinetics (Klugbauer et al 1999, Hobom et al 2000, Yasuda et al 2004, Canti et al 2005). The α2δ subunit is one of the targets of anti-epileptic and anti-allodynic drugs gabapentin and its analogs (Field et al 2006).

To date, eight different Ca,γ subunits (Ca,γ1- Ca,γ8) have been cloned (Freise et al 2000). The Ca,γ subunit is a glycoprotein with four transmembrane domains and intracellular N- and C- termini (Jay et al 1990). So far, only Ca,γ1 has been proven to be part of Ca,1.1 and Ca,1.2; however, the role of Ca,γ1 is still unclear (Curtis and Catterall 1985, Kuniyasu et al 1992). γ2-γ8 have not been identified as the Ca,s subunits, but have been suggested to play a role in AMPA glutamate trafficking (Tomita et al 2003).

1.1.1.2 Modulation of voltage-gated calcium channels

Ca\textsuperscript{2+} is one of the essential second messengers for a variety of cellular activities; however, excessive intracellular Ca\textsuperscript{2+} is very toxic. Therefore, tight regulation of Ca,s
Figure 2 The structure of the core of Cavβ subunit-AID complex. The core protein is made up of a guanylate kinase (GK) domain (purple) and SH3 domain (green). The alpha interaction domain (AID) is shown in red, and the arrow indicates the direction in which the AID connects to transmembrane segment IS6. (Van Petegem et al 2004)
that controls Ca\textsuperscript{2+} entry is crucial for normal cell physiology. A large number of proteins have been identified to regulate the activity of Ca\textsubscript{s} such as channel gating and kinetics (Khanna et al 2007). Here, three major modulations mediated by calmodulin, GPCRs and protein kinases are discussed (Figure 3).

1.1.1.2.1 Regulation of Ca\textsubscript{s} by calmodulin

Calmodulin (CaM) is one of the crucial regulators of HVA Ca\textsubscript{s}, and it serves as the Ca\textsuperscript{2+} sensor for positive and negative Ca\textsuperscript{2+} feedback control. CaM is a ubiquitous Ca\textsuperscript{2+} binding protein that is composed of an N-terminal lobe and C-terminal lobe, and binding of Ca\textsuperscript{2+} to any or all the sites can cause different conformational changes to interact with the target proteins (Hoeflich and IKura 2002, Clapham 2007).

CaM interacts with Ca\textsubscript{s} through an IQ motif (IQXXXRGXXXR) within the C-tail of the channels (Van Petegem et al 2005, Dunlap 2007, Kim et al 2008) (Figure 4). The binding between CaM and Ca\textsubscript{s} drives two opposing Ca\textsubscript{s} feedback modulations: calcium-dependent inactivation (CDI) and calcium-dependent facilitation (CDF) which is the process whereby increased basal Ca\textsuperscript{2+} or repeated depolarization leads to increased Ca\textsuperscript{2+} currents. CDI and CDF are induced by specific lobes of calmodulin. Ca\textsuperscript{2+} binding to C-lobe of CaM (Ca\textsuperscript{2+}/C-lobe) controls CDI in Cav 1.2 but CDF in Cav 2.1; Ca\textsuperscript{2+} binding to N-lobe (Ca\textsuperscript{2+}/N-lobe) governs CDI in Cav 2.1, 2.2 and 2.3 (Van Petegem et al 2005, Kim et al 2008).
Figure 3 The interaction sites of different regulatory proteins of Cav2 α1 subunit. Gβγ binds to N-terminal, C-terminal and I-II loop of Ca,α1 subunit. PKC phosphorylates I-II loop and II-III loop of Ca,α1 subunit. SNARE proteins interacts the Ca,α1 subunit at II-III loop. Ca2+/CaM binds to IM domain (IQ-like domain) and CBD domain within C-terminal. (Catterall and Few 2008)
1.1.1.2 G-protein modulation of $\text{Ca}_\alpha$s

G-proteins usually exist in a heterotrimeric form, which consists of $\text{Ga}$, $\text{Gb}$ and $\text{Gy}$. The activation of the G-protein by G-protein-coupled receptors (GPCR) causes dissociation between $\text{Ga}$ subunit and $\text{GbGy}$ subunit, which can function as a modulator to trigger downstream events in the signaling pathways (Gether et al. 2002, Perez and Karnik, 2005).

G-proteins show inhibitory effect on $\text{Ca}_\alpha$s. The electrophysiological features of voltage-dependent inhibition are a marked current reduction, depolarizing shift of the activation curve, and slower kinetics in both channel activation and inactivation (Artim and Meriney, 2000). The underlying mechanism of voltage-dependent inhibition is the direct binding $\text{GbGy}$ subunit to cytoplasmic regions of $\alpha$1 subunit of $\text{Ca}_\alpha$2 that can render channels difficult to open (Herlitze et al. 1996, Ikeda and Dunlap 1999). Three major interaction sites are the N-terminal region, the intracellular I-II loop, and the C-terminal tail of the $\text{Ca}_\alpha$1 subunit. (Canti et al. 1999, Zamponi et al. 1997, Li et al. 2004, Agler et al. 2005).

1.1.1.3 Modulation of $\text{Ca}_\alpha$s by proteins kinases

Three major kinases that regulate $\text{Ca}_\alpha$s are PKA, PKC and $\text{Ca}^{2+}$/calmodulin-dependent protein kinase II (CaMKII). The activation of cAMP-dependent PKA is under GPCR regulation. The activity of both L-type $\text{Ca}_\alpha$1.1 and $\text{Ca}_\alpha$1.2 is upregulated by PKA (Cachelin et al. 1983). PKA is recruited to L-type calcium channels by A kinase anchoring proteins (AKAPs), which are physically bound to the channels (Fraser et al. 1998, Gray et al. 1998). PKA also potentiates the neuronal $\text{Ca}_\alpha$2.1 (Huang et al. 1998).
Figure 4 Structures of Ca,1 and Ca,2-Ca$^{2+}$/CaM-IQ domain complexes. (a) structure of Ca, 2.1-Ca$^{2+}$/CaM IQ domain complex (b) structure of Ca, 2.2-Ca$^{2+}$/CaM IQ domain complex (c) structure of Ca, 2.3-Ca$^{2+}$/CaM IQ domain complex (d) structure of Ca, 1.2-Ca$^{2+}$/CaM IQ domain complex. The cartoon below the crystal structure is a schematic of the binding mode for each complex and lobe specific function. (Kim et al 2008)
PKC is a family of protein kinases, which can be activated by different second messengers (Nishizuka 1995). PKC plays a dual role in regulating Ca,1.2. The activity of the channel is decreased by N-terminal phosphorylation, but increased by C-terminal phosphorylation (McGee et al 2004, Yang et al 2005). PKC potentiates Ca,2.1 and Ca,2.2 by phosphorylation of the I-II loop of the Ca,α1 subunit, thus preventing Gβγ binding (Zamponi et al 1997). PKC also increases the activity of Ca,2.1 and Ca,2.2 by phosphorylation of the II-III loop to dissociate SNARE protein binding and thus antagonize SNARE protein-mediated inhibition (more elaboration in next two sections) (Jarvis et al 2002). CaMKII is a key downstream effector of Ca,αs. Activation of CaMKII requires interacting with Ca\(^{2+}/CaM\) and autophosphorylation (Lou et al 1989, Hudmon et al 2002). CaMKII phosphorylates and interacts with the α1 subunit of Ca,1.2 to increase calcium-dependent facilitation (Hudmon et al 2005, Lee et al 2006). For Ca,2.1, CaMKII slows down voltage-dependent inactivation, but supports Ca\(^{2+}\)-dependent facilitation by binding instead of phosphorylating the α1 subunit of Ca,2.1 (Jiang et al 2008). CaMKII also increases the activity of LVA Ca,3.2 by phosphorylating the II-III loop of the Ca,α1 subunit (Welshby et al 2003).

1.1.2 Presynaptic proteins in the synaptic vesicle exocytosis

The release of neurotransmitters by synaptic vesicle exocytosis is a key event in neuronal communication, and it requires membrane fusion between vesicles and plasma membranes. The high speed of Ca\(^{2+}\)-triggered synaptic transmission confers the ability of the nervous system to efficiently respond to the immense variety of stimuli. Therefore, synaptic vesicle exocytosis needs to be under precise regulation both spatially and temporally. There are several features in the exocytosis event. Firstly, the synaptic
vesicles filled with neurotransmitters are docked at the specialized sites of presynaptic membranes called active zones, and then a series of priming reactions leave the vesicles in a state that is ready for release. Next, an action potential triggers the opening of Ca, s. The resulting influx of Ca^{2+} then triggers the fusion of the vesicle with the plasma membrane. To date, several presynaptic proteins have been found to be involved in the neurotransmitter release. Due to their involvement with Ca,s, we here describe the major points about SNAREs and synaptotagmin.

