Aus dem Bereich Physiologie

Theoretische Medizin und Biowissenschaften

Der Medizinischen Fakultät

Der Universität des Saarlandes, Homburg/Saar

Role of SNARE regulators - Complexin and Synaptotagmin in Ca^{2+} -triggered exocytosis

Dissertation

zur Erlangung des Grades eines Doktors der Naturwissenschaften

der Medizinschen Fakultät

der UNIVERSITÄT DES SAARLANDES

2019

vorgelegt von: Surya Raj Kumar Gaya, M.Sc (Molecular Biology)

geb. am. 19.06.1980 in Vijayawada, India

Homburg (Saar)

2019

Erklärung gemäß § 7 Abs. 1 Nr. 4

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise unentgeltlich/entgeltlich geholfen:

1. .....
2. ..... usw.

Weitere Personen waren an der inhaltlich-materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater/innen oder anderer Personen) in Anspruch genommen. Außer den Angegebenen hat niemand von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form in einem anderen Verfahren zur Erlangung des Doktorgrades einer anderen Prüfungsbehörde vorgelegt.

Ich versichere an Eides statt, dass ich nach bestem Wissen die Wahrheit gesagt und nichts verschwiegen habe.

Vor Aufnahme der vorstehenden Versicherung an Eides Statt wurde ich über die Bedeutung einer eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung belehrt.

Ort, Datum

Unterschrift der/des Promovierenden

Unterschrift der die Versicherung an Eides statt aufnehmenden Beamtin bzw. des aufnehmenden Beamten

Tag des Promotionskolloquiums:	14.07.2020
Dekan:	Prof. M. D. Menger
Vorsitzender:	--
Berichterstatter:	Prof. D. Bruns
	Prof. F. Schmitz

Dedicated to my family

Mrs. Premavathi & Mr. Vijay Kumar Gaya

Crystal, Alicia & Shawn

Sunith Kumar, Vasavi, Gloria & Olivia

Contents

Table of figures

Summary

Regulated neurotransmitter release is orchestrated by fusogenic SNARE proteins, the regulatory proteins, Complexin and Synaptotagmin. Complexin is believed to hinder premature exocytosis by clamping the membrane-bridging, partially assembled SNARE complex at a partially zippered state whereas Synaptotagmin1 acts as major Ca^{2+} sensor, synchronizing the fusion event to Ca^{2+} stimulus. Despite intensive research, the exact molecular mechanisms of how these SNARE 'regulators' and their potential interplay govern neurotransmitter release have remained enigmatic and are therefore the theme of this dissertation.

Previous studies have demonstrated that Cpx C-terminus clamps premature vesicle secretion and the N-terminus of Cpx accelerates the kinetics of exocytosis. Based on these findings, we set out to pinpoint crucial amino acid residues within the Cpx C-terminus and how they may mediate the molecular 'clamp' action of the protein preventing tonic secretion. In the framework of structure-function analysis, the results illustrate that the last 34 amino acids of Cpx are most responsible for preventing premature vesicular fusion. In particular, a C-terminal stretch of 19 amino acids, forming an amphipathic helix, turned out to be crucial for the clamp action. Mutating specific hydrophobic residues ($Cpx2^{L124,128W}$ mutant) in the C-terminus strongly impaired the clamp action. Based on these results and other corroborating evidences, we propose a model wherein the C-terminal amphipathic region acts as an alternative SNARE motif and thereby hinders progressive zippering of SNARE proteins.

In a separate set of experiments, we investigated the functional relationship between Cpx2 and the secondary Ca^{2+} sensor, Syt7 which is expressed in mouse chromaffin cells. The results show that Syt7 functions as a Ca^{2+} sensor for vesicle secretion, particularly at low Ca^{2+} concentrations. In the absence of Syt7, Cpx2 clamps premature secretion like in wt cells confirming that Cpx directly clamps SNARE proteins and not the Ca^{2+} sensor. Furthermore, in the absence of Cpx2, additional loss of Syt7 strongly aggravates the Cpx2 ko phenotype in contrast to Cpx2-Syt1 dko cells. This result shows that Cpx2 and Syt7 operate in different molecular steps en route to vesicle fusion. The combined set of data supports a scenario wherein Syt7 mediates early stages of vesicle priming and as the vesicles attain full release competence, they require Syt1 mediation for rapid release.

Taken together, this doctoral thesis pinpoints key properties of SNARE regulators shedding light on crucial regions of Cpx and the functional relevance of Syt7 in Ca^{2+} triggered exocytosis.

Zusammenfassung

Die regulierte Neurotransmitterfreisetzung wird durch fusogene SNARE-Proteine, die regulatorischen Proteine Complexin und Synaptotagmin gesteuert. Es wird angenommen, dass Complexin eine vorzeitige Exozytose verhindert, indem der membranüberbrückende, teilweise zusammengesetzte SNARE-Komplex in einem Zustand mit teilweisem Reißverschluss geklemmt wird, während Synaptotagmin1 als Haupt-Ca^{2+} -Sensor fungiert und das Fusionsereignis mit dem Ca^{2+} -Stimulus synchronisiert. Trotz intensiver Forschung sind die genauen molekularen Mechanismen, wie diese SNARE-Regulatoren und ihr mögliches Zusammenspiel die Freisetzung von Neurotransmittern steuern, rätselhaft geblieben und daher das Thema dieser Dissertation.

Frühere Studien haben gezeigt, dass der Cpx-C-Terminus die vorzeitige Vesikelsekretion hemmt und der N-Terminus von Cpx die Kinetik der Exozytose beschleunigt. Basierend auf diesen Erkenntnissen haben wir uns vorgenommen, wichtige Aminosäurereste im Cpx-C-Terminus zu lokalisieren und herauszufinden, wie sie die molekulare "Clamp" -Wirkung des Proteins, das die tonische Sekretion verhindert, vermitteln können. Im Rahmen der Struktur-Funktions-Analyse zeigen die Ergibnisse, dass die letzten 34 Aminosäuren von Cpx am stärksten für die Verhinderung einer vorzeitigen vesikulären Fusion verantwortlich sind. Insbesondere ein C-terminaler Abschnitt von 19 Aminosäuren, der eine amphipathische Helix bildet, erwies sich als entscheidend für die Klemmwirkung. Das Mutieren von spezifischen hydrophoben Resten ($Cpx2^{L124,128W}$-Mutante) im C-Terminus beeinträchtigt die Klemmwirkung stark. Basierend auf diesen Ergebnissen und anderen Beweisen schlagen wir ein Modell vor, bei dem die C-terminale amphipathische Region als alternatives SNARE-Motiv fungiert und dadurch das progressive Zippen von SNARE-Proteinen behindert.

In einer separaten Reihe von Experimenten untersuchten wir die funktionelle Beziehung zwischen Cpx2 und dem sekundären Ca^{2+} -Sensor Syt7, der in Chromaffinzellen der Maus exprimiert wird. Die Ergebnisse zeigen, dass Syt7 als Ca^{2+} -Sensor für die Vesikelsekretion fungiert, insbesondere bei niedrigen Ca^{2+} -Konzentrationen. In Abwesenheit von Syt7 klemmt Cpx2 die vorzeitige Sekretion wie in WT-Zellen und bestätigt, dass Cpx SNARE-Proteine direkt klemmt und nicht den Ca^{2+} -Sensor. Darüber hinaus verstärkt ein zusätzlicher Verlust von Syt7 in Abwesenheit von Cpx2 den Cpx2-ko-Phänotyp im Gegensatz zu Cpx2-Syt1-dko-Zellen. Dieses Ergebnis zeigt, dass Cpx2 und Syt7 auf dem Weg zur Vesikelfusion in verschiedenen molekularen Schritten arbeiten. Der kombinierte Datensatz unterstützt ein Szenario, bei dem

Syt7 frühe Stadien der Vesikelvorbereitung vermittelt und bei Erreichen der vollständigen Freisetzungskompetenz eine Syt1-Vermittlung für eine schnelle Freisetzung erforderlich ist.

Zusammenfassend zeigt diese Doktorarbeit wichtige Eigenschaften von SNARE-Regulatoren auf, die ein neus licht auf die wichtigen Bereiche von Cpx und die funktionelle Relevanz von Syt7 in Ca^{2+} -ausgelösten Exozytose.

Abbreviations

aa:	amino acid
ATP:	adenosine triphosphate
BAPTA:	1,2-bis (*o*-aminophenoxy) ethane- N,N,N',N'-tetraacetic acid
°C:	celsius
Ca^{2+}:	calcium
$CaCl_2$:	calcium chloride
$[Ca^{2+}]_i$:	intracellular free calcium ion concentration
cDNA:	complementary DNA
cm:	centimeter
CM:	membrane capacitance
CO_2:	carbon dioxide
Cpx:	complexin
Cpx2 ko:	complexin2 knock out
Cpx2/Syt1 dko:	complexin2/synaptotagmin1 double knock out
Cs:	cesium
CsOH:	cesium hydroxide
ΔC_M:	delta (change) in membrane capacitance
DDW:	double distilled water
Dko:	double knockout
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
dNTP:	deoxyribonucleotide triphosphate
DPTA:	1,3-diaminopropane-N,N,N',N'-tetraacetic acid
EB:	exocytotic burst
EDTA:	ethylenediamine tetra acetic acid
EGFP:	enhanced Green Fluorescent Protein
EGTA:	ethylene glycol tetra acetic acid
fF:	femtofarad
fF/s:	femtofarad per second
Fig:	figure
Fw-Primer:	forward primer

g:	gram
GM:	membrane conductance
GS:	series conductance
GTP:	Guanosine triphosphate
h:	hour
H_2O:	water
HEPES:	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HCl:	hydrochloric acid
Hz:	Hertz
kb:	kilobase pair
KCl:	potassium chloride
kDa:	kilodalton
kg:	kilogram
KHz:	kilohertz
ko:	knockout
LDCV:	large dense-core vesicles
µF:	microfarad
µg:	microgram
µL:	microliter
mg:	milligram
Mg^{2+}:	magnesium
MgATP:	magnesium ATP
$MgCl_2$:	magnesium chloride
min:	minutes
mL:	milliliter
mm:	millimeter
mM:	millimolar
mOsm:	milliosmol
ms:	milliseconds
Munc-18:	mammalian uncoordinated-18
mV:	millivolt
MΩ:	megaohm
n:	number of elements (e.g., cells)
Na_2EDTA:	sodium EDTA

Na_2GTP:	sodium GTP
$NaHCO_3$:	sodium bicarbonate
NaCl:	sodium chloride
$(NH_4)_2SO_4$:	ammonium sulfate
nm:	nanometer
nM:	nanomolar
NMJ:	neuromuscular junction
NP-EGTA:	nitrophenyl-EGTA
nS:	nanosiemens
NSF:	N-ethylmaleimide sensitive factor
p:	p-value, probability value
PCR:	polymerase chain reaction
pF:	picofarad
pM:	picomolar
pS:	picosiemens
R:	ratio
Rev-Primer:	reverse primer
RNA:	ribonucleic acid
rpm:	revolutions per minute
RRP:	readily-releasable pool
s:	seconds
SDS:	sodium dodecyl sulfate (detergent)
SFV:	semiliki Forest Virus
SNAP:	soluble NSF attachment protein
SNAP-25:	synaptosomal-associated protein of 25 kDa
SNARE:	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SR:	sustained rate
SRP:	slowly releasable pool
Syb:	synaptobrevin
Syt:	synaptotagmin
Syt1 ko:	synaptotagmin1 knock out
Syt7 ko:	synaptotagmin7 knock out
Stx:	syntaxin
t:	time

Tab:	table
TAE:	tris-acetate-EDTA
temp:	temperature
TMD:	transmembrane domain
TMR:	transmembrane region
TRIS:	tris(hydroxymethyl)aminomethane
t-SNARE:	target SNARE
UV:	ultraviolet
V:	volt
VAMP:	vesicle-associated membrane protein
v-SNARE:	vesicular SNARE
wt:	wild type

1 Introduction

The ability of cells to transmit information between each other is a hallmark of multicellular organisms. One of the ways cells perform this 'communication' is by transferring chemical substances from one cell to another. Neurons and neuroendocrine cells contain membranous organelles called secretory vesicles or granules that store neurotransmitter molecules and hormones which are released into the extracellular space by an efficient process known as exocytosis. Classical exocytosis may be distinguished further into constitutive or regulated exocytosis (Fig.1). Constitutive exocytosis occurs continuously in all cells irrespective of any environmental factors or stimuli and involves the transfer of macromolecules such as newly synthesized lipids and proteins to the plasma membrane, soluble proteins, etc. in vesicles. Here, the transport vesicles are simply incorporated into the cell membrane to release their components into the extracellular space. Regulated exocytosis on the other hand, occurs only in secretory cells and the vesicular components (neurotransmitters, hormones, cytokines, etc.) are released only when triggered or stimulated for example by a change in the intracellular signal levels (Burgess and Kelly, 1987).

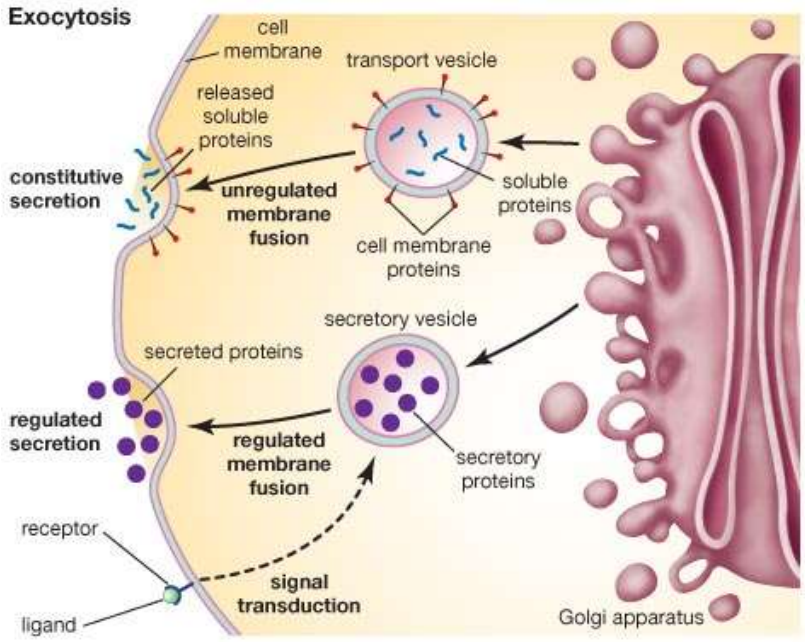


Figure 1. Cells transport proteins actively via exocytosis.
Membrane bound vesicles carrying soluble proteins or neurotransmitter molecules, bud off either from Golgi apparatus or from early endosomes. Vesicles fuse with the plasma membrane to deposit their cargo into the exterior of the cell. Fusion occurs either constitutively, without any external signal or in a regulated fashion, due to an external stimulus *(adapted from Encyclopedia Britannica, 2008).*

1.1 Regulated Exocytosis- A multi-step process

Regulated exocytosis is the very basis of cell-to-cell communication in all living organisms. It is characterized by extremely tight control of Ca^{2+} and a very high speed of execution. Studies have shown that regulated exocytosis proceeds in a step-wise fashion that involves different, molecularly distinct steps (Burgoyne, Morgan 1998). Briefly, after the biogenesis of vesicles, they are translocated along microtubules of the cytoskeleton to the sub-plasmalemmal release sites of the cell where they are tethered or 'docked'. Then they undergo 'priming'- a molecular process by which they acquire fusion competence. The next step is the 'fusion' of vesicular membrane to the plasma membrane delivering the release of neurotransmitters or catecholamines into the extracellular space. As vesicles continue to fuse and hand over their contents, newly synthesized vesicles either by the process of biogenesis or membrane retrieval (endocytosis) arrive at the plasma membrane for another cycle of exocytosis. (Burgoyne 1995). Based on studies of membrane trafficking in yeast, neuronal exocytosis, in vitro fusion reactions of intracellular organelles, members of four protein families (SNARE proteins, sec1/munc18-like (SM) proteins, NSF with adaptor proteins, and Rab proteins) are known to mediate exocytosis in eukaryotes (Gerber and Südhof, 2002) (Fig. 2).

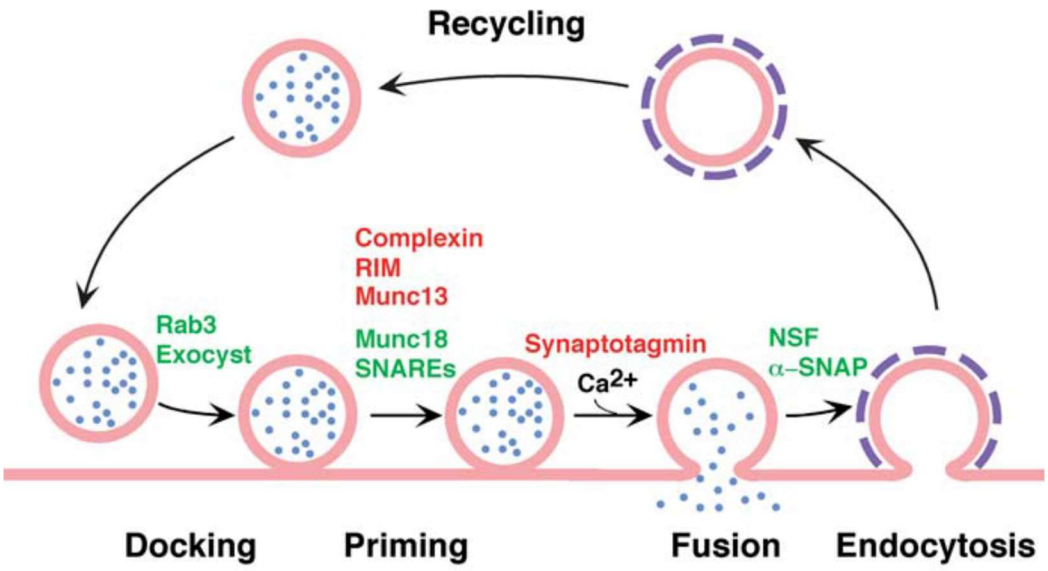


Figure 2. Different stages of exocytosis.
Newly synthesized vesicles migrate to the vicinity of the plasma membrane. Here, they are physically tethered/docked, primed (gain release competence) and then fuse with the plasma membrane. Several proteins coordinate these molecular events, eventually delivering the vesicular contents to the extracellular space and recycling the vesicles for another cycle of exocytosis *(adapted from LiL & Chin LS, 2002).*

1.1.1 Vesicle Docking and Priming

The term ‘docking’ is widely used to describe a state of vesicles in the process of maturation, before they attain fusion-competence. Usually, docking is considered to be an initial ‘tethering’ or ‘anchoring’ of the vesicles (within close proximity) to the plasma membrane (Waters et al, 2000; Pfeffer 1999). However, several criteria have been used to assess and define the docking stage of vesicles. For instance, according to electron microscopy studies, morphological proximity of vesicles to the plasma membrane has been used to define docked vesicles (Gray 1959; Verhage and Sorensen 2008). Some studies consider vesicles to be docked when they are present at a distance of about 40 nm from the plasma membrane (Siksou et al., 2009) while others have considered 30 nm from the plasma membrane to be a docking zone (de Wit et al., 2006). A biochemical definition of docking can refer to a stable interaction between vesicles and plasma membrane. Martin, Kowalchyk et al., 1997 identified vesicle fractions that not only retain their association with the plasma membrane after homogenization but also retain their competence for Ca^{2+}-triggered fusion, if appropriately primed.

In neural cells and neuroendocrine cells, the ‘tethered/docked’ condition of the vesicles does not intrinsically render them ready to fuse with the plasma membrane. Although a relatively large number of vesicles may be docked, only a fraction of these undergo ‘maturation’ events that render the docked vesicles competent for Ca^{2+} triggered fusion. Patch clamp recordings and capacitance measurements in neurons and neuroendocrine cells have shown different kinetic phases of secretion (Rettig and Neher, 2002). For instance, the first kinetic phase, known as the exocytotic burst (EB) occurs rapidly (milli seconds to a few seconds timescale) and represents the ‘fusion-competent’ or readily releasable pool (RRP) of vesicles. The later slower phase (~10 s), known as the sustained component, represents the newly arriving pool of vesicles, refilling the RRP. These studies illustrate that only a privileged subset of docked vesicles is staged in a release ready or primed state waiting only for a Ca^{2+} trigger to initiate membrane fusion. Priming can include reorganization of actin cytoskeleton (Burgoyne, Cheek 1987), formation of trimeric SNARE complexes (Jahn and Südhof 1999; Chen and Scheller 2001; Brunger 2001; Rizo and Südhof 2002; Bruns and Jahn 2002; Fasshauer 2003), modification of SNARE proteins by ATPase NSF (Banerjee, 1996) and maintenance of poly-phosphoinositide levels (Eberhard et al., 1990). Priming is also thought to be a recruiting event in which the vesicles are positioned in the proximity of Ca^{2+} channels (Neher and Sakaba, 2008). Different proteins such as Munc-13 and CAPS have been shown to be crucial in priming vesicles by initiating SNARE complex

formation and to stabilize synaptic vesicles in a fusion competent state (Südhof, 2004, Jockusch et al., 2007).

1.1.2 Membrane Fusion

The final step in exocytosis is membrane fusion, where in two separate lipid bilayers (vesicular membrane and the cell membrane) merge to become a single continuous bilayer. All fusion reactions essentially proceed in two or three steps (Jahn et al., 2003) as described here below. To begin with, the vesicles must be brought into close proximity of the plasma membrane, against several mechanical and molecular energy barriers. For instance, the clearance of proteins (sieving) or the counteracting electrostatic forces need to be overcome. Second, the destabilization of the hydrophobic and hydrophilic portions of the bilayers must take place. Consequently, the lipids present in the proximal leaflets merge with each other, generating non-bilayer transition states known as hemifusion intermediates (Chernomordik et al., 2003). This hemifusion state is described as the formation of an hour-glass-shaped lipid structure or a fusion stalk between two adjacent membrane leaflets (Fig. 3). Hemifusion finally culminates in the formation of an aqueous fusion pore (diameter <0.5-2 nm) that rapidly dilates to fully collapse into the plasma membrane (full fusion).

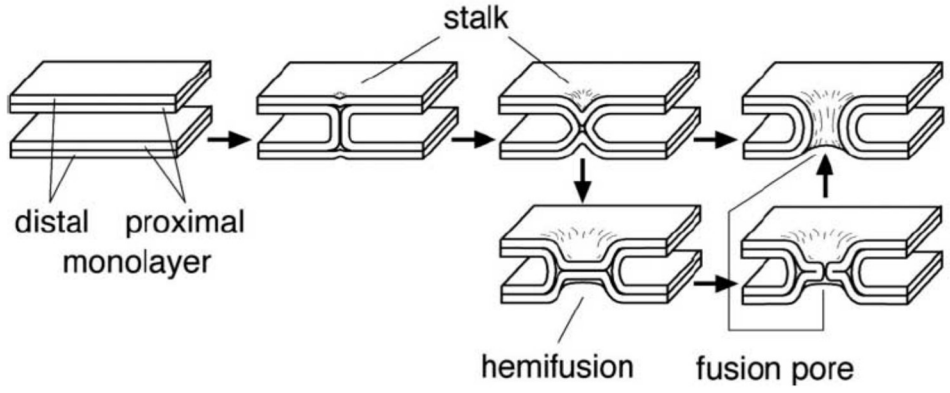


Figure 3. Hemifusion.
A pre-fusion stage where only the proximal lipid layers merge before an initial fusion pore is formed. With the merging of distal leaflets, an aqueous pore is formed which rapidly dilates and collapses into the plasma membrane causing full fusion *(adapted from Jahn R et al., 2003)*.

The whole process of membrane fusion is coordinated with the help of several proteins which essentially work to lower the energy barriers at the right time and place. For instance, Rab/Ypt GTPases (e.g. Rab3A and Rab3B) are actively involved not only in trafficking transport vesicles (donor) to the plasma membrane (acceptor), but also to tether the two membranes together,

thus providing specificity to fusion reactions (Pfeffer and Aivazian, 2004; Zerial and McBride, 2001). Following membrane attachment, fusion is initiated by the combined action of SNARE proteins and SM (Sec1/Munc18-like) proteins.

1.1.3 SNARE proteins – the core of Membrane Fusion Machinery

Although membrane fusion is important for cellular communication, spontaneous membrane fusion is actively opposed by forces such as electrostatic repulsions between two approaching membrane surfaces or the lateral tension of the bilayer interface (Chernomordik et al., 1987; Kozlovsky et al., 2002). The energy needed to overcome such energy barriers for the fusion of two membranes is provided by the assembling of specialized proteins known as the SNARE proteins into α-helical bundles or *trans*-SNARE complexes (Jahn et al., 2003; Rizo et al., 2006; Jahn and Fasshauer, 2012). Both neurons and neuroendocrine cells employ the same fusion machinery composed of SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins and SM proteins (Südhof, Rothman 2009). The core fusion machinery is comprised of three highly conserved proteins known as the SNARE proteins, namely Syntaxin-1, SNAP-25 and Synaptobrevin/VAMP (vesicle associated membrane protein). These proteins belong to a superfamily (>35 members in mammals) of membrane associated proteins that are involved in all trafficking steps of the secretory pathway (Rizo & Südhof 2002, Chen & Scheller 2001, Kavalali 2002, Brunger 2001). SNARE proteins in general have a simple structure consisting of a transmembrane domain towards their C-terminus, a short linker and a homologous sequence called the 'SNARE motif' (Kloepper et al., 2007). The SNARE motif comprises a stretch of ~ 60-70 amino acids which is further characterized by eight heptad repeats of hydrophobic residues that can form coiled-coils at the moment of interaction with other SNARE motifs. These motifs are highly conserved and serve as a defining hallmark of SNARE proteins (Bock et al., 2001).

Distinct SNARE proteins are targeted to distinct membrane domains and compartments. Depending on their sub-cellular localization, SNARE proteins are initially classified as those that are associated with the vesicles (v-SNAREs - predominantly Synaptobrevin) and those that are located on the target, plasma membrane (t-SNAREs - predominantly Syntaxin and SNAP-25) (Söllner et al., 1993b). Furthermore, being present on two adjacent membranes, the SNAREs can interact within their motifs to form extremely stable, even SDS-resistant coiled-coil complexes or helix bundles (Fig. 4). The vesicular protein Synaptobrevin/v-SNARE motif interacts with one t-SNARE motif provided by Syntaxin and two t-SNARE motifs provided by

SNAP-25 (heterodimeric). Thus, SNARE complexes are comprised of four SNARE motifs that zipper from N- to C-terminal (Hanson et al., 1997) forming a 4-helix bundle in parallel orientation. Zippering of the SNAREs generates energy to pull the two membranes (vesicular and plasma membrane) in to close proximity and causes a membrane merger or a fusion pore. Studies have shown that the *trans*-SNARE complexes can zipper all the way into the transmembrane domain (Stein et al., 2009; Risselada et al., 2011). Further dilation of the fusion pore fully integrates the vesicular membrane into the plasma membrane emptying the content of the vesicles into the extracellular space.

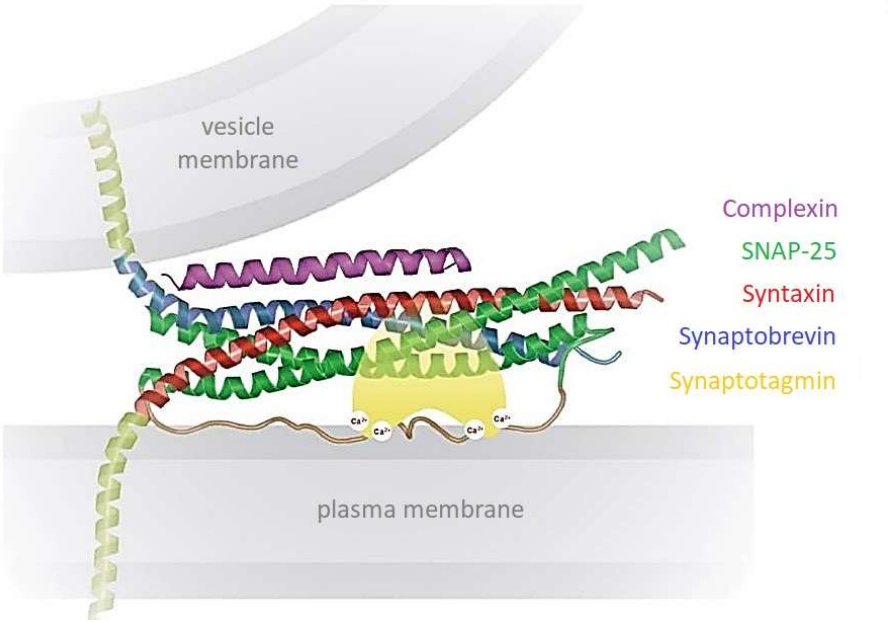


Figure 4. SNARE proteins.
v-SNARE protein, synaptobrevin (blue) and t-SNARE proteins syntaxin (red) and SNAP-25 (green) bind with each other to form a 4-helix bundle thereby linking the vesicular membrane to the plasma membrane as shown here in this trans-SNARE complex. Full zippering of SNARE proteins causes the two membranes to merge and release their contents into the extra cellular space. Transmembrane domains are depicted in yellow and the linker region of SNAP25 in brown. Probable location of Complexin (purple) and Synaptotagmin (yellow) is also shown.

1.2 Exocytosis Regulation

Neurotransmitter release is tightly regulated, spatially accurate and occurs at a very rapid pace in mammalian cells. For instance, at the active zones of nerve cell synapses, an action potential is transduced into vesicle fusion within less than 1 ms (Borst and Sakmann, 1996, Sabatini and Regehr, 1996). Such fast stimulus-secretion coupling is exquisitely dependent on the ambient Ca^{2+} concentration (Schneggenburger and Neher, 2005). However, it has been shown that without any additional factors, the three SNARE proteins operate independently of Ca^{2+} and

induce slow fusion in liposomes *in vitro* (Weber et al., 1998). Moreover, since SNARE proteins are constitutively active and are able to form complexes spontaneously (Sorensen et al., 2006; Jahn, Scheller 2006), it is possible that vesicles fuse spontaneously leading to premature loss of functionally distinct pool of primed vesicles. Apparently, SNARE mediated exocytosis is driven by additional proteins that confer Ca^{2+} sensitivity of the fusion process as well as the speed of stimulus-secretion coupling. The SNARE proteins require regulatory proteins like Complexin (Cpx) (McMahon et al., 1995; Chen et al., 2002) and Synaptotagmin (Syt) (Geppert et al., 1994) to confer speed and precision to the fusion process (Wojcik and Brose 2007). While Cpx is seen to potentially hinder the SNARE proteins from zippering completely, Syt is believed in general to be the Ca^{2+} sensor of the fusion machinery (Chapman, 2008).

1.2.1 Complexins – small but capable

Complexins, also called Synaphins, constitute a family of small (15-20 kDa), cytosolic (hydrophilic) proteins rich in glutamic acid and lysine residues. Four isoforms of complexin (1, 2, 3 and 4) have been identified in mammals and are all predominantly expressed in the central nervous system (Reim et al, 2005; Brose, 2008). Cpx1 and Cpx2 display 86% sequence identity with each other and are widely distributed in the body but predominantly expressed in the brain. Cpx3 and Cpx4 exhibit a different pattern in structure containing 158 and 160 amino acids respectively as well as in their expression. Cpx1 and Cpx2 show limited homology (only 24-28% identity) with Cpx3 and Cpx4. Cpx3 has strong expression in the retina and lesser levels in the brain whereas Cpx4 is exclusively present in retinal ribbon synapses. Interestingly, Cpx3 and Cpx4 also contain on the C-terminus, a functional isoprenylation sequence (CAAX-box) also known as farnesylation site that is absent in Cpx1 and Cpx2.

1.2.1.1 Function of Complexin

Complexins operate by binding to SNARE complexes with high affinity. This binding is thought to stabilize and clamp SNARE complexes in a highly fusogenic state (Reim et al., 2001; Xue et al., 2007) which results in the ‘inhibition’ of primed vesicles from premature, un-triggered spontaneous secretion. Indeed, deletion of complexin in neuromuscular junctions of C. *elegans* has resulted in strong increase in spontaneous exocytosis (Hobson et al., 2011). In Drosophila neuromuscular synapses, genetic ablation of complexin increased spontaneous release by >20 fold (Huntwork et al., 2007). Studies from our lab have demonstrated that in mouse chromaffin cells, loss of Cpx2 results in enhanced tonic secretion at submicromolar Ca^{2+} concentrations (>100 nM). Interestingly, on the other hand, overexpressing complexin in neurons and non-

neuronal cells has provided strong evidence for the inhibition of vesicle secretion due to the 'clamp' action of complexin in mouse (Dhara et al., 2014) and bovine (Archer et al., 2002) chromaffin cells.

Loss of complexin not only leads to increased spontaneous release but also impairs evoked secretion. Therefore, complexin has also been implicated in playing a direct role in 'facilitating' evoked neurotransmitter release, (in the presence of Ca^{2+} stimulus) as revealed from several knock-down and knock-out studies (Xue et al., 2008; Cai et al., 2008; Cho et al., 2010; Yang et al., 2013; Lin et al., 2013). According to a study, complexin facilitates secretion by granting fusogenicity to primed vesicle pool (RRP) (Xue et al., 2010). The absence of complexin therefore exhibits remarkable reduction in evoked secretion because the vesicles reside in a more fusion-reluctant state. Research in our lab has demonstrated that in chromaffin cells lacking complexin, in addition to the diminished magnitude of evoked exocytosis, the rates of RRP and SRP are also slowed down. Moreover, loss of Cpx2 conferred a longer secretory delay between the stimulus and the onset of secretory response (Dhara et al., 2014). This de-synchronization of release was also seen in fly and murine preparations, genetically lacking complexin (Iyer et al., 2013; Jorquera et al., 2012).

In human beings, alteration in the expression levels of Complexin causes deficits in synaptic transmission. Such deficits are associated with symptoms of neurological disorders such as schizophrenia, depression, bipolar disorder, Alzheimer's disease, Parkinson's disease, Huntington's disease, Wernicke's encephalopathy, traumatic brain injury (Brose, 2008).

1.2.1.2 Structural features of complexin: Distinct domains – Distinct functions

Complexins are composed of multiple domains with distinct functions (Fig.5). NMR studies have revealed that Cpx lacks a tertiary structure but has a central helical region (Pabst et al., 2000). The central α-helical region (aa 26-83) that constitutes both the accessory α-helix (aa 28-47) and the SNARE binding (aa 48-70) domain is the 'minimal clamping domain' required for the proteins' regulatory function (Giraudo et al., 2008). The central α-helix of Cpx (aa 48-70) is the main binding interface with which Cpx binds in an antiparallel orientation to the SNARE complex in the groove between Synaptobrevin and Syntaxin-1 helices (Bracher et al., 2002, Hu et al., 2002, Bowen et al., 2005). Mutations in this region diminish the association of Cpx with the SNARE complex (Xue et al., 2007). Complexins are known to colocalize with SNAP receptors and bind very rapidly ($\approx 5x10^{7}M^{-1}.s^{-1}$) to SNARE complexes with high affinity (McMahon et al.,

1995; Ishuzuka et al 1995) and with 1:1 stoichiometry (Cai, Chow 2008). In addition, interaction of Cpx with a 1:1 SNAP-25:Syntaxin1a complex has also been reported (Weninger et al., 2008). Cpxs 1 and 2 are shown to stabilize SNARE complexes and thereby maintain a readily releasable pool (RRP) of granules that is sensitive to Ca^{2+} (Reim et al., 2001; Chen et al., 2002). The interaction of Cpx with SNARE core complexes is crucial for regulating exocytosis.

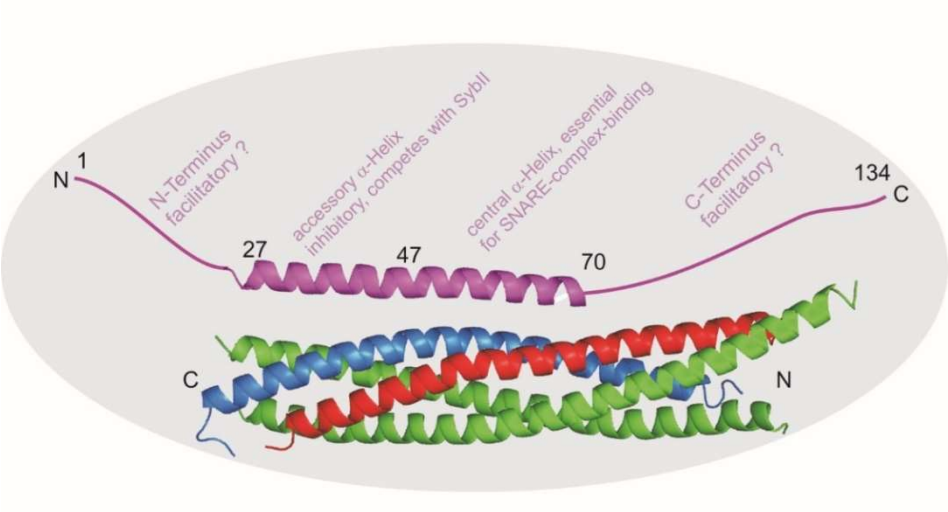


Figure 5. Structure of complexin-SNARE complex.
Ribbon diagram of the mammalian complexin-SNARE complex. The central α-helix (aa 27-71) is flanked by unstructured amino and carboxy terminals. The binding of Cpx to the SNARE complex, between the SyntaxinI (red) and Synaptobrevin (blue) is crucial for all its functions. Note the antiparallel orientation of complexin with respect to the SNARE complex *(adapted from Sutton et al., 1998 and Chen et al., 2002)*.

Adjacent to the central SNARE binding α-helix, Cpx contains an accessory α-helix (aa 28-47) which does not contact the SNARE complex (Chen et al., 2002) but is known to enhance the SNARE binding of the central α-helix (Xue et al., 2007). The accessory α-helix can bind to the C-terminal region of a partially assembled SNARE complex and block the full zippering of the complexes, thereby inhibiting the neurotransmitter release (Giraudo et al., 2008, Krishnakumar et al., 2011). By competing with the binding of Syb2 to its cognate SNARE partners namely SNAP-25 and Syntaxin, the accessory α-helix of Cpx is thought to function as an 'on-off' switch for fusion (Giraudo et al., 2009). Another research group (Kümmel et al., 2011) studied the crystal structure of a complex formed between Cpx1 'superclamp' mutant (having more hydrophobic interface with the SNARE complex leading to increased clamping efficiency) and Syb2 lacking its C-terminus. This study proposed that the accessory α-helix of Cpx may bind to one SNARE complex while the SNARE binding domain may be bound to another SNARE complex, thereby organizing SNARE complexes into a 'zigzag' array that hinders fusion.

Furthermore, Trimbuch et al., 2014 noticed that in the absence of the accessory α-helix, there is a tenfold decrease in the binding affinity of complexin's SNARE binding domain to the SNARE complex. Since the accessory α-helix contains a number of negatively charged amino acids, this region may hinder secretion by enhancing electrostatic repulsions between the vesicle and the plasma membrane. This suggests that the accessory α-helix may indirectly affect the interactions between complexin and the SNARE bundle. Additionally, the helicity of this region may also be crucial to stabilize complexin's binding to the SNARE complex. Using molecular dynamics simulation, another group showed that the accessory α-helix interacts with Syb2 in a way that arrests the zippering of the last (+7 and +8) hydrophobic layers (Bykhovskaia et al., 2013). Yet another study proposed that complexins accessory α-helix putatively clamps an unidentified secondary Ca^{2+} sensor. In the absence of Cpx, activation of this Ca^{2+} sensor would lead to unrestricted vesicle fusion (Yang et al., 2010).

The central helical region is flanked by unstructured sequences namely the N-terminal domain and the C-terminal domain. Pabst et al., 2002 reported that both these regions are susceptible to proteolytic degradation when Cpx-SNARE complex is formed suggesting that these regions are not involved in tight interactions and may stay unstructured. Later studies however showed that the N-terminal domain (aa 1-26) can fully rescue the evoked release in hippocampal neurons genetically devoid of Cpx1 (Xue et al., 2007). They reported that the very N-terminus of Cpx1 contains crucial residues (Phe3, Val4, Met5 and Lys6) that are important for exerting facilitatory function. Based on biophysical experiments and structural considerations, they also proposed that the N-terminus of Cpx has the potential to form amphipathic α-helix that can bind to the C-terminus of SNARE bundle and stabilize its assembly (Xue et al., 2010). Distinct N-terminal sequences of complexin have also been shown to activate synchronous secretion and clamp the force exerted by trans-SNARE complexes on the fusing membranes (Maximov et al., 2009; Yang et al., 2010). Previous work in our lab has shown that the N-terminus is responsible for shortening the secretory delay and accelerating Ca^{2+} -triggered exocytosis. Mouse chromaffin cells lacking the N-terminus (ΔN, aa 27-134) also displayed reduced Ca^{2+}-sensitivity of evoked release indicating a crosstalk between complexin and synaptotagmin, the Ca^{2+} sensor (Dhara et al., 2014).

Regarding the C-terminal domain (aa 72-134) of complexin which was previously thought to be functionally inert (Xue et al., 2007), *in vitro* and *in vivo* experiments have shown that the C-terminus can participate in 'clamping' spontaneous fusion in neurons (Cho et al., 2010; Buhl et

al., 2013; Kaeser-Woo et al., 2012; Martin et al., 2011). Cpx C-terminus was also seen to bind to Synaptotagmin (Tokumaru et al., 2008). Some studies also indicate that C-terminus of Cpx contains an amphipathic helix with which it could bind to phospholipids (Wragg et al., 2013; Seiler et al., 2009; Malsam et al., 2009). The C-terminus of Cpx, with the help of its amphipathic helix has been shown to target the protein to the vesicles in a curvature sensitive manner to inhibit spontaneous exocytosis (Wragg et al., 2013; Snead et al., 2014; Gong et al., 2016). In addition, experiments using C-terminus truncation mutant (ΔC, aa 1-72) have demonstrated that complexin loses its ability to clamp tonic secretion in neuroendocrine cells (Dhara et al., 2014).

Despite intensive research with regard to Cpx, a comprehensive view on the molecular mechanism by which Cpx hinders premature exocytosis is still a matter of discussion. In the present dissertation, we set out to delineate the action of C-terminal domain of Cpx in detail, even to the amino acid level.

1.2.2 Synaptotagmins as Ca^{2+} sensors

Under physiological conditions, an action potential causes the opening of voltage-sensitive Ca^{2+} channels, leading to a transient increase in the local Ca^{2+} concentration. By activating Ca^{2+} sensors, calcium triggers neurotransmitter release within a few hundred microseconds (Sabatini and Regehr., 1996). The two main determinants of neurotransmitter release are therefore the Ca^{2+} -dynamics (properties and location of Ca^{2+} channels, Ca^{2+} concentration, Ca^{2+} affinities and kinetics of local Ca^{2+} buffers, etc.) and the action of Ca^{2+} -sensors that translate the calcium signal into release.

Synaptotagmins (Syts) are a family of membrane-trafficking proteins that are the most likely candidates to function as Ca^{2+} sensors of exocytosis (Chapman 2002; Koh and Bellen 2003). In mammals, at least 15 isoforms of synaptotagmins, all sharing a common domain structure, but different expression patterns and properties have been identified. Only 8 isoforms (-1, -2, -3, -5, -6, -7, -9, -10) can bind Ca^{2+} with different Ca^{2+} affinities (Craxton 2007; Gustavsson and Han, 2009). The other Syt isoforms fail to bind calcium either because they lack the five critical acidic residues or since they cannot form proper calcium binding sites. Syt-1, -2 & -9 have been shown to function as Ca^{2+}-sensors during fast, synchronous secretion in both synaptic and neuroendocrine systems (Xu et al., 2007, Brose et al., 1992, Perin et al., 1990). Syt2 is expressed in synapses such as neuromuscular junctions and calyx of Held and is known to trigger release much faster than Syt1. Syt9 on the other hand operates with a significantly

slower time course. Syt10 acts as a Ca^{2+} sensor secreting IGF-1 in olfactory bulb neurons (Cao et al., 2011, 2013). Syt7 has been shown to contribute to slow, asynchronous release in neuromuscular junction of zebrafish (Wen et al., 2010). In neuroendocrine cells, Syt7 co-localizes with Syt1 and is shown to mediate Ca^{2+} triggered exocytosis with a slower time course (Schonn et al., 2008).

The primary structure of synaptotagmin shows a transmembrane domain (TMD) towards the amino terminus and two calcium sensing domains (C2A & C2B) towards the carboxy terminus accounting for the majority of the protein (Fig.6). The C2 domains, being cytosolic are connected to the TMD by a central linker. Both C2-domains - C2A and C2B are each composed of eight-stranded, stable β-sheets with flexible loops present at the top and bottom. The C2A domain can accommodate 3 Ca^{2+} ions in its loop made of five aspartate residues (D^{172}, D^{178}, D^{230}, D^{232}, S^{235}& D^{238}). The C2B domains can host 2 Ca^{2+} ions in their loops via D^{303}, D^{309}, D^{363}, D^{365} and D^{371} (Shao X et al., 1998; Fernandez et al., 2001).

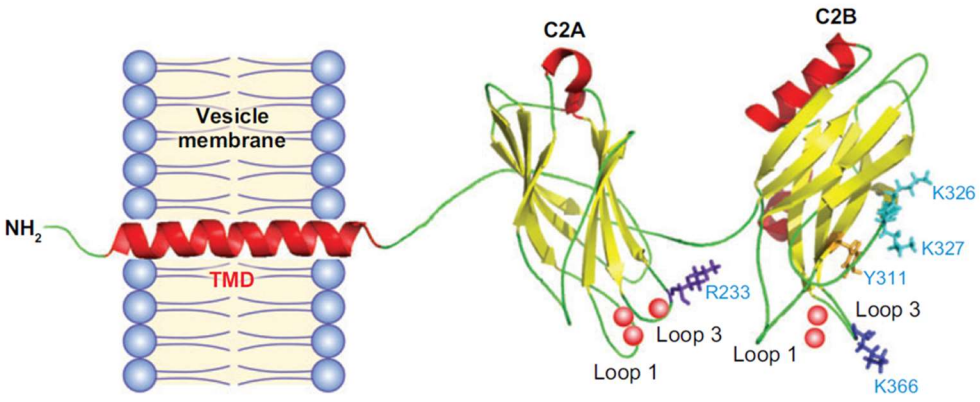


Figure 6. Structure of Synaptotagmin1.
The C2AB domains were rendered from Fernandez et al., 2001; Shao et al., 1998 using PyMOL. The remaining protein segments were added using a drawing program. Ca^{2+} ions are shown as red spheres. The side chains of R233, K366, etc. within the Ca^{2+} binding loops are shown *(adapted from Chapman, 2008)*.

Several studies have shown that although both domains bind Ca^{2+} ions (Bai and Chapman 2004b), they are not equivalent but only cooperate with each other. Mutations within C2A domain have shown ~50% reduction in evoked exocytosis due to changes not only in the overall Ca^{2+} affinity of Syt but also in the overall Ca^{2+} affinity of synaptic exocytosis (Fernandez-Chacon et al., 2001; Shin et al., 2009). However, when Ca^{2+} binding to C2B domain was blocked, synchronous secretion was fully blocked (Mackler et al., 2002). C2B binding is shown to be important for both Ca^{2+}-dependent phospholipid binding and also for dislodging Cpx from

SNARE complexes demonstrating the importance of this domain (Pang et al., 2006). Thus, both C2-domains function cooperatively and the binding of Ca^{2+} to both domains trigger release to achieve the high cooperativity of Ca^{2+} triggering of release (Meinrenken et al., 2003). Initial studies on Syt1 have revealed that both C2 domains not only 'sense' Ca^{2+} ions but also bind to phospholipids (Fernandez et al., 2001; Davletov and Südhof 1992) and SNARE proteins (Chapman et al., 1995; Bennett et al., 1992) in a calcium dependent manner. Synaptotagmin via its interactions with SNAP25 was seen to be implicated in docking of vesicles (Chieregatti et al., 2002).

Both in neurons and neuroendocrine cells, neurotransmitter release is triggered by calcium and is composed of a fast, synchronous and a slow asynchronous component. A calcium concentration in the range of ~10-40 μM and a calcium cooperativity value of ~5 is required for synchronous secretion. However, low Ca^{2+} concentration (~2-9 μM) and low Ca^{2+} -cooperativity value (~2) is sufficient for asynchronous secretion (Südhof, 2004; Sun et al., 2007). Several studies have established that Syt1 is a low affinity Ca^{2+} sensor that is widely expressed in nervous and endocrine systems where it acts as a main calcium sensor during the rapid phase (RRP) of exocytosis. Genetic deletion of Syt1 in mice, Drosophila and *Caenorhabditis elegans* has selectively abolished the fast component (RRP) without affecting the slow, asynchronous secretion, indicating that Syt1 is required for rapid exocytosis (Geppert et al., 1994; Pang et al., 2006; DiAntonio et al., 1993; Littleton et al 1993; Nonet et al., 1993). In chromaffin cells, Syt1 deletion leads to selective loss of RRP (Voets et al., 2001b) and rescuing with different Syt isoforms leads to variable fusion kinetics (Nagy et al., 2006). These studies strongly support the view that Syt1 functions as the major Ca^{2+} sensor for synchronous (RRP) secretion. Although no Ca^{2+} sensor has been identified for the asynchronous SRP secretion, other Syt isoforms might perform this function. For instance, Syt7 has been reported to function as a Ca^{2+} sensor in neurons and neuroendocrine cells (Südhof, 2011; Schonn et al., 2008). Loss of Syt7 alone leads to a 50% reduction in the exocytotic burst in mouse chromaffin cells (Schonn et al., 2008) as well as in insulin-secreting cells (Li et al., 2007; Gauthier et al., 2008). Syt7 is shown to be responsible for the slower phase of exocytotic burst that remains in Syt1 ko and further loss of Syt7 eliminates the residual slow burst, as demonstrated by Syt1/Syt7 dko experiments (Schonn et al., 2008).

1.2.3 Complexin – Synaptotagmin Interplay

The mechanistic interplay between complexin and synaptotagmin is of central importance to better understand the two contrasting (facilitatory and inhibitory) actions of complexin. While the inhibitory action is brought about by Cpx C-terminus, the N-terminus is seen to facilitate fusion by increasing the Ca^{2+} sensitivity and release probability (Dhara et al., 2014). This implies that when cytosolic [Ca^{2+}] reaches above-threshold (μM) levels, the complexin mediated 'fusion clamp' must be rapidly lifted off by an antagonistic action of Ca^{2+}-bound Syt1 leading to full vesicular fusion.

Based on invitro fusion experiments (Schaub et al., 2006; Giraudo et al., 2008; Krishnakumar et al., 2011; Malsam et al., 2012) and biochemical studies (Tang et al., 2006; Dai et al., 2007), it has been proposed that Cpx and Syt1 are mutually exclusive for binding on the SNARE complex and that Syt1 may antagonistically displace Cpx from the SNARE complex. However, other studies (Chicka et al., 2009) presented evidence for simultaneous binding of both proteins to the SNARE complex in a non-overlapping configuration and proposed that the antagonism does not come from competition for binding sites.

Based on amperometry experiments investigating initial fusion pore dynamics, a push-pull mechanism between Cpx2 and Syt1 has been suggested, wherein Cpx restricts fusion pore expansion while Syt accelerates fusion pore dilation (Dhara et al., 2014). Thus, at the moment of Ca^{2+} rise, Syt1 overcomes Cpx2 mediated restraints on force transduction to initiate the formation and accelerates the dilation of fusion pore (even while both proteins remain residing on the SNARE complex).

Another view proposes that conformational changes in the accessory α-helix and the N-terminus of Cpx determine its mode of action. Accordingly, the α-helix converges onto the SNARE complex to get into a closed conformation in order to trigger fusion. The placement of N-terminus close to the C-terminal end of SNARE complex promotes full assembly of SNARE proteins. Cpx acts as an allosteric adaptor for Syt1 where Cpx modulates the Ca^{2+} dependency of release (Neher, 2010).

Overall, the current consensus suggests that Ca^{2+}-bound Syt1 antagonizes Cpx without dislodging it and such antagonism carries on even to very late stages of fusion process, where in Cpx inhibits fusion pore expansion and Syt1 promotes it.

1.3 Mouse Chromaffin cell – A model system to study neuronal signaling

Adrenomedullary chromaffin cells are quite unique in that they are closely related to sympathetic ganglionic neurons. In addition to sharing a common embryonic origin, namely the neural crest (Kobayashi, 1977), they also share some of the biochemical properties of neurons such as the possession of voltage-dependent Ca^{2+} and Na^{2+} channels and the expression of a large number of receptors and neuronal-specific proteins. Chromaffin cells are also excitable and are capable of firing action potentials which result in secretion (Aunis 1998). Chromaffin cells have a diameter of about 10 μM and store catecholamines within small (100-200 nm) membrane-bound, subcellular organelles called chromaffin granules also known as large dense core vesicles (LDCVs) (Burgess and Kelly, 1987) which are analogous to the synaptic vesicles found in neurons. Chromaffin granules also contain soluble proteins such as chromogranins (Kirschner and Kirschner, 1971) and membrane proteins (Winkler, 1971).

Chromaffin cells can synthesize, store, and secrete a wide range of bioactive substances like neuropeptides, growth hormones, endorphins and most importantly epinephrine and norepinephrine. Release of hormones and other proteins in chromaffin cells occurs by the process of 'exocytosis' (Smith and Winkler, 1972; Smith et al., 1973) and is Ca^{2+} dependent (Garcia et al., 2006). Briefly, in response to acetylcholine released by preganglionic sympathetic nerve fibers that innervate the adrenal medulla (Feldberg et al., 1932), nicotinic receptors on the cells' surface are activated, allowing for the entry of Na^{2+} and depolarization of the cell membrane. As a result, voltage gated Ca^{2+} channels open up letting extracellular Ca^{2+} to enter the cell. This elevation in cytosolic Ca^{2+} concentration triggers the delivery of catecholamines into the extracellular space.

The adrenal chromaffin cells are one of the best sources of current knowledge on neurotransmitter release (Rettig and Neher, 2002; Sorensen 2004), the others being the goldfish bipolar ribbon synapses (von Gersdorff and Matthews 1999) and Calyx of Held (Schneggenburger et al., 2002) which are unusually large. Due to the larger size, spherical shape, and lack of extended processes in chromaffin cells, whole-cell patch clamp recordings of changes in membrane capacitance are greatly favored. Increase in membrane capacitance reflects increased surface area of the cell membrane as a result of vesicle fusion. In contrast, neurons permit measurement of post-synaptic currents (mEPSCs) which represent the sum of several presynaptic as well as postsynaptic factors. Several effectors can play a role in this indirect estimation of signal transduction such as diffusion across synaptic cleft, postsynaptic

receptor opening kinetics, etc. (Becherer and Rettig, 2006). Chromaffin cells also permit the use of flash photolysis, a technique that requires the exchange of pipette solution with the cytosolic compartment and uncages caged Ca^{2+}, rapidly and homogenously, increasing intracellular Ca^{2+} levels for fusion stimulation. Furthermore, the combination of carbon fiber amperometry to directly assay the kinetics of fusion from single vesicles released from chromaffin cells provides two independent measures of exocytosis. Finally, chromaffin cells grant more feasibility for tracking and visualizing vesicles near the cell membrane using total internal reflection fluorescence microscopy (TIRFM) (Steyer and Almers 2001).

2 Aim of thesis

Complexin and Synaptotagmin have been implicated to ensure temporal precision and speed delivery of vesicular contents in SNARE mediated exocytosis. The precise molecular mechanism of how these proteins coordinate this process is still unclear. Research in our lab has previously demonstrated that the C-terminus (last 62 amino acids) 'clamps' premature exocytosis and that the N-terminus (first 26 amino acids) shortens the secretory delay and accelerates the kinetics of Ca^{2+} triggered exocytosis. Once thought to be functionally inert, the unstructured C-terminus is now seen as a domain necessary to clamp fusion. But, how does the C-terminus accomplish this? Amounting to almost half of the protein, which motifs and amino acids exactly bring about this clamp activity and how? Given the functional relevance of this region, we set out to perform a detailed structure-function characterization of complexin. The first aim of this thesis work has been to pinpoint crucial 'determinants' by which complexin C-terminus regulates exocytosis. To this end, Cpx2 variants with different truncations and substitutions of potentially crucial amino acid residues were designed. Taking advantage of whole-cell patch clamp recording of membrane capacitance and simultaneous uncaging of caged calcium, both asynchronous and synchronous secretion in mouse adrenal chromaffin cells were investigated.

Ca^{2+} triggered exocytosis also depends on protein-protein interactions. It is believed that the binding of synaptotagmin to SNARE complex antagonizes the inhibitory actions of complexin (Tang et al., 2006). Previous work in our lab has shown that complexin (N-terminus) increases the Ca^{2+} affinity of phasic release, potentially by adapting the major Ca^{2+} sensor Syt1 (Dhara et al., 2014). Double knock out ($Cpx2^{-/-}Syt1^{-/-}$) experiments in chromaffin cells presented a phenotype that was virtually identical to that of Syt1 ko, leaving a moderate exocytosis with slower kinetics. Since mouse chromaffin cells also express a secondary Ca^{2+} sensor - Syt7, we also set out to define the actions of Cpx2 and Syt7 on vesicle release process and clarify how they regulate the Ca^{2+} triggered apparatus. For this, electrophysiology experiments using single null mutants for Cpx2 and Syt7 and double mutants (Cpx2/Syt7 dko) were conducted.

Overall, this study focuses on pinpointing crucial properties of Cpx2 and Syt7 that guide SNARE-mediated exocytosis in a functional ballet from pre- to post fusional stages.

3 Materials and Methods

3.1 Materials

Unless otherwise mentioned, all reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other products such as falcon tubes, six-well plates, Petri dishes, etc. were obtained from Life Technologies (Carlsbad, California, USA).

Cell culture solutions

Locke's solution

NaCl	154 mM
KCl	5.6 mM
$NaHCO_3$	3.6 mM
Hepes	5 mM
Glucose	5.6 mM (pH 7.3)

Enzyme Solution

DMEM	(31966, 4.5g/L glucose and Pyruvate)
l-cysteine	(use free base, 121.14; conc: 1.64 mM)
$CaCl_2$dihydrate	(147.02 MW; conc: 1 mM)
Na_2EDTA	(372.2 MW; conc: 0.5 mM)

pH: 7.3; Osm: 340 mOsm

Inactivation Media

DMEM (31966, 4.5g/L glucose and Pyruvate)
Fetal Calf Serum (heat inactivated)
Trypsin Inhibitor
Albumin

Cell culture medium

Isolated chromaffin cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (4.5g/L glucose and Pyruvate), supplemented with 1% Insulin-Transferin-Selenium-X (Invitrogen, San Diego, CA) and 0.4% penicillin-streptomycin (Invitrogen, San Diego, CA) in a humidified atmosphere (8% CO_2) at 37°C.

Virus Activation solutions

Chymotripsin (2g/ml)

20 mg Chymotrypsin (extracted from bovine pancreas)
10 ml OptiMEM (Gibco, Invitrogen, San Diego, CA)

Aprotinin (6g/ml)

60 mg Aprotinin (extracted from bovine lung)
10 ml OptiMEM

BSA solution – 6.5% (w/v) (6.5g/ml)

65 mg Bovine Serum Albumin
10 ml OptiMEM

Immunocytochemistry solutions

10X PBS:	1.36 M NaCl, 25 mM KCl, 15 mM KH_2PO_4, 65 mM Na_2HPO_4
1X PBS:	100 ml (10X) PBS, 500 µl (1M) $CaCl_2$, 2 ml (1M) $MgCl_2$, final volume made up to 1000 ml with double distilled water (DDW) pH 7.4, 320 mOsm/kg)
4% PFA:	1g PFA in 50ml (1X) PBS, pH 7.3
Quenching buffer:	0.1g NH_4Cl, 40 ml (1X) PBS
Blocking buffer:	15g BSA, 0.75g Triton (100X), 500 ml (1X) PBS

3.2 Transgenic mice

All experiments in this study were performed on isolated chromaffin cells from wild-type (wt), Complexin2 ko (Cpx2 ko), Synaptotagmin7 ko (Syt7 ko), and Complexin2/Synaptotagmin7 double ko (Cpx2/Syt7 ko) mice. Animals were bred, maintained, and prepped according to German Animal Health Care Regulations. Complexin2 ko mice (Reim et al., 2001) were kindly provided by Dr. N. Brose (MPI, Göttingen, Germany). Synaptotagmin7 ko mice were purchased from Jackson Laboratory (Bar Harbor, USA). Both Cpx2 ko and Syt7 ko mice are viable and fertile (Chakrabarti et al., 2003). Double-deficient mouse line (Cpx2-/-, Syt7-/-) used in this study was produced by cross-breeding Syt7 heterozygosity onto a Cpx2 homozygous null background. Corresponding double knock out mutants were recovered at the expected Mendelian ratio (Fig.7).

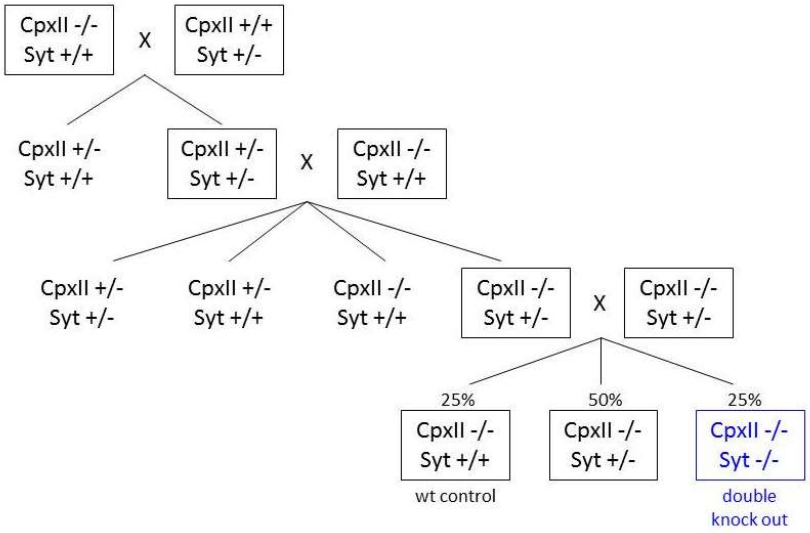


Figure 7. Breeding plan of Cpx2-Syt double knock out mutant animals.
+/+ homozygous wt; +/- heterozygous; -/- homozygous null mutant animal. Paired genotypes are marked with X.

3.3 Genotyping

The genotype of new born pups was routinely determined before isolating chromaffin cells from wt, Cpx2 ko, Syt7 ko, and Cpx2/Syt7 dko. Pups with specific genotype were identified from the offspring/litter, after detecting their genotypes using PCR. For this purpose, new born pups (P0, P1) were labelled and small tail pieces (~2 mm) were collected into prelabelled Eppendorf tubes. To extract genomic DNA, each tail piece was digested for 15 minutes at 75° C in a solution containing 88 µL Sigma water, 10 µL Fast Extract Buffer (KAPPA Mouse Genotyping – PeqLab) and 2 µL Fast Extract Enzyme Buffer (KAPPA Mouse Genotyping – PeqLab). To inactivate the reaction, the tubes were transferred to 95° C for 10 minutes and 100 µL Sigma water was added to dilute the extracted DNA. Following a 13000-rpm centrifugation for 2 mins, 2 µL of the supernatant (DNA extract) was used for further PCR reaction.

3.3.1 Polymerase Chain Reaction (PCR)

The PCR Master Mix contained Green Kappa Mix, $MgCl_2$, DMSO, and the corresponding forward and reverse primers (wt or mutant primers) along with sigma water.

Ingredients for PCR

Green KAPPA Mix	6.25 µl
$MgCl_2$ (25 mM)	0.5 µl
DMSO	1.25 µl

Forward Primer	1.25 µl
Reverse Primer	1.25 µl
H_2O	13.5 µl
DNA	1 µl

All primers used in the PCR were purchased from Eurofins MWG Operon (Ebersberg, Germany) and used at a final concentration of 25 pM/µl.

Cpx2 Primers

Cpx2-/- Forward:	5'- CAG GCA CAC TAC ATC CCA CAA ACA -3'
Cpx2-/- Reverse:	5'- CGC GGC GGA GTT GTT GAC CTC G -3'
Wildtype Forward:	5'- CGG CAG CAG ATC CGA GAC AAG -3'
Wildtype Reverse:	5'- GAG AGG GGC ATG AAG TCA AGT CAG -3'

Syt7 Primers

Syt7 Mutant Forward:	5'- CCT ACC TGA AGC CTG TGT TCA C -3'
Syt7 Neo-Forward:	5'- CAG CTG TGC TCG ACG TTG TCA CTG -3'
Syt7_wt_Forward:	5'- CAT CCT CCA CTG GCC ATG AAT G -3'
Syt7_wt_Reverse:	5'- GCT TCA CCT TGG TCT CCA G -3'

Two genotyping-PCR protocols were designed and run separately – one for wild type and the other for mutant alleles (Cpx2 and Syt7). PCR protocols were carried out in a T1 thermocycler (Biometra).