1.1.2.1 SNAREs

SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are involved in all the steps of exocytotic and endocytotic pathways in eukaryotes (Joseph and John 2003). SNARE proteins are believed to establish the basic frame for the fusion apparatus and provide the necessary force for membrane fusion (Sollner et al 1993). SNAREs can be divided into two groups: vesicle (v-) SNAREs located on the vesicle membranes and target (t-) SNAREs residing on the plasma membranes. The most distinctive and ubiquitous molecular action of SNAREs is the formation of a stable parallel four helix bundle, referred as SNARE core, by SNARE motifs provided by both t- and v-SNAREs (Figure 5b). According to the “zippering” hypothesis, the N-terminal parts of the SNARE motifs begin to interact, then SNARE domains coil towards the C terminus to form a trans-SNARE, and eventually, the carboxy-terminal linker regions and transmembrane domains of SNAREs bind to each other to assemble a cis-SNARE complex, resulting in membrane fusion (Montecucco et al 2005, Sorensen et al 2006, Stein et al 2009).
In neuronal vesicle fusion, syntaxin1A and SNAP-25 serve as t-SNAREs, while synaptobrevin functions as a v-SNARE. Syntaxin1A contains an N-terminal three helical bundle $H_{abc}$ domain, a SNARE motif and a transmembrane segment (Figure 5a). The NMR studies and fluorescence correlation spectroscopy show that syntaxin1A switches between two conformations: in the closed conformation, the $H_{abc}$ domain partially interacts with the SNARE motif to prevent the formation of the SNARE complex, while in the open state, the SNARE motif is available for binding other proteins (Misura et al 2000, Margittai et al 2003, Chen et al 2008). SNAP-25 is an unfolded soluble protein, consisting of two SNARE motifs separated by a linker that has four palmitoylated cysteine residues, which are responsible for plasma membrane anchoring (Figure 5a).

Synaptobrevin is composed of an unfolded N terminus, a SNARE motif and a transmembrane segment (Figure 5a). Upon binding, syntaxin, SNAP-25 and synaptobrevin undergo disorder-to-order transition to form a stable quaternary structure in 1:1:1 ratio (Figure 5b).

Although the idea that SNAREs provide a basic frame for the membrane fusion is widely accepted, the mechanism underlying the membrane fusion driven by SNAREs is still under debate. One of several proposed mechanisms stated here presents how SNAREs could regulate the membrane fusion. Prior to formation of the SNARE complex, syntaxin1A exists in a highly-ordered cluster form (Sieber et al 2007, Lu et al 2008). SNAP-25 is believed to be bound to syntaxin by the interaction of the SNARE motif without significant alteration of the cluster morphology (Richman et al 2005). The advantage of forming clusters is to provide hot spots on the plasma membrane so that assembly of multiple SNARE complexes can take place simultaneously to trigger
membrane fusion. Increasing amounts of evidence show that successful membrane fusion relies on coordination of several SNARE complexes (Chen et al 1999, Hua and Scheller 2001). As the fusion process continues, multiple syntaxin/SNAP-25 clusters simultaneously recruit synaptobrevins to initiate the formation of SNARE complexes, resulting in gap junction-like structures. Lipid mixing then starts to take place to form a lipid stalk intermediate, eventually leading to a hemifusion state: the outer membranes are merged, whereas the inner membrane still remain intact (Reese et al 2005, Yoon et al 2006, Lu et al 2008). Finally, the fusion pore opens, which is a result of merging of the inner membrane and formation of ring-like \textit{cis}-SNARE complexes.

Most of the researchers agree that the formation of the SNARE complex itself can trigger the membrane fusion, which has been proven by \textit{in vitro} reconstitution experiments. However, the fusion kinetics of \textit{in vitro} reconstitution experiments is 1000 times slower than the fusion rate in synaptic neurotransmission (Weber et al 1998, Bowen et al 2004, Liu et al 2005). Therefore, the SNARE assembly alone is not an efficient fusogen. In addition, the timing for the synchronized neurotransmitter release needs to be strictly controlled: the membrane fusion event does not happen only until the entry of Ca$^{2+}$. Apparently, the random membrane fusion stimulated by SNARE complexes cannot serve this role. Hence, there must be more players involved in the process of synaptic neurotransmission.

1.1.2.2 Synaptotagmin

There are strong evidences showing that synaptotagmin, a vesicle protein, acts as the calcium sensor for fusion in calcium-triggered synaptic vesicle exocytosis (Wang et
al 2006, Rizo et al 2006, Chapman 2008). Synaptotagmin consists of a transmembrane segment, two cytosolic C2 domains (C2A and C2B, which bind three and two \( \text{Ca}^{2+} \), respectively) separated by a linker region (Figure 6) (Rizo et al 2006). Upon \( \text{Ca}^{2+} \) binding, both C2A and C2B domains function cooperatively and penetrate into the plasma membrane through electrostatic interaction with the phospholipid head groups (Bai et al 2000, Rhee et al 2005, Herrick et al 2006). This interaction not only brings the vesicle and plasma membrane into close proximity, but also creates a curved dimple that points toward the vesicles, which can reduce the energy barrier for fusion (Monck and Fernandez 1994, Hui et al 2009). The C2B domain interacts with the syntaxin1A/SNAP-25 t-SNARE complex in a \( \text{Ca}^{2+} \)-dependent manner, suggesting that synaptotagmin might initially help formation of the SNARE complex (Pang et al 2006, Bhatta et al 2006). More interestingly, synaptotagmin binds simultaneously to SNARE complex and membranes, indicating that upon \( \text{Ca}^{2+} \) binding, synaptotagmin can cooperate with SNAREs to bring vesicle and plasma membrane into close proximity and accelerate membrane fusion (Arac et al 2006, Dai et al 2007).

1.1.3 Regulation of presynaptic \( \text{Ca}_{\nu}s \) by SNARE proteins

\( \text{Ca}_{\nu}2.1 \) and \( \text{Ca}_{\nu}2.2 \) are the main \( \text{Ca}^{2+} \) channels expressed in the presynaptic terminals, and the \( \text{Ca}^{2+} \) influx through these two channels is primarily responsible for synaptic transmission (Olivera et al 1994). Since neurotransmitter release is a steep exponential function of the \( \text{Ca}^{2+} \) concentration, the regulation of presynaptic \( \text{Ca}^{2+} \) channels provides a sensitive and efficient means to regulate neurotransmitter release (Mintz et al 1995). The neurotransmitter release occurs in two phases: a fast synchronous
component and a slow asynchronous component (Atluri and Regehr 1998). In the synchronous phase, the release is triggered by the timed Ca\textsuperscript{2+} current, resulting in a large and fast synaptic transmission. The asynchronous release is driven by the residual Ca\textsuperscript{2+} remaining in the nerve terminal after an action potential, and provides a basal amount of neurotransmitter release (Sabatini and Regehr 1996, Hagler and Goda 2001).

1.1.3.1 Functional effects of SNARE proteins on presynaptic Ca\textsubscript{v}s

The close proximity between the primed vesicles and the presynaptic Ca\textsubscript{v}s is crucial for the fast synaptic transmission (Heidelberger et al 1994), and the SNARE proteins are one of the presynaptic proteins that serve this role (Mochida et al 1996). Using pull-down experiments, it was shown that syntaxin1A and SNAP-25 interact with Ca\textsubscript{v.1} and Ca\textsubscript{v.2} through the synaptic protein interaction (synprint) site (Figure 1 and Figure 7a), which is localized on the II-III loop of the Ca\textsubscript{v.1} subunit (Rettig et al 1996, Sheng et al 1997, Kim et al 1997). Since SNAREs and vesicles are present at the active zones, this means that the Ca\textsubscript{v}–SNARE interaction keeps the channel and vesicles in close proximity. Besides keeping the close proximity between the channels and the vesicles, syntaxin1A and SNAP-25 also modulate the activity of the presynaptic channels through interaction with the synprint site.

In particular, the interaction between SNARE proteins and the presynaptic channels inhibits the calcium channel activity by stabilizing slow inactivation of the channel and causing a hyperpolarizing shift in the voltage-dependence of inactivation. It has been proposed that the inhibitory effect of the SNARE proteins on the channels prevents the entry of Ca\textsuperscript{2+} when the vesicles are not ready to release. This interaction also
Figure 5 Cartoon representations of neuronal SNARE proteins and the structure of the SNARE complex. (a) v-SNARE Synaptobrevin consists of a SNARE motif and a transmembrane segment. t-SNARE syntaxin is composed of a Habc domain, a SNARE motif and a transmembrane segment. t-SNARE SNAP-25 is made up of two SNARE motifs. (b) SNARE motifs of synaptobrevin, syntaxin and SNAP-25 form a stable four-helical bundle. The Habc domain of syntaxin is not involved in the formation of SNARE complex. (Rizo et al 2006)
Figure 6 The structure of synaptotagmin. Synaptotagmin consists of two C2 domains (C2A and C2B) and a transmembrane segment (cartoon representation). C2A domain binds three Ca\(^{2+}\), while C2B binds two Ca\(^{2+}\). (Rizo et al 2006)
occupies SNARE proteins from the formation of SNARE complex before the vesicle docking, which is suggested by the competition between synaptotagmin and the synprint site to syntaxin1A in a Ca\textsuperscript{2+}-dependent manner. At low Ca\textsuperscript{2+} levels (below 30\textmu M), syntaxin1A is proposed to interact strongly with the synprint site, while at higher Ca\textsuperscript{2+} levels, syntaxin 1A shows a higher affinity for synaptotagmin (Sheng \textit{et al} 1997). This observation may suggest that upon Ca\textsuperscript{2+} entry, syntaxin1A released from Ca\textsuperscript{2+} channel is available for interacting with synaptotagmin and the other SNARE components to drive membrane fusion, and the channels relieved from the inhibition allow for more Ca\textsuperscript{2+} entry. Inhibition of rapid, synchronous synaptic transmission accompanied with enhancement of asynchronous transmitter release by peptides containing the synprint site of Ca\textsubscript{v}2.2 indicates that the binding of the synprint site to SNARE proteins may be essential in regulating docking and priming events for efficient synaptic transmission (Mochida \textit{et al} 1996). Besides Ca\textsuperscript{2+}-triggered exocytosis, there is another exocytotic event that is induced by membrane depolarization in a Ca\textsuperscript{2+}-independent fashion. A peptide containing the synprint site can decrease Ca\textsuperscript{2+}-independent transmitter release by the peptide containing the synprint site of Ca\textsubscript{v}2.2, implying that the interaction between the synprint site and SNARE proteins may transmit a voltage-dependent signal to the SNARE complex (Mochida \textit{et al} 1998). The synprint site may also play a role in the localization of presynaptic Ca\textsubscript{s}s in the nerve terminals (Mochida \textit{et al} 2003). The phosphorylation of the synprint site by PKC and CaMKII regulates the activity of presynaptic Ca\textsubscript{s}s (Yokoyama \textit{et al} 1997, Yokoyama \textit{et al} 2005). Phosphorylation by PKC reduces the binding affinity of syntaxin1A and reverses the regulatory effects (Yokoyama \textit{et al} 2005). In addition, the interaction between syntaxin1A and Ca\textsubscript{v}2.2 allows syntaxin1A to
promote G-protein inhibition of the channel by physically recruiting the Gβγ subunit to
the I-II loop of the channel (Jarvis et al 2001, Jarvis et al 2002).