Wildtype Reaction			**Mutant Reaction**	
Temp (°C)	*Time (mins)*		*Temp (°C)*	*Time (mins)*
94	05:00		94	05:00
94	00:30		94	00:45
64	00:45	41 Cycles	59	00:30
72	01:00		72	01.00
72	07:00		72	07:00

3.3.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments of different lengths. To

prepare the required 1.8% gel, agarose powder (1.8g) was mixed with 100 mL of 1x TAE (Tris-acetate-EDTA) buffer (40 mM TRIS, 20 mM acetic acid and 1 mM EDTA). By heating the solution in a microwave oven, the agarose was completely dissolved. Ethidium Bromide (final concentration 0.5 µg/mL) was added on the surface of the tray and the warm agarose mix (~60 °C) was poured into the casting tray and was allowed to polymerize at room temperature. Once the gel was solidified, the comb was removed and the gel tray was introduced into the electrophoresis chamber (Bio-Rad) and covered with 1X TAE buffer. The PCR products samples, corresponding controls were then loaded next to a standard marker (1 kb DNA ladder). The electrophoresis was performed at a voltage of 5 V/cm and when adequate migration occurred, the gel was visualized and photographed in a gel documentation system. Bands were observed at 600 bps for wild type and 350 bps for mutant (Cpx2 ko) reactions. For Syt7, wild type bands were observed at 400 bps and 800 bps for Syt7 ko mutant reactions.

3.4 Chromaffin cell Preparation

Chromaffin cells were isolated from the adrenal glands of wild type and knock out pups as described by Borisovska et al. (2005). The pups were decapitated and adrenal glands were removed rapidly and placed in ice-cold Locke's solution. The excessive fat and connective tissue around the glands were trimmed off and clean glands were transferred into a sterile 15 ml falcon tube containing enzyme media with activated Cysteine-Papain (20-30 U/ml; Worthington Biochemical Corporation). The 'enzyme-papain' solution was pre-equilibrated with carbogen in a water-bath set at 37 °C for 15 minutes. After incubating the glands for 20-30 min, the papain-enzyme solution was carefully pipetted out and the glands were washed with pre-warmed culture medium (enriched DMEM). To stop the enzyme activity, inactivation solution was added and the glands were further incubated for 5 mins and then washed with culture medium once again under the hood. Finally, 300 µl of freshly prepared enriched DMEM (100 ml DMEM supplemented with 0.4 ml penicillin/streptomycin and 1 ml Insulin-Transferrin-Selenium-X; Invitrogen) was added to each falcon tube and the glands were gently triturated until the tissue was completely dissociated. Additional medium was added to dilute the cell suspension as desired. 100 µl of cell suspension was plated on 25 mm glass coverslips (uncoated and UV irradiated) placed in sterile 6-well plates. The cells were allowed to settle and adhere to the glass surface for about 30 minutes in the incubator at 37°C and 8% CO_2. Then, 3ml of enriched DMEM was added to each of the six-well plates and further cultured for 2 days. All electrophysiology and immunocytochemical experiments were performed on 2-3 days of culture

at room temperature.

3.5 Expressing target gene in chromaffin cells

Semliki Forest Virus (SFV) system was used to infect chromaffin cells and subsequently have a gene of interest expressed. First, the virus containing the mutant gene (stored at -80 C) was thawed and activated by adding chymotrypsin (110 µL) and OptiMEM (350µL). After incubation at RT for 45 min, the reaction is inactivated by adding BSA (110 µL) and Aprotinin (110 µL). Approximately, 60 µL of solution with active virus particles (containing the gene of interest) was added towards the inner walls of the wells containing culture chromaffin cells and incubated (37°C & 8% CO_2). Electrophysiological experiments were done after 5.5 hours of transfection and immunocytochemical experiments were done after 3.5 - 4 hours of transfection.

3.6 Detecting infected cells on the patch setup

Virus infected cells expressing the gene of interest (e.g. $Cpx2^{1-100}$, $Cpx2^{1-115}$, $Cpx2^{E/A}$, etc.) were identified at the patch setup with the help of GFP illumination (480 nm) and their membrane capacitance recorded. Since cDNAs were subcloned into viral plasmid pSFV1 (Invitrogen) upstream of an independent open reading frame that codes for enhanced green fluorescent protein (EGFP), cells expressing GFP also express the upstream protein of interest indicating successful viral transfection in the cell. A list of all Cpx2 mutants expressed by SFV system in this study is listed here below.

1. Cpx2: wt Cpx2 protein (1-134 amino acids) (used for rescue or overexpression)
2. $Cpx2^{1-100}$: last 34 amino acids towards the C-terminus of Cpx2 truncated
3. $Cpx2^{1-115}$: last 19 amino acids towards the C-terminus of Cpx2 truncated
4. $Cpx2^{L124E/L128E}$: Leucines at 124^{th} and 128^{th} positions of Cpx2 exchanged to Glutamic acid (glu, E)
5. $Cpx2^{L124W/L128W}$: Leucines at 124^{th} and 128^{th} positions of Cpx2 exchanged to Tryptophan (trp, W)
6. $Cpx2^{E/A}$: a stretch of 7 glutamic acids starting from 108 through 114 exchanged to alanines (ala, A)
7. $Cpx2^{\Delta 53}$: first 53 amino acids towards the N-terminus of Cpx2 truncated

The structure of Cpx2 as well as its amino acid sequence showing different domains and

indicating the regions and amino acids that are truncated or substituted are shown in the figure below.

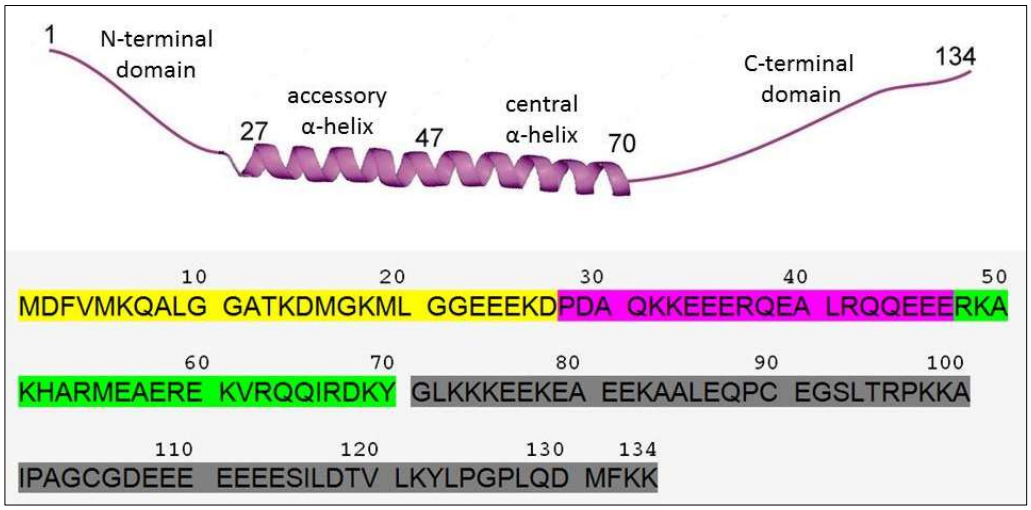


Figure 8. Structure of Complexin2 (mus musculus).
Cpx2 is comprised of a central α-helix flanked by unstructured N-terminus and the C-terminus (Top panel). The amino acid sequence of Cpx2 shows each distinct domain highlighted by a different color. N-terminal domain – yellow; accessory α-helix – pink, SNARE binding domain – green, and the C-terminal domain – grey (Lower panel).

3.7 Patch Clamp experiments - Capacitance measurements

With the help of patch-clamp technique, originally developed to study the conductivity of ion channels in cells (Neher and Sakmann, 1976; Hamill et al., 1981), it is possible to clamp a mouse chromaffin cell membrane at a physiological resting potential (-70 mV) or a defined voltage. Simultaneously, the cell can be perfused with intracellular solution containing a defined Ca^{2+} concentration, via the patch pipette. The resulting small currents (amounting to picoamperes) can be recorded (Neher and Marty, 1982). In order to monitor the changes in membrane capacitance and measure the currents as an assay for granule exocytosis, a particular geometrical arrangement between the pipette and the cell is essential. The 'whole-cell' patch clamp mode is a standard configuration employed to study these changes in chromaffin cells (Fig.9A). To achieve this arrangement, first, a patch pipette is placed in direct contact with the cell, gently pressed onto the cell surface, in order to establish a tight, high resistance 'seal' (giga-Ohm range) against the tip (1-2 µm) of a glass pipette. By applying pulses of suction to the pipette interior, the region of cell membrane, covered by patch pipette is ruptured open.

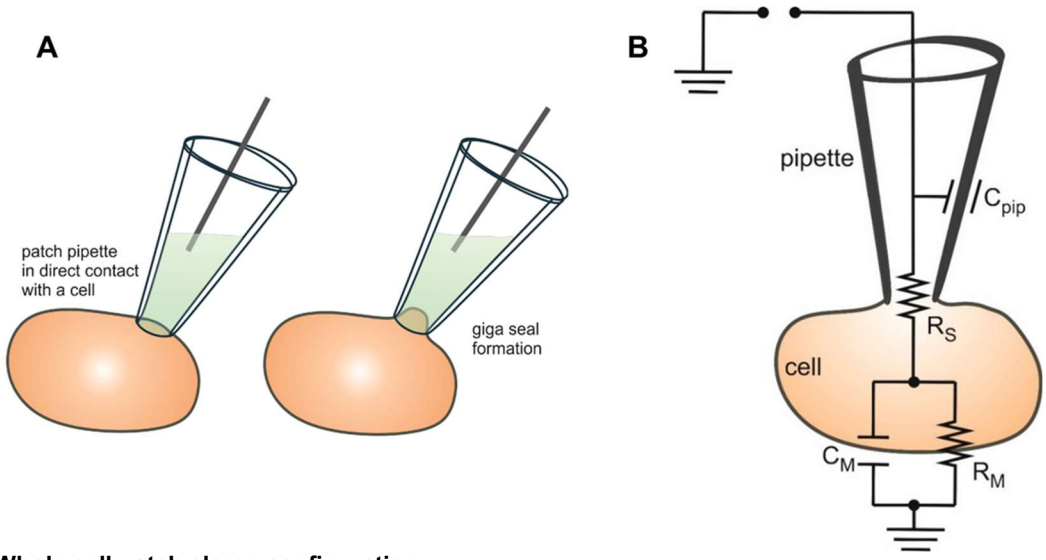


Figure 9. Whole cell patch clamp configuration
(A). A glass pipette (≈ 4 MΩ) filled with intracellular solution is brought near a healthy cell. Releasing the positive pressure, previously applied allows the formation of a tight seal (1 GΩ). Gentle suction through the glass pipette ruptures the cell surface within the tip region thereby allowing the cell membrane to be in continuation with the pipette. (B). **Minimum equivalent circuit of a cell patched in whole-cell configuration.** R_S is the series/access resistance through the patch pipette, whereas R_M and C_M are membrane resistance and membrane capacitance respectively.

3.8 Principles of membrane capacitance measurements

Excitable cells such as neurons and chromaffin cells transfer signals electrochemically. The cell membrane (phospholipid bilayer) serves as an insulator separating two conducting solutions, namely the intracellular fluid and the extracellular fluid (ringer/bath), both containing charged ions. It also carries ion channels or pores which open sporadically allowing the flow of ions (current), maintaining a relatively greater number of negative charged ions inside the cell than on the outside. The difference in voltage of the fluids inside a cell and outside a cell creates a resting membrane potential, which is usually about -70 mV. When there is any change in the voltage across the insulator, charge builds up at the interface, since current cannot flow directly across the insulator. Thus, the cell membrane by accumulating charge resembles a 'parallel plate capacitor' and the capacitance of a membrane is described as membrane capacitance (C_M). The capacitance of a cell is directly proportional to the surface area of the cell, since the more area a cell membrane has, the more charge it can carry. The capacitance of most biological membranes is considered to be 1 μF/cm^2 (Cole., 1968; Fenwick et al., 1982). Furthermore, the cell membrane also acts as a 'resistor', since it resists/blocks the free flow of ions. The resistance given by a cell is expressed as membrane resistance (R_M) and is a measure of how easily ions move across the cell membrane. From an electrical point of view, a typical 'whole cell' patch clamp scenario would involve a connection between two resistors and

a capacitor (a 3-element system). While the cell membrane represents a capacitor (C_M) and a resistor (R_M) connected in parallel, the patch pipette acts as an additional resistor (R_S) that is connected to the cell in series as shown in Fig.9B.

3.8.1 Capacitive and Resistive currents

The main idea behind the measurement of membrane capacitance is that when voltage across the cell membrane changes, current flows proportionally, or in other words, the cell membrane accumulates charge. The flow of current across a capacitor (cell membrane) is described as:

$$I_C = C_M * dV_C/dt$$

When there is no change in voltage (no movement of ions across the membrane), there is no collection of charge and therefore no flow of capacitive current. And the flow of current across a resistor can be calculated using Ohm's Law:

$$I_R = V_C/R_C$$

Thus, when voltage changes, the resulting current signal (I_P) is a sum of two currents, namely, a resistive current (= V_C/R_C) and a capacitive current (= $C_M * dV_C/dt$):

$$I_P = V_C/R_C + C_M * dV_C/dt$$

By knowing the voltage and measuring the charge currents upon a set potential change, it is possible to estimate the membrane capacitance simply by separating the capacitive currents from resistive currents.

3.8.2 Time-domain Technique

Furthermore, charging current decays with a time constant (τ) that is a function of R_S and C_M. Any change in voltage (ΔV) results in a current signal that has two components namely an initial transient current (Io) that relaxes exponentially and a constant current (Ir). The time course is given by the following equation.,

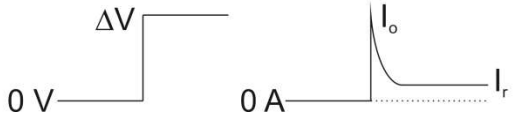
$$i(t) = (I_o - I_r)\exp(-t/\tau)+I_r$$

where t represents time after the voltage step and τ is the time constant of the decay of I_o, as shown in the scheme below. This equation can be fitted to the current response in order to obtain estimates on the circuit parameters indicated in Fig.9B (R_S, R_M, C_M):

$$R_S = \Delta V/I_o$$

$$R_M = (\Delta V - R_S I_r)/I_r$$

$$C_M = t(1/R_S+1/R_M)$$



3.8.3 Stimulation Protocol

The most common approach for estimating changes in membrane capacitance with high-time resolution and sensitivity is to apply a sinusoidal excitation/voltage ($v(t) = V_o \cos\omega t$, where ω is a frequency) and analyze the resulting sinusoidal current (Lindau and Neher, 1988; Chen and Gillis, 2000; Neher and Marty, 1982). This method is based on the principle that ohmic/resistive currents flowing through a membrane conductance are 'in-phase' with the voltage, whereas capacitive currents are 'phase shifted' by 90°, with respect to membrane voltage. Therefore, when a sinusoidal voltage is applied through the pipette to the cell, across a cell membrane that acts both as a resistor and a capacitor, it results in a resistive current that is in-phase with the voltage signal and a capacitive current that is phase shifted by 90° between the applied voltage and the resulting current.

$$I_R(t) = v(t)/R_M = (Vo/ R_M)*\cos\omega t \qquad [R_M = (Vo/I_R(t))* \cos\omega t]$$

$$I_C(t) = Cdv(t)/dt = -\omega C V_o \sin\omega t$$

Membrane capacitance (C_M) can be calculated using the equation:

$$C_M = -I_C(t)/w*V_o \sin\omega t$$

Thus, the differences between the input voltage and the output current signals with respect to amplitude and phase carry the information about the electrical properties of the circuit in a 3-element system described in Fig.9B.

3.8.4 The 'sine wave + DC' technique

The 'sine wave + DC' technique, developed by Manfred Lindau and Erwin Neher (Lindau and Neher, 1988) allows high resolution measurement of tiny changes of membrane capacitance (C_M) and membrane conductance (G_M) by minimizing the amplification of background noise. Most importantly, this method applies DC (direct current) along with sine wave stimulus, to obtain C_M estimates/values that are independent of changes in resistive components ($R_M + R_S$):

$$I_{DC} = V_{DC} – E_R / (R_M + R_S)$$

where E_R is assumed to have a constant value for a given cell and ionic composition of the pipette solution. The current signal produced as a result of sinusoidal voltage is fed into a lock-in amplifier that is phase sensitive. This lock-in amplifier separates the current signal into two components namely, a component 'in-phase' with stimulus voltage and a component 90° 'out-of-phase' with the stimulus voltage. An automated computer routine (Gillis, 2000) is used to calculate the three circuit parameters (R_S, R_M and C_M) from the in-phase component, out of phase component as well as the DC-signal quantities.

An EPC-10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) along with Pulse software (v. 8.53) was used to acquire current signals at a digitization rate of 20 kHz. To calculate Rs, Rm, and Cm, the X-chart extension of Pulse software was used. DC-holding potential was set to -70 mV, sine wave stimulus to 1000 Hz and peak to peak amplitude was set to 35 mV. Electrophysiological recordings were performed on mouse adrenal chromaffin cells (3-5 pF) when they were between 1.5 and 2.5 days old in culture. Cells were bathed in extracellular Ringer's solution containing (mM) 130 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 30 glucose, as well as 10 Hepes-NaOH, pH 7.3., and 320-325 mOsm. Glass pipettes (Science Products) that are Sylgard coated were pulled on a programmable puller (P-1000, Sutter Instruments, CA, USA) and were heat polished. The pipette solution for flash experiments contained (mM) 110 Cs-glutamate, 8 NaCl, 3.5 $CaCl_2$, 5 NP-EGTA, 0.2 fura-2, 0.3 furaptra, 2 MgATP, 0.3 Na_2GTP, 40 Hepes-CsOH, pH 7.3, and 320 mOsm. Whole cell mode was achieved by initially applying a gentle positive pressure through the glass pipette. Pipettes were placed in contact with the chromaffin cells and releasing the pressure ensured the formation of high resistance giga seal between the cell and the patch pipette. Patch pipettes had a tip diameter of about 1-2 µM and a pipette resistance of 4-6 MΩ. A brief suction through the glass pipette break opens the cell and allowed for continuity between the cytosol and the intracellular solution within the pipette. All measurements were carried out at room temperature.

3.9 Flash photolysis and Ca^{2+}measurements

UV-Flash photolysis is a technique in which a biologically relevant molecule (e.g., ATP or Ca^{2+}) is rendered inactive by its linkage to a chemical group (the "cage"). A pulse of intense light in the near ultraviolet range (UV; 350-360 nm) breaks the photolabile bond thereby releasing the active compound. In this study, whole-cell capacitance measurements were performed in combination with flash photolysis of caged Ca^{2+} as well as ratiometric measurements of $[Ca^{2+}]_i$, as described previously (Borisovska et al., 2005). The pipette solution contained nitrophenyl-EGTA (NP-EGTA), a Ca^{2+} chelator (supplied by Dr. Ellis-Davis, MCP Hahnemann University, Philadelphia, PA) and Ca^{2+} indicators FURA-2 and Furaptra (Molecular Probes, Eugene, OR) along with Ca^{2+} and other substances. The concentration of unbound or free Ca^{2+}, for a freshly prepared pipette solution was adjusted to be in nanomolar range (~600-800 nM). A typical measurement of changes in C_M began with attaining whole cell configuration between the pipette and the chromaffin cell (1.5 DIC). With the application of gentle suction, cells were opened and perfused with pipette solution (free Ca^{2+} ~600-800 nM) for 120 s, in order to allow

'priming' of granules, while simultaneously recording the membrane capacitance. Subsequently, a UV-light flash was applied, which uncages caged Ca^{2+}, thereby rapidly increasing cytosolic Ca^{2+} concentration (~ 20 mM). This results in synchronized exocytosis of large dense core vesicles from isolated mouse chromaffin cells. The post-flash recording usually continued for 6 seconds and then stopped.

Intracellular Ca^{2+} concentration was accurately determined with the help of ratiometric, dual excitation Ca^{2+} indicators namely Fura-2 and Furaptra (included in the pipette solution). Fura-2 in unbound/free state gets excited at 380 nm and in bound form at 340 nm. The emitted light is measured at around 510 nm (Fig. 10). Furaptra also exhibits similar spectral properties except that they occur at higher Ca^{2+} concentrations. Since, Fura-2 has a high affinity for Ca^{2+} (Kd ~145 nM) which is comparable to endogenous resting Ca^{2+} levels and Furaptra has a low Ca^{2+} affinity (Kd ~25 μM), both these indicators are ideal to supervise changes in intracellular Ca^{2+} levels from submicromolar to micro molar range.

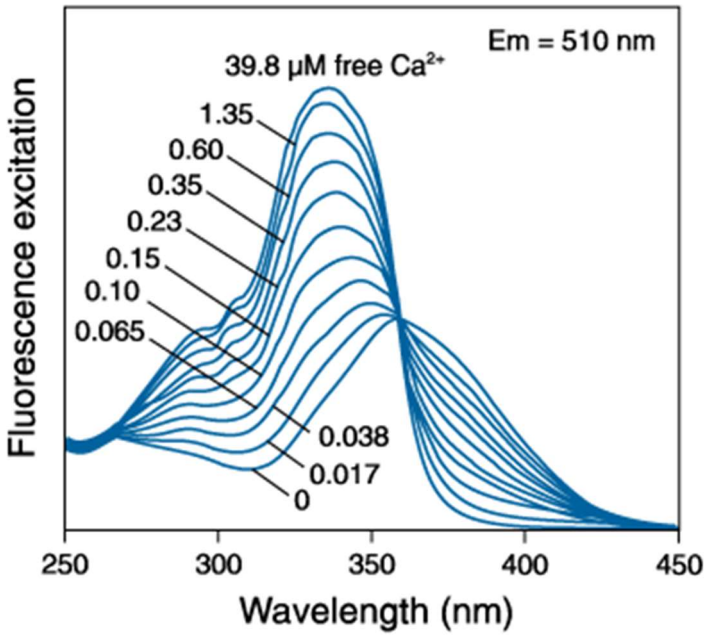


Figure 10. Excitation spectra of Fura-2.
Fura-2 in solutions with different Ca^{2+} concentrations (0 – 39.8 μM) determined at emission wave length of 510 nm. At zero or low Ca^{2+} levels, the fluorescence intensity is highest at 387 nm. However, as Ca^{2+} concentration increases, the fluorescence intensity peaks around 340 nm *(Reference: Molecular Probes)*.

In this study, a multiwavelength illumination system (monochromator), with an integrated light source (Polychrome IV, T.I.L.L. Photonics, Planegg, Germany) was used to quickly (1.5 ms)

switch between 340 and 380 nm to excite Fura-2, Furaptra, while the emitted light was measured at 510 nm. A Ca^{2+} calibration curve was constructed to convert the ratio R of the fluorescence intensities at 340 and 380 nm into intracellular calcium concentration (Fig. 11).

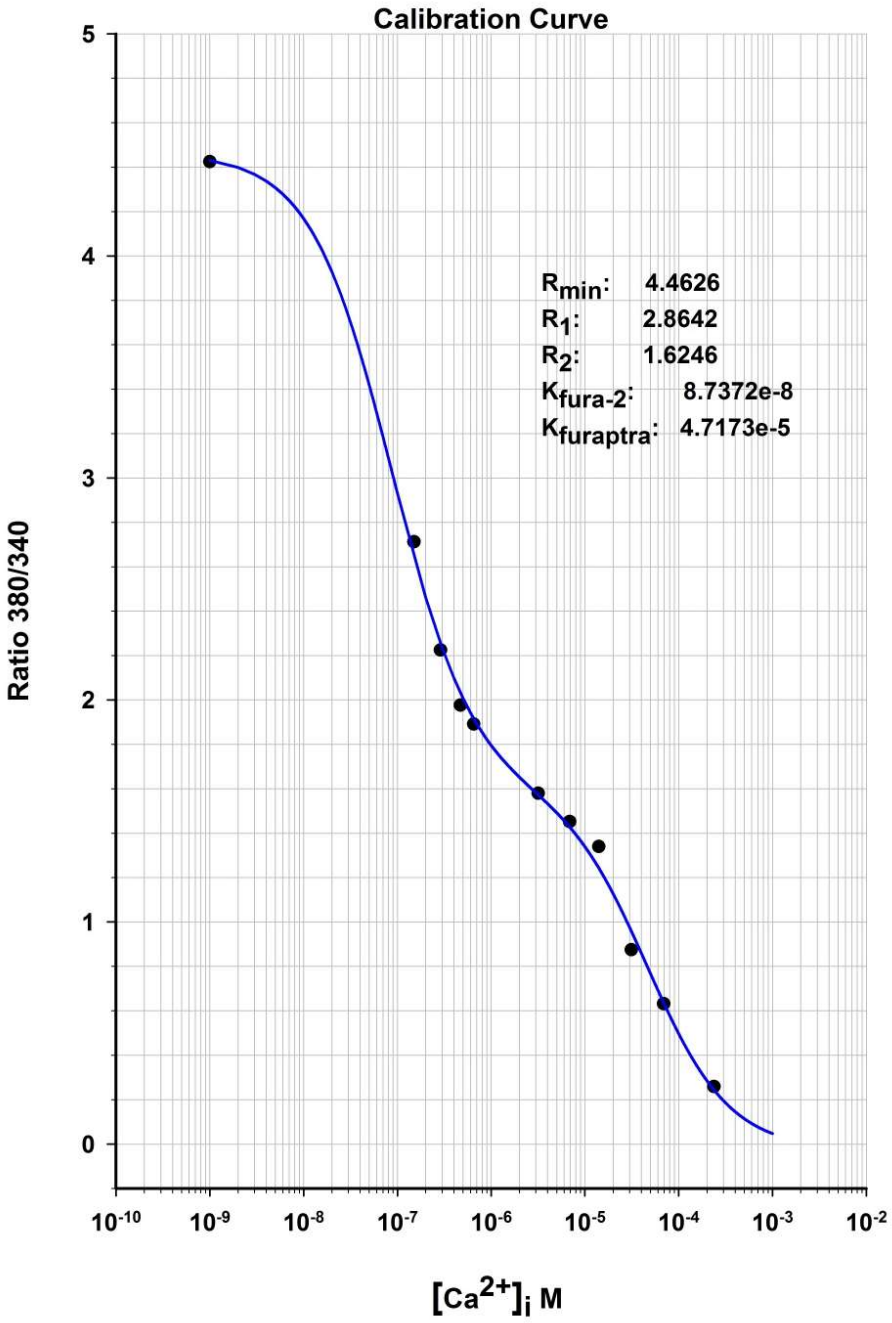


Figure 11. In vivo calibration curve for a Furaptra – Fura-2 mix.
Ratios of fluorescence signals at 380 and 340 nm illumination were measured using pipette solutions containing Ca^{2+} buffered to the indicated concentrations. Each data point represents the mean of three to five experiments.

Each data point in the curve corresponds to the mean ratio obtained during whole-cell patch clamp recordings on chromaffin cells with intracellular solutions buffering free Ca^{2+} of known concentrations (Voets et al., 2000). For this, two sets of stock solutions were made – one containing a high affinity Ca^{2+} chelator, 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetra acetate (BAPTA) and the other containing a low affinity Ca^{2+} chelator namely 1,3-diaminopropane-2-ol-N,N′-tetra acetate (DPTA). Since DPTA is pH sensitive, the pH was adjusted additionally using CsOH for all solutions containing DPTA. Each stock solution (64 µl) was mixed with 10X Mg-ATP/Na_2-GTP solution (8 µl) and 10X Fura-2/Furaptra solution (8µl). A final osmolality of 280 – 320 mOsm/kg and a pH of 7.2 – 7.3 was ensured by supplementing the solution with Cs-glutamate. The ratiometric values (380/340) obtained from each intracellular solution were plotted in a logarithmic scale.

The following equation was used for fitting the experimental data:

$$R = R_{min} - R_1([Ca^{2+}]_i/([Ca^{2+}]_i+K_{fura})) - R_2([Ca^{2+}]_i/([Ca^{2+}]_i+K_{furaptra}))$$

where R_{min} reflects R at $[Ca^{2+}]_i = 0$ (30 mM BAPTA, no $CaCl_2$), ($R_{min} – R_1 – R_2$) reflects the ratio measured with pipette solution containing 10 mM $CaCl_2$ and without any chelator (BAPTA or DPTA). The parameters determined after fitting (R_{min} = 4.46; R_1 = 2,86; R_2 = 1,62; K_{fura} = 87.3 nM; $K_{furaptra}$ = 47.2 µM) were further used to convert ratiometric signal into $[Ca^{2+}]_i$.

3.9.1 The primed vesicles and their kinetics

Ca^{2+}-triggered exocytosis in neurosecretory cells has been well studied using a combination of biophysical techniques such as fluorescent dye based Ca^{2+} imaging, flash photolysis of Ca^{2+}-chelator (NP-EGTA), carbon fiber amperometry, and patch-clamp measurement of membrane capacitance. High time resolution experiments in adrenal chromaffin cells have shown two components in exocytosis, namely an initial fast phasic component (within milliseconds) known as the Exocytotic Burst (EB) and a second component that is slow and sustained (for few seconds) (Voets et al., 1999). The phasic exocytotic burst (EB) includes secretion from two distinct populations of ‘primed’ vesicles known as the readily releasable pool (RRP) and slowly releasable pool (SRP) as shown in Figure 12. Although both RRP and SRP are fusion competent, the rate at which they are released is largely different. The RRP represent fully mature vesicles present very close to the Ca^{2+} channels on the plasma membrane waiting only for the elevation in $[Ca^{2+}]_i$ to undergo fusion. At the moment of Ca^{2+} entry, the RRP is partially or fully depleted rapidly within tens of milliseconds ($\tau \approx 20$ ms), causing a secretory exhaustion until the pool is refilled with newly arrived vesicles. Several studies have suggested that besides

acting as a trigger, increase in [Ca^{2+}] also accelerates the priming reaction as in squid giant synapses (Kusano and Landau 1975), in chromaffin cells (Bittner and Holz, 1992; Smith et al., 1998) and also in neuronal synapses (Dittman and Regehn, 1998). The SRP is also primed part of the EB, but has a slower release rate ($\tau \approx 300$ ms).

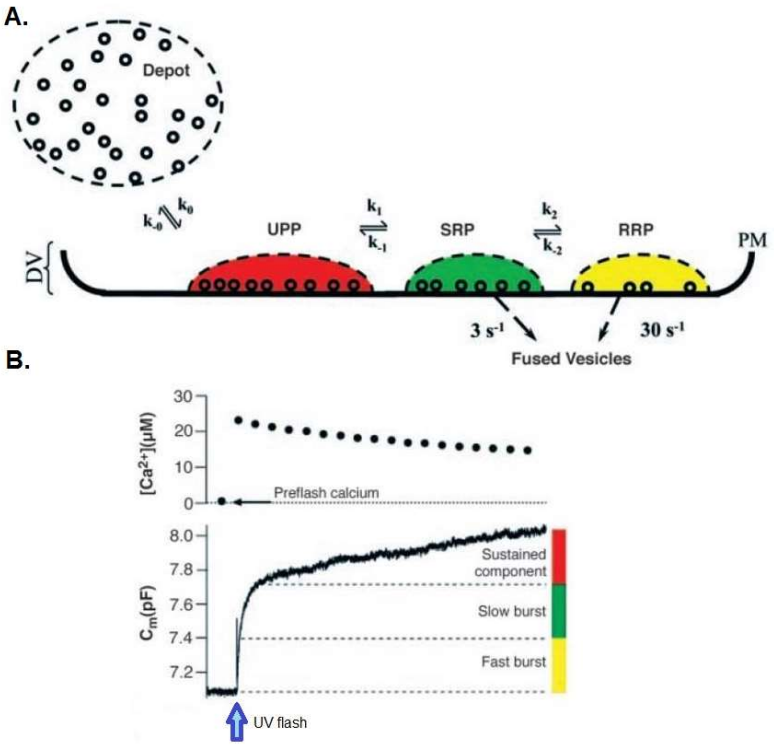


Figure 12. Vesicle pools and Flash response
(A) Newly synthesized vesicles from the depot pool enter the unprimed pool. Some of them dock morphologically at the plasma membrane while others enter the primed pool which includes both the slowly releasable pool (SRP) and rapidly releasable pool (RRP). (B) Application of UV-flash (arrow) causes a step increase of cytosolic [Ca^{2+}] from ~300 nM to ~20 μM (top panel). As the number of chromaffin granules fuse with the plasma membrane, membrane capacitance increases proportionally (lower panel). The color code assigns parts of the trace to the fusion of vesicles from RRP (yellow), SRP (green) or previously unprimed pool (UPP) of vesicles (red). *Modified from Rettig and Neher, 2002)*.