1.1.3.2 Molecular determinates of the interaction between SNARE proteins and the synprint site

The interaction between the synprint site and syntaxin1A and SNAP-25 shows
isoform specificity and Ca^{2+} dependence (Figure 7b) (Retting et al 1996, Kim et al 1997).
The differences in interactions between Ca^{2+} channels and the SNARE proteins may
contribute to the differences in the efficiency of synaptic transmission for Ca^{2+} entry
through these different channels and confer specific functional and regulatory properties
on the process of neurotransmitter release.

An in vitro binding assay has shown that both the N-terminal half (718-895) and
the C-terminal half (832-963) of the Ca_{2.2} synprint bind to syntaxin and SNAP-25
(Yokoyama C T et al, 2005). Moreover, the overlapping region of two halves predicted to
be collagen homolog is also required for the interaction. Mutagenesis experiments show
that the specific peptide sequence LRASCEALY (781-789) and KTSASTPAGGEQDR
(860-873) within the N-terminal and C-terminal regions are crucial for SNARE binding
(Yokoyama C T et al, 2005). Deletion of the N-terminal half, the entire synprint site or
the whole II-III loop results in depolarizing shift of voltage-dependent inactivation.
Furthermore, coexpression of syntaxin1A with those impaired channels fail to cause the
same hyperpolarizing shift compared to the wildtype channels, but the inhibitory effect of
syntaxin still significantly remains (Bezprozvanny I et al, 2000). This may imply that the
Figure 7 Sequence of the synprint site and interaction with SNARE proteins. (a) Sequence alignment of the synprint sites of different isoforms in Cav2.1 and Cav2.2 channels. The first shaded region shows the putative coiled-coil. The second shaded region shows collagen homolog. The “start” and “end” of the synprint sites are indicated by arrows. (Van Petegem 2007) (b) Interaction between the synprint site and SNARE protein shows isoform specificity and Ca\(^{2+}\) dependence.
modulatory effect of syntaxin1A is normally transmitted by the synprint site; however, in the absence of the synprint site, other regions of the channel may partially substitute for this site. Both the N-terminal Habc and the C-terminal H3 domain of syntaxin1A are implicated in interacting with the synprint site (Retting J et al, 1996; Jarvis et al, 2002). However, it seems that only the H3 domain and transmembrane segment of syntaxin1A have functional effects on channel properties.

1.1.3.3 Splicing variants of the synprint site

The distinct Ca\textsuperscript{2+} channel variants from regulated alternative splicing show the specialized channel functions to optimize calcium signaling in different regions of the brain. To date, two human Ca\textsubscript{v}2.2 and two rat Ca\textsubscript{v}2.1 splice variants that lack synprint have been reported (Kaneko et al 2002, Rajapaksha et al 2008). One of the common features for all four variants is that voltage-dependence inactivation is shifted in the depolarizing direction instead of hyperpolarization as a result of syntaxin or SNAP-25 modulation (Kaneko et al 2002, Rajapaksha et al 2008). This is reasonable because without the synprint site, the targeting site, SNARE proteins lose the modulatory effects on the Ca\textsuperscript{2+} channels. Interesting, two rat Ca\textsubscript{v}2.1 variants are the predominant forms in neuroendocrine cells, which may reinforce the idea that the synprint site plays a role in targeting presynaptic Ca\textsubscript{s}s to nerve terminals (Rajapaksha et al 2008). However, this hypothesis has been challenged by the fact that in snail, Ca\textsubscript{v}2.1/2.2 lacking the entire synprint still localize in the nerve terminals (Spafford et al 2003). The observation suggests that other proteins such as the modular adaptor proteins CASK and Mint are also involved in channel targeting (Spafford et al 2003).
1.1.3.4 Other interacting partners of the synprint site

There are other proteins such as Cystein string protein (CSPα) (Swayne et al 2006), Huntingtin (htt) (Swayne et al 2005) and RIM (Coppola et al 2001) have also been proposed to interact with the synprint site. However, no direct functional effect on the channel has been shown.

1.2 Crystallization

X-ray crystallography is an invaluable tool to study protein structures. Since the function of a protein depends on its structure, accurately determining protein structures allows for the study of protein functions at the atomic level and provides reliable answers to the structure-related questions. Here I will discuss a number of methods used to obtain protein crystals.

1.2.1 Overview of protein crystallization

Crystallization is the process that forces a protein into well-ordered three dimensional arrays. The crystal formation involves three steps: nucleation (the initial event in which a small number of molecules become arranged and form a site for additional molecules to be deposited on), growth and cessation of growth. The crystallization process can be explained in the phase diagram (Figure 8a). For a protein to crystallize, it must first be in the state of supersaturation, in which the protein reaches or overcomes the solubility boundary and starts to become insoluble. However, high levels of supersaturation (precipitation zone) cause the protein to precipitate rather than forming
well-ordered crystals. The goal is to keep the protein in the labile zone where the nucleation occurs and then bring it to the metastable zone where the crystal grows.

In the process of protein crystallization, both the environmental parameters and the intrinsic protein properties should be considered and well controlled. Suitable environmental parameters such as pH, temperature and ionic strength need to be adjusted for the optimal growth of crystal. Intrinsic properties include purity and homogeneity. Purity refers to lack of contamination. Homogeneity refers to lack of conformational heterogeneity (flexible domains and polymer formation) and lack of sequence heterogeneity (proteolysis or degradation).

1.2.2 Vapor diffusion crystallization

The vapor diffusion technique is the most frequently used crystallization method. Typically, a small amount of protein (usually 1-10 µl) is mixed with an equal amount of crystallization solution containing for example buffer, salt and precipitant. The drop is suspended and sealed over the well solution which contains the crystallization solution. The different concentration of the salt and precipitant between the drop and the well solution drives water to evaporate from the drop until an equilibrium is established, in which the activity of the water in the drop and the well is equal (Figure 8b).
1.2.3 Counter diffusion crystallization

In the conventional vapor diffusion approach, a supersaturation condition targets a single labile region for nucleation. Therefore, crystals grow out of one single condition. In the counter diffusion method, the protein and the precipitating agent are placed on the opposite ends of a capillary, and diffuse against each other, forming a gradient of conditions along the length of the capillary (Ng et al 2003). A single capillary can thus span a continuum of regions in the phase diagram. The continuous supersaturation gradient makes counter diffusion a good optimization method. One of the disadvantages is that counter diffusion in regular capillaries is not amenable to high throughput. However, chips (Fluidigm) and small counter diffusion plates (microlytics) have been developed to automate the procedure.

1.2.4 Dialysis crystallization

The protein contained within the dialysis membrane is equilibrated against the precipitant in the surrounding solvent to slowly achieve the supersaturation for crystallization. Dialysis is the most effective way for crystallization and works by decreasing the ionic strength. If a protein is less soluble at low ionic strength, it may be possible to crystallize by dialyzing against a low-salt buffer or water to slowly remove the salt. Also, dialysis offers an easy way to screen for different conditions simply by moving the dialysis container from one condition to another. Unlike vapor diffusion, the protein concentration remains constant during dialysis.
1.2.5 Other crystallization techniques

For a protein to crystallize, it must be in the supersaturation state (Figure 8a). In addition, nucleation is also necessary for crystallization. However, sometimes the levels of supersaturation that promote spontaneous nucleation are too high for the slow, accumulative growth of nice single 3D crystals, but often lead to showers of small crystals. Seeding is a powerful tool for crystal optimization, which separates the nucleation event from the growth process. The seed crystals are transferred from the original drops where they nucleated to a new experimental condition, in which the supersaturation level is high enough to support crystal growth, but low enough to prevent spontaneous nucleation (Bergfors 2007). Therefore, protein molecules can accumulate on a ready-made template in order for the crystal to grow bigger. Seeding is often used to reduce showers of crystals, to grow single crystals, to increase the size of crystals and to improve crystal quality.

Crystallization of proteins fused to stabilizing proteins is a relatively new method. Usually, the fusion proteins have enhanced solubility and facilitate protein folding when overexpressed in an expression host. Several successful examples have proven the feasibility of fusion protein crystallization (Smyth et al 2003). One major problem with this method rises from the linker region between the protein tag and the protein of interest. A linker that is too short can cause misfolding of the protein, whereas a long linker increases the flexibility of the fusion protein, resulting in conformational heterogeneity or susceptibility to proteolysis.
Protein crystallization has been proven to be predominantly dependent on entropic effects (Derewenda and Vekilov 2006). Entropic cost from the ordering of protein molecules and the loss of side chain freedom disfavors the protein crystallization (Tidor and Karplus 1994). Burying flexible surface residues such as Lys and Glu at crystal contact regions significantly contribute to the loss of entropy. Therefore, mutating the surface residues with high conformational energy into small amino acids such as Ala would reduce the surface entropy, thus lowering the free energy barrier and promoting protein crystallization.
2 Objectives

There are two experimental goals in this study. Firstly, in order to study the synprint site on the molecular level, we use crystallography to determine the three-dimensional structure of the synprint site. Secondly, we use the biochemical technique isothermal titration Calorimetry (ITC) to further characterize the interaction between the synprint site and t-SNARE syntaxin 1A.
3. Materials and Methods

3.1 Cloning

3.1.1 Cloning of synprint domains from rabbit Ca₂⁺.1 and bovine Ca₂⁺.2

All the synprint domain constructs from rabbit Ca₂⁺.1 and bovine Ca₂⁺.2 were cloned into the pET28HMT vector (Table 2). The templates for the inserts were rabbit Ca₂⁺.1 full-length channel and bovine Ca₂⁺.2. The standard PCR protocol was the initial denaturation at 95°C for 3 minutes and 30 cycles of: denaturation at 95°C for 0.5 minutes, annealing at 55°C for 0.5 minutes and elongation at 72°C for 3 minutes, and 1 cycle to complete the elongation at 72°C for 10 minutes. DMSO (Acros) at a final concentration of 2.5% was necessary for the successful PCR of all the bovine Ca₂⁺.2 constructs.

Ligation independent cloning (LIC) was used to paste the PCR products into the expression vector. Briefly, the PCR products were verified on 1% agarose (Fisher) gels and extracted (Qiagen). The pET28HMT vector was digested by SspI (NEB) for 3 hours at 37°C, verified on a 1% agarose gel and extracted. Both the inserts and the vector were subjected to T4 polymerase (Fermentas) treatment with dCTP (BioLabs) and dGTP (BioLabs), respectively, to create the long overhangs for 40 minutes at room temperature, followed by 20-minute heat inactivation of the polymerase at 75°C. 2μl of T4-treated insert and vector were mixed together, and the overhangs were annealed for 10 minutes at room temperature. Finally, the ligation products were transformed into *E. coli* DH5α under kanamycin selection. The LIC overhang sequence for the forward primer was tacttccaatccaatgca, and the LIC overhang sequence for the reverse primer was ttatccacttccaatgta.
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Table 2 Oligonucleotides used in this study (continued)

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After plasmid extraction (Qiagen), several clones were sent for sequencing (Macrogen).

**3.1.2 Mutagenesis of the synprint constructs**

Normal Quikchange (Stratagene) mutagenesis did not work for the synprint mutagenesis. Phusion site-directed mutagenesis (Finnzymes) was used instead according to the manufacturer’s guidelines. T4 ligase (NEB) was used to ligate the PCR products at room temperature for 5 minutes, followed by transformation.

Another mutagenesis method was used to make the mutant of Bovα1synA8C784A/E794A/E795A/K737A/K739A/E740A (Table 2) using three sequential PCRs and using Bovα1BsynA1C784A/E794A/E795A as a template. The first two PCRs were used to create the K737A/K739A/E740A mutations using primer 16F, 16R and 16F’, 16R’. The two PCR products were verified on a 1% agarose gel, extracted, and mixed together for the template of the third PCR. The third PCR was performed using primer 16F and 16R’. The PCR product was verified on a 1% agarose gel, extracted, and used as insert for LIC cloning into the pET28HMT vector. The standard PCR protocol, described above, was used in all the three PCRs.

**3.1.3 Cloning of the fusion proteins**

The constructs of MBP and GST-(linker)-synA8EE were cloned into the pET28HT vector by LIC (Table 2). The insert consisted of an N-terminal MBP or GST tag fused to synA8EE. The MBP tag and the synA8EE were amplified with primer 17F, 17R and 17F’, 17R’ respectively using the standard PCR protocol (Table 2). Both PCR products were verified by 1% agarose gel, extracted, and digested by BamHI (NEB) for 1 hour at 37°C. The two BamHI-treated ends were ligated by T4 ligase at room temperature.
for 2 hours. The ligation products were run on a 1% agarose gel. This generated a fused construct devoid of any linker. Two additional constructs of MBP-linker synA8EE with 3-alanine and 5-alanine codons were also made. Similarly, GST-linker-synA8EE constructs with different linkers were also made (Table 2). Finally, the gel-purified ligation product was cloned into pET28HT by LIC.

The MBP and GST-(linker)-synA8EE-His constructs were cloned into the pET24a (Novagen) vector using NdeI and XhoI restriction sites. The MBP tag and the synA8EE were amplified with primer 23F, 23R and 23F', 23R' respectively using the standard PCR protocol (Table 2). Both PCR products were verified by 1% agarose gel, extracted, and digested by BamHI for 1 hour at 37°C. The two BamHI-treated ends were ligated by T4 ligase at room temperature for 2 hours. The ligation products were run on a 1% agarose gel. The gel-purified ligation product was digested at 37°C sequentially (16 hours for NdeI (NEB) and 16 hours for XhoI (NEB)). The insert and the vector were ligated using T4 ligase at room temperature for 2 hours, transformed into E. coli DH5α, and plated on ampicillin. This generated a fused construct devoid of any linker. The other MBP-tagged and GST-tagged constructs were generated in a similar way.

3.1.4 Cloning of syntaxin1A constructs

Mouse syntaxin1A (1-189) was cloned into the pET28HMT vector by LIC. The standard PCR protocol was used with a full-length mouse syntaxin1A as template. Mouse syntaxin1A (190-264) was cloned into pGEX6.1 using the BamHI and XhoI restriction sites. The standard PCR protocol was used with a full-length mouse syntaxin1A clone as template. PCR products were verified on a 1% agarose, extracted, and digested at 37°C in
sequential reactions (1 hour for BamHI; 16 hours for XhoI). The vector and insert were ligated using T4 DNA ligase for 2 hours at room temperature, transformed in to *E.coli* DH5α, and plated on ampicillin.

### 3.2 Protein expression and purification

#### 3.2.1 Protein expression.

All the proteins in this study were expressed in *E. coli* Rosetta (DE3) pLacI, and grown in 2xYT medium (Fisher) at 37°C under continuous shaking unless stated otherwise. Typically, 1L of culture was grown in 2.8L Fernbach flasks until OD$_{600}$ of 0.6, induced with 0.4mM IPTG (VWR), and grown further for 3-4 hours. Cells were harvested by centrifugation at 5,000 rpm for 15 minutes using the floor centrifuge (Beckman Coulter). Cells were lysed using Sonic Dismembrator Model 500 (Fisher) at 40% amplitude for 1 minute for four times. The cell lysis buffer contained 250mM KCl (Fisher), 10mM Hepes pH 7.4 (Fisher), 10% glycerol (Fisher), 100mM PMSF (Sigma), 25 mg/ml DNaseI (Sigma) and 25 mg/ml lysozyme (Pierce) unless stated otherwise. Centrifugation was performed at 35,000xg for 30 minutes at 4°C to remove insoluble material.

#### 3.2.2 Purification of the synprint domains from rabbit Ca$_{2.1}$ and bovine Ca$_{2.2}$

All the synprint domain constructs were cloned into the pET28HMT vector, containing, in sequence, a His$_6$ tag, maltose binding protein (MBP) tag, and a cleavage site for TEV protease at the N-terminus of the synprint domains (Table 2).
A two-day purification protocol was used, and all the buffers used for chromatography are listed in Table 3. The clarified supernatant was loaded onto a poros MC (nickel affinity) column (Applied Biosystems) in buffer A and eluted in successive 6% and 60% steps of buffer B. The eluate was dialyzed (3.5K cutoff membrane tubing, VWR) in buffer A for 2 hours at room temperature, loaded onto an amylose (MBP affinity) column (BioLabs) in buffer A, and then eluted in buffer C. The eluate was collected and incubated with TEV protease (3 mg/ml, 1mL) overnight at room temperature. The next day, the cleaved sample was run on a poros MC column to remove the His-MBP tag, His-TEV protease and any uncleaved proteins. The protein of interest was collected in the flow-through, and dialyzed in low ionic strength buffer D, F, or J, depending on the subsequent ion exchange column, for 1 hour at room temperature. The protein sample was then applied to either a Hiload SP (cation exchange) column (GE Healthcare) or a Hiload Q (anion exchange) column (GE Healthcare) for further polishing and eluted using a linear gradient of buffer E, G or K. The eluate was concentrated to approximately 500 µl by centrifugation at 4150 rpm at 4°C using a Amicon Ultra centrifugal filter (3K cut-off, Millipore) and loaded onto a superdex 200 (size exclusion) column (GE Healthcare) in buffer A. The peak fractions were collected. The KCl concentration was reduced to 25mM in the final protein preparation by diluting the protein sample in 10mM Hepes pH 7.4. The protein concentration was determined by reading the absorbance at 280nm under denaturing conditions (Edelhoch, 1967). The final protein preparation was stored in the buffer containing 25mM KCl and 10mM Hepes pH 7.4 at a concentration of 10mg/ml at -80°C.
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3.2.3 Purification of Bova1BsynA8 mutants