The sustained release (SR) phase represents the newly formed vesicles that are refilling the primed pool just secreted (Nagy et al., 2004). It occurs at a much slower rate (about tens of seconds). In neurons and neuroendocrine cells, it has been observed that only a fraction of the docked vesicles that have attained full maturation are released in response to physiological stimuli. This indicates the necessity of additional priming steps (formation of SNARE complexes, optimal Ca^{2+} concentration) before undocked or docked vesicles become release competent. Therefore, there are at least three kinds of vesicle pools namely the undocked, docked – but unprimed and the readily releasable vesicles. (Sorensen, 2004). According to Voets et al., 1999, this difference in the release rates of both biphasic secretion (EB) and sustained component is

due to the proximity of the vesicles to the Ca^{2+} microdomains.

3.10 Immunocytochemistry

Immunostaining was performed to quantify the expression levels of Cpx2 mutant genes in mouse chromaffin cells infected by semliki forest virus system. After 3.5 hours of virus infection, the cells were used for immunostaining (Guzman et al., 2010). Briefly, cells were washed with 1X PBS and fixed in 4% PFA for 35 mins. After washing with PBS three times, cells were quenched with NH_4Cl for 10 mins. Following another washing step, cells were incubated in blocking buffer (2% BSA). Cells were then incubated with primary antibodies (1:1000) in wet chambers at 4°C overnight. After the cells and solutions reach room temperature, primary antibody was washed off with blocking buffer thrice. Cells were then incubated with suitable secondary antibody (1:1000) for 1.5 hours at room temperature in dark. After washing with PBS, cells were finally washed with double distilled water to remove salts and mounted in glycerol for imaging.

A list of primary and secondary antibodies used for immunostaining in this study is given below.

Primary antibodies

- ComplexinII 122102; rabbit-polyclonal (Synaptic systems) – SNARE binding
- ComplexinII 122 002; rabbit-polyclonal (Synaptic systems) – C-terminus binding

Secondary antibodies

- Alexa Fluor 555 Goat Anti-rabbit (Invitrogen)

3.10.1 Epifluorescence microscopy

For epifluorescence microscopy, a Zeiss AxioVert 200 microscope was used. Digital images (8 bit encoded) were acquired with a CCD camera and AxioVert Software (Zeiss, Germany). Virus (Cpx2) transfected cells were processed after 3.5-4 h of transfection to prevent strong protein overexpression. To ensure proper visualization, the cells within a group (e.g. Cpx2 wt and its mutant viruses) were imaged with different exposure time which was later calculated out. Fluorescence intensities were analyzed offline with ImageJ software after subtracting the background fluorescence.

3.11 Data Acquisition, Analysis and Statistics

Data were analyzed by using sigma plot and Igor pro software (Wavemetrics, lake Oswego, OR), and were shown as mean ± SEM. For determining the statistical significance, one-way analysis of variance (ANOVA) and a Tukey–Kramer post test for multiple groups and t-test was used for paired data.

4 Results

Complexins 1 and 2 are composed of four distinct domains which are capable of providing separate functions in the process of SNARE mediated exocytosis. This study is a detailed investigation of “structural and functional” properties of Cpx2 as well as its interactions with Syt7 in mouse chromaffin cells. In the first part of the study, using whole cell patch clamp experiments combined with flash photolysis, the role of different sections of Cpx2 and their role in exocytosis was explored in a loss or gain of function approach. The results depict key amino acids within the C-terminus that are crucial for the clamp action attributed to Cpx2. In the second part, the role of Cpx2 in relation to synaptotagmins 1 and 7, expressed in mouse chromaffin cells has been explored. Experiments on single and double knock out cells (Syt7 ko, Cpx2 ko and Syt7/Cpx2 dko) have shown that Syt7 acts as a Ca^{2+} sensor at low Ca^{2+} concentrations and that Cpx2 efficiently clamps premature vesicle secretion independently of both Syt isoforms. The phenotypical consequences of combined absence of Cpx2 and Syt7 indicate that these proteins operate in different molecular steps unlike Cpx2 and Syt1 which operate together.

4.1 Part I – Cpx C-terminus comprises different motifs

Initial experiments suggested that the C-terminus of Cpx2 is functionally inert (Xue et al., 2007), but later it has been shown to be involved in clamping spontaneous vesicle fusion (Martin et al., 2011) and for synaptic vesicle priming in neurons (Cai et al., 2008; Yang et al., 2010; Diao et al., 2013). Furthermore, there are reports showing that the C-terminus of Cpx binds to phospholipids (Seiler et al., 2009; Malsam et al., 2009) and interacts with membranes in a curvature-dependent fashion (Snead et al., 2014). Based on these experiments, it has been hypothesized that the C-terminus concentrates other inhibitory domains of Cpx at the site of action (Wragg et al., 2013). Recent work from our laboratory (Dhara et al., 2014) has shown that the C-terminal domain of Cpx2 (63 amino acids from position 72 to 134) is essential for clamping tonic secretion in mouse chromaffin cells, independent of its ability to bind to membranes (Dhara et al., 2014; Makke et al., 2018). Given these controversial findings about the attributes of the C-terminus, we set out to delineate the underlying mechanism by which Cpx2 functions by answering the following questions.

1. Which specific region(s) or amino acids within the C-terminus are responsible for the clamp action of Cpx?

2. What is the molecular mechanism by which complexin regulates exocytosis?

The comparative analysis of the primary sequence alignment of mouse Cpx2 with that of other species illustrates on one hand, variability in the sequence and on the other, identifies structural similarities that are conserved through out the animal kingdom, an observation that promises functional relevance (Fig. 13).

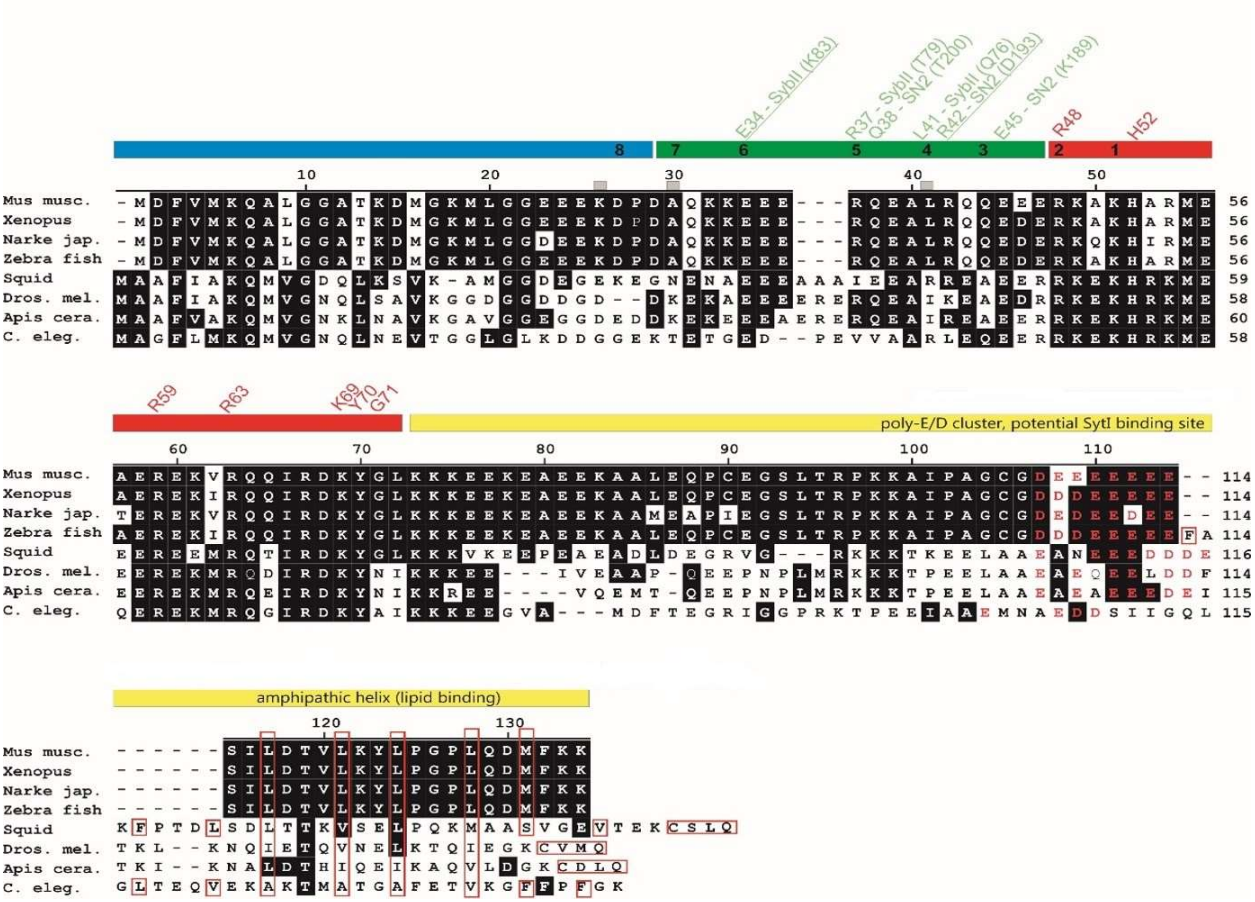


Figure 13. Alignment of primary sequences of mouse Cpx2 and other species.
The alignment illustrates the different domains marked by different colors (N-terminus, blue; accessory α-helix, green; SNARE binding α-helix, red; C-terminus, yellow). Crucial residues within the accessory α-helix that participate in protein-protein interactions and residues within the SNARE binding central α-helix that are important for SNARE binding are indicated (Synaptobrevin, SybII; SNAP-25, SN2; Synaptotagmin1, SytI). The conserved glutamate residues (red letters) and the periodicity of hydrophobic residues (red boxes) in the C-terminus are shown. Notice that some species (Squid, Drosophila, and Apis) also contain a C-terminal CAAX box that provides a farnesylation site and is also involved in lipid binding. *(Figure in collaboration with Prof. Dr. Bruns)*.

All Cpx variants carry a glutamate cluster, which has been proposed to interact with Synaptotagmin (Tokumaru et al., 2008). Moreover, the C-terminal region is characterized by a periodicity of hydrophobic residues that confers amphipathic properties to the C-terminal region. To invesitigate the functional properties of such regions, we systematically mutated the

glutamate cluster as well as certain key residues on the amphipathic region. Whole cell patch clamp experiments in mouse chromaffin cells deficient for Cpx2 and those virally expressing Cpx2 mutant proteins allowed us to study the protein in a gain-of-function approach.

4.1.1 Complexin determines the magnitude of synchronous secretion by hindering premature secretion

Complexins bind rapidly and with high affinity to the SNARE complexes via central α-helix. By this, they are seen to "clamp" premature vesicles leading to the accumulation of primed vesicles (Dhara et al., 2014). In order to allow for Ca^{2+} dependent priming of large dense core vesicles in chromaffin cells, the cells were perfused with intracellular solution containing ~700 nM free $[Ca^{2+}]_i$ for 120 s. Simultaneously, changes in membrane capacitance (ΔC_M) were recorded. The analysis shows significant increase in tonic secretion from Cpx2 ko cells (red, 33±4 fF, n=16,) compared to wild type cells (black, 15±3 fF, n=14) (Fig. 14A-C) indicating that Cpx2 clamps premature secretion. In contrast, the expression of wt Cpx2 protein in Cpx2 ko cells, strongly suppressed the tonic secretion (dark green, -6±1 fF, n=14) indicating that the excess of Cpx2 suppresses premature secretion even beyond the level of wt cells. Overall, these results show that Cpx2 levels are rate-limiting and are of crucial importance to hinder premature secretion.

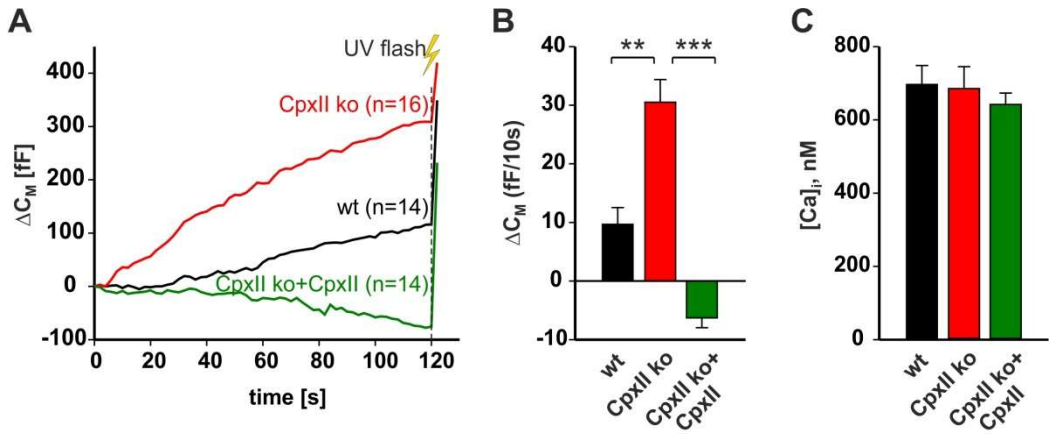


Figure 14. Cpx2 hinders tonic secretion.
(A) Wild type Cpx2 (black, n=14) and the overexpression of Cpx2 (dark green, n=14) hinder tonic exocytosis whereas loss of Cpx2 (red, n=16) show elevated tonic secretion at priming calcium concentration. (B) Mean rate of membrane capacitance before UV flash. ANOVA between the groups. ** P < 0.01; *** P < 0.001. Error bars indicate mean ±SEM.

Upon the application of UV-flash, there is a step-wise increase in the intracellular Ca^{2+} levels from 700 nM to ~20 µM. By fitting individual post flash capacitance traces with a double exponential function, the amplitude and secretory kinetics of synchronous exocytosis were

determined. The analysis shows that wild type cells exhibit an exocytotic burst (RRP 110±8 fF; SRP 114±6 fF) with a sustained rate of 19±2 fF/sec (black trace, n=14). In comparison, the Cpx2 ko cells showed strongly diminished exocytotic burst (RRP 53±5 fF; SRP 78±10 fF, red, n=16) with no significant changes in the sustained component (13±2 fF/s) (Fig 15A-B).

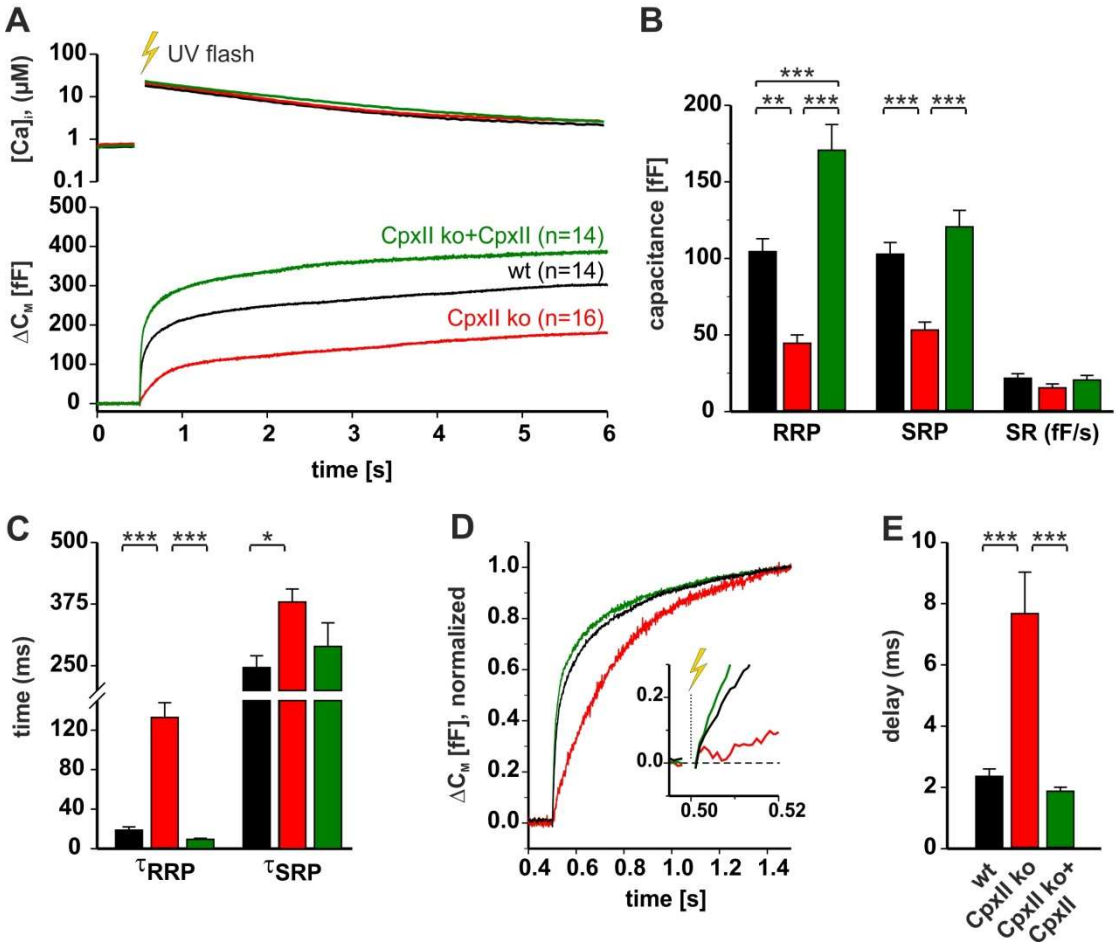


Figure 15. Cpx2 determines the magnitude and kinetics of synchronous exocytosis.
(A) Top panel shows step wise increase in intracellular Ca^{2+} levels due to flash application and lower panel shows corresponding membrane capacitance changes in wt cells (black, n=14), Cpx2 ko cells (red, n=16) and Cpx2 ko cells expressing wt Cpx2 (dark green, n=14). (B) Quantification of different components determined from fitting individual responses (bars with same color code as shown in A. Loss of Cpx2 shows decreased RRP and SRP but is rescued with the expression of wt Cpx2. (C) Analysis of time constants of RRP and SRP reveal that loss of Cpx2 slows down synchronous secretion. (D) Normalized capacitance responses after subtracting sustained component show slow secretion for Cpx2 ko cells. The inset shows an extended scaling of the normalized CM signals during the first 20 ms after flash illustrating the delayed onset of secretion in Cpx2 ko cells. (E) The mean exocytotic delay is higher for Cpx2 ko cells. Error bars represent SEM. **p<0.05. **p<0.01, ***p<0.001;* One-way Anova test with post-hoc tukey's test.

The analysis of release kinetics shows that the secretory rate of RRP and SRP in Cpx2 ko cells was much slower (τRRP=135±14 ms and τSRP=453±41 ms) than that of wt cells (τRRP=20±2

ms, τSRP=244±18 ms). Normalization of the traces corroborate the altered kinetic properties (Fig. 15C-D). Furthermore, loss of Cpx2 also displayed a longer secretory delay between the stimulus and the onset of secretion (Fig. 15E). Thus, loss of Cpx2 in chromaffin cells reduces EB and slows down the secretory kinetics and prolongs the stimulus-secretion coupling. In contrast, the expression of wt Cpx2 protein in Cpx2 ko cells not only rescued the EB, but also increased synchronous secretion beyond the levels found in wt cells. These cells show faster kinetic properties (τRRP=16±2 ms and τSRP=210±37 ms) as well as faster stimulus-secretion coupling (Fig. 15C-E). Taken together, these results indicate that Cpx2 prevents premature secretion at submicromolar [Ca^{2+}] leading to the accumulation of primed vesicles in chromaffin cells. Cpx2 also speeds up stimulus-secretion coupling. While the enhanced amplitude of the flash evoked synchronous response (EB) is a consequence of the increase pool of premature vesicles, the speeding up of exocytosis is due to an increased Ca^{2+} dependency of synchronous secretion. These results are in excellent agreement with our previous findings illustrating a reciprocal relationship between tonic secretion and exocytotic burst (Dhara et al., 2014).

4.1.2 Last 34 amino acids in the C-terminus of Cpx2 determine the clamp action

Previous studies in our lab have also shown that Cpx2 lacking the entire C-terminal domain ($Cpx2^{\Delta C}$ mutant, aa 1-72; a total of 62 out of 134 amino acids truncated) fails to prevent tonic release (Dhara et al., 2014). These original observations led us to hypothesize that the clamp action may reside in the C-terminus of Cpx2. In order to narrow down the region responsible for the clamp action, we designed a truncated version of Cpx2 lacking 34 amino acids ($Cpx2^{1-100}$) at the very C-terminus (Fig. 16A). Such a truncation eliminates the two conserved motifs namely the amphipathic region (aa 116-134) and the glutamate cluster (aa 108-114) (Fig.13). Secretion properties of Cpx2 ko cells expressing either wt Cpx2 or the truncated Cpx2 ($Cpx2^{1-100}$) were comparatively analyzed. The results show that $Cpx2^{1-100}$ cells displayed a similar increase in premature secretion (olive green, 288±29 fF, n=20) as in Cpx2 ko cells (red, 364±39 fF, n=23). This suggests that the truncation of the last 34 amino acids suffice to abolish the clamp activity of Cpx2. In contrast, expression of wt Cpx2 protein strongly suppressed tonic secretion in ko cells (dark green, -42±17 fF, n=22) (Fig. 16B-D). Thus, at submicromolar Ca^{2+} concentrations, premature fusion of vesicles is prevented by the last 34 amino acids at the C-terminal end of Cpx2. In close correlation, the truncated protein $Cpx2^{1-100}$ failed to restore the EB component (RRP=57±5 fF, SRP=65±5 fF) compared to wt Cpx2 expressing cells (RRP=142±16 fF, SRP=97±9 fF). No significant changes in the subsequent sustained phase were noticed in all

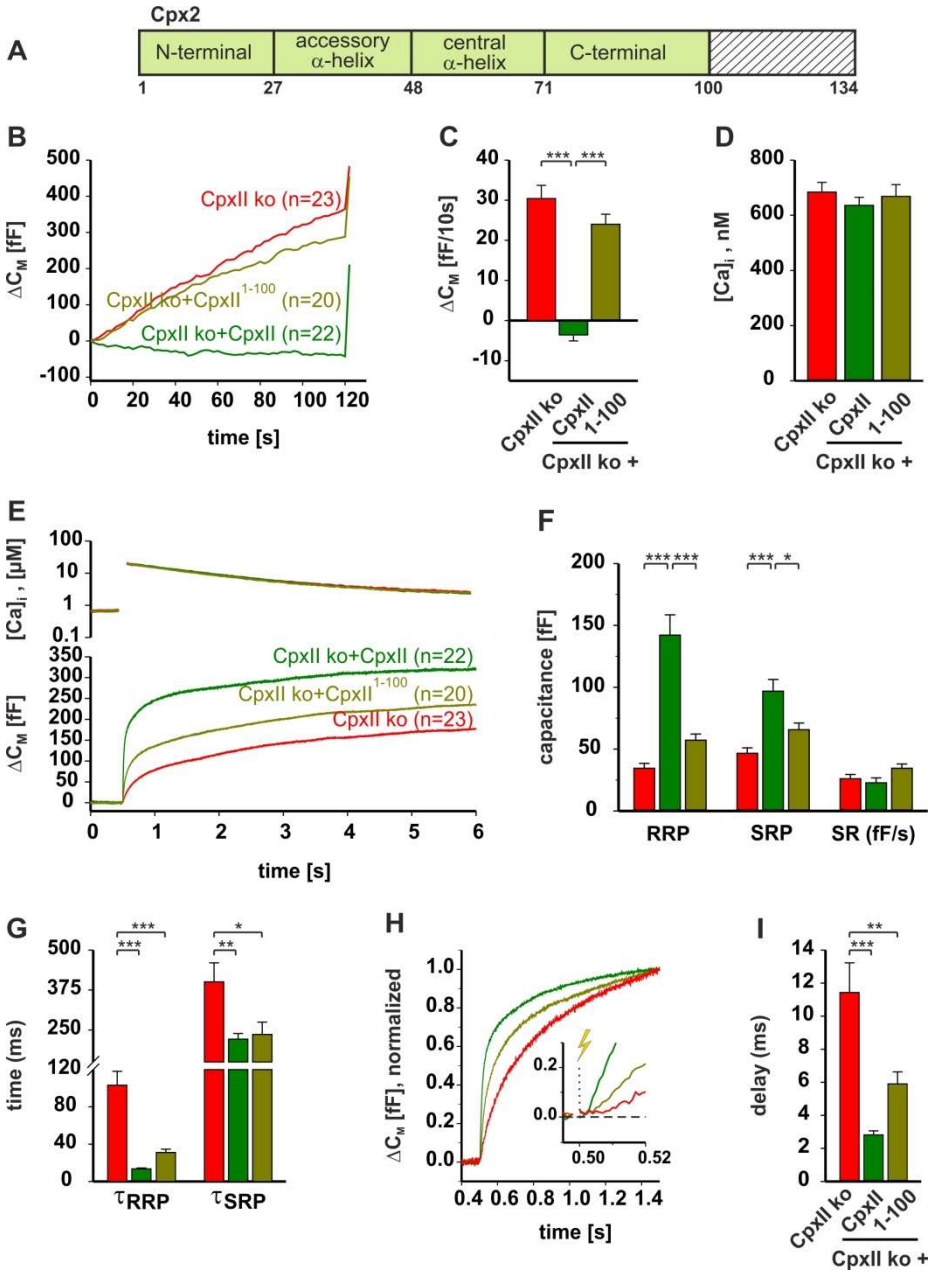


Figure 16. The clamp action of Cpx2 is determined by the farthest 34 amino acids of the C-terminus.
(A) Schematic representation of Cpx2 showing different domains demarcated by amino acid numbers. Striped region represents truncation of the last 34 amino acids. (B-C) Mean capacitance recorded over 120 s in Cpx2 ko cells (red, n=23) and Cpx2 ko cells expressing wt Cpx2 (dark green, n=22) or $Cpx2^{1-100}$ mutant (olive green, n=20). Cells expressing $Cpx2^{1-100}$ exhibit tonic secretion similar to that of Cpx2 ko cells. (D) Similar intracellular pre-flash Ca^{2+} levels in all cell types. (E) Upper panel shows mean $[Ca^{2+}]_i$ before and after UV flash application at t = 0.5 s. Lower panel depicts CM changes in response to flash evoked Ca^{2+} stimulus in above-mentioned cell groups. (F) Quantification of post flash CM traces and the rate of sustained phase. (G) Time constants of RRP and SRP obtained from fitting individual capacitance traces show faster kinetics by $Cpx2^{1-100}$ similar to wt Cpx2. (H) Mean capacitance traces normalized, after subtraction of sustained component. Inset shows a close-up view of the normalized mean CM traces during the first 20 ms depicting the onset of secretion in response to flash stimulation. (I) Quantification of delay indicates that the large delay seen in Cpx2 ko cells is restored even in the absence of the last 34 amino acids. Error bars represent means ±SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; One-way Anova test with post-hoc tukey's test.

three groups (Fig. 16E-F). Fitting individual cellular responses revealed unaltered kinetic properties (Fig. 16G) for both EB components of $Cpx2^{1-100}$ (τRRP=31±4 ms, τSRP=236±39 ms) when compared to those of wt Cpx2 (τRRP=13±1 ms, τSRP=221±18 ms). Normalization of mean CM traces confirms faster kinetics by $Cpx2^{1-100}$ compared to Cpx2 ko (Fig. 16H). A closer look at the first 20 ms of the recording (inset) further depicts a faster and quicker onset of secretion for $Cpx2^{1-100}$ cells (6 ms) than in Cpx2 ko cells (11 ms) (Fig 16I). Interestingly, except for secretory kinetics, the $Cpx2^{1-100}$ mutant cells exhibit a phenotype similar to that observed in Cpx2 ko cells. This result is consistent with the finding that Cpx2 N-terminus (aa 1-27) is responsible for accelerating fusion rate and shortening the exocytotic delay (Dhara et al., 2014). Taken together, loss of the last 34 amino acids specifically impairs the clamp action leading to depletion of primed vesicles, which results in diminished synchronous secretion. Thus, the clamp function of Cpx resides within a stretch of 34 amino acids at the very end of Cpx C-terminal domain.

4.1.3 The amphipathic character of the C-terminus of Cpx2 supports clamping of tonic secretion

Motivated by the above findings, we aimed to investigate which specific properties within the Cpx2 C-terminus (last 34 amino acids) can be held responsible for promoting the proteins' clamp action. As mentioned earlier, the C-terminus of Cpx2 comprises a short stretch of amino acids, that is amphipathic in nature with approximately 3 helical turns within the last 19 amino acid residues (aa 116-134). Previous studies have suggested that this region is required to sense membrane curvature and target Cpx to synaptic vesicles (Wragg et al., 2013; Snead et al., 2014; Gong et al., 2016). By this, they can concentrate other inhibitory domains of Cpx like the accessory α-helix at the site of fusion. They are also known to participate in the process of membrane fusion (Seiler et al., 2009). To assess the functional relevance of the C-terminal amphipathic motif, we designed a Cpx2 variant in which the last 19 amino acids were truncated ($Cpx2^{1-115}$). Analysis of preflash capacitance measurements reveal that Cpx2 ko cells expressing the truncated protein ($Cpx2^{1-115}$) display an intermediate or moderate levels of tonic secretion (ΔC_M=119±39 fF, blue, n=21) compared to Cpx2 ko cells (ΔC_M=400±55 fF, red, n=23) and those rescued by wt Cpx2 (ΔC_M=-21±16 fF, dark green, n=27) (Fig. 17B-D). This result suggests a partial defect in the clamp action due to the loss of the amphipathic helix (last 19 aa). In the same line, at high calcium (~20 µM) concentrations evoked by UV-flash, the mutant cells ($Cpx2^{1-115}$) showed again an intermediate level of synchronous secretion (RRP=58±7 fF,

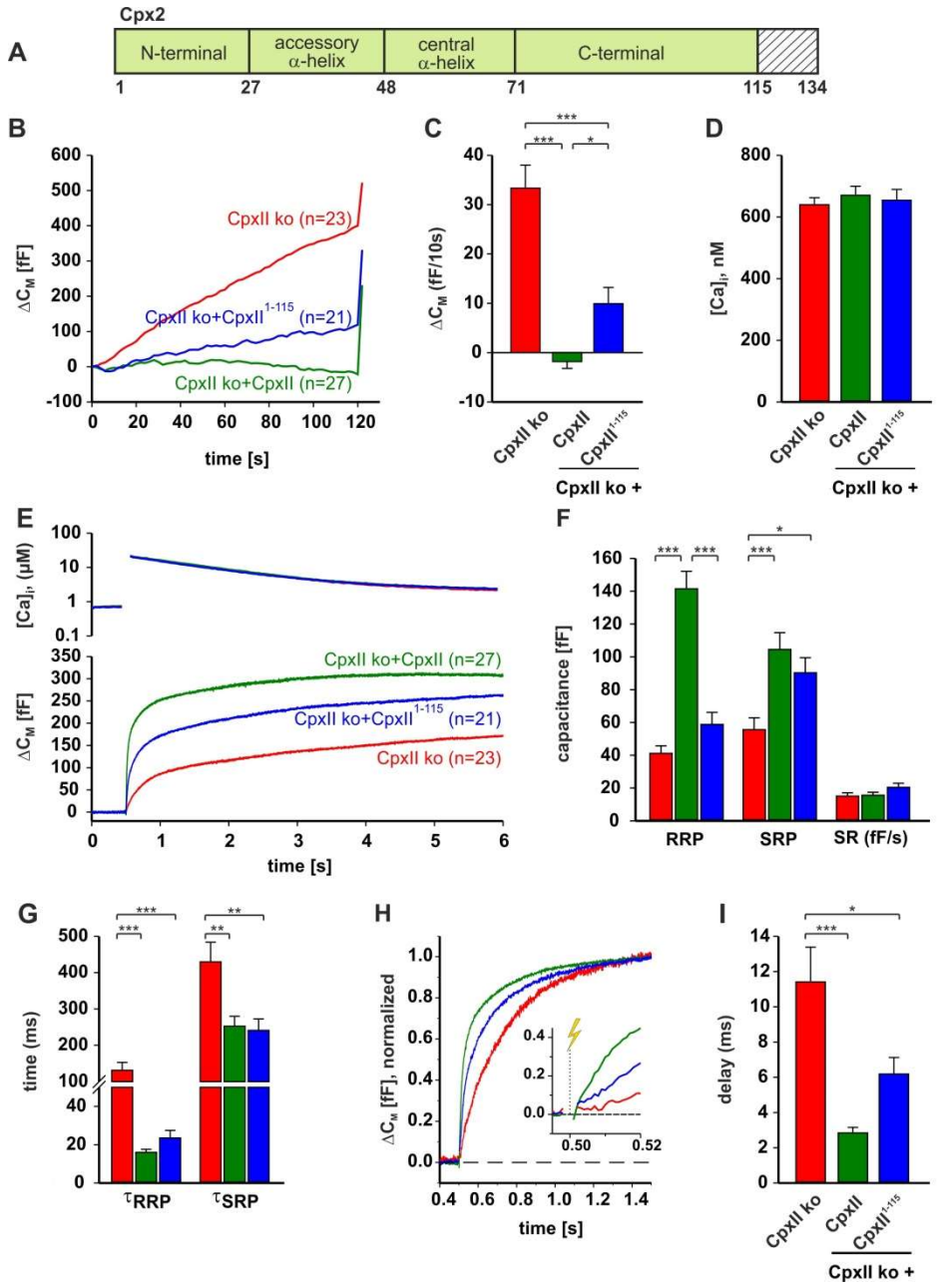


Figure 17. Amphipathic region in the C-terminus of Cpx2 affects clamping and facilitatory actions of Cpx2.
(A) Schematic view of Cpx2 with its different domains and the striped region pointing out the location of amphipathic region. (B) Tonic secretion at submicromolar $[Ca^{2+}]i$ from chromaffin cells – Cpx2 ko (red, n=23), Cpx2 ko+Cpx2 (dark green, n=29) and Cpx2 ko+Cpx2$^{1-115}$ (blue, n=22). (C) Rate of tonic exocytosis in the above groups of cells and (D) Ca^{2+} levels before UV flash is applied. (E) Average $[Ca^{2+}]_i$ after UV flash (top panel) and corresponding evoked capacitance signals of the above groups of cells (lower panel). (F) Amplitudes of exocytotic burst (RRP and SRP) along with rate of sustained component. Notice the major reduction in RRP from cells expressing Cpx2$^{1-115}$ compared to wt cells. (G) Time constants of EB (τRRP and τSRP) associated with Cpx2$^{1-115}$ reflect those of wt cells. (H) Capacitance traces shown in (E, lower panel) normalized to EB (during the first 1.5 s post flash) shows unaltered kinetics in Cpx2$^{1-115}$ cells. Inset shows the first 20 ms of the recordings wherein Cpx2$^{1-115}$ responds significantly faster than the Cpx2 ko cells. (I) Mean exocytotic delay (ms) obtained from fitting responses from individual cells. Error bars represent means $\pm$SEM. *, $p< 0.05$; **, $p < 0.01$; ***, $p < 0.001$; One-way Anova test with post-hoc tukey's test.

SRP=90±9 fF, n=21) (Fig. 17E-F). In comparison, exocytosis was strongly reduced in Cpx2 ko cells (RRP=41±4.5 fF, SRP=55±7 fF, n=23) and enhanced in ko cells expressing wt Cpx2 (RRP=141±10 fF, SRP=104±10 fF, n=27). This shows the reciprocal relationship between the clamping efficiency and the amplitude of synchronous secretion. The sustained component representing the secretion of newly primed vesicles remained unaltered across all groups. Interestingly, secretory kinetics in the $Cpx2^{1-115}$ cells remained fast (τRRP=23±4 ms, τSRP=241±31 ms) similar to Cpx2 ko cells expressing wt Cpx2 (τRRP=16±2 ms, τSRP=252±27 ms) in contrast to the slower release kinetics of Cpx2 ko cells (τRRP=131±22 ms, τSRP=429±54 ms) (Fig. 17G). Furthermore, the slower stimulus-secretion coupling displayed by Cpx2 ko cells (11 ms) was rescued by the expression of wt Cpx2 (3 ms) as well as $Cpx2^{1-115}$ cells (6 ms), as quantified in Fig.17I. These findings reaffirm the role of N-terminal domain of Cpx2 in accelerating the kinetics of release and shortening the secretory delay (Dhara et al., 2014). Taken together, these results demonstrate that the last 19 amino acids in the C-terminus of Cpx2 exert an important, albeit moderate influence in preventing premature vesicle secretion. Given the partial loss of function phenotype it is possible that the C-terminal amphipathic region may act 'in tandem' with other functional domains of Cpx2.

4.1.4 C-terminal hydrophobic amino acids are crucial for clamping tonic secretion

The amphipathic motif present in the very C-terminus of Cpx2 is characterized by the presence of hydrophobic (non-polar) and hydrophilic (polar) amino acids. In order to further investigate the role of conserved residues within the amphipathic helix in preventing premature secretion, we designed Cpx2 variants that exhibit modifications in the hydrophobic face of the helix. We first substituted the leucine (L) residues at 124^{th} and 128^{th} positions either with glutamate residues ($Cpx2^{L124,128E}$) or tryptophan residues ($Cpx2^{L124,128W}$) (Fig. 18A). Replacing leucine residues with charged glutamate (Glu/E) residues makes the C-terminus less hydrophobic whereas exchange with tryptophans maintains the hydrophobic character of the C-terminus. At submicromolar Ca^{2+} concentrations, cells expressing $Cpx2^{L124,128E}$ mutations display an intermediate level of tonic secretion (ΔC_M=202±58 fF, purple, n=24) indicating a failure to effectively clamp premature secretion compared to rescue cells (ΔC_M=-36±40 fF, dark green, n=25) and ko cells (ΔC_M=484±51, red, n=19) (Fig.18B-D). Interestingly, this phenotype is similar to that seen in the $Cpx2^{1-115}$ mutant, where amphipathic helix was completely absent. This suggests that mutating only a few key amino acid residues in the very C-terminus can effectively alter the clamp

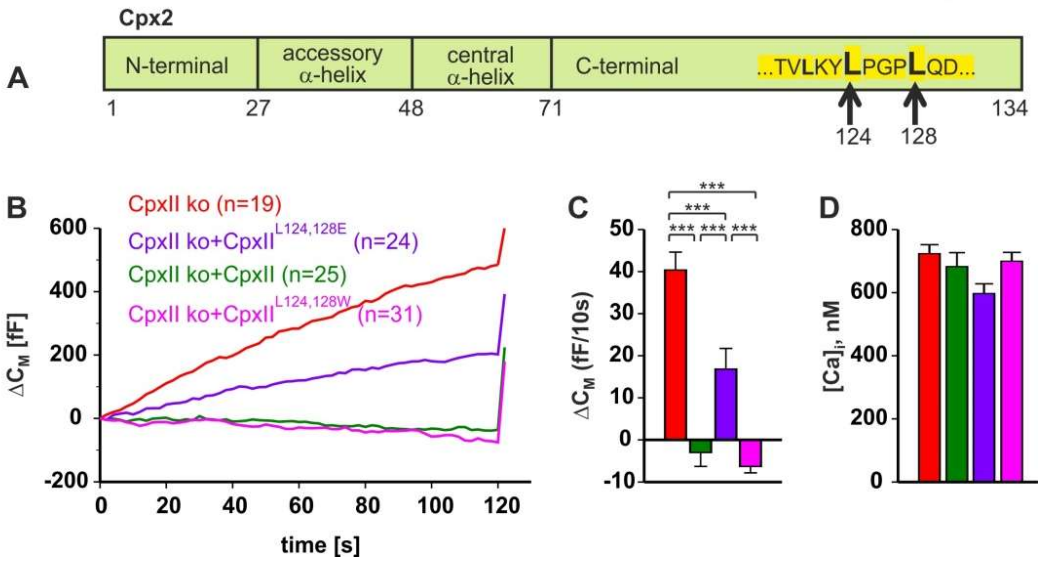


Figure 18. Hydrophobic character of the amphipathic region supports the clamping of tonic secretion.
(A) Schematic view of Cpx2 with four distinct domains. Within the C-terminal domain, a short stretch of amino acid sequence is highlighted (yellow) and indicates the leucine residues at position 124 and 128 (black arrows) that were either mutated to glutamates or tryptophans. (B) Capacitance responses to submicromolar Ca^{2+} concentrations in different cell groups. Chromaffin cells lacking Cpx2 (red, n=19) show elevated levels of tonic secretion but in Cpx2 ko cells expressing wt Cpx2 protein (dark green, n=25) tonic secretion is suppressed. Notice that substitution of leucines with glutamate residues (purple, n=24) does not effectively clamp premature secretion, but tryptophan substitutions (pink, n=31) prevent tonic secretion as in cells expressing Cpx2. (C) Quantified rate of tonic secretion of the above groups of cells (identical color code). (D) Similar 'pre-flash' calcium levels at which effective priming of vesicles occurs, before evoking a flash-induced synchronous secretion. Error bars represent mean ±SEM. ***, $p < 0.001$; One-way Anova test with post-hoc tukey's test.

function of Cpx2. The disruption in the clamp action of Cpx2, may be a consequence of altered protein-protein interactions since a C-terminal peptide comprising the last 34 amino acids of Cpx2 has been shown to bind to other SNARE proteins (Makke et al., 2018). On the other hand, the tryptophan mutant which conserves the hydrophobic character of the C-terminal domain effectively arrests tonic secretion (ΔC_M=-75±18 fF, pink, n=31) similar to cells expressing wt Cpx2 protein. This indicates the specific requirement for hydrophobic residues at 124th and 128th positions within the C-terminus of Cpx2 to hinder premature secretion in mouse chromaffin cells.

Post flash capacitance measurements show that the Cpx2^{L124,128E} mutant cells (Fig.19A-B, purple) failed to fully rescue the diminished synchronous secretion present in Cpx2 ko cells (RRP 45±9 fF, SRP 77±7 fF, n=19, red) but cells expressing wt Cpx2 fully restored exocytosis (RRP 127±13 fF, SRP 120±11 fF, n=25, dark green). Although both vesicle pools show a tendency to be reduced, the RRP (53±5 fF) was more reduced than the SRP (104±10 fF) similar to the Cpx2$^{1-115}$ mutant (Fig. 17F) demonstrating a failure to support fast releasable vesicle pool (n=24, purple).

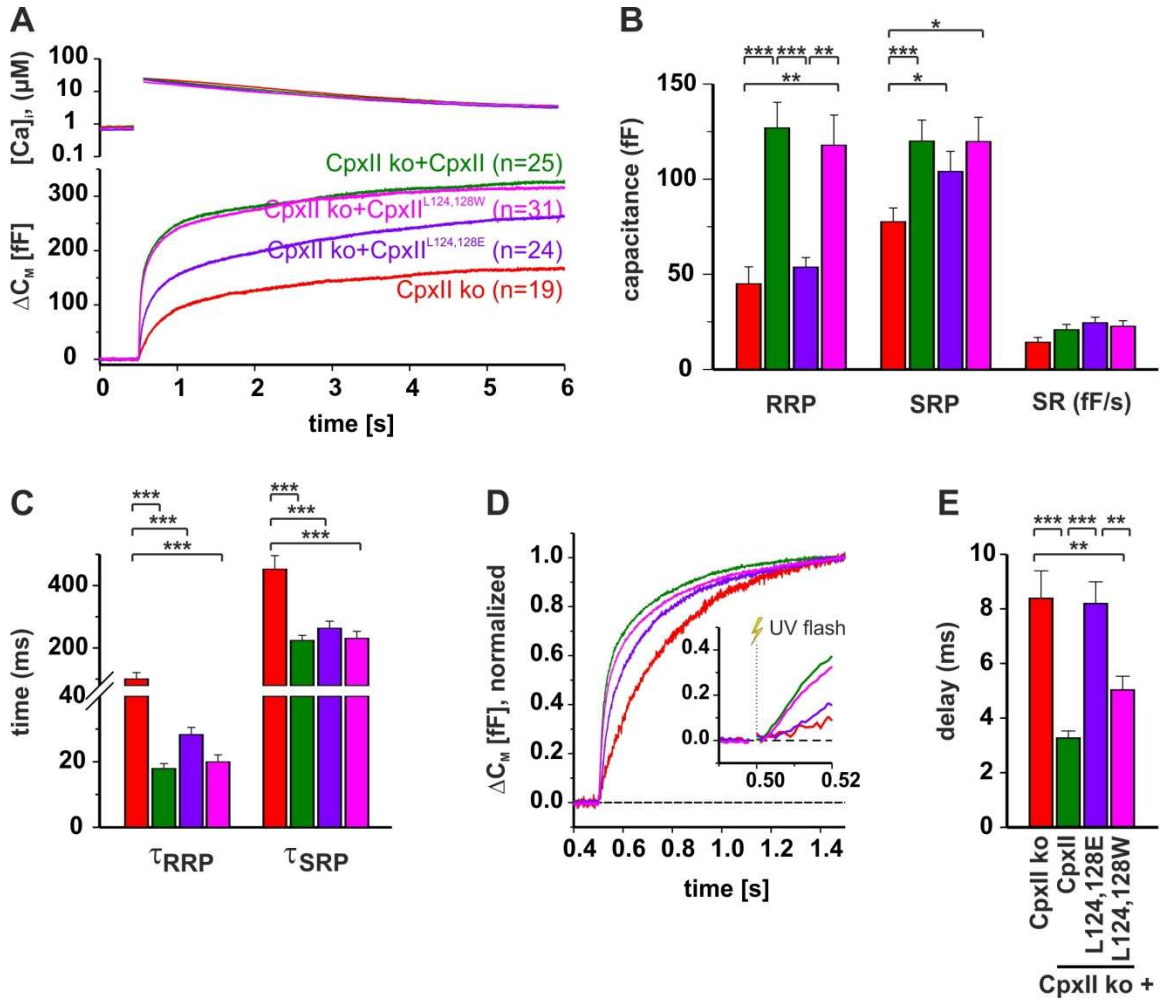


Figure 19. Hydrophobic character in the amphipathic region supports synchronous secretion
(A) Average intracellular Ca^{2+} levels pre and post flash (top panel) and flash evoked secretory responses (lower panel). (B) Relative magnitudes of exocytotic burst (RRP & SRP) and rate of sustained component (fF/s). (C) Time constants of RRP and SRP. (D) Mean capacitance traces of Cpx2 ko and the double mutants normalized to that of wt Cpx2 during the first 1.5 s. Inset shows the normalized traces rescaled to demonstrate the differences in stimulus-secretion coupling among the above groups of cells during the first 20 ms of the flash application. (E) Quantification of the onset of secretion (delay) in milliseconds show faster stimulus-secretion coupling by tryptophan mutants than by glutamate mutants. Error bars represent mean ±SEM. **, $p < 0.01$; ***, $p < 0.001$; One-way Anova test with post-hoc tukey's test.

In contrast, the tryptophan double mutant ($Cpx2^{L124,128W}$) fully restores the exocytotic burst (RRP=118±16 fF, SRP=120±13) similar to wild type rescue cells (Fig.19A-B, pink). These results show that the hydrophobic character at 124th and 128th amino acid residues of mouse Cpx2 is of central importance in restoring synchronous exocytosis. The sustained component of exocytosis remained unaffected in both glutamate and tryptophan mutant cells. No significant changes were seen in the secretory kinetics of the $Cpx2^{L124,128E}$ mutant cells (purple, τRRP=28±2 ms, τSRP=263±23 ms) as well as the tryptophan mutations (pink, τRRP= 20±2 ms,

τSRP= 230±28 ms) compared to cells expressing wt Cpx2 (Fig. 19C). Normalization of the average capacitance traces (Fig. 19D) further confirmed faster secretory kinetics and faster stimulus-secretion coupling by $Cpx2^{L124,128W}$ cells than the $Cpx2^{L124,128E}$ cells. Interestingly, the $Cpx2^{L124,128E}$ cells exhibited a delay similar to Cpx2 ko cells indicating that the triggering of synchronous exocytosis might be severely affected by the introduction of charged amino acids in the very C- terminus (Fig. 19E). These results show the importance of hydrophobic character in the very C-terminus of Cpx2 in determining the magnitude as well as onset of synchronous secretion.

4.1.5 The Glutamate cluster within the C-terminus of Cpx2 enhances the magnitude of RRP

The primary sequence of Cpx C-terminal domain shows upstream of the amphipathic region, a cluster of negatively charged hydrophilic amino acids (glutamate residues, aa 108-114), a feature that is conserved through out the animal kingdom. This cluster has been reported to be essential for interacting with Synaptotagmin1 to recruit it to the fusion complex and mediate fast synaptic vesicle exocytosis (Tokumaru et al., 2008). In order to examine the function of such molecularly significant amino acids, we designed a Cpx2 variant wherein 7 glutamate residues (negatively charged) in the C-terminus were exchanged to neutral alanines ($Cpx2^{E/A}$). Cells that virally express the $Cpx2^{E/A}$ mutant protein show undiminished clamping of tonic secretion (teal, -59±33 fF, n=20) similar to cells expressing wt Cpx2 protein (dark green, -67±26 fF, n=19) (Fig. 20B-C). Since the primed vesicle pool is intact, a full rescue of synchronous secretion can be expected. Surprisingly, the mutant was not able to fully restore the exocytotic burst (Fig. 20E-F). Instead, the RRP component was significantly reduced in the absence of the glutamate cluster. Fitting of the capacitance traces show no significant changes in the release kinetics of exocytotic burst in $Cpx2^{E/A}$ mutant cells (τRRP 34±6 ms; τSRP=229±17 ms) compared to cells expressing wt Cpx2 (τRRP 23±3 ms; τSRP=210±25 ms) (Fig. 20G) as also confirmed by the normalization of the CM traces (Fig. 20H). Furthermore, loss of glutamate cluster in the C-terminus did not confer any additional secretory delay between the stimulus and the onset of secretion (Fig. 20I). These data indicate that the $Cpx2^{E/A}$ is involved in regulating the magnitude of triggered exocytosis. In order to verify our findings, we also expressed $Cpx2^{E/A}$ mutant protein and the wt Cpx2 in wild type cells (Fig. 21A-E). The experiments show no changes in tonic secretion, but an intermediate response by the $Cpx2^{E/A}$ mutant, including again a significant reduction in the RRP component.

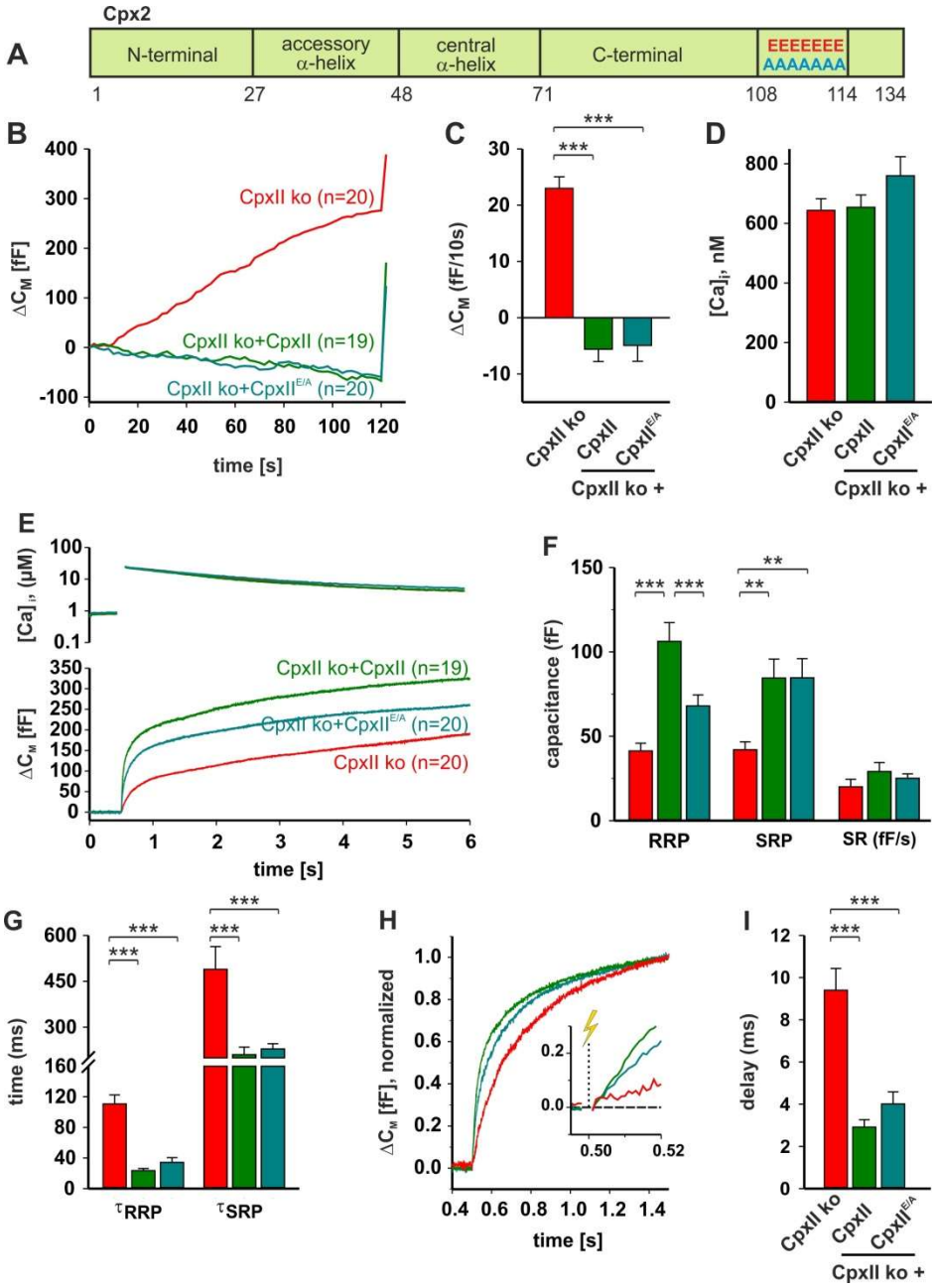


Figure 20. Glutamate cluster in Cpx C-terminus does not affect clamping of tonic secretion.
(A) Schematic representation of distinct domains of Cpx2 including approximate location of 7 glutamic acids within the C-terminal domain. (B) Hindered tonic secretion due to the clamp action of Cpx2 even in the absence of 7 glutamates in the C-terminus. Cpx2 ko cells (red, n=20), and those expressing Cpx2 (dark green, n=19) and the mutant protein $Cpx2^{E/A}$ (teal, n=20). (C) Quantified tonic secretion for every 10 s. (D) Pipette solutions with similar free Ca^{2+} levels were maintained before applying flash. (E) Average $[Ca^{2+}]_i$, (top panel) and flash-evoked capacitance responses (lower panel) in all the above-mentioned cell groups. (F) Quantitative analysis of RRP, SRP and SR (fF/s). Notice the reduced RRP in the mutant cells. (G) Assessment of kinetics across the mentioned cell groups reveals no changes in the secretory rates due to the loss of glutamate cluster in the C-terminus. (H) Normalized capacitance traces show faster secretion in cells expressing wt Cpx2 and the mutant protein in comparison with that of Cpx2 ko cells. The first 20 ms of flash stimulus (inset) reflect this phenotype in detail (I) Analysis of stimulus-secretion coupling show delayed response only in Cpx2 ko cells but not in the mutant cells. Error bars represent mean ±SEM. **, $p < 0.01$; ***, $p < 0.001$; One-way Anova test with post-hoc tukey's test.

These results indicate a dominant negative phenotype of the mutant protein suggesting that the mutant protein competes with the endogenous Cpx2 for binding to productive SNARE complexes. No significant changes were seen either in the secretory kinetics or in the delay of triggered exocytosis in Cpx2$^{E/A}$ mutant cells. Overall, these results indicate that the glutamate residues in the C-terminus have no clamping function but they are essential in determining the magnitude of the fast component (RRP) of synchronous secretion.

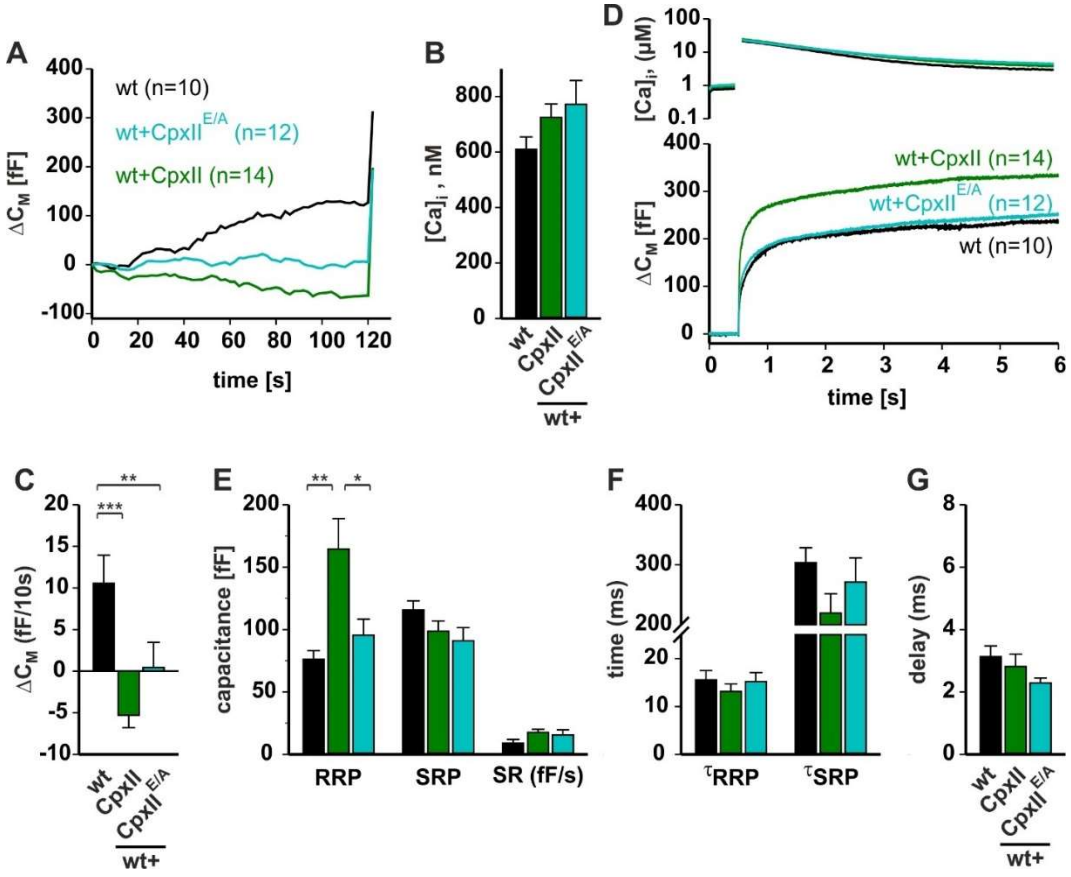


Figure 21. Glutamate cluster (aa 108-114) in the C-terminus affects the fast component of exocytotic burst. (A) Tonic secretion displayed by wt cells (black, n=10), wt cells overexpressing Cpx2 (green, n=14) and those expressing the mutant Cpx2$^{E/A}$ gene (mint, n=12). (B) similar submicromolar Ca^{2+} levels preflash. (C) Average rate of tonic exocytosis (D) Flash-induced CM responses in the above groups of cells. (E) Quantified mean CM. Notice the significant reduction in the RRP component in comparison to SRP (F) unaltered time constants of exocytotic burst. (G) No change in onset of secretion in response to stimulus ***, P < 0.001, one-way ANOVA. Error bars indicate means ± SEM. Error bars represent means ±SEM. *, p< 0.05; **, p < 0.01; ***, p < 0.001; One-way Anova test with post-hoc tukey's test.

4.1.6 Stable binding of Cpx2 to the ternary SNARE complex is crucial for its functions

Previous studies on mouse chromaffin cells in our lab have established that the N-terminus of Cpx2 (aa 1-26) speeds up the exocytosis by way of increasing Ca^{2+} affinity of synchronous secretion. Consequently, the loss of N-terminal domain of Cpx2 not only leads to slower kinetics of exocytosis but also to a much delayed exocytotic response (Dhara et al., 2014). Lying downstream of the N-terminus, the accessory α-helix also has crucial functions. It has been shown to exert inhibitory effect due to its helical conformation (Xue et al, 2007) and to clamp vesicle fusion by inserting into the space between the v- and t -SNAREs (Maximov et al, 2009). It can prevent full zippering of partially assembled SNARE complexes (Giraudo et al, 2009) or displace the C-terminus of VAMP2 (Lu et al., 2010). In addition, the accessory α-helix also harbors several amino acids residues that interact with Synaptobrevin and SNAP25 (Bykhovskaya et al., 2013) (Fig. 13). It has also been reported to stabilize the secondary structure of the central α-helix (Radoff et al., 2014; Xue et al, 2010). In order to investigate the importance of the N-terminal domain and the accessory α-helix of Cpx2 in mouse chromaffin cells, we designed a variant of Cpx2 that lacks the first 53 amino acids ($Cpx2^{\Delta 53}$) (Fig. 22A). At submicromolar Ca^{2+} concentration, cells expressing $Cpx2^{\Delta 53}$ failed to fully clamp tonic secretion (ΔC_M=166±30 fF, violet, n=23) compared to the cells expressing Cpx2 (dark green, -75±16 fF) (Fig. 22B-C). Furthermore, the $Cpx2^{\Delta 53}$ cells exhibited a strong reduction in synchronous secretion (RRP=31±4 fF, SRP=44±5 fF) when compared to wt Cpx2 (RRP=142±15 fF, SRP=112±10 fF) (Fig. 22E-F). It should be noted that the truncation includes the amino acids R48 and H52, which are known to be crucial for binding to the SNARE complex (Xue et al., 2007). The $Cpx2^{\Delta 53}$ truncation may interfere with the SNARE binding properties of the protein and therefore affects all the functions of Cpx2. Interestingly, fitting analysis showed that the secretory kinetics of $Cpx2^{\Delta 53}$ cells (τRRP=55±10 ms, τSRP=482±97 ms) are slower than Cpx2 ko cells overexpressing Cpx2 (τRRP=11±2 ms, τSRP= 298±40 ms) (Fig. 22G-H). In the same line, $Cpx2^{\Delta 53}$ cells like Cpx2 ko cells, exhibited a significant delay in the stimulus-secretion coupling in contrast to wt and cells rescued with Cpx2 (Fig.22I). These results corroborate previous findings that the N-terminal domain is responsible for accelerating the kinetics of synchronous exocytosis (Dhara et al., 2014). Thus, the observed phenotype may be a consequence of a dysfunctional, misfolded protein.

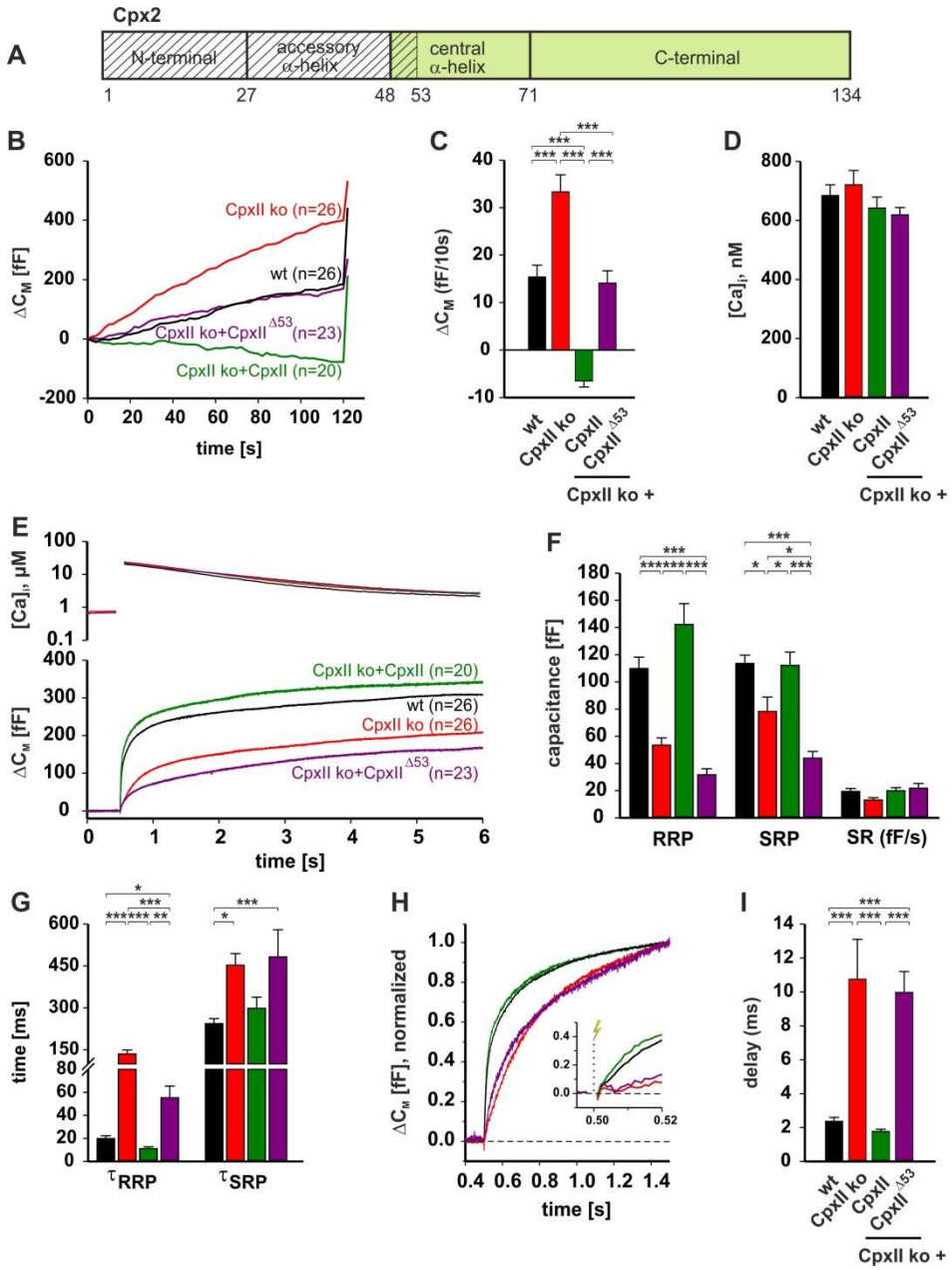


Figure 22. Proper SNARE binding is necessary for Cpx functions

(A) Graphical representation of distinct domains of Cpx2. Striped region illustrates the truncation well into the SNARE binding central α-helix. (B) Tonic secretion at submicromolar $[Ca^{2+}]_i$, recorded from wt cells (black, n=26), Cpx2 ko cells (red, n=26), rescue cells (dark green, n=20) and Cpx2$^{\Delta 53}$ cells (violet, n=23). (C) Quantified tonic secretion per 10 sec (D) Similar $[Ca^{2+}]_i$ were maintained across all the groups of cells before the application of flash. (E) Top panel depicts rapid flash-evoked Ca^{2+} change from ~700 nM to ~20µM. CM responses due to high calcium stimulus across all the above-mentioned groups of cells (lower panel). (F) Quantification of the different components of exocytosis (RRP, SRP and Sustained phase). Notice the severe reduction of the EB exhibited by the truncated protein, Cpx2$^{\Delta 53}$ (violet). (G) Time constants of exocytotic burst across tested groups of cells. Kinetics of the truncated protein are significantly slower than the wt protein expressing cells, owing to the loss of N-terminus that is known to be responsible for speeding up of exocytosis. (H) Normalization of the average capacitance traces confirms the slow secretory behavior by the Cpx2$^{\Delta 53}$ cells. Inset displays the close-up view of the first 20 ms of mean CM traces post flash indicating delayed secretion from the onset of stimulus. (I) Quantified delay shown by the above cell groups. Error bars represent mean ±SEM. *, $p < 0.05$; ***, $p < 0.001$; One-way Anova test with post-hoc tukey's test.