The purification scheme of the Bova1BsynA8 mutants was the same as the two-day purification protocol described above with the following modifications: three additional columns, a shorter time of TEV cleavage and all the chromatography buffers contained Tris pH 8.0 as the buffer. On the first day, between the poros MC column and amylose column, the protein sample was dialyzed in buffer H at room temperature for 1 hour, loaded onto a Hiload Q column, and eluted using a linear gradient of buffer I. After the amylose column, the protein sample was concentrated to approximately 2 ml and run on a Hiload superdex 200 (size exclusion) column in buffer A overnight. On the second day, the peak fractions from the superdex 200 were collected and subjected to TEV cleavage for 3 hours at room temperature. Following the poros MC and Hiload Q columns, another amylose column was used to further remove the cleaved His-MBP tag.

3.2.4 Purification of MBP (GST)-(linker)-synA8EE fusion proteins

A one-day protocol was used for the purification. The clarified supernatant was loaded onto a poros MC column in buffer A and eluted in successive 6% and 60% steps of buffer B. The eluate was dialyzed in buffer A supplemented with 14mM βME for 3 hours at room temperature along with TEV protease. The cleaved protein sample was then loaded on a second poros MC column, and the flow-through was collected in buffer A. The protein sample was applied to either an amylose column or a glutathione sepharose column (GE Healthcare) in buffer A depending on the affinity tag. The MBP or GST tagged proteins were then eluted in buffer C or L, respectively. After a one-hour dialysis in buffer F, the protein sample was loaded onto a Hiload Q column and eluted
using a linear gradient of buffer G. Since the fusion protein was sensitive to degradation, the protein sample was not prepared for crystallization.

3.2.5 Purification of MBP (GST)-(linker)-synA8EE-His fusion proteins

The buffers used for chromatography all contained Tris pH 8.0. The clarified supernatant was loaded onto a poros MC column in buffer A and eluted in successive 6% and 60% steps of buffer B. The eluate was dialyzed in buffer A for 2 hours at room temperature, loaded onto an amylose column in buffer A, and then eluted in buffer C. The eluate was directly loaded onto a poros MC column in buffer A and eluted in 60% buffer B. After a one-hour dialysis in buffer H, the protein sample was loaded onto a Hiload Q column, and eluted using a linear gradient of buffer I. The KCl concentration was reduced as before (section 2.2.2). The final protein prep stored in the buffer containing 25mM KCl and 10mM Tris pH 8.0 at a concentration of 30mg/ml at -80°C.

3.2.6 Purification of stx1-189, synA1 and synB1 for ITC

stx1-189, synA1 and synB1 (Figure 20) were purified with the same purification protocol. The clarified supernatant was loaded onto a poros MC column in buffer A and eluted in successive 6% and 60% steps of buffer B. The eluate was dialyzed in buffer A plus 14mM βME for 3 hours at room temperature along with TEV protease. The cleaved protein sample was then loaded on a second poros MC column, and the flow-through was collected in buffer A. synA1 or synB1 was dialyzed in buffer J, while stx1-189 was dialyzed in buffer F. synA1 or synB1 was loaded onto a Hiload Q column and eluted using a linear gradient of buffer K. stx1-189 was applied onto a Hiload Q column and
eluted using a linear gradient of buffer G. The protein preparations were stored with 20% glycerol at -80°C prior to use.

3.2.7 Expression and purification of stx190-264 for ITC

6L of cell culture was grown at 37°C until the OD<sub>600</sub> reached 0.4, and then growth was continued at 25°C until an OD<sub>600</sub> of 0.6. After induction with 0.4mM IPTG, the cell culture was grown at 25°C overnight.

The clarified supernatant was loaded onto a glutathione sepharose column in buffer L and eluted in buffer M. After dialysis in buffer H at room temperature for 1 hour, the protein sample was loaded onto a Hiload Q column and eluted in a linear gradient of buffer I. The eluate was collected and dialyzed in buffer A along with Prescission protease (kindly provided by the lab of Dr. N. Strynadka) for two days at 4°C. The protein sample was applied onto a glutathione sepharose column in buffer L, and the flow-through was collected. The final protein preparation was stored in 20% glycerol at -80°C prior to use. All the chromatography buffers used contained Tris pH 8.0 and 14mM βME.

3.3 Crystallization

3.3.1 Vapor diffusion crystallization

Both sitting drop and hanging drop setups were used in this study. The sitting drop was used for screening random conditions using the following crystallization kits from Qiagen: PEGs, Classics, Classics II, pH clear, pH clear II, JSCG+ and ProComplex in 96-well plates (low profile Greiner, Hampton Research). A multichannel pipette was
used to mix 1μl from 60μl of the well solution with 1μl of the protein drop. The plates were sealed with the crystal clear tape (Hampton Research). The hanging drop method was used for optimization in 24-well plates (VDX, Hampton Research). 1μl of the protein sample was mixed with 1μl of the precipitant on a cover slip, and the cover slip was inverted over 500μl of the precipitant solution.

3.3.2 Counter diffusion crystallization

In the counter diffusion method, the Crystal Former (Microlytic) was used. The Crystal Former is a capillary device with 16 crystallization channels, and each provides access to two inlets for loading proteins and precipitant solutions. 0.5μl of protein was loaded into the right inlet, while 0.5μl of the precipitant solution was loaded into the left inlet. The inlets were sealed by crystal clear tape.

3.3.3 Dialysis crystallization

Dialysis crystallization was carried out in 24-well plates. 5μl of the protein samples were loaded into 5μl dialysis buttons (Hampton Research) and sealed with a 1000 Da MWCO membrane. The dialysis buttons were placed in 1.5 ml of the well solution. The 24-well plates were sealed with tape.

3.3.4 Seeding

In the seeding experiments, the natural fiber Seeding Tool (Hampton Research) was used to transfer small seeds from the spherulites or the needle clusters to a clear drop that was set up one day before by streaking a line in the drop.
3.4 Isothermal titration calorimetry (ITC)

Both VP-ITC (MicroCal) and ITC200 (MicroCal) instruments were used to test the interaction between stx1-189 and synA1 or synB1. Samples were concentrated and dialyzed in the buffers containing the different experimental conditions. When using VP-ITC, samples were degassed for 5 minutes, and injections of 10µl of stx1-189 were titrated into 1.4 ml of synA1 or synB1. When using ITC200, injections of 2µl of stx1-189 were titrated into 200µl of synA1 or synB1. For all the measurements, stx1-189 was titrated into the experimental buffers as a control.

The ITC200 instrument was used to test the interaction between stx190-264 and synA1 or synB1. The protein samples were dialyzed against 150mM KCl, 10mM Hepes pH 7.4, 20µM CaCl₂ and 10mM βME. Injections of 2µl of stx190-264 were titrated into 200µl of synA1 or synB1. For all the measurements, stx190-264 was titrated into the experimental buffer as a control.

The protein concentration was determined by reading the absorbance at 280nm under denaturing conditions (Edelhoch, 1967). The experimental temperature was 25°C. Data were processed with MicroCal Origin 7.0.

3.5 Circular dichroism (CD)

The CD spectra were measured for synA1 and synB1 using a Jasco Model J-810 spectrometer (Jasco). Wavelength scans from 195 to 280 nm were taken at 25°C in a 2 mm path length quartz cuvette. Both synA1 and synB1 spectra were measured at a protein concentration of 10µM. The proteins were prepared in 10mM phosphate pH6.5. The
protein concentration was determined by reading the absorbance at 280nm under denaturing conditions (Edelhoch, 1967).
4. Results

4.1 Crystallization of the synprint site from Ca,2.1 and Ca,2.2

In the past 15 years, the interaction between the synprint site and SNAREs has been intensively studied by multiple biochemical and physiological techniques (Catterall and Few 2008, Kisilevsky and Zamponi 2008). However, some fundamental questions still remain, including how exactly the synprint site binds to SNARE proteins, why the interaction shows isoform specificity, and how the Ca$^{2+}$ dependence occurs. A detailed description of the interaction at the molecular level is needed to address these questions. In order to obtain a molecular template for further dissection of the interactions, we attempted to use protein crystallography to determine the three-dimensional structure of the synprint site.