4.1.1 Complexin and its mutant variants have similar protein expression levels

To study whether the observed phenotypes are not simply the consequence of different expression levels, we analyzed the amount of expressed Cpx2 proteins using an antibody directed towards the SNARE binding domain of Cpx2. Since Cpx2 is a cytosolic protein, fluorescence signals were quantified throughout the cytoplasm (excluding the nucleus). No signals were detected in Cpx2 ko cells indicating the specificity of the antibody (Fig. 23A). No significant differences in the protein expression could be observed for any Cpx2 mutant variants when compared with the expression of the wt protein. Furthermore, the level of mutant protein expression was similar to that of wt Cpx2 which is significantly higher than the endogenous protein levels (Fig. 23B). Thus, the observed phenotypes are the consequence of the loss of functionally important motifs and amino acid residues.

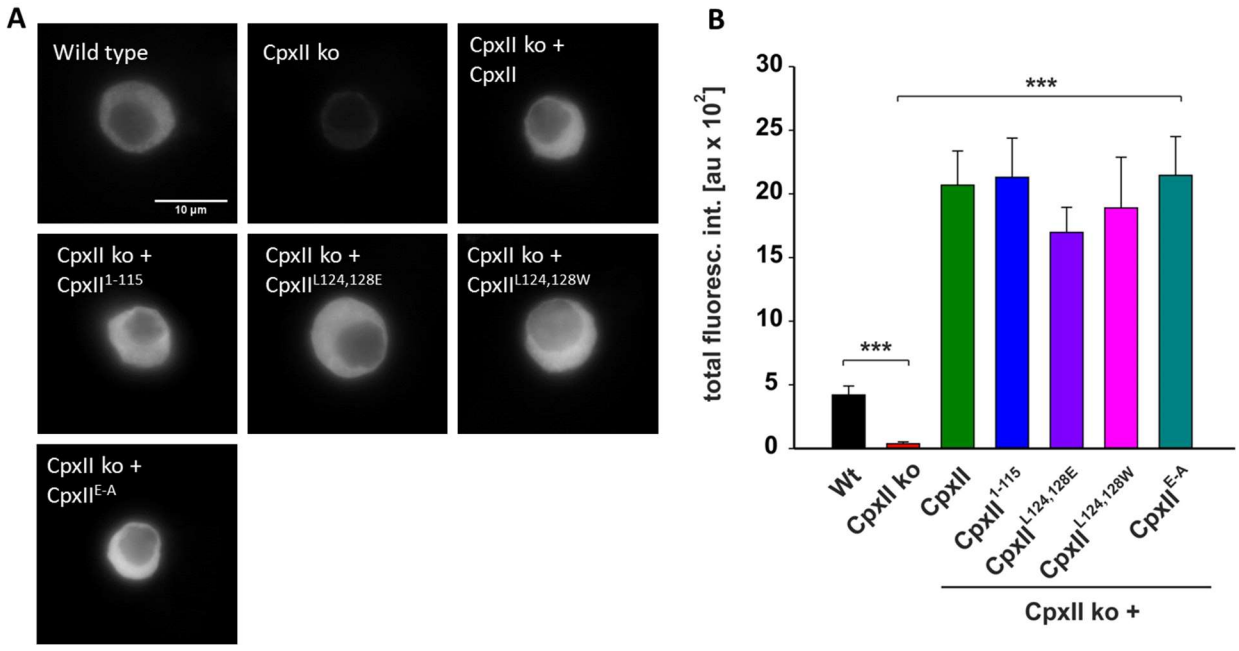


Figure 23. Immunosignals reveal similar expression levels of Cpx2 and its mutants.
(A) Exemplary fluorescence images of wt, Cpx2 ko and Cpx2 ko cells expressing either wt Cpx2 or the mutated Cpx2 proteins. Immunosignals were detected with a polyclonal Cpx2 antibody that binds to SNARE binding domain of Cpx. (B) Mean total fluorescence intensity of the above-mentioned cell groups (acquired 3.5 h after Semliki forest viral transfection). All mutant proteins were expressed in higher levels compared to the endogenous protein (wt, n=23; Cpx2 ko, n=19; Cpx2, n=22; $Cpx2^{1-115}$, n=13; $Cpx2^{L124,128E}$, n=20; $Cpx2^{L124,128W}$, n=9; $Cpx2^{E/A}$, n=11). Error bars indicate means ± SEM. au, arbitrary unit; int, intensity. Error bars represent means ±SEM. ***, $p < 0.001$; One-way Anova test with post-hoc tukey's test.

4.1.1.1 Summary for Part I

Our experiments with the C-terminal domain of Cpx2 demonstrate that the last 34 amino acids of Cpx2 provide a site that is involved in clamping premature vesicles. This region of Cpx2 comprises crucial motifs such as the glutamate cluster and the amphipathic region that are well conserved across the animal kingdom. While the glutamate cluster is not involved in clamping tonic secretion, it is required to maintain the magnitude of readily releasable vesicles, most likely by being a potential site for interacting with Syt1. The amphipathic helix at the very end of C-terminal domain is important for inhibiting premature secretion. This inhibition may arise due to its ability to participate in protein-protein interactions, for example with other SNARE proteins. Mutating only few key residues that are hydrophobic in nature within the amphipathic region can dramatically alter the clamp function of the whole protein. Lastly, mutations that disturb the SNARE binding properties of Cpx2 can render the protein dysfunctional. The N-terminal domain and the accessory α-helix contribute properties (accelerating and stabilizing) that are essential for facilitating fast synchronous secretion in mouse chromaffin cells.

4.2 Part II – Complexin2 and Synaptotagmin7

SNARE proteins (Syb, Stx and SN25) along with regulatory proteins (Syt and Cpx) constitute the basic machinery for Ca^{2+} triggered exocytosis. Although both Syt and Cpx are known to interact with the SNARE complexes, it is unclear how their functions are coupled. Previous studies from our lab have proposed that Cpx2 hinders premature vesicle exocytosis and facilitates synchronous secretion with its N-terminus acting as an adaptor for the Ca^{2+} sensor, Syt1. Amperometric measurements of single vesicle release furthermore indicated that Syt1 antagonises Cpx2 clamp action at moment of Ca^{2+} rise (Dhara et al., 2014). Since individual loss of Syt1 impairs (fast) synchronous but not the (slow) asynchronous exocytosis (Chen et al., 2002; Pabst et al., 2002; McMahon et al., 1995; Bracher et al., 2002), it is likely that other Ca^{2+} sensors may be present. Indeed, chromaffin cells are equipped with two functionally overlapping Ca^{2+} sensors (Schonn et al., 2008; Wang et al., 2005; Segovia et al., 2010), each having different Ca^{2+} affinities (Südhof, 2002; Sugita et al., 2002; Bhalla et al., 2005). Ongoing experiments in our lab have comparatively analyzed granule exocytosis from null mutants (Cpx2 ko and Syt1 ko) as well as the combined double knock outs (Cpx2/Syt1 dko). Loss of Syt1 abolished RRP without altering tonic secretion. In the absence of Cpx2, additional loss of Syt1 did not further aggravate the Cpx2 ko phenotype (other than the selective reduction of the RRP), indicating that both Cpx2 and Syt1 operate in the same pathway. No additional changes were observed either in tonic or evoked secretion of Cpx2/Syt1 dko cells in comparison with Cpx2 ko cells. These findings provide the basic framework for this part of the PhD thesis. Here, we investigate the role of secondary Ca^{2+} sensor, Syt7 together with Cpx2 in regulating granule exocytosis in mouse chromaffin cells. To this end, we performed patch-clamp experiments to identify changes in membrane capacitance from null mutants (Cpx2 ko and Syt7 ko) and double mutants (Cpx2/Syt7 dko).

4.2.1 Synaptotagmin7 promotes fusion at low Ca^{2+} concentrations

Syt7 is known to be a high affinity Ca^{2+} sensor (Wang et al., 2005) and is also responsible for slow exocytosis (Schonn et al., 2008). Syt7 has been reported to be the Ca^{2+} sensor for asynchronous secretion in zebrafish neuromuscular synapse (Wen et al., 2010), in hippocampal neurons (Bacaj et al., 2013) and at calyx of Held synapses (Luo & Südhof, 2017). Capacitance recordings in chromaffin cells cells lacking Syt7 show that tonic secretion is significantly reduced (28±13 fF, n=20) in comparison with wt cells (152±38 fF, n=17) (Fig.24A-B). This indicates that Syt7 supports tonic secretion at submicromolar Ca^{2+} concentrations (Fig.

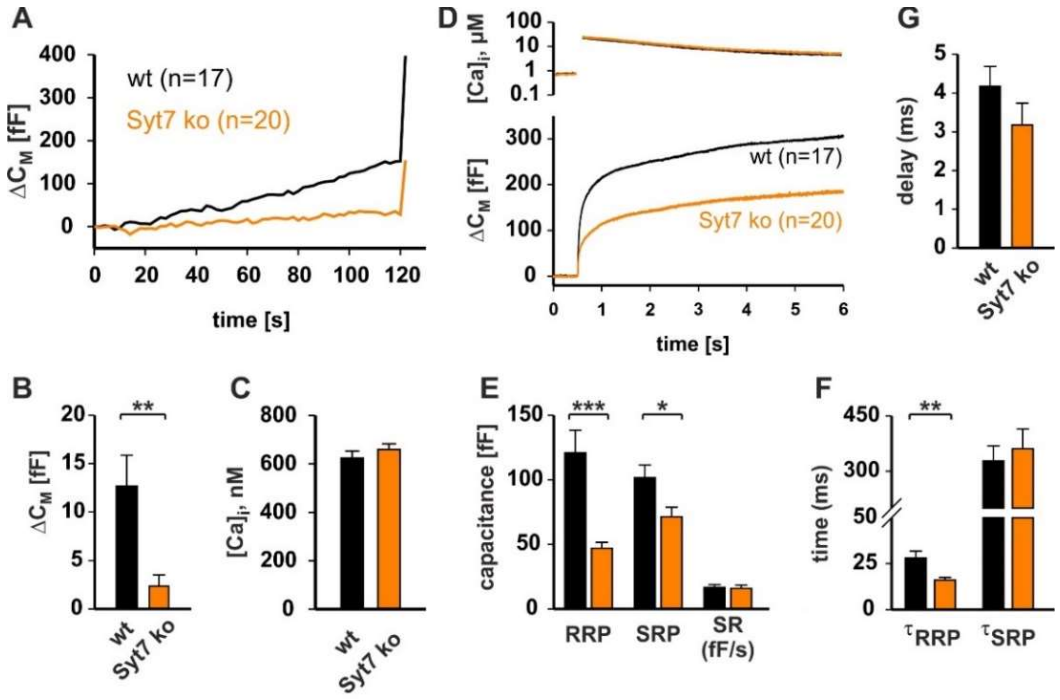


Figure 24. Syt7 serves as Ca^{2+} sensor at low Ca^{2+} concentrations.
(A). Averaged capacitance traces of wt cells (black, n=17) and Syt7 ko cells (orange, n=20) (B). Quantified rate of premature secretion. (C). Similar submicromolar Ca^{2+} concentrations during preflash measurements of ΔC_M. (D). Average Ca^{2+} concentrations recording before and after flash application (upper panel) Flash evoked synchronous secretion recorded over 6 sec (lower panel). (E). Quantification of different components of triggered exocytosis shows that Syt7 ko cells exhibit significantly reduced exocytosis. (F). Kinetics of EB components reveals that Syt7 ko cells exhibit faster secretion than wt cells. (G). the onset of secretion from the point of stimulation is similar in both the cell groups. *, $p< 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Student's T-test.

24A-C). Flash evoked synchronous secretion in Syt7 ko cells also displayed a significant reduction in the amplitude of exocytotic burst (RRP= 47 fF, SRP=71 fF, n=17) compared to that of wt cells (RRP=120 fF, SRP=102 fF, n=20) (Fig. 24D-E). This reduction in fusion competent vesicles mirrors a defect in vesicle replenishment in Syt7 ko cells (Liu et al., 2014). The thorough analysis of the fitted capacitance traces revealed that chromaffin cells genetically deficient of Syt7 display significantly faster release kinetics (τRRP=16±1 ms, τSRP=360±54 ms) than those of the wt cells (τRRP=28±4 ms, τSRP=327±40 ms) (Fig.24F), indicating that Syt7 mediates slow release. The accelerated kinetics of EB seen in Syt7 ko are most likely due to the other Syt isoform, Syt1 found in chromaffin cells that mediates fast synchronous secretion (Liu et al., 2009; Nishiki and Augustine, 2004). No changes were observed in the sustained phase of release. Taken together, this result indicates that Syt7 not only acts as a Ca^{2+} sensor, for LDCV fusion of vesicles at submicromolar Ca^{2+} concentrations but also contributes in part to the magnitude of synchronous fusion in chromaffin cells.

4.2.2 Cpx2 regulates exocytosis independently of Syt7

Previous experiments in our lab have established that on wt background, expression of Cpx2 hinders premature secretion and boosts exocytotic burst (Dhara et al., 2014). In order to test whether Cpx can also boost exocytosis in the absence of Syt7, Cpx2 was overexpressed in Syt7 ko cells. The results show that tonic secretion was strongly suppressed by Syt7 ko cells overexpressing Cpx2 (-44±18 fF, n=18) compared to both wt cells (92±18 fF, n=17) and Syt7 ko cells (27±19 fF, n=18) (Fig. 25A-B). This confirms that the expression of Cpx2 strongly hinders premature vesicle exocytosis with similar efficiency as in wt and Cpx2 ko backgrounds (compare with Fig. 14). Thus, Cpx2 arrests tonic secretion independently of Syt7. Upon rapid uncaging of Ca^{2+} due to UV flash, the synchronous secretion seen in Syt7 ko cells (RRP=46±7 fF, SRP=38±4 fF) was strongly boosted by the expression of Cpx2 (RRP=94±9 fF, SRP=59±5 fF) (Fig. 25D-E).

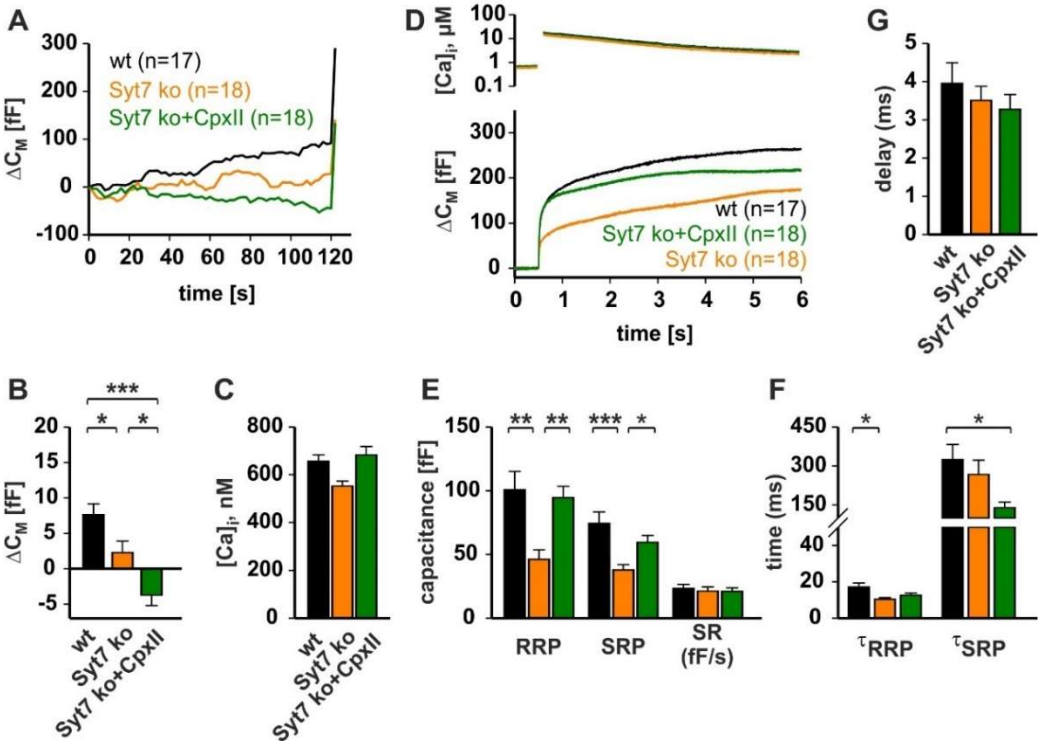


Figure 25. Cpx2 clamps premature vesicle secretion independently of Syt7.
Expression of Cpx2 in Syt7 ko cells suppresses premature secretion and enhances synchronous secretion with similar efficiency as in wt cells (A) Pre-flash membrane capacitance signals showing tonic secretion from wt cells (black, n=17), Syt7 ko cells (orange, n=18) and Syt7 ko cells overexpressing Cpx2 (dark green, n=18). (B) Relative magnitudes of tonic secretion per 10 s. (C) mean cytosolic Ca^{2+} signals during 120 sec of measuring CM pre-flash. (D) Step-wise intracellular Ca^{2+} changes due to flash application (upper panel) and corresponding CM traces (lower panel). (E) Quantification of exocytotic components. (F) time constants of exocytotic burst and (G) delay in onset of secretion in response to stimulus. *, $p< 0.05$; **, $p < 0.01$; ***, $p < 0.001$; One-way Anova test with post-hoc tukey's test.

Secretory kinetics displayed by Syt7 ko cells overexpressing Cpx2 were indeed faster (τRRP=12±1 ms, τSRP=139±22 ms) than in wt cells (τRRP=17±2 ms, τSRP=324±58 ms) and comparable to Syt7 ko cells (τRRP=10±1 ms, τSRP=266±55 ms) (Fig. 25F). No significant changes in the stimulus-secretion coupling were found indicating that the triggering of exocytosis was unchanged in all the groups. Taken together, these results indicate that Cpx2 arrests premature vesicle secretion in the absence of Syt7 and also boosts synchronous exocytosis as previously reported in wt cells (Dhara et al., 2014), most likely by cooperating with Syt1. Therefore, the functions of Cpx2 are independent of Syt7 in regulating exocytosis.

4.2.3 Cpx2 and Syt7 operate in different molecular steps en route to fusion

As mentioned earlier, in the absence of Cpx2, additional loss of Syt1 does not aggravate the Cpx2 ko phenotype indicating that both Cpx2 and Syt1 operate in the same pathway. To understand the functional relationship between Cpx2 and Syt7, we took advantage of a novel double knockout mouse (Cpx2/Syt7 dko) generated in our lab and performed capacitance measurements on Cpx2 ko cells and Cpx2/Syt7 dko cells. For the sake of comparison with Syt7 ko and wt phenotypes, CM traces from Fig. 25 were superimposed in Fig. 26. The comparative analysis shows that Cpx2/Syt7 dko cells display diminished tonic secretion (150±30 fF, n=24, purple) compared to Cpx2 ko cells (349±36 fF, n=20, red) (Fig. 26A-C). This outcome is in line with our work on single knock out (Syt7 ko) which showed that Syt7 acts as a Ca^{2+} sensor at submicromolar Ca^{2+} concentrations (Fig. 24). Strikingly, the flash evoked responses of Cpx2/Syt7 dko cells (n=24, purple) were fully abolished compared to that in Cpx2 ko cells (RRP=42±8 fF, SRP=72±12 fF, n=20, red) (Fig. 26D-E). Only the sustained component of exocytosis was present meaning that the general fusion competence is not abolished. Overall, these results show that in the absence of Cpx2, additional loss of Syt7 leads to additive effects, indicating that Cpx2 and Syt7 operate independently. Both Cpx2 and Syt7 support synchronous secretion but do so in molecularly different steps.

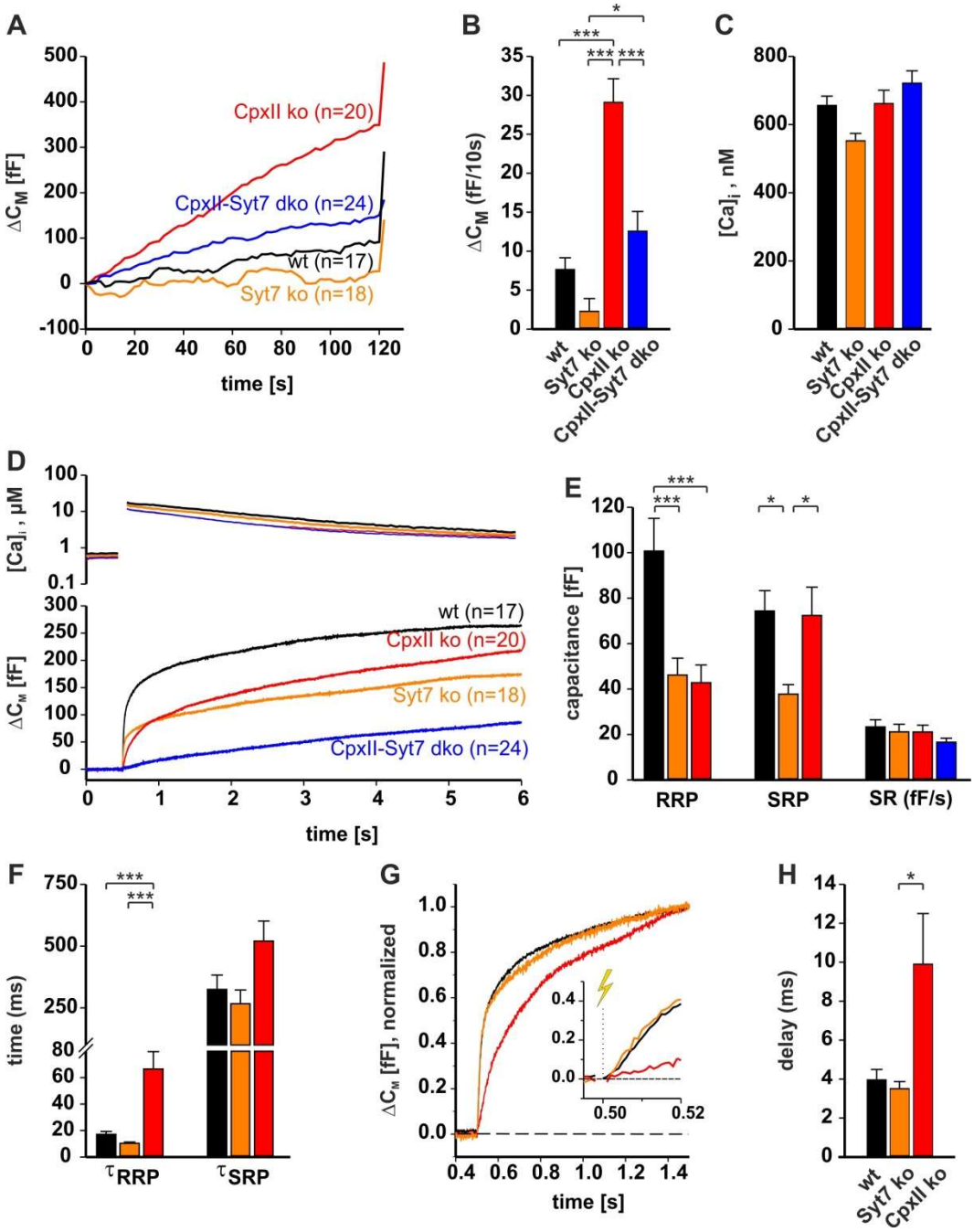


Figure 26. Cpx2 and Syt7 support synchronous exocytosis by independent mechanisms.
(A) Tonic secretion of Cpx2ko cells (red, n=20), Cpx2/Syt7 dko cells (blue, n=24), Orange (Syt7 ko cells) and black traces (wt cells) (B) Quantification of tonic exocytosis. Dko cells show diminished tonic secretion compared to Cpx2ko cells. (C). Similar intracellular Ca^{2+} levels before the application of flash. (D). Step-like rapid rise in $[Ca^{2+}]_i$ upon flash application and average Ca^{2+} traces for 6s (upper panel).and corresponding CM changes (lower panel). (E). Quantification of magnitudes of membrane capacitance of the above-mentioned groups of cells. Notice the abolished RRP and SRP for dko cells in comparison with Cpx2ko cells. No changes were observed in the sustained phase across all the measured cell groups. (F). Secretory time constants of wt, Syt7 ko and Cpx2 ko. (G). Normalization of average capacitance traces during the first 1.5 s of the recording. The inset shows expanded scaling for the first 20 ms of the flash stimulus. The $Syt7^{-/-}Cpx2^{-/-}$ dko does not exhibit any exocytotic burst. (H). Quantified analysis of stimulus-secretion coupling. *, $p< 0.05$; ***, $p < 0.001$; One-way Anova test with post-hoc tukey's test.

4.2.3.1 Summary of Part II

In summary, Syt7 plays an active role as a Ca^{2+} sensor at submicromolar Ca^{2+} concentrations. The fast kinetics of synchronous secretion seen in Syt7 ko cells suggest a possible intervention by Syt1, a major Ca^{2+} sensor expressed in chromaffin cells. Secondly, Cpx is able to clamp premature granule exocytosis and supports synchronous secretion independent of Syt7. Consequently, the overexpression of Cpx is able to hinder tonic secretion and boost synchronous exocytosis even in the absence of Syt7. Furthermore, loss of Syt7 in the absence of Cpx2 results in dramatic additive consequences (both RRP and SRP abolished) in contrast to no additive changes in Cpx2/Syt1 dko cells. This shows that Cpx2 and Syt7 support synchronous exocytosis of vesicles in two different pathways en route to fusion, whereas Cpx2 and Syt1 operate in the same pathway. Our data support the possibility of different secretory pathways where Cpx and Syt isoforms impose their regulatory roles as the granules pass from one state of maturity to another. Thus, an attractive model could be that Syt7 serves as the Ca^{2+} sensor in the slow release pathway whereas Syt1 assumes the Ca^{2+} sensing role in the downstream fast release pathway.

5 Discussion

Several studies have demonstated that Complexin inhibits fusion of vesicles in neurons and neuroendocrine cells (Maximov et al., 2009; Yang et al., 2010; Kaeser-Woo et al., 2012; Wragg et al., 2013; Dhara et al., 2014; Mohrmann et al., 2015) but the precise mode of Cpx2 action is not clearly understood. Complexin comprises a central helix flanked by the N-terminus and the C-terminus (Fig. 27). The first part of this study pinpoints crucial amino acid residues and motifs in the C-terminus of Cpx2 that are responsible for maintaining a tight control over premature vesicle secretion. Our experiments demonstrate that the last 34 amino acids of Cpx (aa 100-134), play a crucial role in clamping tonic secretion (Makke et al., 2018). This section comprises the glutamate cluster and the amphipathic region both of which appear to be involved in protein-protein interactions in order to inhibit fusion of vesicles. Using an N-terminal truncation that potentially disturbs SNARE binding we could also show that the N-terminus and the accessory α-helix lying upstream of the SNARE binding domain of Cpx2 are necessary for proper functioning of Cpx.

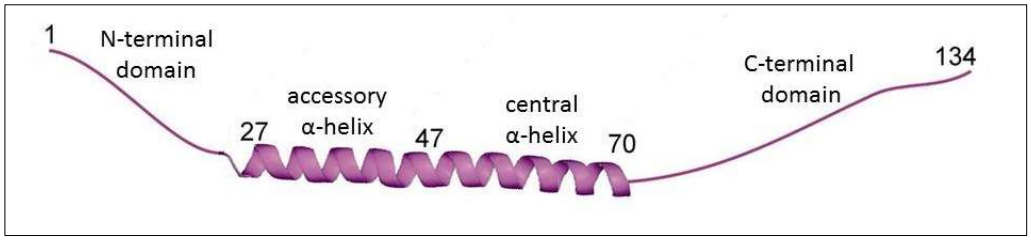


Figure 27. Domain structure of Complexin2 (Cpx2)
Numbers indicate the domain boundaries. The N-terminus (aa 1-27) is believed to facilitate exocytosis and the accessory α -helix (aa 27-47) is thought to stabilize the central α-helix (aa 48-70) also known as the SNARE binding domain. The binding of the SNARE binding domain to the SNARE complex is a prerequisite for the proteins' function. The C-terminus (aa 71-134) clamps premature vesicle secretion.

5.1 The C-terminus of Cpx2 prevents premature secretion

The C-terminus of Cpx2, is composed of about 60 amino acids constituting almost half of the protein. Previous studies in our lab have specifically shown that the C-terminal domain (aa 72-134) lying downstream of the SNARE-binding domain of Cpx2, is responsible for arresting tonic secretion (clamp function) (Dhara et al., 2014). By hindering the depletion of release-ready vesicles at priming Ca^{2+} concentrations, Cpx2 builds up a pool of release ready vesicles that may be released synchronously. In order to identify the exact site of 'clamp' action of Cpx and decode its precise mode of action we employed different Cpx2 variants with different truncations or amino acid substitutions. Using whole cell patch clamp technique combined with flash

photolysis, both tonic and synchronous exocytosis in chromaffin cells were evaluated quantitatively in front of well-defined intracellular Ca^{2+} concentrations.

5.1.1 Clamp action of Cpx2 is determined by the far C-terminus

The truncation of the last 34 amino acids ($Cpx2^{1-100}$) resulted in increased tonic secretion indicating impaired 'clamping' of primed vesicles (Fig. 16). Such an increase in tonic secretion was also observed in Cpx2 ko cells and those expressing the $Cpx2^{\Delta C}$ mutant where the entire C-terminus (aa 73-134,) was truncated (Dhara et al., 2014). Our data suggest that the very C-terminal amino acids (last 34) constitute the crucial region for clamping tonic exocytosis in mouse chromaffin cells. Our observation that the 'clamp' action resides in the very C-terminus is consistent with a previous study that showed enhanced spontaneous release in hippocampal neurons, when only the last 48 amino acids of Cpx1 C-terminus were truncated (Kaeser-Woo et al., 2012) Furthermore, with the $Cpx2^{1-100}$ mutant, the increase in tonic secretion was accompanied by a decrease in flash evoked synchronous exocytosis (Fig. 16E-H) suggesting a direct relationship between the loss of primed vesicles and diminished exocytosis as proposed by Dhara et al., 2014. An increase in tonic secretion and a decrease in evoked synaptic vesicle fusion was also found at the neuromuscular junctions of *C. elegans* due to the loss of Cpx1 (Martin et al., 2011). The unchanged release kinetics are also in agreement with previous findings showing that the N-terminal domain (aa 1-27) is responsible for increased Ca^{2+} -affinity of synchronous secretion and therefore for faster release kinetics (Xue et al., 2007; Maximov et al., 2009; Dhara et al., 2014). The $Cpx2^{1-100}$ mutant, when expressed in wt cells, also enhanced tonic secretion and as a result decreased synchronous release illustrating its dominant negative phenotype (Makke et al., 2018). Thus, our experiments demonstrate that it is the very C-terminus (last 34 amino acids) that actively clamps tonic secretion at submicromolar $[Ca^{2+}]_i$, building up a pool of primed vesicles that can be released rapidly by the Ca^{2+} -trigger.