4.1.1 Designing different synprint constructs from Ca,2.1 and Ca,2.2

In crystallographic studies, designing suitable protein constructs is a crucial step in the success of the experiment. Based on sequence alignment of different isoforms from different species (Figure 7a), the synprint site can be divided into four parts: a putative coiled-coil domain, a conserved region, a region corresponding to homology with collagen, and a C-terminal non-conserved region. Seven amino acid residues (DNLANAQ) before the start of the proposed synprint site (indicated as “start arrow”) are also predicted to be part of the coiled-coil domain (Figure 7a). We therefore included this part in our study. Moreover, the entire synprint site was already shown to be sensitive to degradation, being cleaved rapidly into two parts. Given this experimental result, we also divided the synprint site into two halves: an N-terminal half that starts from the
coiled-coiled domain and ends at a "proline" residue indicated as "red arrow" in Figure 7a, and a C-terminal half that expands the rest of the non-conserved region. This experimental design strongly agrees with the previous predication that the synprint site consists of two domains (N-terminal and C-terminal) (Yokoyama et al, 2004). In addition, the C-terminus of Ca,2.1 synprint contains 7 consecutive glycine residues, which has already been shown to be unstable to proteolysis. According to the information listed above, 11 synprint constructs from rabbit Ca,2.1 and bovine Ca,2.2 have been made for crystallization trails (Table 2).

4.1.2 Purification of different synprint constructs from Ca,2.1 and Ca,2.2

All the synprint constructs were cloned into the pET28HMT vector, and were fused with a His6 tag, a maltose binding protein (MBP), and a specific protease cleavage site for the Tobacco etch virus protease. The result of the purification, as described in the materials and methods, is shown in Figure 9a. We successfully purified all of the synprint constructs listed in Table 2 except one construct, Rab α1AsynA7. The protein seemed to precipitate after the second poros MC column. The reason is likely that the short construct did not fold properly, thus precipitating after removal of the MBP tag. The rest of the synprint constructs all remained stable throughout the entire purification and were well-behaved. However, all of them shared a small degradation problem, and we were unable to completely remove the degradation products (Figure 9a Lane 6). Nevertheless, the degree of degradation was different among these constructs. An interesting
Figure 9 Purification of the synprint domains. BovulbsynA8C784A was used as an example. Bands in Lane 2 to Lane 6 (a) represented the elution of the first poros MC column (b), the elution of the amylose column (c), the flow-through of the second poros MC column (d), the elution of the Hiload Q column (e) and the major peak of the superdex column (f).
observation was that all the constructs with the additional seven amino acids (DNLANAQ) at the N-terminus seemed to be more stable than the others. We speculated that these residues were indeed part of the coiled-coil and that their presence could stabilize the structure of the entire protein.

4.1.3 CD experiment of the N- and C-terminal of the synprint site

A CD experiment was performed to test the secondary structures of the N-terminal part (synA1) and the C-terminal part (synB1) of the synprint site. Figure 10 showed that the N-terminal part of synprint indeed showed some α helical feature (48.4% α helix), which confirmed the prediction, while the C-terminal non-conserved part did not display any secondary structure but only random coils (13.4%α helix) (Greenfield and Fasman 1969). Therefore, we focused our efforts into crystallizing the N-terminal part because the C-terminal part without a well-organized structure would not have much chance to crystallize.

4.1.4 Crystallization of different synprint constructs from Ca,2.1 and Ca,2.2

All well-behaved proteins were used for random condition crystallization screening. Spherulites appeared for almost all of the synprint constructs (Figure 11a). This is an indication that the synprint domains from both Ca,2.1 and Ca,2.2 had a good chance to be crystallized. Moreover, there were needle-like small crystals growing on the top of spherulites from Bovα1BsynA4C784A and Bovα1BsynA8C784A constructs (Figure 11b). The cysteine to alanine mutation could prevent potential non-specific di-
Figure 10 CD spectra of synA1 (N-terminal) and synB1 (C-terminal). (a) CD spectrum of synA1 showed α helical feature. (b) CD spectrum of synB1 displayed random coils.
Figure 11 Spherulites and small needle-like crystals from crystallization trials of the synprint domains. (a) Spherulites or small crystalline materials. (b) The red arrow indicated the thin hair-like crystals growing on the spherulites.
sulfide bond formation. These small needle-like crystals tended to grow separately from each other, which was also a good sign for potential growth of single crystals.

From these constructs, we discovered that the collagen homology region was important for crystallization. The needle-like crystals showed up in Bovα1BsynA4C784A but not its homolog Rab α1AsynA4. The difference between Bovα1BsynA4C784A and Rab α1AsynA4 was that Bovα1BsynA4C784A contained a collagen homology domain, but Rab α1AsynA4 contained a non-conserved sequence at this site (Figure 7a). However, unfortunately neither the spherulites nor the needle-like crystals appeared large enough for X-ray diffraction experiments. More effort was focused on fine screening of the two constructs (Bovα1BsynA4C784A and Bovα1BsynA8C784A) around the conditions where the needle-like crystals showed up. However, the improvement in protein crystal quality was limited, and no single three-dimensional crystals were found in the fine screening.

4.2 Crystallization of synprint domains with surface engineering

Protein crystallization predominantly depends on entropic effects. Mutational surface engineering, creating patches with low conformational entropy, is an effective tool to enhance the success of protein crystallization (Derewenda and Vekilov 2006).

Bovα1BsynA8C784A was chosen as the template for the surface engineering. There were three reasons for picking this synprint construct. Firstly, Bovα1BsynA8C784A starts from the sequence DNLANAQ, which we found to play a potential role in stabilizing the synprint domain. Secondly, both the N-terminal region (coiled-coil) and the C-terminal region (collagen homolog) of this construct are predicted to be alpha
helices, which could coil around one another. Thirdly, small needle-like crystals were observed for this construct (Section 3.1.3). The SER on-line server (http://nihserver.mbi.ucla.edu/SER/) was applied to predict the potential “hot spots” that are most suitable for mutations designed to enhance crystallizability by surface engineering. There were three hits: 737KAKE740, 794EE795, and 820EAGE823.

4.2.1 Purification of Bovα1BsynA8C784A/E794A/E795A

Previous work has shown that degradation is a big issue for purification and crystallization of the synprint domains. To minimize degradation, three procedures were implemented. Firstly, a protease stability test was performed on synA8EE (short for Bovα1BsynA8C784A/E794A/E795A) (Figure 12a). The basic pH 8.0 was chosen for protein purification. At the acidic pH 5.0, the degree of degradation was the smallest, but this condition was not chosen because the poros MC column would not be functional at this pH. Furthermore, given the pI 5.35 of synA8EE, the ion exchange columns would have poor separation. Secondly, the TEV cleavage time was shortened to 3 hours instead of overnight incubations. Thirdly, three additional columns were used for protein purification, and all the purification steps were performed as fast as possible, within two days (Section 2.2.3).

As a result, one can see that this greatly reduced the amount of degradation (Lane 9 in Figure 13a versus Lane 6 in Figure 9a). In addition, the chromatogram of the second
Figure 12 Stability tests. (a) Stability test of synA8EE at different pHs. (b) Stability test of MBP-5A-synA8EE at different pHs.
size exclusion column displayed a single symmetrical peak, indicating that the protein was well-behaved (Figure 13i).

4.2.2 Crystallization of synA8EE

synA8EE protein preparations were used to set up random condition crystallization screening. The best outcome from these screenings was needle-like small crystals growing on spherulites (Figure 14a). These needles were bigger and thicker than observed before. A lot of effort was put into the optimization of these synA8EE crystals. The best result from these fine screens was the appearance of long thin needle clusters sharing the same origin (Figure 14b). This was a large improvement. These long needle crystals (~0.2mm) were well separated from each other. However, the weakness of the needle crystals was that they were flexible and only grew in one dimension instead of three dimensions. Unfortunately, because of these problems, these needle crystals showed no visible diffraction at the CMCF beamline of the Canadian Light Source (Saskatoon Canada).

There might be two underlying causes for this unsatisfying crystal quality. Firstly, the flexibility of the needle crystals suggested weak crystal contacts. Therefore, surface engineering of the other two “hot spots” was performed on synA8EE. However, synA8EE combined with the E820A surface mutation did not improve crystal quality. The synA8EE with K737A/K739A/E740A exhibited too much degradation during purification, indicating that the lysine to alanine mutations compromised the protein integrity. Secondly, we suspected that protein degradation was also influencing crystal
Figure 13 Purification of synA8EE. Bands in Lane 2 to Lane 9 (a) represented the elution of the first poros MC column (b), the elution of the HQ column (c), the elution of the amylose column (d), the major peak of the superdex column (e), the elution of the second poros MC column (f), the elution of the Hiload Q column (g), the flow-through of the amylose column (h), and the peak of the superdex column (i).
Figure 14 The needle-like crystals obtained from synA8EE. (a) The short needle-like crystals from the random screen. (b) The long needle-like crystals after optimization.
quality. Mass spectrometry was used to identify the degradation sites (quadrupole ESI, UBC); however, it turned out that only one species representing synA8EE was identified (Figure 15a), even though small degradation bands could even be seen on Coomassie-stained SDS-PAGE gel (Figure 13a Lane 9). This unexpected result might be due to the fact that the signal of major species of synA8EE was so strong that the signals of minor species of degradation products were masked in the mass spectrum.

Seeding is a very useful crystallographic tool for small crystals to grow high-quality crystals. Several conditions around the best crystallization hit for synA8EE were set up using lower concentrations of the protein. Seeding experiments were performed on the next day. However, along the streaking, only small needle clusters were observed without any improvement of crystal quality (Figure 15b).

4.2.3 Crystallization of synA8EE using different crystallization methods

If one crystallization method has been exhausted, the quality of crystals may be improved by using other crystallization techniques.

4.2.3.1 Crystallization of synA8EE using dialysis crystallization method

In crystallographic studies, the existence of salt/buffer in the final protein preparation could always be a problem because one never knows if the salt/buffer prevents crystal formation. Obviously, H₂O would be the best choice for the solvent. However, most of the time proteins need some salt and buffer for stability.
Figure 15 Mass spectrum of synA8EE and the result of seeding experiment. (a) The mass spectrum of synA8EE. According to the mass spectrum, the protein preparation contained a single peak represented synA8EE. No other protein contaminants were
detected. (b) The result from seeding experiments. Only spherulites with small needle-like crystals were observed.

In this study, when the concentration of salt (KCl) was reduced below 10mM, synA8EE started to precipitate. This observation led to the idea that if the desalting process could be slowed down, it might be possible for synA8EE to crystallize during the process. Dialysis experiments were therefore set up (Figure 16a). In the horizontal direction, KCl concentration was gradually increasing from 1mM to 100mM, and in the vertical direction, different pHs (3.0, 4.0, 5.0 and 6.0) were screened. In this screen, only spherulites were observed (Figure 16c). Therefore, this was unsuccessful for improving synA8EE crystallization.

4.2.3.2 Crystallization of synA8EE by counter diffusion crystallization method

In the counter diffusion method, a gradient was formed of both protein and precipitant concentrations mixture is formed along the length of the capillary (Ng et al 2003). Therefore, the counter diffusion is a good optimization technique. In this study, the synA8EE protein was placed on the right side of the capillary, while the best condition with different pHs (3.0-10.0) was loaded on the left side of the capillary (Figure 16b). However, only spherulites were observed (Figure 16d). Therefore, the counter diffusion method failed to grow better 3D crystals.

4.3 Crystallization of fusion proteins

Previous studies have shown that large-affinity tags can help stabilize the proteins of interest for crystallization (Smyth et al 2003). In this study, both MBP and GST tags
Figure 16 Results of dialysis and counter diffusion crystallization. (a) The setup of dialysis crystallization. (b) The setup of counter diffusion crystallization. (c) The spherulites were obtained from the dialysis crystallization. (d) The spherulite seen in the counter diffusion method.
were used as fusion tags for synA8EE, and 0, 3-alanine and 5-alanine linkers were used to test for the optimal length for crystallization.

4.3.1 Purification of MBP-(linker)-synA8EE-His and GST-(linker)-synA8EE-His

Initially, the fusion proteins with only MBP or GST tags had degradation issues during purification (Figure 17). Therefore, we chose to put affinity tags (an MBP or GST tag and a His tag) on both ends, and used poros MC and amylase/glyutathione sepharose columns to select only intact fusion proteins (Section 2.2.5). As expected, this purification scheme indeed increased the purity and completely removed degradation products (Figure 18a). A protease stability test showed that the fusion protein was stable at all the tested pHs except pH3.0 (Figure 12b). The size exclusion column also confirmed that the fusion protein was well-behaved (Figure 18g).

We observed that the GST-fusion proteins experienced more degradation than MBP-fusion proteins (Figure 17 and Figure 18). It appeared that instead of stabilizing the synA8EE, the GST-affinity tag interfered with the folding of synA8EE and made synA8EE more vulnerable to proteolysis.

4.3.2 Crystallization of MBP-(linker)-synA8EE-His

All three MBP-fusion proteins with linkers of different lengths were successfully purified (30mg/ml) and subjected to random screening. We observed a correlation between the linker length and quality and quantity of spherulites. The fusion protein
Figure 17 Hiload Q results of MBP-tagged synA8EE and GST-tagged synA8EE. (a) The result of the MBP-tagged synA8EE from the Hiload Q column. Degradation was observed. (b) The result of the GST-tagged synA8EE from the Hiload Q column. The amount of degradation was more than that of MBP-tagged synA8EE. (c) The Hiload Q profile of MBP-tagged synA8EE. (d) The Hiload Q profile of GST-tagged synA8EE.
Figure 18 Purification of MBP (GST)-linker-synA8EE-His. (a) The purification of MBP-5A-synA8EE-His. Bands in Lane 2 to Lane 5 represented the elution of the first poros MC column (c), the elution of the amylase column (d), the elution of the second poros MC column (e) and the elution of the Hiload Q column (f). (b) a large amount of degradation was seen during the purification of GST-tagged fusion protein. (g) the superdex profile of the MBP-5A-synA8EE-His indicated that the protein was well-behaved.
without the linker gave spherulites for only 12 conditions, but no needle-like crystals. In contrast, the 5 alanine-linker fusion protein yielded 73 conditions with spherulites, and small needle-like crystals appeared in 17 conditions. The best results, obtained after fine screening of MBP-5A-synA8EE-His, are shown in Figure 19. However, the quality of these crystals is still poor and more optimization is needed.

4.4 Interaction between the synprint site and syntaxin1A

It is well established that the modulation of the presynaptic voltage-gated calcium channels by SNARE proteins is mainly through the interaction between the synprint site and SNARE proteins. However, the precise binding sites are still under debate. For example, the Catterall group suggested that the H3 domain (SNARE motif) of syntaxin1A is responsible for interaction with the synprint site (Yokoyama C T et al, 2005), while the Zamponi lab showed that the Habc domain of syntaxin1A is sufficient for the binding (Jarvis S E et al, 2002). In this study, the biochemical technique isothermal titration calorimetry (ITC) was used to further map the binding sites between the synprint site and syntaxin1A.

Isothermal titration calorimetry (ITC) is an exquisite biochemical tool to study protein-protein interactions by determining the thermodynamic parameters (enthalpy, entropy, stoichiometry). In this study, ITC was used to test if the N-terminal part of syntaxin1A stx1-189 could bind to the N-terminal half (synA1 from Rabbit Ca,2.1) and the C-terminal half (synB1 from Rabbit Ca,2.1) of the synprint site (Figure 20). The purity
Figure 19 The needle-like crystals obtained from MBP-5A-synA8EE-His. (a) These needle-like crystals were short but thicker. (b) These needle-like crystals were long but thinner.
of the proteins used is shown in the figures 21-23. 500μM stx<sub>1-189</sub> was titrated into either
50μM synA1 or synB1. The S-shaped curves from the ITC profiles indicated that stx<sub>1-189</sub>
bound to both synA1 and synB1 in 1:1 fashion with $K_d=152 \pm 12 \text{nM}$ ($\Delta H=-8.09 \pm 0.26$
$\times 10^3 \text{cal/mol}$, $\Delta S=-0.859 \text{cal/mol/K}$) and $K_d=1.81 \pm 0.34 \mu\text{M}$ ($\Delta H=-4.45 \pm 0.02 \times 10^3 \text{cal/mol}$,
$\Delta S=-16.3 \text{cal/mol/K}$), respectively (Figure 25 a and b). This result suggested two
different aspects. Firstly, both the N-terminal half and the C-terminal half of the synprint
site contributed to the interaction. Secondly, the N-terminal part of syntaxin1A
containing H<sub>abc</sub> domain itself showed strong affinity for the synprint region.

However, the initial positive result was not reproducible using the original
conditions (150mM KCl, 10mM Hepes pH 7.4, 20μM CaCl<sub>2</sub>, and 10mM βME);
moreover, the negative results suggested that there was no binding between stx<sub>1-189</sub>
and the synprint site whatsoever. Then, a set of ITC experiments with different conditions
was carried out to try to reproduce the initial positive result. The conditions were chosen
to test which parameters might be crucial for the interaction, but which may have been
recorded incorrectly. We changed the concentration of KCl (10mM versus 250mM), and
CaCl<sub>2</sub> (0 versus 1M). All four ITC results were negative and showed no binding. The
Chapman group reported that the non-specific binding of DNA to syntaxin 1A, formed
during lysis of the host cells, hindered syntaxin 1A from interacting with other protein
partners. Therefore, we removed DNA by MnSO<sub>4</sub> precipitation. Unfortunately, we still
obtained the same negative result. In previous pulldown or Co-IP experiments showing
interaction between syntaxin 1A and the synprint site, only crude cell lysates were used.
Therefore, it is possible that some other proteins in the cell lysates assist in the binding.
To address this point, relatively “dirty” protein preparations of both synA1 and stx<sub>1-189</sub>
Figure 20 The cartoon representation of the synprint site and syntaxin 1A. (a) the different domains of the synprint site from rabbit Cav2.1. (b) the intracellular part of syntaxin 1A.
Figure 21 Purification of stx1-189. Bands in Lane 2 to Lane 4 (a) represented the elution of the first poros MC column (b), the flow-through of the second poros MC column (c), and the elution peak of the Hiload Q column, which is indicated by the arrow (d).
Figure 22 Purification of synA1. Bands in Lane 2 to Lane 4 (a) represented the elution of the first poros MC column (b), the flow-through of the second poros MC column (c), and the elution of the Hiload Q column (d).
Figure 23 Purification of synB1. Bands in Lane 2 to Lane 4 (a) represented the elution of the first poros MC column (b), the flow-through of the second poros MC column (c), and the elution peak of the HiLoad Q column (d).
Figure 24 Purification of stx190-264. Bands in Lane 2 to Lane 4 (a) represented the elution of the glutathione sepharose column (b), the elution of the Hiload Q column (c), and the flow-through of the second glutathione sepharose column (d).
Figure 25 The interaction between domains of syntaxin 1A and parts of the synprint site using ITC. (a) The interaction between stx₁₁₈₉ and synA₁. (b) The interaction between stx₁₁₈₉ and synB₁. (c) The interaction between stx₁₉₀-²₆₄ and synA₁. (d) The interaction between stx₁₉₀-²₆₄ and synB₁.
which had only experienced the poros MC column were used for ITC. The result indicated that there was no binding as well. Another experimental parameter that could affect the ITC result was the concentration of glycerol. The residual glycerol from the incomplete dialysis in the protein preparations might help the binding by maintaining the protein integrity. Thus, keeping glycerol in the protein preparations might solve the reproducibility problem. Indeed, a positive ITC result was seen by including 20% glycerol in the protein samples (Figure 26c). However, the $K_d$ was only 7.94$\mu$M, about 40 fold weaker compared to the initial ITC result. It is highly possible that this last ITC result is simply a false positive due to the heat of diluting glycerol.

Another aspect that was under consideration was the effect of protein degradation. Previous studies have shown that presynaptic protein Munc 18-1 interacts with syntaxin 1A/SNAP-25 heterodimer through the small N-terminal end (residues 1-27) of syntaxin 1A (Dulubova et al 2007). If the small N-terminal end of syntaxin 1A was important for interacting with the synprint site, but cleaved off during purification, then it would explain the negative results. Therefore, mass spectrometry was used to determine if both synA1 and stx1-189 were intact (MALDI-TOF, NAPS). The result showed that the MS signals of both synA1 and stx1-189 were bigger than the calculated intact proteins (due to imprecision of MALDI for proteins this size), thus suggesting that proteolysis was not the cause of the latter negative results (Figure 26a and b).

ITC was used to test if the C-terminal part of syntaxin stx190-264 (the H3 domain), the coiled coil region bound to synA1 or synB1. The purity of the proteins used is shown
in the figures 19, 20 and 22. The ITC results revealed that there was no interaction between stx190-264 and both halves of the synprint site (Figure 25 c and d).

In all, there is no binding between the H3 domain of syntaxin 1A and the synprint site, and whether the N-terminal part of syntaxin 1A interacts with the synprint site is still inconclusive.
Figure 26 Mass spectrometry of synA1 and synB1 and ITC result in the presence of glycerol. (a) The molecular mass of synA1 obtained from mass spectrometry was 19100.03 Da, greater than the theoretical molecular weight 19020 Da. (b) The molecular mass of stx_{1-189} obtained from mass spectrometry was 22172.0 Da, greater than the theoretical molecular weight 22071.5 Da. (c) ITC result of the interaction between stx_{1-189} and synA1 in the presence of 20% glycerol.
5. Discussion

5.1 Crystallization

The synprint site from Ca2.1 and Ca2.2 has been proposed to be involved in channel gating, kinetics, and localization. Solving the synprint structure will provide further information at a molecular level to study how the synprint site is involved in channel regulation.

In this study, 11 synprint domain constructs have been made, and 10 of them have been successfully purified and set up for crystallization trails except for the very minimal construct (Rabα1AsynA7). We did not focus on the C-terminal non-conserved region (Rab α1AsynB1) because both CD experiments (Figure 10b) and the sequence (absence of hydrophobic residues) (Figure 7a) suggested it to be unfolded. However, because the diversity, the C-terminal region may be responsible for the isoform specificity and Ca^{2+} dependence of the synprint sites from different isoforms to interact with SNARE proteins (Figure 7). Unfortunately, our structural study was not able to provide the answer to these questions. The rest of the 9 constructs, made up of the different regions of the N-terminal part of the synprint site, were all well-behaved (Figure 9f). Out of the 9 constructs, the best outcome came from the thin needle-like crystals (Figure 11b). Moreover, from these constructs, we observed that the conserved DNLANAQ sequence at the N-terminus appeared to help stabilize the synprint site, and the collagen homology region was important in protein crystallization. It was thought that the predicted α-helices of both the N-terminal (coiled-coil) and the C-terminal (the collagen homolog) could coil together and improve the stability of the protein.
It was suspected that the flexible surface residues and protein degradation might prevent the growth of single 3D crystals. In order to solve these two problems, surface engineering and a new two-day purification scheme with additional chromatography columns and different buffers were applied. Indeed, after minimizing the degradation effects and creating low-entropy patches on the protein surface, long, well-separated needle-like crystals from synA8EE were obtained (Figure 14b). However, the problem with these crystals was that they were flexible, displayed growth in one dimension, and did not diffract X rays. There might be several reasons for this result. Firstly, the flexibility of the needle-like crystals indicated that the crystal contacts were not strong. Therefore, further surface engineering might solve the problem. However, it turned out that mutating more surface residues did not help with crystallization. Secondly, the proteolysis issue still remained. Even though different procedures were used to protect the protein integrity, degradation products were still present in the final protein preparation (Figure 13a Lane 9). Furthermore, the long needle-like crystals appeared at pH 3.0. According to the stability test, degradation still occurred at pH 3.0, and during the crystallization process, some proteins were still undergoing proteolysis, which could interfere with the crystal growth. Therefore, mass spectrometry was used to map the cleavage sites. Hopefully, by mutating the protease cleavage sites, the degradation issue could be resolved, and the crystal quality could be improved. Unfortunately, mass spectrometry was not able to identify any degradation products (Figure 15a).

We also tried different crystallization techniques such as seeding, counter diffusion, and dialysis to obtain better crystals; however, none of them improved crystal quality. It thus seemed impossible to obtain good crystals of the synprint domains by themselves.
Therefore, we tried to crystallize the synprint site with attached protein tags (MBP or GST and His tags) because proteins tags can stabilize the structure of the protein of interest. Indeed, the protein degradation was substantially limited (Figure 18a). One major problem of this method is the conformational heterogeneity allowed by the flexible linker region. To solve this problem, we change the length of the linkers (no linker, 3 alanines and 5 alanines). We observed that the longer linkers resulted in crystals with improved quality. Given this trend, in the future, fusion proteins with even longer linkers should be tested. Moreover, if we cannot obtain high-quality crystals, we should try to solve the structure of the synprint site by NMR.

5.2 Interaction between the synprint site and syntaxin 1A

It has been well established that the interaction between the synprint site and SNARE proteins (syntaxin 1A and SNAP-25) is responsible for the modulation of presynaptic Ca$_{\alpha}$s by SNARE proteins (Bezprozvanny et al 2000, Yokoyama et al 2004, Evans and Zamponi 2006, Keith et al 2007). Moreover, researchers have proposed that the binding of the different domains of syntaxin 1A to the synprint site regulates different types of channel behaviour. The interaction between the H$_3$ domain (SNARE motif) of syntaxin 1A and the synprint site is responsible for syntaxin1A-mediated channel inhibition (Rettig et al 1996, Bezprozvanny et al 2000). The interaction between the H$_{abc}$ domain of syntaxin 1A and the synprint site anchors syntaxin 1A on the Ca$_{\alpha}$.2.2. Because of the G$\beta$$\gamma$ subunit-H$_3$ domain interaction, syntaxin 1A recruits G$\beta$$\gamma$ subunit to the Ca$^{2+}$ channel to promote G protein-mediated channel inhibition (Jarvis et al 2002).
In this study, ITC experiments were used to map the binding sites between the synprint site and syntaxin 1A. The ITC experiment showed no binding between each half of the synprint site and the H₃ domain of syntaxin1A (Figure 25c and d). However, in the previous study, a strong interaction was detected by pulldown assays (Sheng et al 1996). Perhaps, the pulldown/western blotting is a more sensitive biochemical technique than ITC. However, it is unlikely that ITC could not detect the reported nanomolar binding. One explanation is that either half of the synprint site shows weak affinity for the H₃ domain of syntaxin1A, but the two halves bind cooperatively to the H₃ domain. However, the ITC experiment using the full-length synprint site and the H₃ domain of syntaxin also displayed the negative result (personal communication: Kelvin Lau). Therefore, it is reasonable to say that there is no strong interaction between the synprint site and the H₃ domain of syntaxin. Whether the N-terminal part of syntaxin1A interacted with the synprint site was inconclusive because our initial positive result could not be reproduced. The positive ITC result showed strong binding between both the N-terminal half and the C-terminal half of the synprint site to N-terminal part of syntaxin1A (Figure 25 a and b). The two S-shaped titration curves with 1:1 stoichiometry did not seem to be an artifact. However, the subsequent ITC using different experimental conditions all showed negative results. We put a lot of effort into finding a reasonable scientific explanation for the conflicting results, but to date it still remains inconclusive. Therefore, it is difficult to conclude whether the N-terminal part of syntaxin binds to the synprint site.

Given these ITC results, it can be concluded that binding between the synprint site and syntaxin1A is not as strong as reported. However, the correlation between SNARE-mediated channel inhibition and the interaction between SNAREs and the synprint site
has been proven by several research groups. Therefore, we believe that there is still binding between SNAREs and the synprint site, but it is too weak to be detected. In the nerve terminal, syntaxin1A and SNAP-25 are anchored on the plasma membrane to act as t-SNAREs for the vesicle fusion, and neuronal Ca_{2.1} and 2.2 are “restricted” in the active zones by several adaptor proteins, such as Mint1 and CASK (Spafford et al 2003). The colocalization and high local concentration of SNAREs and Ca_{2.1}s can thus increase the chances for them to interact, thereby allowing modulation of calcium channels through the synprint site.
References