5.1.2 The C-terminal amphipathic helix is essential for clamping premature secretion

Previous studies have shown that the C-terminal region of mammalian and invertebrate Cpx1 contains a motif that may form an amphipathic helix (Seiler et al., 2009; Wragg et al., 2013). Motivated by the results obtained for the $Cpx2^{1-100}$ mutant, we systematically explored the functional consequences of the amphipathic motif and the glutamate cluster in the C-terminus of mouse Cpx2.

The Cpx2$^{E/A}$ mutant lacking seven negatively charged glutamate residues (aa 108-114) located upstream of the amphipathic region (aa 116-134) arrested premature vesicle secretion similar to the cells expressing Cpx2 indicating no changes in the clamping function of the protein. However, the Cpx2$^{E/A}$ mutant could not boost synchronous exocytosis up to the level seen with wt protein as expected (Fig. 20). Interestingly, the RRP was significantly reduced resembling a phenotype observed in Syt1 ko cells (Dhara et al., 2014). According to another previous study, Syt1 binds to the C-terminus of Cpx mainly by electrostatic interactions of negatively charged glutamates in the C-terminus of Cpx (Tokumaru et al., 2009). Mutating these glutamate residues in the C-terminal of Cpx to alanines or glutamines dramatically reduced the binding of Cpx to Syt1. Thus, the specific loss of RRP observed with the Cpx2$^{E/A}$ mutant, points to the possibility that the activity of Syt1 may have been affected due to the loss of glutamate cluster. These observations also affirm the view that the far C-terminus of Cpx2 is involved in protein-protein interactions.

The Cpx2$^{1-115}$ mutant wherein the amphipathic region (aa 116-134) was truncated, failed to clamp premature tonic secretion causing a corresponding decrease in the size of exocytotic burst. In the same line, experiments with Cpx2^{L124,128E} mutant where the hydrophobic character of the amphipathic C-terminus is decreased caused similar phenotype (compare Fig. 17 with Figs. 18 & 19). In contrast, restoring hydrophobicity by replacing leucines with tryptophan residues (Cpx2^{L124,128W}) did exhibit strong clamp activity and enhanced synchronous secretion like the wt protein. This indicates that the hydrophobic character in the C-terminus is of crucial importance in clamping premature secretion. Our results are in line with mutation experiments in neurons, where replacing a leucine within the putative amphipathic region with a charged residue, lysine (Cpx^{L117K}) or a helix breaker, proline (Cpx^{V120P}) failed to suppress the elevated mEPSC frequency (Kaeser-Woo et al., 2012). This study also showed that substitution of L117 and I101 with bulky hydrophobic residues, tryptophan (Cpx^{I101W} and Cpx^{L117W}) displayed decrease in spontaneous mEPSC signaling. Our data by highlighting the significance of key residues that maintain the amphipathic character at the end of C-terminus, demonstrate that the C-terminal amphipathic helix of Cpx2 is crucial for clamping premature vesicle secretion in mouse chromaffin cells.

5.1.3 The far C-terminus of Cpx2 participates in protein-protein interactions

In a recent study, we have demonstrated that Cpx2-CTD shows a high degree (50%) of sequence similarity to the hydrophobic layers +2 to +7 of SNAP25-SN1 motif (Makke et al.,

2018). Biochemical analyses have shown that an isolated C-terminal peptide co-precipitates with other SNARE proteins and have also implicated the C-terminal domain in transiently hindering SNARE complex formation (Makke et al., 2018). These studies support our results, illustrating the importance of the last stretch of Cpx2 C-terminus (amphipathic region) in protein-protein interactions. These observations are in contrast to a simple targeting role of the C-terminal amphipathic region for concentrating other domains of Cpx2 at the site of fusion, as proposed by Wragg et al., 2013. Taken together, our results support a mechanism approach wherein the amphipathic region may act as an alternative SNARE motif that binds to half-zippered SNARE complexes.

5.2 Mechanistic model for clamp action of Complexin

A large amount of experimental data now supports the view that the Cpx C-terminus which was once thought to be functionally inert (Xue et al., 2007) is actively involved in clamping fusion of vesicles (Cho et al., 2010; Martin et al., 2011; Kaeser-Woo et al., 2012; Dhara et al., 2014). In a recent study, our experiments provide evidence that the very C-terminal region, particularly the amphipathic helix present within the last 19 amino acids of Cpx2 are crucial for clamping tonic exocytosis (Makke et al., 2018). Here, we could also show that a C-terminal peptide (aa 101-134) co-precipitates with SNARE proteins and is able to transiently hinder the formation of the ternary SNARE complex. This indicates that the C-terminus can serve as an alternative SNARE binding motif. Given that the zippering of SNARE proteins starts from the N-terminus to the C-terminus of SNARE motifs and is arrested halfway, there is scope for the membrane-proximal layers of SNARE proteins to be unzippered and free for other protein-protein interactions (Li et al., 2007; Gao et al., 2012; Zhou et al., 2017). While Cpx2, with its central SNARE binding α-helix positions on the partially assembled SNARE complex in an antiparallel orientation, the downstream regions of Cpx2 may provide enough structural flexibility for the far C-terminus to fold back on membrane-proximal regions of the SNARE proteins and thus hinder their progressive zippering (Fig. 28). Under these conditions, the C-terminus of Cpx could bind to the SNAREs in a parallel orientation. Furthermore, lipid binding of the CTD could define the state of tonic secretion whereas SNARE binding allows the accumulation of release competent vesicles. This scenario is possible because the intramolecular distance between the SNARE binding domain and the far C-terminal domain of Cpx2 appears long enough for this flexibility to occur (Fig. 28 right panel). Indeed, FRET studies have shown that Cys105 of the Cpx C-terminal domain interacts with Syntaxin (Stx-1a) near the zero-layer providing evidence for the proposed

back folding of the Cpx C-terminus onto membrane proximal regions of the SNARE complex and interfere with their zippering (Bowen et al., 2005). Thirdly, the sequence similarities between the C-terminus of Cpx2 and the +2 to +7 hydrophobic layers of the SN1 motif of SNAP25 (Makke et al., 2018) suggest that Cpx C-terminus may compete with the SN1 domain of SNAP25 for binding to the SNARE complex. Thus, full zippering of the SNARE complex is hindered and exocytosis is halted effectively.

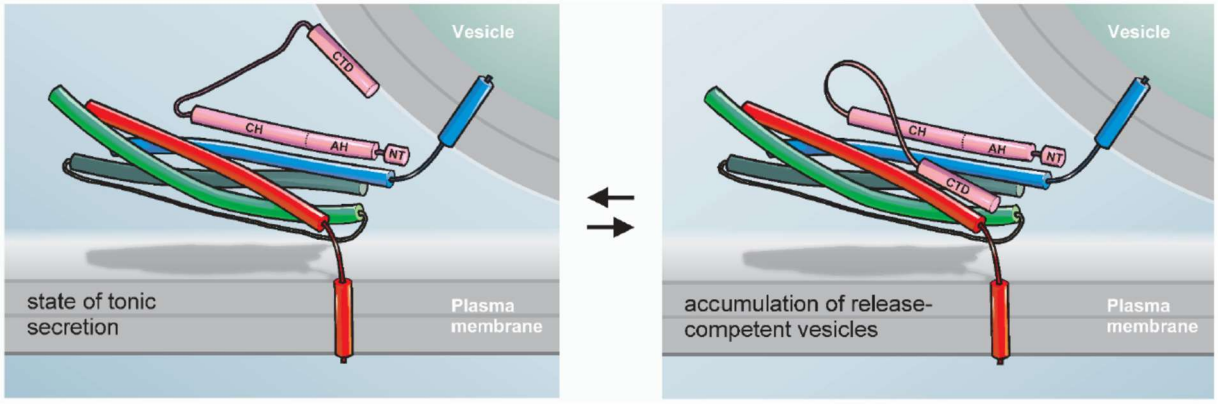


Figure 28. Hypothetical model for clamping mechanism by Cpx C-terminal domain.
Complexin (Cpx, violet/pink) binds with high affinity to partially zippered trans-SNARE complexes composed of vSNAREs (Syb2, blue) and tSNAREs (Stx, red/orange; SNAP25, green). Cpx C-terminus folds back to either interact with the vesicular membrane where it does not inhibit tonic release (left panel) or clamps the partially assembled SNARE complex (right panel), leading to accumulation of primed vesicles (*adapted from Mohrmann et al., 2015*). Thus, the amphipathic region provides both lipid interactions and protein-protein interactions.

5.3 N-terminal domains are necessary for proper Cpx functions

Previous experiments in our lab have shown that the N-terminally truncated Cpx2 variant (aa 28-134) could arrest tonic secretion effectively but slowed down the evoked release due to reduced Ca^{2+} -sensitivity of synchronous secretion (Dhara et al., 2014). In the current study, truncating the N-terminal domain, the accessory α-helix, and a few amino acids into the SNARE binding domain resulted in severe loss of synchronous secretion accompanied with slow release kinetics and prolonged secretory delay typical in Cpx2 ko cells. The overall phenotype indicates that the protein may be dysfunctional. Since it is well established that stable binding of Cpx's central α-helix to the SNARE complex is crucial for all functions of Cpx, deletion of the first 53 amino acids ($Cpx2^{\Delta 53}$) may hinder stable binding of the remaining C-terminal fragment ($Cpx2^{54-134}$) to the SNARE complex. Previous studies have shown that the amino acids within the accessory α-helix stabilize the secondary structure of the central α-helix (Pabst et al., 2000; Xue et al., 2007; Radoff et al., 2014). The loss of crucial amino acids (R48, H52) within the central

SNARE binding α-helix has been shown to perturb the binding of Cpx to the SNARE complex (Xue et al., 2007). Furthermore, we have shown that the isolated C-terminus of Cpx2 clamps premature secretion and boosts synchronous secretion when expressed in wt cells but not in Cpx2 ko cells (Makke et al., 2018). This indicates that other domains of Cpx2 are important either for cooperating with the C-terminus or for bringing the exocytotic machinery into a state where the C-terminus of Cpx2 exerts its inhibitory function. Therefore, we conclude that the $Cpx2^{\Delta 53}$ lacking the N-terminal domain and the accessory α-helix domain has no clear functional phenotype most likely because of impaired SNARE binding.

5.4 The Complexin – Synaptotagmin Interplay

Cpx2 and Syt1 control the magnitude and timing of synchronous exocytosis by different molecular mechanisms. Cpx with its C-terminus clamps partially zippered SNARE complexes, hindering premature vesicle secretion, leading to the accumulation of vesicles (Makke et al., 2018). Syt1, with its Ca^{2+} binding kinetics determines the timing of fusion events (Voets et al., 2001; Nagy et al., 2006). Recent amperometric data showed that Ca^{2+} bound Syt1 exhibits an antagonistic action by which Cpx2-mediated restraints are overcome leading to full vesicle fusion (Dhara et al., 2014). Intriguingly, chromaffin cells express two isoforms of Synaptotagmin namely Syt1 and Syt7 (Schonn et al., 2008), each having different affinities for Ca^{2+} (Bhalla et al., 2005). In the present study, we investigated the role of the secondary Ca^{2+} sensor, Syt7 in relation to Cpx2 using a combination of null mutants for Syt7 and Cpx2 as well as Cpx2/Syt7 dko cells.

5.4.1 Role of Syt7 and Syt1 in chromaffin cells

Several studies in neurons have shown Syt7 mediates slow asynchronous secretion (Wen et al., 2010; Bacaj et al., 2013; Luo & Südhof, 2017). In this thesis, we show that Syt7 promotes premature secretion at submicromolar Ca^{2+} concentrations in chromaffin cells (Fig. 24). In contrast, loss of Syt1 does not affect tonic exocytosis at similarly low Ca^{2+} concentrations as shown by earlier experiments in our lab (Dhara et al., 2014). Viewed together, these data indicate a functional distinction between both the co-existing Ca^{2+} sensors. Furthermore, the flash evoked synchronous secretions in Syt7 ko cells showed an exocytotic burst of reduced magnitude but with significantly faster release kinetics. While the reduced EB amplitude may be due to defective vesicle replenishment (Liu et al., 2014), the more rapid exocytosis of vesicles is most likely due to the presence of the remaining Ca^{2+} sensor, Syt1 which is known to have

faster Ca^{2+} binding kinetics. This conclusion is corroborated by the observation that loss of Syt1 selectively abolishes the fast component (RRP) in chromaffin cells (Dhara et al., 2014). Collectively, these data indicate that the secretion in chromaffin cells is functionally divided, wherein the slow premature secretion is mediated by Syt7 and fast synchronous secretion is mediated by Syt1. Interestingly, the two Ca^{2+} sensors expressed in chromaffin cells can function as stand-alone Ca^{2+} sensors in accordance with their Ca^{2+} binding properties. Thus, Syt7 is active Ca^{2+} sensor at submicromolar Ca^{2+} concentrations, consistent with its higher Ca^{2+} affinity whereas Syt1 is active at higher Ca^{2+} concentrations, in line with its lower Ca^{2+} affinity.

5.4.2 Cpx2 does not clamp Ca^{2+} sensors

It is possible that Cpx2 prevents premature secretion either by directly clamping the SNARE assembly or alternatively by clamping the triggering molecules like Syt7 or Syt1. Indeed, previous literature has suggested that loss of Cpx unclamps the secondary Ca^{2+} sensor indicating direct interaction (Yang et al., 2010). To explore this scenario, we expressed Cpx2 in Syt7 ko background. The results show that overexpression of Cpx2 suppressed tonic secretion with similar efficiency as in wt cells or in Cpx2 ko cells, providing no evidence for such functional interaction of Cpx2 with the secondary Ca^{2+} sensor. Therefore, the clamp action or its loss does not seem to be mediated by the functional interaction of Cpx2 with Syt7. In the same line, overexpression of Cpx2 in Syt1 ko cells also suppressed premature release (Dhara et al., 2014) indicating that Cpx interacts with neither Syt7 nor Syt1. Thus, Cpx2 hinders exocytosis independently of both the Ca^{2+} sensors (Syt7 and Syt1) in chromaffin cells. Furthermore, our results also indicate that Cpx2 clamps SNAREs directly but not the Ca^{2+} sensors, as pointed out in a previous study on hippocampal neurons (Yang et al., 2010). At this point, it also interesting to note that the clamping of tonic secretion is an action specific to Cpx2, because no increase in premature exocytosis was observed in Syt1 ko cells (Dhara et al., 2014). Thus, clamping premature exocytosis is not a general feature of SNARE regulators.

5.4.3 Friends and Foes of Cpx

Our observations with single knock out cells (Syt7 ko, Syt1 ko, Cpx2 ko) and double ko cells (Cpx2/Syt1 dko) provide interesting information about how Cpx works together with either of the Syt isoforms in chromaffin cells. The results indicate that Cpx2 cooperates with Syt1 but not with Syt7. Several lines of evidence support this conclusion. First, the over expression of Cpx2 in Syt7 ko cells enhanced the EB with similar efficacy as in wt cells (Fig. 25) but failed to do so in

Syt1 ko cells (Dhara et al., 2014). Second, previous experiments in our lab have shown that in comparison to Cpx2 ko, additional loss of Syt1 (Cpx2/Syt1 dko) does not further aggravate the Cpx2 ko phenotype, as expected for proteins acting in the same molecular pathway. Third, additional loss of Syt7 (Cpx2/Syt7 dko) fully abolished both the RRP and SRP components. Overall, these experiments provide strong evidence that Cpx2 and Syt1 cooperate in the RRP pathway. In contrast, Syt7 acts independently in different molecular steps/pathways. Previous studies suggested that the two Ca^{2+} sensors act in a functionally redundant manner (Schonn et al., 2008). Our comparative analysis of both double deficiencies together with expression experimients instead showed that Syt1 and Syt7 fulfill distinct functions.

Furthermore, another interesting aspect of the Cpx2/Syt7 dko phenotype is the undiminished component of sustained release, although priming of vesicles is fully abolished. This indicates that in the absence of Syt7 and Cpx2, not all types of fusion are similarly affected. It suggests that sustained release is molecularly distinct and may reflect exocytosis from a less mature state of release readiness (Bruns., 2002).

Based on the observations above, we propose a possible working model of Cpx2 and Synaptotagmins (Fig. 29) wherein Cpx inhibits premature granule exocytosis thereby allowing the accumulation of vesicles in a release competent state. Most likely, Cpx with the help of its C-terminus clamps the partially zippered membrane proximal layers of SNARE proteins. The N-terminus of Cpx2 fulfills a facilitatory function either by promoting full assembly of SNARE proteins or by regulating the binding configuration of Syt1. The N-terminus may act as an allosteric adaptor for Syt1 and modulate the Ca^{2+} -dependency of synchronous secretion (Neher, 2010; Dhara et al., 2014). The different modes of cooperation of Cpx with Syt7 and Syt1 points to the possibility that Cpx shifts the balance between the two Ca^{2+} sensors, from a more immature, Syt7 driven exocytosis to a more mature Syt1 based secretion. Accordingly, Cpx inhibits vesicles in both secretory pathways (tonic and phasic) and also shifts the usage of Syt variants from Syt7 to Syt1. When the secretory vesicle is able to recruit Syt1, instead of Syt7, it has the chance to be ‘super-primed’ bringing the vesicle to a very quickly releasable state. This implies that either pathway seems to engage a predominant Ca^{2+} sensor. While Syt1 promotes preferentially RRP exocytosis, Syt7 plays a major role in the exocytosis of vesicles from the SRP as well as for the tonic secretion at submicromolar Ca^{2+} concentrations. Therefore, the kinetics of Syt1 dependent RRP are overall faster than those of Syt7 mediated SRP. In the absence of one Ca^{2+} sensor (Syt1), the exocytotic machinery relies on the alternative Ca^{2+}

sensor (Syt7). With the arrival of Ca^{2+} trigger, Syt antagonizes Cpx2 action on half-zippered SNARE complexes leading to full zippering of the proteins and thus to full fusion.

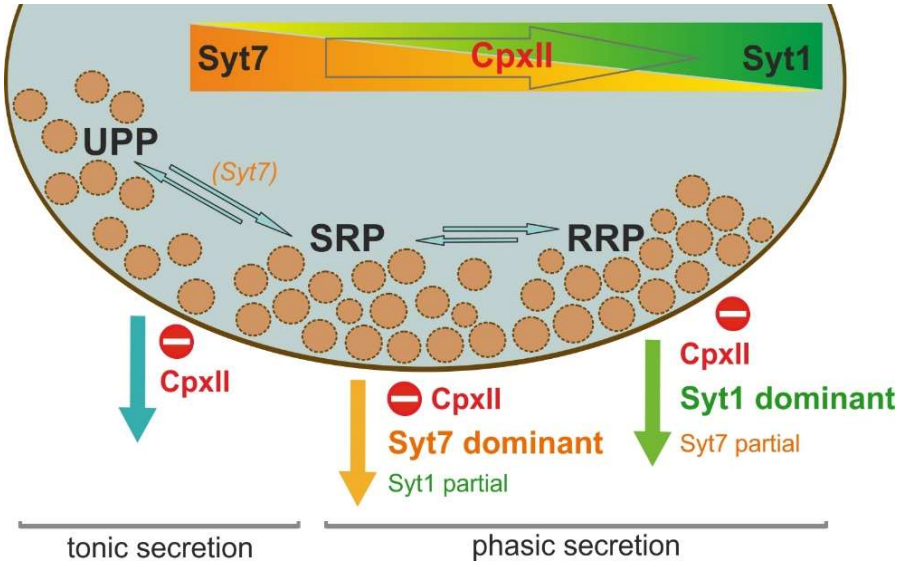


Figure 29. Hypothetical model describing vesicle maturation and Synaptotagmin - Complexin interplay. Abbreviations: UPP, unprimed pool; SRP, slowly releasable pool; RRP, readily releasable pool. Syt7 serves as a priming agent at earlier stages of vesicle maturation as well as Ca^{2+} sensor at submicromolar Ca^{2+} concentrations. The clamp function of Cpx2 allows vesicles to progressively attain release competence. As the vesicle matures, its release becomes more and more Syt1 mediated leading to rapid release upon Ca^{2+} entry. Loss of Syt1 impairs the RRP pathway and the loss of Syt7 disables the replenishing of vesicles from UPP through SRP.

5.4.4 Outlook

Despite a wealth of information on the mechanism of neurotransmitter release, several questions still remain open about the mechanism of SNARE regulators (Cpx and Syt). Future experiments addressing the interactions between the N-terminal domain of Cpx2 and Syt1 and/or Syt7 will be helpful in elucidating the role of Cpx and Syt in governing regulated exocytosis. Functional consequences of mutations in the N-terminal amino acids of Cpx2 as well as the Ca^{2+} -binding domain of Syt1 and Syt7 could be verified. The role of Syt7 in regulating fusion pore dynamics using amperometry can be employed. Analysis of sequence and structural differences between Syt1 and Syt7 can be instrumental in understanding the molecular mechanism behind the triggering functions of Ca^{2+} sensors both in neurons and neuroendocrine cells. Chimera experiments that modulate the Ca^{2+} affinities or the membrane binding properties of Cpx and Syt may also provide substantial information regarding the structure-function relationships of these Ca^{2+} sensors. Structural studies, imaging techniques and electrophysiological studies will continue to be essential to test the emerging models and to better understand the mechanism behind the release machinery.

6 Conclusions

Complexin and Synaptotagmin are known to regulate SNARE mediated exocytosis, but their precise mode of action and how they interact with each other has remained unclear. While the C-terminus of Cpx2 is seen to clamp premature secretion and consequently boosts exocytosis, the N-terminal domain of Cpx2 is responsible for speeding up of exocytosis. This dissertation narrows down the territory of relevant amino acids for clamp action of Cpx to the very C-terminus (last 34 amino acids) which comprises two important regions. The glutamate cluster (aa 108-114) is expendable for clamping premature exocytosis but is important for maintaining the integrity of the readily releasable pool of vesicles. The amphipathic motif (aa 116-134) is critical for clamping premature exocytosis, most likely by participating in protein-protein interactions. Given the intramolecular distance between the amphipathic helix and the SNARE motif, it is possible that the very C-terminus flips over to interfere with the assembling SNARE complex. By this it prevents full zippering of SNARE motifs and thus hinders fusion of vesicles. Furthermore, an intact SNARE binding α-helix (aa 42-71) and the upstream domains are crucial for maintaining proper functions of Cpx. Our experiments also illustrate that Syt7 acts a Ca^{2+} sensor at submicromolar Ca^{2+} concentrations. Consistent with the proposed clamp action of Cpx2, the protein hinders tonic secretion independently of both Syt1 and Syt7. The double deficiency of Cpx2 and Syt7 leads to additive effects in contrast to the non-additive effects observed with the Cpx2/Syt1 dko. This indicates that Cpx2 affects Syt1 and Syt7 dependent synchronous secretion differentially. It seems to cooperate with Syt1 in the fast release pathway but not with Syt7, which mediates the slow release of vesicles.

7 References

1. Alabi AA, Tsien RW (2013) Perspectives on kiss-and-run: role in exocytosis, endocytosis, and neurotransmission. *Annual review of physiology* 75: 393-422.
2. An S, Zenisek D (2004) Regulation of exocytosis in neurons and neuroendocrine cells. *Curr Opin Neurobiol* 14: 522-530.
3. Archer DA, Graham ME, Burgoyne RD (2002) Complexin regulates the closure of the fusion pore during regulated vesicle exocytosis. *The Journal of biological chemistry* 277: 18249-18252.
4. Ashery U, Betz A, Xu T, Brose N, Rettig J (1999) An efficient method for infection of adrenal chromaffin cells using the Semliki Forest virus gene expression system. *European journal of cell biology* 78: 525-532.
5. Aunis, D. (1998) Exocytosis in chromaffin cells of the adrenal medulla. *Int Rev Cytol* 181, 213–320.
6. Bacaj T, Wu D, Yang X, Morishita W, Zhou P, Xu W, Malenka RC, Südhof TC (2013) Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release. *Neuron* 80: 947-959.
7. Bai J, Wang CT, Richards DA, Jackson MB, Chapman ER (2004b) Fusion pore dynamics are regulated by synaptotagmin·t-SNARE interactions. *Neuron* **41**: 929-942.
8. Banerjee A, 1996 Banerjee A, Barry VA, DasGupta BR, Martin TF (1996) N-Ethylmaleimide-sensitive factor acts at a prefusion ATP-dependent step in Ca^{2+}-activated exocytosis. *The Journal of biological chemistry* 271: 20223-20226.
9. Becherer U, Rettig J (2006) Vesicle pools, docking, priming and release. *Cell Tissue Res* 326:393–407.
10. Bennett MK, Calakos N, Scheller RH (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* 257: 255-259.
11. Bhalla A., Tucker W.C., and Chapman E.R. 2005. Synaptotagmin isoforms couple distinct ranges of Ca^{2+}, Ba^{2+}, and Sr^{2+} concentration to SNARE-mediated membrane fusion. *Mol. Biol. Cell.*16:4755–4764. 10.1091.
12. Bittner, M.A., and Holz, R.W. (1992). Kinetic analysis of secretion from permeabilized adrenal chromaffin cells reveals distinct components. *J. Biol. Chem.*267, 16219-16225.
13. Bock JB, Matern HT, Peden AA, Scheller RH (2001) Feb 15 A genomic perspective on membrane compartment organization. *Nature.* 409 (6822): 839-41.
14. Borisovska M, Zhao Y, Tsytsyura Y, Glyvuk N, Takamori S, Matti U, Rettig J, Sudhof T,

Bruns D (2005) v-SNAREs control exocytosis of vesicles from priming to fusion. *The EMBO journal* 24: 2114-2126.

15. Borst JGG, Sakmann B. Calcium influx and transmitter release in a fast CNS synapse. *Nature.* 1996; 383:431–434.
16. Bowen ME, Weninger K, Ernst J, Chu S, Brunger AT (2005) Single-molecule studies of synaptotagmin and complexin binding to the SNARE complex. *Biophysical journal* 89: 690-702.
17. Bracher A, Kadlec J, Betz H, Weissenhorn W (2002) X-ray structure of a neuronal complexin-SNARE complex from squid. *The Journal of biological chemistry* 277: 26517-26523.
18. Brose N, Petrenko AG, Sudhof TC, Jahn R (1992) Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science* 256: 1021-1025.
19. Brose N. Altered complexin expression in psychiatric and neurological disorders: cause or consequence? *Mol Cells* 2008; **25**:7–19.
20. Brose, N. (2008). "For better or for worse: complexins regulate SNARE function and vesicle fusion." *Traffic* 9 (9): 1403- 1043.
21. Brunger AT. (2001) Structure of proteins involved in synaptic vesicle fusion in neurons. *Annu. Rev. Biophys. Biomol. Struct.*30, 157–171.
22. Bruns D, Jahn R (2002) Molecular determinants of exocytosis. Pflugers Arch: Eur J Physiol 443:333–338.
23. Buhl LK, Jorquera RA, Akbergenova Y, Huntwork-Rodriguez S, Volfson D, Littleton JT (2013) Differential regulation of evoked and spontaneous neurotransmitter release by C-terminal modifications of complexin. *Molecular and cellular neurosciences* 52: 161-172.
24. Burgess TL, Kelly RB (1987) Constitutive and regulated secretion of proteins. *Annual review of cell biology* 3: 243-293.
25. Burgoyne R.D., Cheek T.R., (1987) Reorganisation of peripheral actin filaments as a prelude to exocytosis. *Biosci. Rep.* 7, 281-288.
26. Burgoyne R.D., Morgan A. (1998) Analysis of regulated exocytosis in adrenal chromaffin cells: insights into NSF/SNAP/SNARE function. *BioEssays;*20: 328–335.
27. Burgoyne. R.D., Morgan A. (1995) Ca^{2+} and secretory-vesicle dynamics *Trends in Neurosciences,* 18 pp. 191-196.
28. Bykhovskaia M, Jagota A, Gonzalez A, Vasin A, Littleton JT (2013) Interaction of the complexin accessory helix with the C-terminus of the SNARE complex: molecular-

dynamics model of the fusion clamp. *Biophysical journal* 105: 679-690.

29. Cai H, Reim K, Varoqueaux F, Tapechum S, Hill K, Sorensen JB, Brose N, Chow RH (2008) Complexin II plays a positive role in Ca^{2+}-triggered exocytosis by facilitating vesicle priming. *Proceedings of the National Academy of Sciences of the United States of America* 105: 19538-19543.
30. Cannon WB (1929) Organization for Physiological Homeostasis. *Physiol Rev* 9: 399–431.
31. Cao P, Yang X, Südhof TC (2013) Complexin activates exocytosis of distinct secretory vesicles controlled by different synaptotagmins. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 33: 1714-1727.
32. Cao P., Maximov A., Südhof T. C. (2011). Activity-dependent IGF-1 exocytosis is controlled by the Ca^{2+}-sensor synaptotagmin-10. *Cell* 145, 300–311.
33. Chakrabarti S, Kobayashi KS, Flavell RA, Marks CB, Miyake K, Liston DR, Fowler KT, Gorelick FS, Andrews NW (2003) Impaired membrane resealing and autoimmune myositis in synaptotagmin VII-deficient mice. *The Journal of cell biology* 162: 543-549.
34. Chapman ER (2008) How does synaptotagmin trigger neurotransmitter release? *Annu Rev Biochem* 77:615–641.
35. Chapman ER, Davis AF (1998) Direct interaction of a Ca^{2+}-binding loop of synaptotagmin with lipid bilayers. *The Journal of biological chemistry* 273: 13995-14001
36. Chapman ER, Hanson PI, An S, Jahn R (1995) Ca^{2+} regulates the interaction between synaptotagmin and syntaxin 1. *The Journal of biological chemistry* 270: 23667-23671.
37. Chapman, E. R. (2002). Synaptotagmin: a Ca^{2+} sensor that triggers exocytosis? *Nat. Rev. Mol. Cell Biol.* 3, 1-11.
38. Chen P, Gillis K.D (2000) The noise of membrane capacitance measurements in the whole-cell recording configuration *Biophys. J.,* 79, pp. 2162-2170.
39. Chen X, Tomchick DR, Kovrigin E, Arac D, Machius M, Südhof TC, Rizo J (2002) Three-dimensional structure of the complexin/SNARE complex. *Neuron* 33:397–409.
40. Chen YA, Scheller RH (2001) SNARE-mediated membrane fusion. *Nature reviews Molecular cell biology* 2: 98-106.
41. Chernomordik LV, Melikyan GB, Chizmadzhev YA. Biomembrane fusion: a new concept derived from model studies using two interacting planar lipid bilayers. *Biochim Biophys Acta.* 1987;906: 309–352.
42. Chernomordik, L.V., and M.M. Kozlov. (2003) Protein-lipid interplay in fusion and fission

of biological membranes*. Annu. Rev. Biochem.* 72:175–207.

43. Chicka MC, Chapman ER (2009) Concurrent binding of complexin and synaptotagmin to liposome-embedded SNARE complexes. *Biochemistry* 48:657–659.
44. Chieregatti E, Witkin JW, Baldini G. SNAP-25 and synaptotagmin 1 function in Ca2 + -dependent reversible docking of granules to the plasma membrane. *Traffic.* 2002;3:496–511.
45. Cho RW, Song Y, Littleton JT (2010) Comparative analysis of Drosophila and mammalian complexins as fusion clamps and facilitators of neurotransmitter release. *Molecular and cellular neurosciences* 45: 389-397.
46. Cole KS (1968) Membrane watching. *The Journal of general physiology* 51: Suppl:1S-7S
47. Craxton M (2007). *BMC Genomics.* 8:259.
48. Dai H, Shen N, Arac D, Rizo J (2007) A quaternary SNARE-synaptotagmin-Ca^{2+}-phospholipid complex in neurotransmitter release. *J Mol Biol* 367:848–863.
49. Davletov BA, Südhof TC 1993. A single C2-domain from synaptotagmin I is sufficient for high affinity $Ca^{2+/-}$phospholipid-binding. *J Biol Chem*268: 26386–26390.
50. de Wit H, Cornelisse LN, Toonen RF, Verhage M (2006) Docking of secretory vesicles is syntaxin dependent. *PloS one* 1: e126.
51. Dhara M, Yarzagaray A, Schwarz Y, Dutta S, Grabner C, Moghadam PK, Bost A, Schirra C, Rettig J, Reim K, Brose N, Mohrmann R, Bruns D (2014) Complexin synchronizes primed vesicle exocytosis and regulates fusion pore dynamics. *J Cell Biol* 204:1123–1140.
52. DiAntonio A, Parfitt KD, Schwarz TL (1993) Synaptic transmission persists in synaptotagmin mutants of Drosophila. *Cell* 73: 1281-1290.
53. Diao J, Cipriano DJ, Zhao M, Zhang Y, Shah S, Padolina MS, Pfuetzner RA, Brunger AT (2013) Complexin-1 enhances the on-rate of vesicle docking via simultaneous SNARE and membrane interactions. *Journal of the American Chemical Society* 135: 15274-15277.
54. Dittman J.S, Regehr W.G. (1998) Calcium-dependence and recovery kinetics of presynaptic depression at the climbing fiber to Purkinje cell synapse *J. Neurosci.,* 18, pp. 6147-6162.
55. Eberhard, D.A., C.L. Cooper, M.G. Low, and R.W. Holz. (1990) Evidence that the inositol phospholipids are necessary for exocytosis. Loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal

of ATP. *Biochem.* J.268: 15–25.

56. Feldberg W and Mintz B. (1932) Die wirkung von azetylcholin auf die nebennieren. *Arch Exp Path Pharmak* 168: 287-291.
57. Fenwick EM, Marty A, Neher E. Sodium and calcium channels in bovine chromaffin cells. *J Physiol. 1982*; 331: 599–635.
58. Fernandez I, Arac D, Ubach J, Gerber SH, Shin O, Gao Y, Anderson RG, Südhof TC, Rizo J (2001) Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine. *Neuron* 32: 1057-1069.
59. Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Sudhof TC (2001) Synaptotagmin I functions as a calcium regulator of release probability. *Nature* 410: 41-49
60. Gao Y, Zorman S, Gundersen G, Xi Z, Ma L, Sirinakis G, Rothman JE, Zhang Y. 2012. Single reconstituted neuronal SNARE complexes zipper in three distinct stages. *Science* 337:1340–1343.
61. García AG, García-De-Diego AM, Gandía L, Borges R, García-Sancho J (2006) Calcium signaling and exocytosis in adrenal chromaffin cells. *Physiol Rev* 86: 1093–1131.
62. Gauthier, B.R., D.L. Duhamel, M. Iezzi, S. Theander, F. Saltel, M. Fukuda, B. Wehrle-Haller, and C.B. Wollheim. 2008. Synaptotagmin VII splice variants alpha, beta, and delta are expressed in pancreatic beta-cells and regulate insulin exocytosis. *FASEB J.* 22:194–206.
63. Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Südhof TC (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. *Cell* 79: 717–727.
64. Gerber, S. H., and Südhof, T. C., 2002. Molecular determinants of regulated exocytosis. *Diabetes* v. 51 Suppl 1 p. S3-11.
65. Gillis, K.D. (2000). Admittance-based measurement of membrane capacitance using the EPC-9 patch-clamp amplifier. *Pflugers Arch439*, 655-664.
66. Giraudo CG, Eng WS, Melia TJ, Rothman JE (2006) A clamping mechanism involved in SNARE-dependent exocytosis. *Science* 313: 676–680.
67. Giraudo CG, Garcia-Diaz A, Eng WS, Chen Y, Hendrickson WA, Melia TJ, Rothman JE (2009) Alternative zippering as an on-off switch for SNARE-mediated fusion. *Science* 323: 512-516.
68. Giraudo CG, Garcia-Diaz A, Eng WS, Yamamoto A, Melia TJ, Rothman JE (2008)

Distinct domains of complexins bind SNARE complexes and clamp fusion in vitro. *The Journal of biological chemistry* 283: 21211-21219.

69. Gong J, Lai Y, Li X, Wang M, Leitz J, Hu Y, Zhang Y, Choi UB, Cipriano D, Pfuetzner RA, Su¨ dhof TC, Yang X, Brunger AT, Diao J. 2016. C-terminal domain of mammalian complexin-1 localizes to highly curved membranes. *PNAS* 113:E7590–E7599.
70. Gray EG. Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J Anat. 1959*; 93(Pt 4):420–433.
71. Gustavsson, N.; Wei, S.H.; Hoang, D.N.; Lao, Y.; Zhang, Q.; Radda, G.K.; Rorsman, P.; Südhof, T.C. and Han, W. (2009). Synaptotagmin-7 is a principal Ca2+ sensor for Ca^{2+}-induced glucagons exocytosis in pancreas. *J Physiol*587, 1169 - 1178.
72. Guzman, R.E., Y.N. Schwarz, J. Rettig, and D. Bruns. 2010. SNARE force synchronizes synaptic vesicle fusion and controls the kinetics of quantal synaptic transmission. *J. Neurosci.* 30:10272–10281.
73. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Archiv: European journal of physiology* 391: 85-100.
74. Hanson PI, Roth R, Morisaki H, Jahn R, Heuser JE (1997 Aug 8) Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell.* 90(3): 523-35.
75. Hobson RJ, Liu Q, Watanabe S, Jorgensen EM (2011) Complexin maintains vesicles in the primed state in C. elegans. *Current biology: CB* 21: 106-113.
76. Hoffman BB, Taylor P. (2001) Neurotransmission. the autonomic and somatic motor nervous system. In: Hardman JG, Limbird LE, Gilman AG, eds. Goodman & Gilman's The Pharmacological Basis of Therapeutics. 10^{th} ed. New York: McGraw-Hill; 115-154
77. Hu K, Carroll J, Rickman C, Davletov B (2002) Action of complexin on SNARE complex. *J Biol Chem* 277: 41652–41656.
78. Hui, E., Bai, J., Wang, P., Sugimori, M., Llinas, R.R., and Chapman, E.R. (2005). Three distinct kinetic groupings of the synaptotagmin family: Candidate sensors for rapid and delayed exocytosis. *Proc. Natl. Acad. Sci.* USA 102, 5210–5214.
79. Huntwork S, Littleton JT (2007) A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth. *Nature neuroscience* 10: 1235-1237.
80. Ishizuka T, Saisu H, Odani S, Abe T (1995) Synaphin: a protein associated with the docking/fusion complex in presynaptic terminals. *Biochemical and biophysical research*

communications 213: 1107-1114.

81. Iyer J, Wahlmark CJ, Kuser-Ahnert GA, Kawasaki F (2013) Molecular mechanisms of COMPLEXIN fusion clamp function in synaptic exocytosis revealed in a new Drosophila mutant. *Molecular and cellular neurosciences* 56: 244-254.
82. Jahn R, Lang T, Sudhof TC (2003) Membrane fusion. *Cell* 112: 519-533.
83. Jahn R, Scheller RH (2006) SNAREs--engines for membrane fusion. *Nature reviews Molecular cell biology* 7: 631-643.
84. Jahn, R., T. Lang, and T.C. Südhof. 2003. Membrane fusion. *Cell.* 112:519–533.
85. Jahn, R. and Fasshauer, D. (2012). Molecular machines governing exocytosis of synaptic vesicles. *Nature* 490, 201–207.
86. Jockusch WJ, Speidel D, Sigler A, Sorensen JB, Varoqueaux F, Rhee JS, Brose N (2007) CAPS-1 and CAPS-2 are essential synaptic vesicle priming proteins. *Cell* 131: 796–808.
87. Jorquera RA, Huntwork-Rodriguez S, Akbergenova Y, Cho RW, Littleton JT (2012) Complexin controls spontaneous and evoked neurotransmitter release by regulating the timing and properties of synaptotagmin activity. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 32: 18234-18245.
88. Kaeser-Woo YJ, Yang X, Südhof TC (2012) C-terminal complexin sequence is selectively required for clamping and priming but not for Ca^{2+} triggering of synaptic exocytosis. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 32: 2877-2885.
89. Kavalali ET. SNARE interactions in membrane trafficking: a perspective from mammalian central synapses. *Bioessays.* 2002; 24(10):926–936.
90. Kesavan J, Borisovska M, Bruns D (2007) *Cell,* 131, pp. 351-363.
91. Kirshner N., and Kirshner A.G., (1971) Chromogranin A, dopamine-β-hydroxylase and secretion from the adrenal medulla, *Phil. Trans. Roy. Soc. Lond. Ser.* B, 261: pp. 279-289.
92. Kloepper TH, Kienle CN, Fasshauer D (2007) An elaborate classification of SNARE proteins sheds light on the conservation of the eukaryotic endomembrane system. *Mol Biol Cell* 18:3463–3471.
93. Kobayashi S. (1977) Adrenal medulla: chromaffin cells as paraneurons. *Arch Histol Jpn.*;40 (Suppl): 61–79.

94. Koh TW, Bellen HJ (2003) Synaptotagmin I, a Ca^{2+} sensor for neurotransmitter release. *Trends Neurosci* 26:413-422.
95. Kozlovsky, Yonathan & V Chernomordik, Leonid & Kozlov, Michael. (2002). Lipid Intermediates in Membrane Fusion: Formation, Structure, and Decay of Hemifusion Diaphragm. *Biophysical journal.* 83. 2634-51. 10.1016/S0006-3495(02)75274-0.
96. Krishnakumar SS, Radoff DT, Kummel D, Giraudo CG, Li F, Khandan L, Baguley SW, Coleman J, Reinisch KM, Pincet F, Rothman JE (2011) A conformational switch in complexin is required for synaptotagmin to trigger synaptic fusion. *Nature structural & molecular biology* 18: 934-940.
97. Kummel D, Krishnakumar SS, Radoff DT, Li F, Giraudo CG, Pincet F, Rothman JE, Reinisch KM (2011) Complexin cross-links prefusion SNAREs into a zigzag array. *Nature structural & molecular biology* 18: 927-933.
98. Kusano K, Landau E.M. (1975) Depression and recovery of transmission at the squid giant synapse *J. Physiol.,* 245, pp. 13-31.
99. Li C, Davletov BA, Südhof TC (1995a) Distinct Ca^{2+} and Sr^{2+} binding properties of synaptotagmins. Definition of candidate Ca^{2+} sensors for the fast and slow components of neurotransmitter release. *The Journal of biological chemistry* 270: 24898-24902.
100. Li C, Ullrich B, Zhang JZ, Anderson RG, Brose N, Südhof TC (1995b) Ca(2+)-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature* 375: 594-599.
101. Li, F., Pincet, F., Perez, E., Eng, W. S., Melia, T. J., Rothman, J. E., et al. (2007). Energetics and dynamics of SNAREpin folding across lipid bilayers. *Nat. Struct. Mol. Biol.* 14, 890–896.
102. Li, Y., P. Wang, J. Xu, F. Gorelick, H. Yamazaki, N. Andrews and G.V. Desir. (2007) Regulation of insulin secretion and GLUT4 trafficking by the calcium sensor synaptotagmin VII. *Biochem. Biophys. Res. Commun.* 362:658–664.
103. Liang K., Wei L., Chen L. (2017). Exocytosis, endocytosis, and their coupling in excitable cells. *Front. Mol. Neurosci.*10:109.
104. Lin MY, Rohan JG, Cai H, Reim K, Ko CP, Chow RH (2013) Complexin facilitates exocytosis and synchronizes vesicle release in two secretory model systems. *The Journal of physiology* 591: 2463-2473.
105. Lindau M, Neher E (1988) Patch-clamp techniques for time-resolved capacitance measurements in single cells. *Pflugers Archiv: European journal of physiology* 411: 137-

146.

106. Littleton JT, Bai J, Vyas B, Desai R, Baltus AE, Garment MB (2001) Synaptotagmin mutants reveal essential functions for the C2B domain in Ca^{2+} -triggered fusion and recycling of synaptic vesicles in vivo. *J Neurosci.*21:1421–33.

107. Littleton JT, Stern M, Schulze K, Perin M, Bellen HJ (1993) Mutational analysis of Drosophila synaptotagmin demonstrates its essential role in Ca(2+)-activated neurotransmitter release. *Cell* 74: 1125-1134.

108. Liu H, Dean C, Arthur CP, Dong M, Chapman ER. 2009. Autapses and networks of hippocampal neurons exhibit distinct synaptic transmission phenotypes in the absence of synaptotagmin I. *The Journal of Neuroscience* 29:7395–7403. 10.1523.

109. Liu H., Bai H., Hui E., Yang L., Evans C.S., Wang Z., Kwon S.E., and Chapman E.R. 2014. Synaptotagmin 7 functions as a Ca^{2+}-sensor for synaptic vesicle replenishment. *eLife.* 3: e01524 10.7554.

110. Lu B, Song S, Shin YK. (2010) Accessory alpha-helix of complexin I can displace VAMP2 locally in the complexin-SNARE quaternary complex. *Journal of Molecular Biology.*396: 602–609.

111. Luo, F., and Südhof, T.C. (2017). *Neuron* 94, 826–839.

112. Mackler JM, Drummond JA, Loewen CA, Robinson IM, Reist NE (2002 Jul 18) The C(2)B $Ca^{(2+)}$-binding motif of synaptotagmin is required for synaptic transmission in vivo. *Nature.* 418(6895): 340-4.

113. Makke, M., Mantero Martinez, M., Gaya, S., Schwarz, Y., Frisch, W., Silva-Bermudez, L., Jung, M., Mohrmann, R., Dhara, M., and Bruns, D. (2018) A mechanism for exocytotic arrest by the Complexin C-terminus. *Elife 7.*

114. Malsam J, Parisotto D, Bharat TA, Scheutzow A, Krause JM, Briggs JA, Sollner TH (2012) Complexin arrests a pool of docked vesicles for fast Ca^{2+} -dependent release. *EMBO J* 31:3270–3281.

115. Malsam J, Seiler F, Schollmeier Y, Rusu P, Krause JM, Sollner TH (2009) The carboxy-terminal domain of complexin I stimulates liposome fusion. *Proceedings of the National Academy of Sciences of the United States of America* 106: 2001-2006.

116. Martin JA, Hu Z, Fenz KM, Fernandez J, Dittman JS (2011) Complexin has opposite effects on two modes of synaptic vesicle fusion. *Current biology: CB* 21: 97-105.

117. Martin TF, Kowalchyk JA (1997) Docked secretory vesicles undergo Ca^{2+}-

activated exocytosis in a cell-free system. *The Journal of biological chemistry* 272: 14447-14453.

118. Maximov A, Südhof TC (2005) Autonomous function of synaptotagmin 1 in triggering synchronous release independent of asynchronous release. *Neuron* 48: 547-554.

119. Maximov A, Tang J, Yang X, Pang ZP, Südhof TC (2009) Complexin controls the force transfer from SNARE complexes to membranes in fusion. *Science* 323: 516-521.

120. McMahon HT, Missler M, Li C, Südhof TC (1995) Complexins: cytosolic proteins that regulate SNAP receptor function. *Cell* 83: 111-119.

121. Meinrenken CJ, Borst JG, Sakmann B 2003. Local routes revisited: The space and time dependence of the Ca^{2+} signal for phasic transmitter release at the rat calyx of Held. *J Physiol* 547: 665–689.

122. Mohrmann, R., Dhara, M., and Bruns, D. (2015). Complexins: small but capable. *Cell. Mol. Life Sci.* 72, 4221–4235.

123. Nagy G, Reim K, Matti U, Brose N, Binz T, Rettig J, Neher E, Sørensen JB (2004) Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. *Neuron* 41: *417*-429.

124. Nagy, G.; Kim, J.H.; Pang, Z.P.; Matti, U.; Rettig, J. and Südhof, T.C. (2006). Different effects on fast exocytosis induced by synaptotagmin 1 and 2 isoforms and abundance but not by phosphorylation. *J Neurosci* 26, 632–643.

125. Neher E, Marty A (1982) Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proceedings of the National Academy of Sciences of the United States of America* 79: 6712-6716.

126. Neher E, Sakaba T. (2008) Multiple roles of calcium ions in the regulation of neurotransmitter release. *Neuron.* 59: 861–872.

127. Neher E, Sakmann B (1976) Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* 260: 799-802.

128. Nishiki T, Augustine GJ. 2004. Synaptotagmin I synchronizes transmitter release in mouse hippocampal neurons. *The Journal of Neuroscience* 24:6127–6132. 10.1523.

129. Nonet ML, Grundahl K, Meyer BJ, Rand JB (1993) Synaptic function is impaired but not eliminated in C. elegans mutants lacking synaptotagmin. *Cell* 73: 1291-1305.

130. Pabst S, Hazzard JW, Antonin W, Südhof TC, Jahn R, Rizo J, Fasshauer D

(2000) Selective interaction of complexin with the neuronal SNARE complex. Determination of the binding regions. *The Journal of biological chemistry* 275: 19808-19818.

131. Pabst S, Margittai M, Vainius D, Langen R, Jahn R, Fasshauer D (2002) Rapid and selective binding to the synaptic SNARE complex suggests a modulatory role of complexins in neuroexocytosis. *The Journal of biological chemistry* 277: 7838-7848.

132. Pang ZP, Shin OH, Meyer AC, Rosenmund C, Südhof TC (2006 Nov 29) A gain-of-function mutation in synaptotagmin-1 reveals a critical role of Ca^{2+}-dependent soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex binding in synaptic exocytosis. *J Neurosci.* 26(48):12556-65.

133. Pang ZP, Südhof TC (2010) Cell biology of Ca^{2+}-triggered exocytosis. *Current opinion in cell biology* 22: 496-505.

134. Pang ZP, Sun J, Rizo J, Maximov A, Südhof TC (2006) Genetic analysis of synaptotagmin 2 in spontaneous and Ca^{2+} – triggered neurotransmitter release. *EMBO J* 25: 2039–2050.

135. Perin MS, Fried VA, Mignery GA, Jahn R, Südhof TC (1990) Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. *Nature* 345: 260-263.

136. Pfeffer S. R. (1999). Transport-vesicle targeting: tethers before SNAREs. *Nat. Cell Biol.1*, E17–E22. 10.1038/8967.

137. Pfeffer, S and Aivazian, D. (2004). Targeting Rab GTPases to distinct membrane compartments. *Nat. Rev. Mol. Cell. Biol.* 5, 886-896.

138. Radoff DT, Dong Y, Snead D, Bai J, Eliezer D, Dittman JS (2014) The accessory helix of complexin functions by stabilizing central helix secondary structure. *eLife* 3

139. Reim K, Mansour M, Varoqueaux F, McMahon HT, Südhof TC, Brose N, Rosenmund C (2001) Complexins regulate a late step in Ca^{2+}-dependent neurotransmitter release. *Cell* 104: 71-81.

140. Reim K, Wegmeyer H, Brandstatter JH, Xue M, Rosenmund C, Dresbach T, Hofmann K, Brose N (2005) Structurally and functionally unique complexins at retinal ribbon synapses. *J Cell Biol* 169: 669–680.

141. Rettig J, Neher E (2002) Emerging roles of presynaptic proteins in Ca^{2+}-triggered exocytosis. *Science* 298:781-785.

142. Rizo J, Südhof TC (2002) Snares and Munc18 in synaptic vesicle fusion. *Nature*

reviews Neuroscience 3: 641-653.

143. Rizo J., Chen X., Arac D. Unraveling the mechanisms of synaptotagmin and SNARE function in neurotransmitter release, *Trends Cell Biol.*, 2006, vol. 16 (pg. 339-350)

144. Rizzoli, S. O., and Jahn, R. (2007) Kiss-and-run, collapse and 'readily retrievable' vesicles. *Traffic* 8, 1137–1144.

145. Sabatini BL, Regehr WG. 1996. Timing of neurotransmission at fast synapses in the mammalian brain. *Nature* 384, 170–172.

146. Schaub JR, Lu X, Doneske B, Shin YK, McNew JA (2006) Hemifusion arrest by complexin is relieved by Ca^{2+}-synaptotagmin I. *Nature structural & molecular biology* 13: 748-750.

147. Schneggenburger R, Neher E. Presynaptic calcium and control of vesicle fusion. *Curr. Opin. Neurobiol.,* 15 (2005), pp. 266-274.

148. Schneggenburger R, Sakaba T, Neher E (2002) Vesicle pools and short-term synaptic depression: lessons from a large synapse. *Trends Neurosci* 25:206–212.

149. Schonn JS, Maximov A, Lao Y, Südhof TC, Sorensen JB (2008) Synaptotagmin-1 and -7 are functionally overlapping Ca^{2+} sensors for exocytosis in adrenal chromaffin cells. *Proceedings of the National Academy of Sciences of the United States of America* 105: 3998-4003.

150. Segovia M, Ales E, Montes MA, Bonifas I, Jemal I, Lindau M, Maximov A, Südhof TC, Alvarez de Toledo G (2010) Push-and-pull regulation of the fusion pore by synaptotagmin-7. *Proceedings of the National Academy of Sciences of the United States of America* 107: 19032-19037.

151. Seiler F, Malsam J, Krause JM, Sollner TH (2009) A role of complexin-lipid interactions in membrane fusion. *FEBS letters* 583: 2343-2348.

152. Shao X, Fernandez I, Südhof TC, Rizo J (1998) Solution structures of the Ca^{2+}-free and Ca^{2+}-bound C2A domain of synaptotagmin I: does Ca^{2+} induce a conformational change? *Biochemistry* 37: 16106-16115.

153. Shin OH, Xu J, Rizo J, Südhof TC. (2009) Differential but convergent functions of Ca^{2+} binding to synaptotagmin-1 C2 domains mediate neurotransmitter release. *PNAS*.106: 16469–16474.

154. Siksou, L., Triller, A. and Marty, S. (2009) An emerging view of presynaptic structure from electron microscopic studies. *Journal of Neurochemistry,* 108: 1336–

1342.

155. Smith C, Moser T, Xu T, Neher E (1998) Cytosolic Ca^{2+} acts by two separate pathways to modulate the supply of release-competent vesicles in chromaffin cells. *Neuron* 20: 1243-1253.

156. Smith U, Smith DS, Winkler H, Ryan JW. (1973) Exocytosis in the adrenal medulla demonstrated by freeze-etching. *Science* 179:79-82.

157. Smith, A.D. and Winkler, H. (1972) Fundamental mechanisms in the release of catecholamines. In: (ed. by) Blaschko, H and Muscholl, E: *Catecholamines, Handbook of experimental Pharmacology, Vol. 33, Springer, Berlin*, pp. 538-617.

158. Snead D, Wragg RT, Dittman JS, Eliezer D (2014) Membrane curvature sensing by the C-terminal domain of complexin. *Nature communications* 5: 4955.

159. Söllner T et al., 1993 Sollner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE (1993a) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 75: 409-418.

160. Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993b) SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362: 318-324.

161. Sorensen JB, Wiederhold K, Muller EM, Milosevic I, Nagy G, de Groot BL, Grubmuller H, Fasshauer D (2006) Sequential N- to C-terminal SNARE complex assembly drives priming and fusion of secretory vesicles. *The EMBO journal* 25: 955-966.

162. Sørensen, J.B. (2004). Formation, stabilisation and fusion of the readily releasable pool of secretory vesicles. *Eur. J. Physiol. 448*, 347–362.

163. Steyer JA, Almers W. A real-time view of life within 100 nm of the plasma membrane. *Nat Rev Mol Cell Biol.* 2001; 2:268–75.

164. Südhof T.C. Synaptotagmins: Why So Many? J. Biol. Chem. 2002; 277: 7629–7632.

165. Südhof TC (2004) The synaptic vesicle cycle. *Annual review of neuroscience* 27: 509-547.

166. Südhof TC (2012) Calcium control of neurotransmitter release. *Cold Spring Harb Perspect Biol* 4: a011353.

167. Südhof TC, Rothman JE (2009) Membrane fusion: grappling with SNARE and

SM proteins. *Science* 323: 474–477.

168. Sugita S, Shin OH, Han W, Lao Y, Südhof TC (2002) Synaptotagmins form a hierarchy of exocytotic Ca(2+) sensors with distinct Ca(2+) affinities. *The EMBO journal* 21: 270-280.

169. Sun J, Pang ZP, Qin D, Fahim AT, Adachi R, Südhof TC (2007) A dual-Ca^{2+}-sensor model for neurotransmitter release in a central synapse. *Nature* 450: 676-682

170. Tang J, Maximov A, Shin OH, Dai H, Rizo J, Südhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. *Cell* 126: 1175-1187.

171. Taraska, J.W., Perrais, D., Ohara-Imaizumi, M., Nagamatsu, S., and Almers, W. (2003). Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. *Proc. Natl. Acad. Sci. USA* 100, 2070–2075.

172. Tokumaru H, Shimizu-Okabe C, Abe T (2008) Direct interaction of SNARE complex binding protein synaphin/complexin with calcium sensor synaptotagmin 1. *Brain cell biology* 36: 173-189.

173. Trimbuch T, Xu J, Flaherty D, Tomchick DR, Rizo J, Rosenmund C (2014) Re-examining how complexin inhibits neurotransmitter release. *eLife* 3: e02391.

174. Tsuboi. T, McMahon H.T, Rutter G.A. (2004) Mechanisms of dense core vesicle recapture following kiss and run (cavicapture) exocytosis in insulin-secreting cells. *J. Biol. Chem.,* 279 (45), pp. 47115-47124.

175. Verhage M, Sorensen JB (2008) Vesicle docking in regulated exocytosis. *Traffic* 9: 1414-1424.

176. Voets T, Moser T, Lund PE, Chow RH, Geppert M, Südhof TC, Neher E (2001) Intracellular calcium dependence of large dense-core vesicle exocytosis in the absence of synaptotagmin I. *Proceedings of the National Academy of Sciences of the United States of America* 98: 11680-11685.

177. Voets T., Toonen R. F., Brian E. C., de Wit H., Moser T., Rettig J., Südhof T. C., Neher E., Verhage M. (2001b). Munc18-1 promotes large dense-core vesicle docking. *Neuron* 31, 581–591

178. Voets, T.; Neher, E. and Moser, T. (1999). Mechanisms underlying phasic and sustained secretion in chromaffin cells from mouse adrenal slices. *Neuron 23*, 607–615.

179. von Gersdorff, H., and Matthews, G. (1999). Electrophysiology of synaptic vesicle cycling. *Annu. Rev. Physiol.* 61, 725–752.

180. Wang P, Chicka MC, Bhalla A, Richards DA, Chapman ER (2005) Synaptotagmin VII is targeted to secretory organelles in PC12 cells, where it functions as a high-affinity calcium sensor. *Molecular and cellular biology* 25: 8693-8702.

181. Waters MG, Hughson FM (2000) Membrane tethering and fusion in the secretory and endocytic pathways. *Traffic* 1: 588-597.

182. Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. *Cell* 92: 759-772.

183. Wen H, Linhoff MW, McGinley MJ, Li GL, Corson GM, Mandel G, Brehm P (2010) Distinct roles for two synaptotagmin isoforms in synchronous and asynchronous transmitter release at zebrafish neuromuscular junction. *Proc Natl Acad Sci U S A* 107: 13906–13911.

184. Wen H, Linhoff MW, McGinley MJ, Li GL, Corson GM, Mandel G, Brehm P (2010) Distinct roles for two synaptotagmin isoforms in synchronous and asynchronous transmitter release at zebrafish neuromuscular junction. *Proceedings of the National Academy of Sciences of the United States of America* 107: 13906-13911.

185. Weninger K, Bowen ME, Choi UB, Chu S, Brunger AT (2008) Accessory proteins stabilize the acceptor complex for synaptobrevin, the 1:1 syntaxin/SNAP-25 complex. *Structure* 16: 308–320.

186. Winkler H. (1971) The membrane of the chromaffin granule*. Philos Trans R Soc Lond. B Biol Sci.* 261(839): pp. 293-303.

187. Wojcik SM, Brose N (2007) Regulation of membrane fusion in synaptic excitation-secretion coupling: speed and accuracy matter. *Neuron* 55: 11-24.

188. Wragg RT, Snead D, Dong Y, Ramlall TF, Menon I, Bai J, Eliezer D, Dittman JS (2013) Synaptic vesicles position complexin to block spontaneous fusion. *Neuron* 77: 323-334.

189. Xu J, Brewer KD, Perez-Castillejos R, Rizo J (2013) Subtle Interplay between synaptotagmin and complexin binding to the SNARE complex. *Journal of molecular biology* 425: 3461-3475.

190. Xu J, Pang ZP, Shin OH, Südhof TC (2009) Synaptotagmin-1 functions as a Ca^{2+} sensor for spontaneous release. *Nat Neurosci* 12:759–766.

191. Xu, J.; Mashimo, T. and Südhof, T.C. (2007). Synaptotagmin-1, -2, and -9: Ca2+-sensors for fast release that specify distinct presynaptic properties in subsets of

neurons. *Neuron54*, 567–581.

192. Xue M, Craig TK, Xu J, Chao HT, Rizo J, Rosenmund C (2010) Binding of the complexin N terminus to the SNARE complex potentiates synaptic-vesicle fusogenicity. *Nature structural & molecular biology* 17: 568-575.

193. Xue M, Reim K, Chen X, Chao HT, Deng H, Rizo J, Brose N, Rosenmund C (2007) Distinct domains of complexin I differentially regulate neurotransmitter release. *Nature structural & molecular biology* 14: 949-958.

194. Xue M, Stradomska A, Chen H, Brose N, Zhang W, Rosenmund C, Reim K (2008b) Complexins facilitate neurotransmitter release at excitatory and inhibitory synapses in mammalian central nervous system. *Proceedings of the National Academy of Sciences of the United States of America* 105: 7875-7880.

195. Yang X, Cao P, Südhof TC (2013) Deconstructing complexin function in activating and clamping Ca^{2+}-triggered exocytosis by comparing knockout and knockdown phenotypes. *Proceedings of the National Academy of Sciences of the United States of America* 110: 20777-20782.

196. Yang X, Kaeser-Woo YJ, Pang ZP, Xu W, Südhof TC (2010) Complexin clamps asynchronous release by blocking a secondary Ca(2+) sensor via its accessory alpha helix. *Neuron* 68: 907-920.

197. Yoon TY, Lu X, Diao J, Lee SM, Ha T, Shin YK (2008) Complexin and Ca2+ stimulate SNARE-mediated membrane fusion. *Nature structural & molecular biology* 15: 707-713.

198. Zerial M, McBride H. Rab proteins as membrane organizers. *Nature Reviews Molecular Cell Biology. 2001*; 2: 107–117.

199. Zhang X, Kim-Miller MJ, Fukuda M, Kowalchyk JA, Martin TF (2002) Ca^{2+}-dependent synaptotagmin binding to SNAP-25 is essential for Ca^{2+}-triggered exocytosis. *Neuron* 34: 599-611.

200. Zhou Q, Zhou P, Wang AL, Wu D, Zhao M, Su¨ dhof TC, Brunger AT. 2017. The primed SNARE-complexin-synaptotagmin complex for neuronal exocytosis. *Nature* 548:420–425.

8 Acknowledgements

My sincere gratitude to my advisor Prof. Dr. Dieter Bruns for his continuous guidance and support during the course of this PhD study here in Homburg (Saar), Germany. His working style, vast knowledge in neuroscience and research experience have been very inspirational. Thanks for the helpful discussions and feedback during lab meetings and data analysis, and thesis correction.

Thanks to Dr. Ralf Mohrmann whose insights, suggestions as well as help with the patch setup has greatly helped me during my research work. Dr. David Stevens for his help and support with the patch setup and feedback during progress reports and synapse clubs.

My sincere thanks to my colleague and friend, Dr. Antonio Yarzagaray. He had been a great help during my doctoral research. He taught me how to troubleshoot the problems with the patch-clamp setup, helped with calibration curves, analyzing the data and had been a great company during long recording hours, pulling pipettes and transfecting the cells for me. I cherish those philosophical discussions, trips to Paris and Saarschleife and many other fun times.

Thanks to Dr. Yvonne Schwarz who has been a great inspiration. Her expertise in science is awesome and her feedback during my experiments, lab meetings and also with the correction of my thesis is sincerely appreciated. I wish the best for all her endeavors in science.

My sincere appreciation and thanks to my colleagues, Ahmed Shabaan (Egypt), Mazen Makke (Lebanon), who have been very friendly and supportive during my experiments, data analysis and helpful discussions during lab meetings, Synapse club presentations etc. Thanks also to Dr. Madhurima Dhara.

My colleagues from the ‘neuron’ group – Barbara Schindeldecker, Ali Harb, Soumyajit Dutta, Evgenii Bogatikov made a great team to work alongside. Thank you for all your support.

Special thanks to Marina Wirth who does a great job organizing all the bureaucratic stuff as well as her direct contribution to ensure timely availability of lab animals for my experiments. Birgit Bimperling and Martina Didion for taking good care of mice in the animal house.

Thanks also to my Indian friends – Halimani Mahantappa, Vinod Babu, Praneeth Chitirala & Keerthana, Ramesh Vandanapu for great company during my time in Homburg.

I am grateful to friends from church and my family for filling my life with joy all the way and being super supportive. Truly a blessing.

Above all, thanks to my Lord and Savior Jesus Christ for all the grace He granted me to live in Germany, explore neuroscience, meet people, make friends and memorable experiences.

THANK YOU

Surya Gaya

9 Curriculum vitae

The curriculum vitae was removed from the electronic version of the doctoral thesis for reasons of data protection.

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht.