Calcium Dependent Activator Protein for Secretion 1 (CAPS1): A Structural Functional Analysis

By

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1. Abstract

The CAPS1 protein was initially discovered as a cytosolic soluble 145kDa protein which was necessary to restore calcium dependent norepinephrine secretion in cracked PC12 cells. Recent findings suggest that CAPS may also play a role in synaptic and dense core vesicle exocytosis as well as in the loading of monoamine neurotransmitters. Recently, studies have implicated CAPS1 in the binding to syntaxin-1. However, no studies have identified the key residues of CAPS1 that facilitate this interaction with syntaxin-1. I show that the binding mode of CAPS1 is independent from that of Munc13 such that CAPS1 requires the full length of syntaxin-1 to bind. Moreover, CAPS1 favors the open conformation of syntaxin-1. Interestingly, the Munc homology (MH) domain of CAPS1 is not critical for this interaction while the C-terminal dense core vesicle binding (DCVB) domain plays an important role. Moreover, truncations to this DCVB domain result in decreased binding to syntaxin-1.
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Abbreviations

(H³)NA-Tritium Labeled Noradrenaline
ATP- Adenosine Triphosphate
BDNF- Brain-derived neurotrophic factor
BSA-Bovine serum albumin
C-Control
CAG-Cytomegalovirus (CMV) early enhancer element and chicken beta-actin promoter
CAPS1-Calcium Dependent Activator Protein for Secretion 1
CHO- Chinese hamster ovary
CMV- Cytomegalovirus
DAG-Diacylglycerol
DCV-Dense core vesicle
DKD-Double Knock Down
ER-Endoplasmic Reticulum
GABA- γ-Aminobutyric acid
GDP- Guanosine diphosphate
GFP- Green fluorescent protein
GTP- Guanosine triphosphate
KD- Knock Down
MSH- Melanocyte-stimulating hormone
NE-Norepinephrine
NPY-Neuropeptide Y
NSF- N-ethylmaleimide sensitivity factor
OD600- Optical Density 600
ONPG- ortho-Nitrophenyl-β-galactoside
PC12-PheoChromocytoma 12
PIP- Phosphatidylinositol (4)-bisphosphate
PIP2- Phosphatidylinositol (4,5)-bisphosphate
PM-Plasma Membrane
PSS-Physiological Saline Solution
RIM- Rab interacting molecule
RRP-Readily releasable pool
SG2-Secretogranin 2
SNAP25-Synaptosomal-Associated Protein 25kDa
SNARE-Soluble NSF Attachment Protein Receptor
SNM-Silent nucleotide mutation
SRP-Slowly releasable pool
SV-Synaptic Vesicle
-UTL-excluding Uracil, Tryptophan and Leucine
VAMP-Vesicle Associated Membrane Protein
2. INTRODUCTION

2.1 Exocytosis

Exocytosis is the process by which an intracellular vesicle fuses its own membrane with the plasma membrane resulting in two lipid bilayers merging to become one (Söllner, 2003). This event accomplishes two functions; the addition of the vesicular membrane and its components into the plasma membrane and the release of the vesicular content to the outside outer environment (Lin and Scheller, 2000). Exocytosis may occur via two generally accepted pathways; constitutive and regulated. Constitutive exocytosis takes place in all eukaryotic cells, and occurs when vesicles fuse with the plasma membrane in the absence of any external signals (Lin and Scheller, 2000). This process is thought to aid in the maintenance of the components of the plasma membrane (lipids) as well as those of the extracellular environment (Südhof and Rothman, 2009a). On the other hand, regulated exocytosis occurs through a specialized pathway in which coordination is required (Söllner, 2003). In general, vesicles undergoing this pathway require stimuli to initiate and maintain the process. Typically an initial electrical stimulus (action potential) is converted to a chemical signal upon vesicular release leading to receptor recognition (Söllner, 2003). Regulated exocytosis plays a critical role as all eukaryotic life depends on it in a variety of ways. It is involved in the release of neurotransmitters, cytokines, hormones and enzymes (Li and Chin, 2003; Lin and Scheller, 2000). This event allows for communication between cells; our nervous system which is critical for our survival, learning and memory relies on this chemical synaptic stimulation for cell to cell communication. Hence, this field of vesicular exocytosis is prominent and highly established.

2.2 Secretory Vesicles

Secretory vesicles account for the release storage and release of neurotransmitters as well as peptide hormones/neuromodulators (Sugita, 2007). These chemicals are released upon stimulation (electrical) in presynaptic neurons, (neuro)endocrine cells and other secretory cells. They function to relay and/or modulate information in post-synaptic neurons, muscles, and various other cell types. Secretory vesicles can be categorized into two distinct groups; synaptic vesicles (SV) and dense core vesicles (DCVs) (Sugita, 2007). Synaptic vesicles contain and release “classical” neurotransmitters such as glycine, glutamate and gamma-Aminobutyric acid (GABA) (Sugita, 2007). These vesicles are about 40nm in size and are localized at active zones in the presynaptic terminal (Schoch and Gundelfinger, 2006). On the other hand, DCVs are about 300nm and are synthesized in the cell body (Schoch and Gundelfinger, 2006). Moreover, these vesicles contain neuromodulators such as
peptides, neutrophins and biogenic amines which are released slowly (Sugita, 2007). For example, catecholamines such as norepinephrine are released from DCVs in adrenal chromaffin cells and sympathetic ganglion neurons as part of the fight-or-flight response resulting in increased heart rate and blood pressure. Likewise, DCVs in the pancreas are critical for insulin release, thus playing an important role in glucose metabolism and diabetes. Hence, it is of utmost importance to better understand the mechanisms behind secretory vesicle release as it is critical for our well being and existence.

2.3 Discovery and Function of SNARE Proteins in Exocytosis

The main players in membrane fusion/exocytosis are the SNARE family of proteins (Figure 1). These proteins were discovered from the purification of cytosolic proteins that were observed to promote vesicular fusion during Golgi transport. SNARE proteins are categorized by a ~60 amino-acid α-helical coiled-coil domain called the SNARE motif which is likely to provide the energy needed to cause membrane fusion (Parpura and Mohideen, 2008). These neuronal SNARE proteins were identified to be SNAP25, synaptobrevin2 (VAPM2) and syntaxin-1 (Sollner et al., 1993). VAMP-2 (Vesicle Associated Membrane Protein) was originally found on synaptic vesicles and thus termed vesicle SNARE (V-SNARE) (Parpura and Mohideen, 2008). On the other hand syntaxin-1 and SNAP25 are found on the target membrane, and thus termed target SNAREs (T-SNAREs) (Parpura and Mohideen, 2008). The SNARE complex consists of these proteins in a 1:1:1 stoichiometric ratio consisting of four SNARE domains one from syntaxin-1 and VAMP and two from SNAP 25 (Parpura and Mohideen, 2008). They form a tight four helix bundle which overcomes the energy barrier for lipid bilayer fusion by “zipping” from their N to the C terminus. The SNARE proteins are the targets of clostridial neurotoxins, as it was shown that the application of tetanus toxin and botulinum neurotoxin B, D, F, and G cleaved VAMP-2. SNAP 25 is a target for botulinum toxin A and E, while Syntaxin-1 serves as the target for botulinum toxin C (Blasi et al., 1993; Schiavo et al., 1992; Sugita, 2007). DCV secretion was shown to be blocked with the application of the active subunit of botulinum toxin C or E in chromaffin cells. (Xu et al., 1998). Furthermore, in vitro fusion assays such as lipid mixing assays or content mixing assays demonstrate that the SNAREs are indeed necessary and sufficient for fusion (Bock and Scheller, 1999; Schuette et al., 2004).
2.4 Secretory Vesicle Cycle

The synaptic vesicle cycle (mainly focusing on DCVs) can be broken down into five steps; i) Biogenesis, ii) Docking, iii) Priming, iv) Triggering, and v) Fusion (Figure 2)

i) Biogenesis

Initially some peptide hormones and neuromodulators are synthesized as larger precursor molecules in the rough endoplasmic reticulum (RER) and transported to the Golgi apparatus (Kim et al., 2006). These precursor molecules are subsequently packaged along with other necessary proteins into secretory granules in the trans-Golgi network (Kim et al., 2006). It is thought that immature DCVs form via active budding from the trans-Golgi network which then is followed by a number of maturation steps leading to a final mature DCV. Protein kinase D is thought to play a important role in the initial budding step as it facilitates the conversion of phosphatidylcholine to diacylglycerol (DAG) (Jamora et al., 1999). DAG, and other phosphotidic acids facilitate the formation of negative...
curvature in the Golgi bilayer and thus promoting to fission/budding (Siddhanta and Shields, 1998). Cholesterol is also known to play a major role since it creates tightly packed lipid microdomains known as lipid rafts which are thought to serve as the budding point in the trans Golgi network (Keller et al., 2004). Cholesterol also facilitates negative curvature along with granulogenic proteins known as granins (Kim et al., 2006). Hence, these budded vesicles are composed of high levels of cholesterol with tightly packed secretory proteins.

These immature granules must further mature in order to become functional DCVs. A critical step in the process is the acidification of the granules. This is facilitated by proteins known as V-ATPase(s) which are essentially proton pumps serving to acidify the intracellular compartment of the vesicles to a pH of ~5.5-5.0 (Taupenot et al., 2005). Acidification is also critical for the processing of pro-hormones inside the granule itself (Blazquez et al., 2001; Molloy et al., 1994). For example, for pro-hormone convertase (PC) which converts pro-insulin to insulin to be active, acidification of the granule must occur (Kim et al., 2006). Maturation also involves the removal of lysosomal enzymes and other membrane proteins that were inadvertently packed inside the granule (Kim et al., 2006). Moreover, the proton gradient generated allows for the packaging of classical neurotransmitters by vesicular transporters such as VGluT (Vesicular Glutamate Transporter) (LiguZ-Leczna and Skangiel-Kramski, 2007). Likewise, monoamines such as epinephrine and norepinephrine use VMATs (Vesicular Monoamine Transporters) for the loading inside these vesicles (Fei et al., 2008).

ii) Docking

Generally speaking, DCVs are defined as docked if they appear to be within 50nm of the plasma membrane (Sugita, 2007). This ensures that the vesicles are anchored to the proper location in the cell. Hence, the vesicles are transferred from a depot pool (<200nm from PM) to a unprimed pool (Schoch and Gundelfinger, 2006). Through electron microscopy analysis, it was observed that approximately 30% of DCVs appear to be docked in the mouse chromaffin cell (Stevens et al., 2011).

The exact mechanism of docking is unclear, however it is thought that this process may involve a variety of proteins. One such protein is Munc18-1; the deletion of Munc18-1 in chromaffin cells resulted in a reduction in the number of DCVs docked at the PM as well as a reduction in secretion (Gulyas-Kovacs et al., 2007; Voets et al., 2001b). However, Munc18-1 deletion in neurons resulted in a loss of secretion but no localization (docking) defect was observed (Toonen et al.,

Munc18-1 is known to bind to syntaxin-1 and stabilize it in a closed conformation by which its N-terminal Habc domain is folded back on its SNARE domain thus preventing any interaction with VAMP or SNAP 25 in the formation of the SNARE complex (see below) (Dulubova et al., 1999; Hata et al., 1993b; Misura et al., 2000b). Intuitively speaking, a deletion of Munc18 should increase secretion since there would be less closed syntaxin-1, however, the opposite has been observed. This is probably due to the fact that Munc18 also interacts with other proteins such as Mint (Munc 18 interacting) protein and DOC2 (double C2 like domain containing protein) (Smyth et al., 2010). The Mint protein is hypothesized to form an intermediate complex with Munc18 and syntaxin-1 while DOC2 may serve as a calcium sensor during this process. Therefore, Munc18 may play an important role in the docking of DCVs however; syntaxin-1 itself may also play a role.

Botulinum toxin C1 which cleaves syntaxin-1a,1b, 2 and 3 was shown to strongly reduce docking the of DCVs in chromaffin cells (de Wit et al., 2006). In addition, a large protein complex known as the exocyst complex may play a role in the process. This complex consists of sec3,5,6,8,10 and 15 was well as exo70 and exo 84 proteins (Li and Chin, 2003). The exocyst complex was first identified in yeast and was shown to localize at fusion sites. Sec4 a vesicular protein is thought to bind to sec15 of the exocyst complex in order to mediate the initial docking of the vesicle (He and Guo, 2009). In mammalian cells, this complex was found to be highly localized in developing neurons at areas of neurite outgrowth (Li and Chin, 2003).

Moreover, the Rab family of proteins is thought to play a role in vesicle trafficking and docking. There are at least 60 Rab proteins in humans but the main one thought to be involved in regulated exocytosis is Rab3. Rab proteins are a family of GTPases which exist in a synaptic vesicle bound GTP form and a cytosolic GDP form (Grosshans et al., 2006). Rab3 deletion in mice has been shown to be involved in the replenishment of docked vesicles after excessive stimulation (Schluter et al., 2004). Additionally, Rab proteins may be involved downstream in vesicle exocytosis as Rab3a null mice exhibit an increase in paired pulse facilitation and decrease in long term potentiation (LTP) (Schluter et al., 2004). Docking may be further facilitated by other large proteins in the cell matrix of the active zone such as bassoon, piccolo and Rab interacting molecule (RIM) (Li and Chin, 2003).

iii) Priming
After docking, vesicles undergo a maturation state by which they become releasable or primed. Priming itself can be divided into an ATP dependent and independent mechanism (Sugita, 2007). In ATP dependent priming the key step is the generation of phosphatidylinositol 4,5 bisphosphate (PIP2) from phosphatidylinositol 4- phosphate (PIP) (Hay et al., 1995; Hay and Martin, 1993). This occurs via the enzyme phosphatidylinositol phosphate kinase I (PIPKI) which uses ATP in order to transfer the second phosphate group from PIP to PIP2 (Hay and Martin, 1993). The generation on PIP2 is a key step since it is thought to be the binding site of the calcium sensor synaptotagmin-1 and CAPS1 (calcium dependent activator protein for secretion 1) (Sugita, 2007). NSF (N-ethylmaleimide Sensitive Factor) protein is also thought to play a role in priming as it itself being an ATPase binds to and disrupts the cis SNARE complex formation (SNAREs located on same membrane) which in itself is inactive for exocytosis (Weber et al., 2000). Hence, the trans formation (VAMP on vesicular membrane and SNAP25 and syntaxin-1 on PM) is favoured and exocytosis is able to occur.

The ATP independent mechanism of priming involves the Munc13 family of proteins. Through yeast two hybrid analysis it was shown that the C-terminal amino acids of Munc13-1 interact with syntaxin-1 (see below) (Betz et al., 1997c). Munc13-1 and Munc18 bind overlapping regions of syntaxin-1 indicating their ability to compete for syntaxin-1 binding. Munc18-1 binds to the closed conformation of syntaxin-1 while Munc13-1 is thought to bind to syntaxin-1 and displace Munc18-1 and thus favouring the open conformation of syntaxin-1 (Ma et al., 2011b; Sassa et al., 1999). This allows VAMP and SNAP25 to bind and fusion to occur. Contrary to this it has been shown in liposome fusion experiments that pre-incubation with Munc18-1 facilitates SNARE mediated fusion (Shen et al., 2007b; Tareste et al., 2008). Hence, Munc13 and 18 may both regulate vesicle priming together.

iv) Triggering

The triggering of exocytosis can be done via a calcium dependent mechanism or independent (GTP) pathway. However, the main focus here is with respect to a calcium dependent process. The current model suggests that depolarization of the presynaptic membrane leads to the opening of voltage gated calcium channels causing intracellular calcium concentrations to rise. This triggers vesicular fusion and release of vesicular contents into the synaptic cleft. The sensor of this calcium change is synaptotagmin-1 a 65 kDa vesicular protein (Fernandez-Chacon et al., 2001). There are at least 14 isoforms of synaptotagmin, all of which are thought to perform the role of a calcium sensor.
This protein contains two C2 domains which bind to phospholipids in a calcium dependent manner (Fernandez-Chacon et al., 2001). The C2A domain binds 3 calcium ions while the C2B domain binds 2 but is critical for calcium sensing (Nishiki and Augustine, 2004; Stevens and Sullivan, 2003). Synaptotagmins are also known to bind to syntaxin-1 and SNAP25. The synaptotagmin-1- SNAP25 interaction plays an important role in DCV as secretion mutations to SNAP25 which alter synaptotagmin-1 binding completely abolish regulated exocytosis (Zhang et al., 2002). However, upon calcium binding synaptotagmin-1 switches from SNARE complex binding to phospholipid binding creating a mechanical force causing the plasma membrane to positively curve and thus promoting fusion (Sudhof, 2004).

v) Fusion

The SNARE complex (SNAP25, syntaxin-1 and VAMP2) has two distinct features that of SDS (sodium dodecyl sulfate) detergent resistance and high temperature stability (Parpura and Mohideen, 2008). According to the zipper model; the SNARE complex assembles from its N terminus to its transmembrane containing C terminus (Südhof and Rothman, 2009b). As this “zippering” gets closer and closer to the C-terminus the vesicle slowly approaches the plasma membrane. Studies have shown the C-terminal of region of this complex as being critical for exocytosis. Mutations to this region of SNAP25 cause a decrease in vesicular fusion while N-terminal mutants did not seem to be as critical (Fang et al., 2008). It is believed that syntaxin-1 and SNAP 25 bind first and a 1:1 ratio which in-turn serves as an acceptor site for VAMP2 (Südhof and Rothman, 2009b). This then forms a coiled complex consisting of four SNARE domains held together by hydrophobic bonds existing in a α-helical bundle (Südhof and Rothman, 2009b). One SNARE domain is contributed by syntaxin-1 and VAMP2 while two domains come from SNAP25. The zippering of this complex provides the energy source required for exocytosis due to the asymmetry of the lipid bilayer.

Moreover, neuronal SNAREs interact with a variety of proteins, the best characterized being Munc13, Munc18 and synaptotagmin-1. Recently, the Calcium Dependent Activator Protein for Secretion 1 (CAPS1) has also been shown to interact with syntaxin-1 a member of the SNARE complex (James et al., 2009b).
Figure 2: The Secretory Vesicle Cycle

The secretory vesicle cycle consists of five steps: i) biogenesis, ii) docking, iii) priming, iv) triggering, and v) fusion. This process is critical for the release of neurotransmitters and neuromodulators thus, facilitating communication between cells. The proteins thought to be involved are listed under each stage.
2. 5 CAPS1-Calcium Dependent Activator Protein for Secretion

My research mainly focuses on the structural-functional relationship of the CAPS1 protein. Below is a literature overview of the CAPS1 protein and its possible function in exocytosis. The headings are divided based on the model system used in each study.

2.5.1- CAPS1 Discovery and Structure

The calcium dependent activator protein for secretion 1 (CAPS1) was initially identified as a protein which was able to reconstitute calcium dependent regulated secretion in permeabilized pheochromocytoma 12 (PC12) cells (Walent et al., 1992). In this approach, PC12 cells were permeabilized and washed extensively. It was then determined what specific factors that must be added to reconstitute calcium dependent secretion. Through this experiment a cytosolic factor of 145kDa (p145) in size was required along with calcium and MgATP (Martin and Walent, 1989). Moreover, the anti-p145 serum when added to the cytosol it inhibited the reconstitution of secretion (Walent et al., 1992). It was also shown through gel filtration and sucrose gradient centrifugation that this protein exists in a dimer (~300kDa) conformation under native conditions (Walent et al., 1992). In addition, this protein was distributed in the cytosolic fraction of the brain, pancreas, adrenal medulla and pituitary. The p145 protein was later sequenced and found to be similar (75% similar, 54% identical) to the UNC31 protein of *C.elegans* (Ann et al., 1997). This UNC31 protein is a nervous system protein in which mutations cause uncoordinated movements, hence the name UNC31(Ann et al., 1997).

Moreover, there is one CAPS gene present in *drosophila* (dCAPS) and *C. elegans* (UNC31) (Speidel et al., 2003). In mice and humans there are at least two isoforms of CAPS present, they are referred to as CAPS1 and CAPS2 respectively (Speidel et al., 2003). CAPS1 is mainly thought to regulate catecholamine release from neuroendocrine cells, whereas CAPS2 is involved in the release of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 from cerebellar granule cells (Grishanin et al., 2002; Sadakata et al., 2006; Sadakata et al., 2004; Walent et al., 1992). CAPS1 and CAPS2 are predominantly expressed in brain yet CAPS1 was observed in the islets of Langerhans, the spleen, stomach, and adrenal medulla, whereas CAPS2 was present in bronchial epithelial cells, thyroid cells, chief cells of the stomach, ductal epithelium of the salivary gland, kidney proximal tubules, spleen, thymus, and colon (Sadakata et al., 2007b; Speidel et al., 2003). Hence, the CAPS proteins may play a role during secretion is numerous cell types indicating a conserved and critical function.
Structurally the CAPS protein is relatively large consisting of approximately 1400 amino acids (~145kDa) (Figure 3). The N-terminus region consists of a dynactin 1 binding domain (DBD). It is proposed that this domain plays a role in sorting and transport of vesicles. Dynactin may link CAPS with dynein or kinesin II which are motor proteins used by microtubules for bidirectional transport (Sadakata et al., 2007a). CAPS also contains a C2 domain which like the ones present in synaptotagmin-1 serve as calcium dependent membrane (phospholipid) interactors (Grishanin et al., 2002). It is proposed that this region is responsible for the calcium sensing required for the function of CAPS. Next to the C2 domain there is a plekstrin homology (PH) domain. PH domains are known to interact with acidic phospholipids such is PIP2 (Grishanin et al., 2002). Mutation of the PIP2 binding pocket in the PH domain did not abolish CAPS1 interaction with PIP2 (Grishanin et al., 2002). However, using fluorescently labeled PIP2 it was shown that a majority of CAPS1 binding co-insides with these PIP2 micro-domains (Grishanin, Klenchin et al. 2002). Likewise CAPS1 was shown to accelerate secretion in a calcium as well as a PIP2 dependent manner (James et al., 2008). CAPS1 mutants with PIP2 binding mutations in the PH domain completely abolished norepinephrine secretion (James et al., 2008). This suggests that in full length CAPS the PH domain is involved in PIP2 binding and is important for protein function. In addition, this domain may play a role in recruiting proteins to the plasma membrane (James et al., 2008).

CAPS1 also contains a Munc13 homology domain (MHD) which is thought to be required for the priming activity of Munc13 in mice and C. elegans (UNC13)(Koch et al., 2000). Munc13-1 itself has two MHD that may be responsible for promoting the open conformation of syntaxin-1 which is critical for SNARE formation and vesicle exocytosis (Madison et al., 2005; Misura et al., 2000a). The C-terminal end of CAPS contains a dense core vesicle domain (DCV). This domain is thought to be required for CAPS to bind to dense core vesicles (Grishanin et al., 2004). It is hypothesized that CAPS uses this domain to bind to DCVs and its PH domain to bind to PIP2, thus bridging the gap between the plasma membrane and the DCV.
Figure 3: Domain Structure of CAPS1 Protein

A) The CAPS1 protein is a relatively large protein approximately 145 kDa in size. It consists of 5 domains; a N-terminal dynactin binding domain (DBD), a C2 domain, a pleckstrin homology (PH) domain, a Munc homology (MH) and a C-terminal dense core vesicle binding (DCVB) domain.

B) The CAPS1 protein consists of two isoforms CAPS1 and CAPS2. Both isoforms are similar in function but differ in their tissue distribution.
2.5.2- CAPS1 Role in DCV Secretion

i) Synaptosomes

Synaptosomes are isolated terminals from neurons obtained after homogenization and fractionalization of nervous tissue. Moreover, synaptosomes are formed from the phospholipid layer of the cell membrane and synaptic proteins. They are commonly used to study synaptic transmission because they contain the molecular machinery necessary for the uptake, storage, and release of neurotransmitters (Berwin et al., 1998). In synaptosomes isolated from rat brains it was determined that CAPS1 was unable to reconstitute the secretion of glutamate in a calcium dependent manner, yet rat brain cytosol was indeed capable of restoration (with the addition of calcium) (Tandon et al., 1998). However, when synaptosomes were incubated with radiolabelled norepinephrine (NE) both CAPS and brain cytosol were capable of reconstituting NE release (Tandon et al., 1998). Also, anti-CAPS1 antibody was able to reduce calcium dependent NE release by at least 75% where as there was no effect on glutamate release (Tandon et al., 1998). Moreover, the inhibition WITH the anti-CAPS1 antibody was proportional to the size of the NE pool hence; the function of CAPS1 may be at a stage late in the maturation of dense core vesicles. Hence, it is proposed that the docking and fusion machinery controlling SV and DCVs may be divergent with CAPS1 acting specifically on DCVs. Additionally, using synaptosomes CAPS1 was shown to be greatly enriched in the DCV pool after centrifugation followed by sucrose gradient separation (Berwin, Floor et al. 1998). It was shown that CAPS1 binds to DCVs with high affinity and this bind was ATP and calcium independent (Berwin, Floor et al. 1998). Moreover, after fractionalization of rat brain homogenate CAPS1 was shown to be partially membrane associated as it was localized with NCAM a neuronal cell adhesion molecule (Berwin, Floor et al. 1998. Additionally, calcium activated secretion of labeled NE was CAPS1 dependent and it was hypothesized that the requirement of CAPS1 in DCV exocytosis occurs after the ATP dependent priming step (Berwin, Floor et al. 1998).

ii) Rat Melanotrophs

Melanotrophs are cells in the pituitary gland which secrete melanocyte stimulating hormone (MSH) and its precursor pro-opio-melanocortin via DCVs. Using capacitance and caged calcium measurements it was shown that the rapid component of dense core vesicle release required the CAPS1 protein (Rupnik et al., 2000). It was illustrated that a neutralizing CAPS1 antibody inhibited the rapid but not the slow component of the $\text{Ca}^{2+}$-dependent capacitance increase (Rupnik, Kreft et al. 2000). In dense core vesicle release there are three phases for secretion, the slow and fast initial burst phase as well as the sustained phase. Primed vesicles can exist in two states a slowly releasable
pool (SRP) and a rapidly releasable pool (RRP) (Sorensen, 2004). It is thought that priming may be sequential in that the docked vesicles being primed to the SRP further move to the RRP. The sustained phase consists of previously unprimed vesicles becoming primed and then released as long as the intracellular calcium concentration is high enough to support release (Rettig and Neher, 2002; Sorensen, 2004). Hence, CAPS1 may as a protein regulating the release of the rapidly releasable pool. Moreover, it was shown through immunogold electron microscopy and immunofluorescence that CAPS was localized to a distinct subset of DCVs in these melanotrophs (Rupnik, Kreft et al. 2000). Hence, the authors propose there to be two distinct calcium dependent pathways involved in the fast and slow release of DCVs respectively with CAPS only acting on the fast component (Rupnik, Kreft et al. 2000. As predicted the fast and slow components were inhibited by botulinum toxin B (targets VAMP) and E (targets syntaxin-1) (Rupnik, Kreft et al. 2000). However, the rapid component was less sensitive to the botulinum neurotoxin treatment which suggests that vesicles entering the slow exocytosis form a loose SNARE complex and those that undergo rapid exocytosis a tight form of the SNARE complex (Rupnik, Kreft et al. 2000. Hence, it was concluded that CAPS is a key component of dense-core vesicles that rapidly fuse at low calcium concentrations.

iii) Chromaffin Cells

Chromaffin cells are neuroendocrine cells found in the medulla of the adrenal gland. These cells secrete catecholamines such as epinephrine and nor-epinephrine directly into the bloodstream. When anti-CAPS antibody was injected via patch pipette into calf adrenal chromaffin cells it was observed that the antibody inhibited catecholamine release by as much as 80% (Elhamdani et al., 1999). Moreover, the number of residual amperimetric spikes and kinetics were affected (Elhamdani et al., 1999). The spikes were much smaller (by 85%) and broader (by 3.5-fold) than those in control cells (Elhamdani et al., 1999). Such kinetic alterations in spike morphology suggest that CAPS1 plays a role at the final stage of calcium triggering secretion, possibly during the fusion of these vesicles. Moreover, the authors concluded that CAPS1 in involved in a post priming step as incubation with its antibody slowed the maximal rate of exocytosis (Elhamdani et al., 1999).

iv) Knock Out Mice

**CAPS1 Single Knockout**
Since CAPS1 was thought to play a role in DCV exocytosis CAPS1 knockout mice were generated and used as a model for calcium dependent DCV secretion. Using homologous recombination, two CAPS1 coding exons were removed and replaced with a neomycin resistant cassette. Phenotypically, these CAPS1 homozygous KO mice breathed slowly and moved only after tactile stimulation (Speidel et al., 2005). Moreover, these mutant mice die within 10-30 minutes after birth however, there was no change in brain morphology or body plan (Speidel et al., 2005). Using high performance liquid chromatography (HPLC) it was shown that in these mice there was no change in catecholamine levels, however dopamine levels were reduced (Speidel et al., 2005). Hence, CAPS1 is essential for postnatal survival but not in the regulation of development.

As mentioned previously, CAPS1 is present in other areas of the body as well leading to the possibility that lethality may be due to the loss of the widespread expression of the protein. Due to the lethality of the homozygous mice heterozygous knockout mice was studied. It was determined that these mice exhibited a significant reduction in CAPS1 in the adrenal gland (Speidel et al., 2005). Using primary cultured chromaffin cells from embryonic day 18-19 (E19) there was no difference in secretion in homozygous knockout versus wild type cells (Speidel et al., 2005). However, using primary cultured chromaffin cells from P30 heterozygous KO mice it was shown that there was a 30-35 percent decrease in catecholamine release when compared to wild type (Speidel et al., 2005). Using amperometric measurements it was determined that E19 homozygous knockout mice exhibited a 60% decrease in amperometric spikes (Speidel et al., 2005). Combining this with the fact that there was no secretion defect seen using capacitance it was suggested that CAPS1 plays a role in the loading of dense core vesicles. Moreover, since there were no other changes in the amperiometric evens (rise time, half width and foot duration) and with only the frequency decreasing many of the fused vesicles were hypothesized to contain no catecholamines (Speidel et al., 2005).

This result however was later determined to be invalid because CAPS2 which is highly expressed in the adrenal medulla in the embryonic stages of birth but decreases in expression postnatally was performing the same function as CAPS1 in the E19 cells. Moreover, looking at the levels of vesicular monoamine transporters (VMATs) which are thought to be responsible for the loading of catecholamines in DCVs there was no change in the expression of these proteins (Speidel et al., 2005). As for the phenotype seen in the heterozygous KO mice the authors proposed that the 25-30 % reduction seen is due to a selective loss of secretory granules (Speidel et al., 2005). That is during the development of the adrenal gland the empty vesicles generated by a partial loss of CAPS1 are moved to a different granule population resulting in less fusion events.
**CAPS1 and CAPS2 Double Knockout**

CAPS1 single KO studies revealed no defect on DCV release, this however is likely due to the fact that CAPS2 was present. Hence, CAPS1 and CAPS2 double knockout (DKO) mice were generated and used as a model system for calcium dependent exocytosis. DKO mice were generated by breeding CAPS1 mutant mice into the CAPS2 mutant background mice (Jockusch et al., 2007; Liu et al., 2008). Using primary cultured chromaffin cells E18/E19 and flash photolysis the authors demonstrated that capacitance decreased by 55% and amperiometric spikes by 62% in DKO versus wild type cells (Liu et al., 2008). By using flash photolysis and ratiometric calcium monitoring there authors were able to ensure that intracellular calcium levels were similar in the DKO and wild type mice (Liu et al., 2008).

As mentioned previously the release of dense core vesicles from chromaffin cells is highly regulated and consists of a number of stages. Likewise, there are different pools of vesicles; the burst phase contains the release of rapid releasable pool and slowly releasable pool and the sustained pool consists of priming DCVs that were unprimed at the initial time of calcium release. In the DKO cells there was a significant decrease in the fast component of release (RRP) and the sustained release was all but abolished (Liu et al., 2008). There are two ways to interpret this data, firstly there could just be a reduction in DCVs at the plasma membrane or secondly there may be a reduction in primed DCVs. Using electron microscopy the number of morphologically docked vesicles (within 80nm of PM) did not differ significantly from wild type and DKO mice (Liu et al., 2008). Hence, the reduction in secretion was attributed to a defect in primed vesicles, indeed a defect in priming would affect the RRP and sustained pool however it should also reduce the rate of vesicle pool recovery after excessive stimulation. Normally, using dual flash photolysis experiments combined with capacitance, an inter-stimulation interval of one to two minutes is enough for the recovery of the releasable vesicle pool such that a second stimulus gives the same result as the first (Liu et al., 2008). By varying the inter-stimulus interval it was determined that the filling of the RRP was greatly reduced in the CAPS DKO however the SRP filling was similar to wild type levels. This could be explained by a lack of stability in the RRP (Liu et al., 2010; Liu et al., 2008). Hence, it is proposed that CAPS plays a role in the filling of the RRP which occurs very slowly in these DKO cells leading to the loss of vesicles in the SRP and eventually a decrease in exocytosis. Lastly, re-expression of CAPS1 was able to rescue all the mutant phenotypes seen.

Additionally, upon using CAPS DKO mice CAPS2 was observed rescue the secretion defect to levels similar to wild type indicating a similar function to that of CAPS1 (Jockusch, Speidel et al.
Also, using wild type chromaffin cells in which CAPS1 and CAPS2 were overexpressed capacitance studies were conducted (Liu et al., 2010). Interestingly, both individually and together (albeit to a higher level) increased the fast component of the burst phase (the RRP) of exocytosis (Liu et al., 2010). This indicates that CAPS1 and CAPS2 both promote priming into the RRP in chromaffin cells. Using the DKO cells the authors were also able to show that the open conformation of syntaxin-1 (L165A/E166A -LE mutant) was capable of rescuing the RRP defect seen in the DKO mice (Liu et al., 2010). However, the open syntaxin-1 expression lead to a rapid exhaustion of release such that there was a reduction in the sustained release (Liu et al., 2010). Using electron microscopy, it was determined that the open conformation of syntaxin-1 lead to a defect in docking in these DKO cells (Liu et al., 2010). Hence, the open conformation syntaxin-1 bypasses the requirement of CAPS for the priming into the RRP however it also results in the reduction in the transporting and docking of DCVs to the plasma membrane.

The CAPS protein shares a structural similarity to the Munc13-1 protein in that they both have at least one Munc13 homology domain. Munc13-1 has two of these domains both of which have been shown to be essential for the priming activity involving the promotion of the open conformation of syntaxin-1 (Liu et al., 2010). Interestingly, Munc13-1 is unable to restore secretion in CAPS DKO cells, yet it can enhance secretion in the presence of CAPS1 but not CAPS2 (Liu et al., 2010). Numerous studies have shown Munc13-1 to be critical for the priming of synaptic vesicles in neurons. Hence, the failure to cross rescue indicates that CAPS1 and Munc13-1 may have different functions although there is some similarity in their domain structures. The proposed model suggests that Munc13 acts upstream of CAPS1 by opening syntaxin-1 which is then followed by CAPS binding to the open syntaxin-1 and possibly serving as a positive enhancer for SNARE formation (Liu et al., 2010).

Knockout Studies in Pancreatic Beta Cells

Using CAPS2 knockout and CAPS1 heterozygous knockout in a null CAPS2 background studies were able to compare the function of CAPS1 and CAPS2 individually. Both mice were shown to be glucose intolerant which was attributed to the reduction in glucose induced insulin secretion (Speidel et al., 2008). Compared to wild type insulin secretion from cultured beta cells
secretion was reduced by ~30-40% in CAPS2 KO mice and by 50-60% in CAPS1 KO mice (in null CAPS2 background) when evoked by glucose or high potassium (Speidel et al., 2008). Moreover, insulin content was not affected in CAPS2 KO but decreased by 25% in the CAPS1 KO (Speidel et al., 2008). In addition, there was a reduction in the size of the morphologically docked pool of vesicles; however this effect was greater in the CAPS1 knockout which had a 44% decrease whereas the CAPS2 knockout had only a 17% reduction. (Speidel et al., 2008). Also CAPS1 removal affected the total granule density and decreased islet content indicating that it is important to the maintenance of granule stability (Speidel et al., 2008). Hence, CAPS1 and CAPS2 play a role in the exocytosis of insulin, however this role seems to be more of a modulatory one such that exocytosis can still take place in the absence of these proteins but in their presence is more efficient. One model suggests that CAPS1 may regulate the trafficking of the insulin granules to the plasma membrane whereas CAPS2 facilitates more distal events such as the maintenance of the RRP (Speidel et al., 2008).

v) PC12 Cell Line Studies

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla (see below) the CAPS1 protein was initially discovered as a cytosolic factor that could reconstitute calcium dependent secretion in permeabilized PC12 cells (Walent et al., 1992). Using PC12 cell lines which naturally do not express CAPS2 the effect of CAPS1 in DCV secretion has been analyzed extensively. Initially, mutant CAPS1 proteins were expressed in COS-1 cells and then used to determine their ability to rescue secretion in permeabilized PC12 cells (Ann et al., 1997). It was determined that mutations in the PH domain of CAPS which abolished its interaction with PIP2 in liposome fusion assay did not affect the ability of CAPS to rescue calcium dependent secretion (Grishanin et al., 2002). Interestingly, some of these triple mutants R558D/K560E/K561E exhibited decreased the interaction with acidic phospholipids such as phosphatidylserine and caused a reduction in membrane localization (Grishanin et al., 2002). In this case, regulated secretion was not inhibited but exhibited a 50 fold reduction in potency (more protein required to trigger NE release from permeabilized PC12 cells) (Grishanin et al., 2002). The C-terminus of CAPS1 contains an acidic cluster (MKDSDEDEDEEDD) consisting of a sorting like motif which is similar to that found in proteins involved in membrane trafficking such as furins and herpes virus envelop proteins (Grishanin et al., 2002). A 135 amino acid truncation of the CAPS C-terminus completely abolished the activation of regulated secretion from permeabilized PC12 cells (Grishanin, Klenchin
et al. 2002). Moreover, this C-terminal fragment fused to GFP was shown to be sufficient to co-localize with DCVs when expressed in PC12 cells (Grishanin, Klenchin et al. 2002).

A more robust test of CAPS1 function in PC12 cells was done in which CAPS1 was downregulated using short hairpin RNA (shRNA) and the exocytosis phenotype was observed. It was shown that stable CAPS1 knockdown cells have a defect in catecholamine release (Fujita et al., 2007). Secretion was consistently decreased in the knockdown clones by 45–80% in both high potassium and alpha latrotoxin-x stimulation experiments when compared with control (Fujita, Xu et al. 2007). Latrotoxin forms pores in the membrane allowing for depolarization of the cell, the fact that both high potassium and latrotoxin mediated secretion are similar indicates that the defects in high potassium-induced secretion are not the result of defects upstream of calcium but rather in the stages leading up to secretion. Moreover, in permeabilized knock-down cells brain cytosol was able to rescue secretion, this confirms that the secretion defect in not due to the loading of catecholamine as a loading defect would not result in the rescue of the permeabilized knockdown cells (Fujita, Xu et al. 2007). Hence, the defect seen is indeed related to the secretion process itself. In addition, the CAPS1 knockdown cells exhibited a decrease in the secretion of exogenously expressed neuropeptides. Although neuropeptides and catecholamines are released from DCVs their mechanism of loading differs. Catecholamines are thought to be incorporated into DCVs via vesicular monoamine transporters which take advantage of the proton gradient in acidified vesicles to transport catecholamines inside (Mahata et al., 1993; Whim, 2006). On the other hand, neuropeptides such as NPY (neuropeptide Y) are incorporated into DCVs as these vesicles bud from the Golgi complex (Dannies, 2001). Thus, if CAPS was involved in the loading of catecholamines, the secretion of NPY from knockdown PC12 cells should not be affected. However, it was shown that the secretion of both NE and NPY is indeed affected in these cells even with proper localization of NPY at the tip of the neuritis in differentiated PC12 cells (Fujita, Xu et al. 2007). Using capacitance to measure exocytosis from these knockdown cells it was shown that the slow burst phase and sustained phase were significantly decreased. Hence, CAPS is thought to play a role in the priming of DCVs and in the mobilization/refilling of the releasable pool (Fujita, Xu et al. 2007).

Interestingly, it was observed that there was a higher accumulation of endogenous NE inside the knockdown cells which was attributed to a decrease in constitutive secretion (Fujita, Xu et al. 2007). Hence, the authors concluded CAPS1 plays a role in both regulated and constitutive exocytosis and this role is independent of loading and is likely a priming defect. Additionally, it has been shown that CAPS1’s activity in priming vesicles requires phosphorylation by protein kinase
CK2. It is proposed that the N-terminal serine residues Ser-5,6,7 must be phosphorylated in order for CAPS1 to function (Nojiri et al., 2009). Inhibition of phosphorylation by tetrabromo-2-benzotriazole (TBB) a protein kinase CK2 inhibitor inhibited secretion in permeabilized PC12 cells and decreased the amount of radiolabelled phosphorylation (Nojiri, Loyet et al. 2009). Moreover, protein kinase CK2 was also shown to phosphorylate CAPS1 in vitro by mass spectrometry analysis. CAPS1 is thought to exist as a dimer ~300kDa, however the de-phosphorylation promoted a tetramer formation (confirmed through immunoprecipitation) which may be responsible for the loss of secretion seen as dephosphorylated CAPS is unable to stimulate norepinephrine release (Nojiri, Loyet et al. 2009). In addition, the phosphorylation itself may be responsible for the stability of the protein such that de-phosphorylated CAPS is only 23% alpha helical whereas the phosphorylated version is 33% alpha helical (Nojiri, Loyet et al. 2009). Moreover a S5A/S6A/S7SA mutation in PC12 cells was unable to rescue the CAPS1 knockdown secretion defect whereas re-expression of the wild type CAPS1 did as measured by total internal reflection fluorescence microscopy indicating the importance of CAPS1 phosphorylation for its role in secretion (Nojiri, Loyet et al. 2009).

2.6-CAPS1 Role in Synaptic Vesicle Secretion

The CAPS family of proteins has been considered candidate proteins for vesicle priming as they contain a sequence similarity to the priming domain of Munc13. The majority of the data has suggest CAPS to play a role in DCV secretion yet CAPS1 and CAPS2 seem to be enriched at presynaptic compartments of neurons which contain mostly synaptic vesicles (SV) and only a few DCVs (Jockusch et al., 2007). Using hippocampal glutamatergic autaptic cultures from wild type, CAPS1 KO, CAPS 2 KO and CAPS1/CAPS2 DKO, it was shown that SV priming and transmitter release was indeed defective. In CAPS KO neurons the excitatory post synaptic current (EPSC) was reduced by 51% of the wild type value and the RRP pool size by 42% (Jockusch, Speidel et al. 2007). In the DKO neurons 39% were totally unable to elicit any EPSC and the RRP was only 15% of the wild type (Jockusch, Speidel et al. 2007). CAPS2 KO neurons were less severe in nature but there was still a small change (Jockusch, Speidel et al. 2007). This suggests that a the CAPS family of proteins may be involved in not only DCV exocytosis but also SV’s as well. Like previously demonstrated, there was no defect in the ultrastructure of the synapse or in the number of synapses.

Interestingly, high frequency stimulation was shown to trigger a process that turns the poorly secreting CAPS DKO neurons into neurons that secrete at levels to that of the wild type (Jockusch, Speidel et al. 2007). Hence, the SV seem to be loaded but require a boost provided by the high
frequency stimulation to become release competent. Further, clamping the intracellular calcium concentration to 12mM caused a massive release of glutamate release which was shown to be caused by an increase in the RRP size (Jockusch, Speidel et al. 2007). This observation is similar to the effect seen in Munc13 KO neurons. However, when Munc13 was overexpressed in the CAPS DKO cells it failed to rescue the mutant phenotype. Likewise when CAPS1 was overexpressed in the Munc13 KO neurons and the same result was observed, hence their failure to cross rescue indicate they may be essential components of the same pathway/apparatus but acting at different stages (Jockusch, Speidel et al. 2007). This was further confirmed with the use of phorbol esters, which cause an increase in Munc13 dependent stimulation of neurotransmitter release. It was shown that phorbol ester application lead to a robust increase in exocytosis in CAPS DKO cells (Jockusch, Speidel et al. 2007). Hence, it is hypothesized that CAPS is a priming protein like that of Munc13 with either the two proteins acting in series such that Munc13 generates a primed pool of vesicles that must be stabilized in a rapidly releasable state by CAPS in order for calcium dependent release.

2.7 CAPS1 Role in Monoamine Uptake

The CAPS1 protein has been shown to be critical for the secretion in neurons and neuroendocrine cells. It is thought that CAPS1 functions as a regulator during the priming step of exocytosis, however recent data suggests that CAPS1 may have completely different function such as in the uptake/loading of vesicular catecholamines. CAPS1-deficient chromaffin cells display strongly reduced vesicular catecholamine levels (Brunk et al., 2009). Moreover, CAPS1 and CAPS2 have been shown to promote monoamine uptake and storage, a process mediated by the vesicular monoamine transporters VMAT1 and VMAT2 (Brunk et al., 2009). Firstly, the overexpression of CAPS1 and CAPS2 is Chinese hamster ovary (CHO) cells expressing VMAT1 and VMAT2 caused an upregulation of monoamine uptake which was reduced by the addition of anti-CAPS1/2 antibodies (Brunk et al., 2009). Additionally, monoamine (serotonin) uptake from embryonic brains of CAPS1 deletion mutants is decreased as compared with wild type brains (Brunk et al., 2009). Previously, it was shown the alpha-subunit of heterotrimeric G-protein Go2 are involved in monoamine uptake and storage (Brunk et al., 2009). The GTP bound active Go2 alpha subunit is thought to contribute to the uptake of catecholamines through an unknown mechanism. Hence, G(o2)alpha knockout mice exhibit decreased monoamine loading (Brunk et al., 2009). Interestingly, the expression of CAPS1 is decreased in brain membrane preparations from mice lacking G(o2)alpha
Moreover, anti-CAPS1 antibody did not further reduce monoamine uptake by G(0)alpha-deficient synaptic vesicles, whereas antibodies directed against CAPS2 whose expression is not altered in G(0)alpha-deficient brain, still reduce monoamine uptake (Brunk et al., 2009). Hence, both CAPS isoforms are thought to play a role in vesicular monoamine uptake though an interplay with VMAT1 and VMAT2 and this occurs through a G(0)alpha mediated mechanism.

2.8 CAPS1 Role in C. elegans

CAPS1 was first identified as a homologue of the C. elegans gene Unc-31 (Ann et al., 1997). A mutation of this gene leads to an uncoordinated phenotype consisting of defects in locomotory function. Moreover, this mutation causes a variety of phenotypic defects such as prolongation of a larval state, egg laying defects, lethargy and constitutive feeding (Speese et al., 2007). The unc31 protein was found to be expressed throughout nervous system in the nerve ring, ventral nerve cord motor neurons and the head ganglion (Speese, Petrie et al. 2007). Moreover, this protein was localized at sites of synaptic contact in presynaptic terminals (co-localized with VAMP) yet, it was not required for neuronal development as no gross defects in axonal projections were seen (Speese, Petrie et al. 2007). Cultured neurons from unc-31 mutants exhibited normal synaptic vesicle recycling as measured my FM143 dye uptake. However, these mutants exhibited reduced release of pre-proatrial natriuretic factor (ANF) (Speese, Petrie et al. 2007). ANF release into the cell body is dependent on fusion of DCVs. Thus, ANF tagged GFP (ANF-GFP) was used as a marker for DCV exocytosis, it was seen that Unc31 mutants release less ANF-GFP into the body cavity (Speese, Petrie et al. 2007). Additionally, to confirm this DCV dependent action Unc13 (Munc13) mutants were used, in these mutants synaptic vesicle exocytosis is all but abolished leading to no synaptic transmission in the motor neurons (Speese, Petrie et al. 2007). Synaptic vesicles at synaptic terminals undergo stimulated exocytosis which can be measured by the stimulated uptake of a membrane bound dye FM 4-64. However, Unc13 mutants cannot undergo synaptic transmission; hence there is no stimulated uptake of FM4-64 (Speese, Petrie et al. 2007). Interestingly, the stimulation of FM4-64 from unc-31 mutants was similar to that of the wild type (Speese, Petrie et al. 2007). This combined with the lack of ANF release indicates that the CAPS homologue Unc31 is required for DCV exocytosis and not SV exocytosis (Speese, Petrie et al. 2007).

Additionally, it was shown that the application of forskolin which leads to elevated cAMP levels was able to rescue DCV secretion in unc31 mutants indicating a G-protein coupled mechanism (Speese, Petrie et al. 2007). Using TIRF microscopy on these unc31 mutants there was
evidence of a docking defect of DCV which was confirmed with using electron microscopy (Hammarlund et al., 2008). In the motor neurons of these mutants, DCV docking was all but eliminated yet the SV docking was normal (Hammarlund, Watanabe et al. 2008). The lack of docking was not caused by the reduction in the total number of DCVs as Unc31 mutants had more total DCVs than wild type (Hammarlund, Watanabe et al. 2008). On the other hand, Unc13 mutants exhibited a large defect in SV docking with only a moderate change in DCV docking (Hammarlund, Watanabe et al. 2008). It is important to note that the open conformation of syntaxin-1 was able to completely rescue this docking defect while the overexpression of the wild type syntaxin-1 had no effect (Hammarlund, Watanabe et al. 2008). Hence, it is proposed that Unc31 (CAPS) functions upstream of syntaxin-1 in DCV docking supposedly by promoting an open conformation of syntaxin-1 while Unc13 is acting in the same way on SVs. In support of this hypothesis, it was shown that Unc64 (syntaxin-1) and Unc31 double mutations are involved in the control of aging in C. elegans (Ailion et al., 1999). Interestingly, weak unc-31 mutants, as well as unc-64 mutants, were found to increase life span. Since Unc-64 is an orthologue of mammalian syntaxin-1-1, an essential protein for neurosecretion, this finding suggests the involvement of neurosecretory pathways in life span regulation. Furthermore, double mutation of unc-31 and unc-64 prolongs life span synergistically, suggesting that these two genes genetically interact with each other (Ailion et al., 1999).

Recently, it has been shown that all of the Unc31 domains may support distinct sequential steps in exocytosis. Using ANF-GFP in the cell body as a measurement of DCV release various Unc31 (CAPS1) truncation mutants were tested on their ability to rescue secretion (Lin et al., 2010). Mutations in the C2, PH, MHD and DCV binding domain all failed to rescue secretion (Lin et al., 2010). Moreover, using TIRF all of these mutants expressed a defective docking phenotype as well. Thus, it is proposed that Unc31 functions in both DCV docking and priming such that it’s N-terminal, C2 and PH domains bind to the plasma membrane while its C-terminal DCV domain binds to dense core vesicles. Hence, Unc31 tethers and docks DCVs to the plasma membrane while its MHD initiates the formation of the SNARE complex and synaptotagmin-1 and further primes vesicle release by its interaction with syntaxin-1.
2.9 CAPS1 Role in Drosophila

Drosophila CAPS (dCAPS) is a 153 kDa protein with 59% sequence identity to rat CAPS1 (Renden et al., 2001). dCAPS is found in the central and peripheral nervous system but is restricted to synapses. This protein is expressed presynaptically and in the neuromuscular junction (Renden, Berwin et al. 2001). Interestingly, it was found to be expressed similarly in boutons that contain glutamatergic vesicles as well as those containing dense core vesicles (Renden, Berwin et al. 2001). The dCAPS null mutants tend to die in the late embryo stage with only a small minority surviving further, these mutants die no more than 6 hours after hatching (Renden, Berwin et al. 2001)). Moreover, there were no detectable morphological abnormalities or developmental defects in body plan. These null mutants suffer a 50% loss of glutamatergic transmission and the accumulation of synaptic vessels at the active zone in the neuromuscular junction (Renden, Berwin et al. 2001). As a result they suffer locomotory defects which are thought to be caused by the defective calcium dependent evoked fusion of synaptic vesicles. Additionally, there was a threefold accumulation of synaptic vesicles and dense core vesicles at the synaptic terminal (Renden, Berwin et al. 2001). This differed from the drosophila Munc13 (dUNC13) in that dUNC13 null mutants only affect the accumulation of synaptic vesicles and not dense core vesicles (Renden, Berwin et al. 2001). It was also observed that the motor neuron specific re-expression in these null mutants did not rescue synaptic function (Renden, Berwin et al. 2001). Hence, it was proposed that CAPS has a primary role in the release of DCVs which is likely to affect its secondary role in the release of glutamate from synaptic vesicles. dCAPS functions in DCV release in drosophila but at what stage it is still unclear. Moreover, it is likely that the loss of the release of these neuromodulatory molecules from DCVs severely affects the release of glutamate from synaptic vesicles.

2.10 Known Protein-Protein Interactions in Literature

2.10.1-Munc18-1 Syntaxin-1 Interaction
The discovery of the Munc18-syntaxin-1 interaction was first established in 1993 with the use of GST pull down assays (Hata et al., 1993a). Munc18 is a 60-70kDa (~600 amino acids) protein consisting of three domains (1, 2, 3a and 3b) characterized by α helices and β sheets (Han et al., 2010c). In mammals, there are three isoforms of Munc18 1, 2, and 3, with Munc18-1 being expressed in neurons and neuroendocrine cells (Han et al., 2010c). Munc18-1 and 2 share binding preferences to syntaxin isoforms 1-3 whereas only Munc18-3 binds syntaxin-4 (Hata and Südhof, 1995). Crystal structure has shown that Munc-18 forms an arc shaped structure with a cavity in the middle to which syntaxin-1 is bound (Misura et al., 2000a). Munc18-1 knockout mice exhibit a complete abolition of both spontaneous and evoked neurotransmitter release (Verhage, 2000). These mice are completely paralyzed with the embryos dying at birth since they cannot breathe however, brain formation is normal (Verhage, 2000). Moreover, null mutant orthologues of this protein demonstrate an 85% reduction in neurotransmitter release in C. elegans and Drosophila (Harrison et al., 1994; Hosono et al., 1992). There are three proposed roles for Munc18; the first being it serving as a chaperone for syntaxin-1, second a role in the docking of vesicles (mentioned previously) and third a proposed interaction with the SNARE complex and regulation of membrane fusion (Han et al., 2010a).

Firstly, Munc18 may act as a chaperone as it is required for the localization of syntaxin-1 at the plasma membrane as without it syntaxin-1 is mis-localized as seen in knock out studies and PC12 knock-down studies (Arunachalam et al., 2008; Han et al., 2009). However, C. elegans (unc18) mutants did show a decreased docking phenotype which is inconsistent with the knockout mice study which did not show any synaptic vesicle docking changes despite the loss of secretion (Toonen and Verhage, 2007). Interestingly, in Munc18-1 deficient neurons and chromaffin cells there was a 50% decrease in syntaxin-1 expression (Voets et al., 2001a). Munc18-1 forms a tight complex with syntaxin-1 which is folded into a closed conformation which involves intramolecular binding of its N-terminal Habc domain to the SNARE domain (H3) and is different from the open conformation of syntaxin-1 that forms the SNARE complex (Misura et al., 2000a). Munc18-1 also binds to the assembled SNARE complexes containing open syntaxin-1 which may enable cooperation between Munc18-1 and the SNAREs in membrane fusion. In liposome fusion assays pre-incubation of the SNARE proteins with Munc18 facilitated liposome fusion (Shen et al., 2007a). More recently, the pre-incubation with VAMP2 alone was sufficient to stimulate liposome fusion (Hu et al., 2011). This suggests that Munc18 may bind VAMP2 in a partially assembled SNARE complex such that it enhances SNARE complex stability resulting in increased membrane fusion.
Syntaxin-1 is a key neuronal SNARE protein; it is about 33kDa in size (~288aa) with many isoforms (Teng et al., 2001). There are at least 15 syntaxin genes in the human genome but for our purpose the main isoforms are syntaxin 1a and 1b which are localized in the pre-synaptic plasma membrane (Teng et al., 2001). Although syntaxin-1 is believed to be essential for secretion, conducting experiments in neuroendocrine cells has been difficult. This is due to the fact that the two isoforms present syntaxin-1a and 1b are closely related. Syntaxin-1a knockout mice show a normal neurotransmission mainly due to the presence of syntaxin-1b (Fujiwara, 2006). These mice appear to be normal and fertile however impaired LTP in hippocampal slices was observed (Fujiwara, 2006). The phenotype of syntaxin-1b knockout mice remains unknown, however using knock in studies of the open (L165A/E166A) conformation mutant causes these mice to experience seizures which become lethal at two weeks of age (Gerber et al., 2008). Moreover, this open conformation mutant reduces but does not completely abolish the interaction with Munc18 and is more effective at forming the SNARE complex compared to wild type syntaxin-1 (Gerber et al., 2008). Hence, although this mutant may reduce the expression and trafficking of syntaxin-1 to the plasma membrane exocytosis still occurs.

The N-terminus of syntaxin-1 consists of a Habc domain which can collapse onto its own H3 (SNARE) helix domain to forms an inactive "closed" syntaxin-1 conformation (Misura et al., 2000a). In between the Habc and H3 domain there is a linker region which is also required for syntaxin-1 interaction. Hence, the first binding mode between Munc18 and syntaxin-1 involves all of the conserved regions of syntaxin-1 such that amino acids 1-25 are required to bind to the outer surface of Munc18 domain 1 while amino acids 25-263 interact with the concave surface formed my domains 1 and 3a (Misura et al., 2000a) (Figure 4). However, the deletion of the C-terminal 36 amino acids of syntaxin-1 (leaving amino acids 1-226) considerably weakens the syntaxin-1-Munc18 interaction (Burkhardt et al., 2008). It is known that syntaxin-1 exists in two conformations an open and closed conformation. In the open conformation it is able to interact with the other SNARE molecules and form the SNARE complex which is required for exocytosis. However, when bound to Munc18 syntaxin-1 adopts a closed conformation in which the H3 (SNARE) domain folds back onto its Habc domain making it in accessible to its partner SNARES. However, this interaction may be critical for secretion as Munc18 is known to be critical for the localization of syntaxin-1 at the active zone (Arunachalam et al., 2008; Burkhardt et al., 2008; Han et al., 2009). By binding to syntaxin-1’s closed conformation and chaperoning it to the active zone Munc18 prevents any ectopic SNARE complex formation (with SNAP25).
Recently, the discovery of an N-peptide region of syntaxin-1 consisting of amino acids 1-19 it thought to as serves a second binding site for Munc18 (Burkhardt et al., 2008; Hu et al., 2007; Shen et al., 2007a). (Burkhardt et al., 2008) The binding between the N-peptide and syntaxin-1 may occur in order to facilitate Munc18-1s interaction with the SNARE complex and thus facilitating exocytosis. By binding to the N-terminus of syntaxin-1 this allows Munc18- to bind to an intermediate conformation of syntaxin-1, allowing it to enter SNARE complexes with SNAP-25 and synaptobrevin-2 (Burkhardt et al., 2008). Hence, the N-terminal binding mode promotes the ability of the SNARE motif of syntaxin-1 to be accessible for SNARE complex formation. Another theory suggests that this interaction further strengthens the initial interaction between the two proteins by serving as a second binding site for Munc18. Subsequently, the formation of the SNARE complex occurs by the removal of the N-peptide of syntaxin-1 (Burkhardt et al., 2008; Han et al., 2009).
When bound to Munc18, syntaxin 1a adopts a 'closed' conformation in which the H3 domain folds back onto Habc, making it inaccessible to its partner SNAREs. All conserved regions of syntaxin 1a interact with Munc18a: the N-peptide (residues 1-25) binds to the outer surface of Munc18a domain 1 and the Habc, linker, and H3 regions (25-262) interact with the concave surface formed by domains 1 and 3a. Deletion of the C-terminal 36 amino acids (1-226) weakens interaction considerably. The N-peptide of syntaxin-1a serves as a second binding site in the Munc18a/syntaxin 1a complex: When the syntaxin-1a N-peptide is bound to Munc18a, SNARE complex formation is blocked. Removal of the N-peptide enables binding of syntaxin-1a to its partner SNARE SNAP-25, while still bound to Munc18-1.
2.10.2-Munc13-1 Syntaxin-1 Interaction

Munc13 is a large 220kDa protein (~1735) with three C2 domains and one C1 domain (Basu et al., 2005a). The C2 domains bind to phospholipids in a calcium dependent manner (Betz et al., 1998; Shin et al., 2010b), while the C1 domain is involved in phorbol-ester binding and stimulation of protein Kinase C (Shin et al., 2010b). In addition, there is a diacylglycerol binding domain and two Munc13 homology domains (Augustin et al., 1999a). Munc13-1 knockout mice once born do not feed and die within a few hours of birth while brain formation is completely normal (Augustin et al., 1999b; Varoqueaux et al., 2002). Munc13-1 binds to the open conformation of syntaxin-1 and is a critical priming factor as knockout mice suffer a complete loss of spontaneous and evoked exocytosis (Varoqueaux et al., 2002). In *C. elegans*, this priming defect was partially rescued by overexpression of the open syntaxin-1 LE mutant (L165A/E166A) (Liu et al., 2010). It is also proposed that Munc13 undergoes homo-dimerization which is inhibitory to its priming function; hence RIM scaffold proteins are required to bind (Deng et al., 2011). This binding causes a hetero-dimer formation of RIM and Munc13 which in-turn activates Munc13 allowing it to bind to syntaxin-1 (Deng et al., 2011).

Through GST pull down assays and yeast two hybrid studies it was shown that the C-terminus of Munc13-1 (residues 1181-1736) binds to the N terminal coiled coil domain of syntaxin-1 consisting of residues 1-80 for syntaxin-1a and 1-79 for syntaxin1b (Betz et al., 1997b). Moreover, residues 1181-1345 of Munc13 were identified as being necessary for syntaxin-1 interaction (Betz et al., 1997a). Chromaffin cells have also been used to test the important domains of Munc13-1 as there is no secretion defect in null mutant cells (Stevens et al., 2005). In these gain of function assays the C-terminal residues 1100-1735 spanning the Munc13 homology domain and C2 domain were critical for syntaxin-1 interaction and DCV exocytosis (Stevens et al., 2005). Priming appeared to require syntaxin-1 interaction as point mutants that abolish syntaxin-1 binding also prevent vesicle priming; hence, supporting the hypothesis that Munc13-1 displaces Munc18-1 from syntaxin-1 directly (Stevens et al., 2005). Moreover, residues 859–1531 of Munc13 form an autonomously folded domain, called the MUN domain (Guan et al., 2008). More recently, the Munc13-1 MUN domain has been shown to rescue the secretion defect seen in cultured excitatory and inhibitory hippocampal autaptic neurons from Munc13-1 and Munc13-2 double-knockout mice (Ma et al., 2011a). Additionally, through NMR and fluorescence experiments it has been shown that the MUN domain binds weakly to the syntaxin-1 SNARE motif and accelerates the opening of syntaxin-1 (Ma, Li et al.
The residues thought to be involved in this binding are amino acids 200-226 in the SNARE (H3) domain of syntaxin-1 (Ma, Li et al. 2011). It is proposed Munc13-1 “extracts” the SNARE domain of syntaxin-1 from its closed conformation when bound to Munc18-1 promoting the open conformation even with Munc18-1 bound. This model agrees with the previous data that show Munc18-1 being capable of binding to the open L165A/E166A mutant of syntaxin-1. Lastly, it must be stated this mechanism is still unclear and more work need to be done on the physiologic mechanism of priming.

Modified from: (Ma et al., 2011a; Rizo and Rosenmund, 2008a)

**Figure 5: Two Proposed Binding Modes Between Syntaxin-1 and Munc13-1**

The C-terminus of Munc13 (residues 1181-1736) was originally shown to interact with the N-terminus of syntaxin 1a (residues 1-80) and syntaxin 1b (residues 1-79). In this model Munc13-1 displaces Munc18-1 from syntaxin-1. More recently, it has been shown that the MUN domain of Munc13-1 (residues 859-1531) interacts with the SNARE domain of syntaxin (residues 200-226). In this binding mode both Munc13-1 and Munc18-1 can be bound to syntaxin-1 at once.
2.10.3 CAPS1 Interaction with SNARE Complex

CAPS1 has been shown to interact with the SNARE complex in particular to syntaxin-1. It was shown through liposome fusion assays that CAPS1 facilitates the formation of the trans-SNARE complex through its interaction with syntaxin-1 (James et al., 2009a). In liposomes where VAMP2 is located on a donor and syntaxin-1 and SNAP25 on the acceptor liposome CAPS1 was shown to accelerate the fusion process (James, Kowalchyk et al. 2009). Interestingly, this process was independent of calcium yet this dependence was restored upon the inclusion of synaptotagmin-1 (James, Kowalchyk et al. 2009). Also PIP2 was required on the acceptor liposomes for fusion to take place (James, Kowalchyk et al. 2009). CAPS1 stimulation of lipid mixing occurred immediately, this was opposite to that of Munc18-1 which required a pre-incubation period with syntaxin-1, VAMP2 and SNAP25 for fusion to take place (James, Kowalchyk et al. 2009). Fusion was also dependent on the density of the SNARE proteins such that lower densities promoted more efficient fusion (James, Kowalchyk et al. 2009).

Additionally, when the fused liposomes were subjected to buoyant density gradient separation CAPS1 was retained on the liposomes that contained syntaxin-1-1/SNAP 25 but not the ones that contained VAMP2 (James, Kowalchyk et al. 2009). Moreover, liposomes containing only syntaxin-1 were also shown to retain CAPS1 indicating that CAPS is a syntaxin-1 binding protein (James, Kowalchyk et al. 2009). It was also shown that fusion proteins that contained just the MHD (859-1073) or an overlapping C-terminus (655-1289) part of CAPS1 did not stimulate fusion but exerted an inhibitory effect on CAPS stimulation on liposome fusion (James, Kowalchyk et al. 2009). This indicated that CAPS promoted SNARE dependent fusion via direct interaction with the syntaxin-1 and SNAP25. Recently, building upon the liposome fusion assays CAPS1 was shown to bind to syntaxin-1 and SNAP25 with high affinity and VAMP2 with lower affinity (Daily et al., 2010). The interaction with syntaxin-1 was shown to be mediated through syntaxin-1 SNARE (H3) and linker domain (amino acids 191-266) (Daily, Boswell et al. 2010). CAPS1 failed to interact with the closed full length syntaxin-1 and with the N-terminal region up to the SNARE domain (amino acids 1-177) (Daily, Boswell et al. 2010). Moreover, it was also isoform specific as syntaxin isoforms 1, 2 and 4 interacted with CAPS1 while isoforms 3 and 6 did not (Daily, Boswell et al. 2010). Also membrane proximal mutations of syntaxin-1 K252A, K260Q, K260Q/K265L and Y244H/K260Q/K265L all lead to a reduction in interaction with CAPS1 (Daily, Boswell et al. 2010).
The open conformation of syntaxin-1 (L165A/E166A mutant) was more efficient at binding CAPS1 and out-competed the wild type syntaxin-1 for CAPS binding (Daily, Boswell et al. 2010). Interestingly, Munc18 did not compete with CAPS for syntaxin-1 binding but by contrast promoted CAPS1 binding to syntaxin-1 by two fold (Daily, Boswell et al. 2010). This suggests that Munc18 may alter syntaxin-1 to promote CAPS1 C-terminal binding as CAPS1 and Munc18 were found to co-reside on syntaxin-1 containing liposomes. It is hypothesized that Munc18 may bind VAMP2 resulting in a more stabilized SNARE complex leading to increased vesicular fusion. Additionally, complexin which binds to the C-terminal groove of syntaxin-1 (residues 214-242) did not alter CAPS binding to syntaxin-1 indicating that this binding occupies a more C-terminal site on syntaxin-1 (Daily, Boswell et al. 2010). This was further confirmed as alpha SNAP which binds to the SNARE domain of syntaxin-1 was found to effectively inhibit CAPS1 binding to syntaxin-1 (Daily, Boswell et al. 2010). CAPS1 also interacts with SNAP 25 containing liposomes with high affinity and this interaction was mediated by the SN1 domain of SNAP 25 (Daily, Boswell et al. 2010). Also, there was a weak interaction between CAPS1 and VAMP2 which was mediated by the N-terminal domain of VAMP2 (Daily, Boswell et al. 2010). Hence, CAPS plays a role in the interaction of the SNARE complex, however it must be stated that these experiments are non-physiological and the interactions seen may not indeed take place in a living cell.

2.11 PC12 Cells: Model for DCV Secretion

The PC12 (pheochromocytoma 12) is a cell line derived from a single cell colonial line from a transplanted rat tumor of the adrenal gland (pheochromocytoma) (Martin and Grishanin, 2003; Westerink and Ewing, 2008). PC-12 cells are small (5–10 µm), have a limited amount of cytoplasm, and have a relatively long doubling time typically two to three days (Tischler and Green, 1977). These cells have been used extensively in the study of neurosecretion as they serve as a good model for primary neuronal cells. PC12 cells have the ability to reversibly differentiate into neuronal like cells in response to nerve growth factor (NGF) (Huff and Guroff, 1979). Undifferentiated PC12 cells are like that of immature adrenal chromaffin cells in many ways more specifically in their ability to secrete neurotransmitters (Westerink and Ewing, 2008). These cells contain large (~100 nm) dense core vesicles (DCVs) that store monoamines as well as smaller (~40 nm) vesicles known as synaptic vesicle-like microvesicles (SLMVs) (Martin and Grishanin, 2003). Upon elevations in cytosolic calcium vesicle fusion with the plasma membrane and the secretion of catecholamines (norepinephrine, epinephrine, and dopamine) and neuropeptides from DCVs and acetylcholine from
SLMV takes place (Martin and Grishanin, 2003). More importantly, neurotransmitter release from DCVs in these cells can be regulated by calcium and GTP dependent exocytotic pathways hence, PC12 cells serve as a strong model for studying regulated neurotransmitter release (Martin and Grishanin, 2003; Westerink and Ewing, 2008). When treated with NGF, PC12 cells stop proliferation, project neuritis, become electrically excitable, increase the number of calcium channels and synthesize more neurotransmitters (Huff and Guroff, 1979; Westerink and Ewing, 2008). Removal of the NGF results in the degradation of the processes whereas the cell body largely remains unaffected.

PC12 cells were used in this study for a variety of reasons, but mostly importantly because they contain only CAPS1 (and not CAPS2) which unlike the mouse model makes it much easier to study the function of the protein (Fujita et al., 2007). Moreover, these cells serve as an excellent model in DCV secretion as have numerous advantages over primary neurons. Firstly; we have the ability to grow PC12 cells in continuous culture with a well-defined secretory cell phenotype. CAPS1 knockdown cells have been created and these cells can be maintained for a long period of time and can be frozen and defrosted when needed (Fujita et al., 2007). Hence, their ease of maintenance and robustness allow for a wide range of genetic manipulations. In fact, studies on the discovery of CAPS were initially conducted on “cracked” PC12 cells where they are permeabilized but still retain calcium dependent secretion since many (40-60%) of the DCVs are still docked at the plasma membrane and the intra-cellular organelles remain intact (Martin and Grishanin, 2003). Moreover, PC12 cells are neuroendocrine in origin and exhibit a well-developed DCV secretory pathway thus, a variety of assays that measure secretion can be preformed. One such assay is the measure in the release of radioactive norepinephrine ([^3]H-NE) from pre-labeled cells which serves as the basis for the measurement of secretion in these cells (Fujita et al., 2007).

2.12 Yeast Two Hybrid Overview

The yeast two hybrid system was initially discovered by Sanley Fields over two decades ago but is still a powerful tool in determining protein-protein interactions (Fields and Song, 1989). The basis of this assay hinges on the GAL4 transcription factor in yeast which is critical galactose metabolism (Brachmann and Boeke, 1997). The intact Gal4 protein is a transcriptional activator which has two domains a DNA binding domain and a transcription activation domain (Rosamond, 1998). The GAL4 transcription factor DNA binding domain binds to the promoter a region situated upstream from the lacZ gene (Rosamond, 1998). Once the transcription activator has bound to the
promoter, it is then able to activate transcription via its activation domain which recruits the transcription machinery. Hence, the activity of a transcription activator requires both a DNA binding domain and an activation domain (Figure 6). Interestingly, the binding domain and the activation domain do not have to be on the same protein. In fact, a protein with a DNA binding domain can activate transcription when bound to another protein containing an activation domain; this principle forms the basis for the yeast two-hybrid technique (Rosamond, 1998). Generally, two fusion proteins are created: the protein of interest (bait) is constructed to have a DNA binding domain attached to its N terminus, and its potential binding partner (prey), which is fused to an activation domain. If the bait protein interacts with the prey protein they will form an intact and functional transcriptional activator. This newly formed transcriptional activator will then go on to transcribe a reporter gene, in our case lacZ which encodes the expression of β-galactosidase. The presence of β-galactosidase can be identified in our assay by using the substrate ortho-Nitrophenyl-β-galactoside (ONPG), this compound is normally colorless, however if β-galactosidase is present, it hydrolyzes the ONPG molecule into galactose and ortho-nitrophenol (Mockli and Auerbach, 2004). Ortho-nitrophenol has a yellow color that can be used to check for enzyme activity by the use of a colorimetric assay (at 420 nm wavelength) (Mockli and Auerbach, 2004). This technique has numerous strong points in that yeast host can be considered an advantage since it has a greater resemblance to higher eukaryotic systems than a system based on a bacterial host (Sobhanifar S, 2003). Moreover, classical biochemical approaches tend to require high quantities of purified proteins or good quality antibodies, however in this technique only the cDNA of the gene of interest is needed (Sobhanifar S, 2003). Also this system can detect weak interactions since there is amplification of the reporter gene response which in itself is a slippery slope (Sobhanifar S, 2003). However, the biggest advantage with this system lies in the fact that it is useful for analysis of known interactions, which then can be tested by the modification important residues and observing this effect on binding leading to discoveries of important domains of interaction (Sobhanifar S, 2003).
A transcriptional activator has two domains a DNA binding domain and a transcription activation domain. The DNA binding domain binds to the promoter a region situated upstream from the gene. The activation domain then recruits the transcription machinery leading to the expression of the desired gene.

Figure 6: Yeast Two Hybrid Assay Overview
3.1 Rationale of Study

The release of catecholamines plays a variety of roles in regulating our bodily functions. Catecholamines such as norepinephrine produce effects such as increased heart rate and blood pressure, dilation of pupils and the respiratory passage as well as narrowing blood vessels in nonessential organs (Rang et al., 2008). Additionally, the catecholamines norepinephrine and dopamine are capable of restoring blood pressure back to normal levels in cases where it has dropped to abnormally low levels (Brown et al., 1992; Floras et al., 1988). Likewise, the regulation of release is critical as over production of catecholamines may lead to abnormally high blood pressure as seen in pheochromocytoma; a tumor originating in the adrenal medulla which results in elevated norepinephrine secretion and hence hypertension (Lenders et al, 2005). Heart failure is a major cause of death in our society today and it is also associated with over-secretion of norepinephrine. Thus, there is a need for a solid foundation in our knowledge in the regulation of catecholamine secretion which in the long run may provide us with the right information to manage the treatment of a variety of medical conditions such as hypertension.

One protein that has been shown to be involved in the regulation catecholamine release is CAPS1 (calcium-dependent activator protein for secretion 1). This protein was initially identified as a cytosolic factor required for the release of norepinephrine in PC12 cells ((Walent et al., 1992). However, as discussed, there have been numerous studies all yielding a variety of actions for CAPS1. CAPS1 has been associated with loading s as well as the docking and priming of dense core vesicles. Moreover, other studies have shown it to be involved in the regulation of synaptic vesicle secretion which further complicates the issue at hand. Perhaps the biggest insight to its function comes from the discovery that CAPS1 interacts with members of the SNARE complex. Through liposome fusion assays CAPS was shown to accelerate liposome fusion and associate with syntaxin-1 containing liposomes (James et al., 2009a). Further studies have shown CAPS to interact with
syntaxin-1 and the specific residues of syntaxin-1 that are involved (Daily et al., 2010). However, it has yet to be shown what residues of CAPS1 are important for syntaxin-1 interaction and moreover, how this interaction effects norepinephrine secretion.

The goal of my project is to elucidate the actions of CAPS1 in terms of a structural-functional relationship with specific emphasis on the CAPS1 syntaxin-1 interaction.
3.2 Hypothesis

The calcium dependent activator protein for secretion 1 (CAPS1) preferentially binds to the open conformation of syntaxin-1 regulating SNARE mediated catecholamine secretion. Moreover, critical mutations that effect this interaction will negatively affect catecholamine secretion.

Aims

Specific Aim 1: To elucidate the molecular mechanisms that controls the physical interaction between CAPS1 and Syntaxin-1

→ Identification of the critical domains and residues in CAPS1 for binding to syntaxin-1

→ Identification of the critical domains and residues in syntaxin-1 for binding to CAPS1

Specific Aim 2: To determine the physiological significance of the CAPS1-Syntaxin-1 interaction in Catecholamine release through analysis of CAPS1 mutants

→ Elucidation of the roles for the Munc Homology (MH) and Dense Core Vesicle Binding (DCVB) Domains of CAPS1 required for syntaxin-1 binding in regulated norepinephrine secretion (NE) secretion using biochemical assays
4. Experimental Procedures

4.1-General Materials—We obtained monoclonal antibodies against CAPS1 from BD Bioscience (Mississauga, Ontario, Canada), syntaxin1 (clone HPC-1) from Sigma (Oakville, Ontario, Canada), and SNAP-25 (clone SMI 81) from Covance (Berkeley, CA); polyclonal antibodies against secretogranin II from QED Bioscience (San Diego, CA). Monoclonal antibody against synaptobrevin2 (Cl69.1) and synaptotagmin1 (Cl41.1) were kind gifts from Dr. Reinhard Jahn (Max Planck Institute for Biophysical Chemistry, Germany). Plasmid pVenus-N1-NPY was a kind gift from Dr. Atsushi Miyawaki (RIKEN, Wako, Saitama, Japan).

4.2- Construction of CAPS1 Knockdown Plasmids—To knockdown the rat CAPS1 gene, we targeted the 19-nucleotide sequence of (residues 464-482, GGACCGATTCCAGGCTTTC) in rat CAPS1. We used TTCAAGAGA as a linker sequence. 64-bp oligonucleotides containing sense and antisense of the target sequences were annealed and subcloned into the BamHI-HindIII sites of pSuper, generating the CAPS1 knockdown plasmid (pSuper-rCAPS1-3). Inserted sequences were verified by sequencing.

4.3- Isolation of Stable CAPS1 Knockdown PC12 Cells—Wildtype PC12 cells (which were kind gifts from Dr. Thomas Martin, University of Wisconsin and Dr. Erik Schweitzer, University of California at Los Angeles) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 5% calf serum, 5% horse serum (both from HyClone, Logan, UT), and penicillin/streptomycin (Sigma). To establish the CAPS1-knockdown clone cells, PC12 cells were co-transfected with non-linearized pSuper-rCAPS1-3 (10 µg) and pBabe-puro (1 µg) by electroporation. We conducted two independent transfections to isolate knockdown clones and control clones. This is to ensure that the phenotypes observed are independent of screening artifacts. Transfected cells were maintained in growth medium containing 2.5 µg/ml puromycin for more than
a month. The growing colonies were picked up with pieces of Whatman paper soaked with Hanks' buffer containing 1 mm EDTA. Isolated colonies were grown in the growth medium without puromycin in 24-well plates. When the cells became confluent, they were transferred into 2 wells of 6-well plates that contained the growth medium with 2.5 µg/ml puromycin. The cells in one of the 6-well plates were subjected to immunoblot analysis using an anti-CAPS1 monoclonal antibody. Once the down-regulation of CAPS1 was confirmed, the cells in the replica wells were transferred into 10-cm dishes containing growth medium with puromycin. These cells were grown, frozen, and kept in a liquid nitrogen tank until use. CAPS1

(Isolation and Knockdown Procedures were conducted previously in the laboratory)

4.4-Expression Construct for the Rescue Experiments—We used mouse CAPS1 cDNA (mKIAA1121, kind gift from Dr. Takahiro Nagase, Kazusa DNA Research Institute, Japan) to generate the expression construct for CAPS1. A 5.4-kb Munl/BamHI fragment from mKIAA1121 clone was subcloned into EcoRI/BamHI site of pCMV5 generating pCMV-mCAPS1-1. mKIAA1121 encodes a long-splice form of CAPS1. To generate a short-splice form (a more common form) of CAPS1, we conducted PCR and generated pCMV-mCAPS1-2. Mouse cDNA (AGACCGGGTTCCAGGCTTTT) differs from rat cDNA (GGACCGATTCCAGGCTTTT) within the knockdown sequence in three bases (indicated by italics). To further protect the mRNA transcripts transcribed from the mouse CAPS1 expression plasmid from being degraded by the anti-CAPS1 RNA interference machineries already induced within the CAPS1 knockdown cells, we introduced three additional silent nucleotide mutations (SNMs) (AGACCGGTTTCAGGCTTTT, underlining indicates SNM) within the target sequence in the CAPS1 gene. But the mutation from G to C is to remove the AgeI site but not to increase silent mutations. The resulting mouse CAPS1 sequence in the expression plasmid (pCMV-mCAPS1(SNM)-2) differs from the rat CAPS1
sequence by five bases (all are SNMs) within the knockdown sequence. pCMVmCAPS1 (SNM-2) was then subcloned into pLVX-IRES-Blast using EcoRI and BamHI restrictions sites to generate pLVX-IB-mCAPS1 (SNM) WT-2. The CMV promoter in pLVX-IB-mCAPS (SNM)1 WT-2 was replaced with a CAG promoter using NdeI and EcoRI restriction sites. Subsequent CAPS1 truncation were generated by PCR using the template pCMVmCAPS1 (SNM-2) and subcloned into pLVX-IB-mCAPS1(SNM) WT-2 with restriction enzymes SpeI-BamHI.

4.5-Lentivirus-mediated Expression of mCAPS1 Wild type and Variants in Knockdown Cells—Clonal lines of CASP1 knockdown cells (KD4) were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 5% calf serum, 5% horse serum (both from HyClone Laboratories, Logan, UT), penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Sigma Chemical), 250 ng/ml amphotericin B (Sigma Chemical), puromycin (2.5 µg/ml) and G418 (700 µg/ml). We have generated the lentivirus-mediated expression constructs of various CAPS1 mutants (and wild type) so that these proteins stably express in the KD4 knockdown cells. The parental expression plasmid was developed by modifying pLVX-IRES-puro. Firstly, the puromycin resistance gene was replaced by a blasticidin resistance gene so that the infected cells could be selected with blasticidin. Secondly, cDNA sequence for emerald green fluorescent protein (EmGFP) was subcloned into BamHI site, generating pLVX-EmGFP-IRES-blast. The CAPS1 knockdown cells that were infected with lentiviruses expressing rescue proteins were selected with blasticidin (5 µg/ml).

4.6-[3H]Noradrenaline Release Assays from PC12 Cells—PC12 cells were plated in 24-well plates; 3-4 days after plating, the cells were labeled with 0.5 µCi of [3H]NA (GE Healthcare, Montreal, Quebec, Canada) in the presence of 0.5 mm ascorbic acid for 12-16 h. The labeled PC12 cells were incubated with the fresh complete Dulbecco's modified Eagle's medium for 1-5 h to remove unincorporated [3H]NA. The cells were washed once with physiological saline solution (PSS)
containing 145 mm NaCl, 5.6 mm KCl, 2.2 mm CaCl₂, 0.5 mm MgCl₂, 5.6 mm glucose, and 15 mm HEPES, pH 7.4, and NA secretion was stimulated with 200 µl of PSS, High K⁺-PSS (containing 81 mm NaCl and 70 mm KCl), or PSS containing 3 nm of α-latrotoxin (α-LTx, Alomone Laboratories, Jerusalem, Israel). Secretion was terminated after a 15-min (High K⁺) or 10-min (α-LTx) incubation at 37 °C by chilling to 0 °C, and samples were centrifuged at 4 °C for 3 min. Supernatants were removed, and the pellets were solubilized in 0.1% Triton X-100 for liquid scintillation counting.

4.7-Yeast Two-Hybrid Assays- mCAPS1 truncations were cut with EcoRI and BamHI and subcloned with, pLexN. A cytoplasmic domain (residues1–261) of rat syntaxin-1A as well as N-terminal syntaxin 1A (1-80) and syntaxin 1B (1-79), were subcloned into EcoRI-BglII site of a prey vector, pVP16-3 (Okamoto and Sudhof, 1997). Full-length wild-type Munc18-1 with SNM (see above) or indicated mutant Munc18-1(SNM) was subcloned into Smal-PstI site of a bait vector, pLexN.

Residues 1181-1736 of Munc13-1) was constructed by amplifying the corresponding DNA fragment from pCMV-Munc13-1 using engineered oligonucleotide primers and subcloning the resulting 1.65-kb fragment into the EcoRI-SalI sites of pLexN ((Hata and Südhof, 1995)). Yeast strain L40 (Vojtek et al., 1993) was transfected with bait and prey vectors by using the lithium acetate method (Schiestl and Gietz, 1989). Transformants were plated on selection plates lacking uracil, tryptophan, and leucine. After 2d of incubation at 30°C, colonies were inoculated into supplemented minimal medium lacking uracil, tryptophan, and leucine and placed in a shaking incubator at 30°C for 2d.

β-β-Galactosidase assays were performed as follows. Yeast cells were chilled on ice and harvested by centrifugation (2000rpm for 5min). The collected yeast cells were resuspended in 250µl of breaking buffer (100mMTris-Cl, pH8.0, 1mM dithiothreitol [DTT], and 20%glycerol). Then, glassbeads (0.45–0.5mm; SigmaChemical) were added to the yeast suspension to a level just below the meniscus of the liquid, followed by 12.5 µl of phenylmethylsulfonyl fluoride stock solution (40mM in 100% isopropanol stored at -20°C). The mixture was then vortexed six times at top speed.
in15-s bursts. After that, another 250 µl of breaking buffer was added, mixed well, and centrifuged for 1 min. The liquid extract was withdrawn and transferred to new tubes. The extracted liquid was further clarified by centrifuging for 15min in a micro-centrifuge. To perform the assay, 80µl of the extract was added to 720µl of Z buffer (60mM Na₂HPO₄, 40mM NaH₂PO₄, 10mM KCl, 1mM MgSO₄, and 2.7ml/l β-mercaptoethanol, pH7.0). The mixture was then incubated in a water bath at room temperature for 5 min. The reaction was initiated by adding 0.16ml of stock solution (4mg/ml o-nitrophenyl-β-D-galactoside in Z buffer; -20°C), and the reaction mixture was incubated at room temperature. The reaction was precisely terminated at the end of 7-min incubation by addition of 0.4ml of 1M Na₂CO₃ stock solution in distilled water, and the optical density of the reaction mixture was measured at 420nm by using a spectrophotometer. At the same time, the protein concentration in the extract was measured using Bradford dye-binding assay. A standard curve was prepared using serial dilutions of BSA dissolved in breaking buffer. 10µL of the extract was added to 1ml of the Bradford reagent (Bio-Rad Laboratories, Hercules, CA), and the blue color formed was measured at 595nm by using a spectrophotometer. The specific activity of β-galactosidase in the extract was calculated according to the following formula: (OD₄₂₀ x 1.36)/[0.0045 x protein concentration [mg/ml] x extract volume [0.08ml] x 7min], where OD₄₂₀ is the optical density of the product o-nitrophenol at 420nm. The factor 1.36 corrects for the reaction volume, and the factor 0.0045 is the optical density of a1nmol/ml solution of o-nitrophenol. The unit of β-galactosidase–specific activity is therefore expressed as nanomoles per minute per milligram of protein.

4.8-Cell preparation for confocal immunofluorescence microscopy—Confocal immunofluorescence microscopy was performed for mCAPS1 KD4 cells that stably express –EmGFP control as well as MCAPS1-WT-2 GFP Sterilized circular glass cover slips (0.25 mm width, 1.8 cm diameter) were placed in 2.2 cm wells within 12-well cell culture plates. The cover slips were then coated for 30 minutes with poly-D-lysine (0.1 mg/ml) at room temperature. Cells were allowed to adhere to the
cover slips overnight and then differentiated on the cover slips for 3-4 days in DMEM containing 100 ng/ml nerve growth factor (NGF) (Sigma), 1% horse serum, 1% calf serum and penicillin/streptomycin. After the differentiation period cells were washed with Phosphate Buffered Saline (PBS). Subsequently, permeabilization of the cells was achieved by incubating for 15 minutes with 0.1% SDS, 0.4% saponin, 1% normal goat serum (NGS), and 1% BSA in PBS. Primary antibodies against EmGFP (rabbit polyclonal, 1:1000 dilution) or rabbit anti-Secretogranin II polyclonal antibody (SgII, 1:1000 dilution) in PBS containing 0.4% saponin, 1% NGS, and 1% BSA were applied overnight to the permeabilized cells. The next day cells were washed three times (10 minutes each time) with PSB containing 0.4% saponin, 1% NGS, and 1% BSA. Secondary antibodies against EmGFP (Alexa-488-conjugated goat anti-rabbit antibody, 1:1000 dilution) and synaptotagmin-1 or Secretogranin II (rhodamine red-x-conjugated goat anti-rabbit antibody, 1:1000 dilution) in PBS containing 0.4% saponin, 1% NGS and 1% BSA were applied for 1 hr in the dark. Cells were then washed three times (10 minutes each time). All samples were mounted onto microscope slides using Fluoromount-G reagent (SouthernBiotech, Birmingham, AL). Immunofluorescence staining was recorded with a Zeiss laser confocal scanning microscope (LSM 510) with an oil immersion objective lens (63x).

**Statistical Analysis**

Statistical significance was calculated where applicable by one way AVOVA or two tailed Student’s t-test. P values of <0.05 were considered significant.
5. Results:

5.1 -CAPS1 Domain Structure and that of Munc13

Previous studies into the function of CAPS1 have linked it as a priming factor involved in dense core vesicle exocytosis (Jockusch et al., 2007). One of the main reasons for this hypothesis is the domain homology of CAPS1 with that of the priming factor Munc13-1 (Figure 7). Munc13-1 knockout mice demonstrate a complete loss of synaptic vesicle priming. Moreover, Munc13-1 and CAPS knockout mice have been shown to exhibit similar priming deficits in hippocampal cultures (Ashery et al., 2000). CAPS1 knockdown pancreatic β-cells expressed a similar phenotype to that of Munc13-1 deficient β-cells in that they both show a reduction of insulin release upon prolonged stimulation (Dong et al., 2007; Speidel et al., 2008). Munc13-1 contains three C2 domains which may function in calcium dependent phospholipid binding, a CI domain that binds to phorbol esters and DAG, and two Munc homology (MH) domains (Rizo and Rosenmund, 2008a; Shin et al., 2010a). The Munc13-1 and CAPS proteins both contain an alpha-helical region termed the "MUN" domain (which contains the MH domain) as well as a C2 domain. The C-terminus of Munc13-1 containing the two MHDs was shown to bind to the N-terminus of syntaxin-1a/1b. Hence, I decided to determine if the similar region of CAPS1 interacted with syntaxin-1 using yeast two hybrid analyses.
Figure 7. Domain structure of Munc13 and CAPS1 as well as the alternative splicing sites in CAPS1. The CAPS1 protein consists of four domains; a C2 calcium dependent phospholipid binding domain, a PIP2 binding pleckstrin homology (PH) domain, a Munc homology (MH) domain and a C-terminal dense core vesicle binding (DCVB) domain. CAPS1 contains numerous alternative splicing sites as well as domain similarity to the priming protein Munc13-1. Like that of CAPS1, Munc13-1 also contains the C2 domain as well as not one but two MH domains.
5.2- Yeast Two Hybrid Analysis: CAPS1-Syntaxin Interaction

To examine if there was an interaction between CAPS1 and syntaxin-1I employed the use of yeast two hybrid analyses. This technique provided a robust method to test if such an interaction existed and allowed for the creation of numerous CAPS1 constructs with various modifications that might effect this interaction.

Firstly, a CAPS1 construct consisting is residues 919-1355 (mCAPS1-1) was created to mimic the region of interaction previously seen with that of Munc13 and N-terminal syntaxin (Figure 8A). Previously, the C-terminal region of Munc13-1 containing its MH domain was shown to bind to the N-terminus of syntaxin 1a/1b through yeast two hybrid analysis (Betz et al., 1997b). The mCAPS1-1 constructs also contains the MH of CAPS1-1 as well as it complete C-terminal dense core vesicle binding (DCVB) domain. This construct (mCAPS1-1) was tested on its ability to bind to full length syntaxin-1 lacking the transmembrane region consisting of residues 1-264. The binding affinity was measured upon the amount of B-galactosidase activity (see experimental procedures). It was determined that this construct did indeed interact with full length syntaxin-1 (lacking the transmembrane region) (Figure 8B). To further clarify the mode of this interaction a N-terminal construct (mCAPS1-2) spanning the C2 domain of CAPS1 consisting of residues 1-493 was created (Figure 8A). However, this region did not show any interaction with syntaxin-1 as no B-galactosidase activity was seen (Figure 8B).

The C-terminus of CAPS1 contains a dense core vesicle binding (DCVB) domain which was previously shown to be important for the localization of CAPS1 with dense core vesicles (Grishanin et al., 2002). Moreover, it was previously shown that a 135 residue truncation of this region resulted in the complete loss of CAPS1 function such that this protein was unable to reconstitute calcium dependent secretion in cracked PC12 cells (Grishanin et al., 2002). To test the importance of the
DCVB domain to syntaxin-1 binding two C-terminal truncations were initially made. The first (mCAPS1-3) was a 70 amino acid truncation consisting of residues 919-1286 (Figure 8A). Additionally, the 135 residue truncation (mCAPS1-4) that was previously shown to effect CAPS1 localization and consisted of amino acids 919-1221 was generated (Figure 8A). Both of these truncations completely abolished the CAPS1 syntaxin-1 interaction (Figure 8B).

Moreover, CAPS1 and Munc13 both contain a MH domain, hence another construct was created to “chop” this region in half (mCAPS1-5) only consisting of residues 1003-1355 (Figure 8A). CAPS1 also has numerous alternatively spliced versions with one splice site being within the MH domain itself (residues 1011-1059). To test if this splicing would affect the syntaxin-1 interaction with CAPS1 I created a spliced version of mCAPS1-5 (residues 1003-1355) termed mCAPS1-5 (+) (Figure 8A). It was seen that both of these constructs interacted with full length syntaxin-1 and interestingly this interaction was stronger than that of mCAPS1-1 which contained the entire MH domain (Figure 8B). Since CAPS1’s function is thought to be related to a priming function I completely removed the MH domain and tested the binding ability to syntaxin-1. Hence, a mCAPS1-6 construct was made which completely removed the MH domain (residues 1114-1355) (Figure 8A). Remarkably, this construct completely abolished the CAPS1 syntaxin-1 interaction (Figure 8B).
Figure 8. CAPS1 binds to syntaxin-1A through the C-terminal region containing part of the MH domain and DCVB domain.

A) CAPS1 truncations were made and tested with their ability to interact with full length syntaxin-1 (residues 1-264) excluding the transmembrane region (TMR).

B) CAPS1-1 containing the MH and DCVB binding domain interacts with syntaxin-1. β-galactosidase activity was used to measure the strength of interaction between the various CAPS1 deletion mutants with syntaxin-1.

Error bars indicate ±S.E. (n = 12)
5.3- Munc18-1 Syntaxin-1 Interaction vs CAPS1 Syntaxin-1 Interaction

The Sec1/Munc18 (SM) protein family is known to be expressed in *C. elegans*, Drosophila yeast, and mammals. There are seven SM proteins expressed in mammals: Munc18-1, -2, -c, sly-1, Vps45, Vps33a and Vps33b (Toonen and Verhage, 2003). Munc18-1 is expressed in neuronal and neuroendocrine cells and is thought to have a variety of functions during the exocytotic process including a syntaxin-1 chaperoning, docking and a priming function (Han et al., 2010c). Munc18-1 is known to bind to syntaxin-1 in its "closed" conformation, where the N-terminal Habc domain of syntaxin-1 folds onto its H3 domain/SNARE motif (Dulubova et al., 1999). All conserved regions of syntaxin-1α are known to interact with Munc18-1: the N-peptide of syntaxin-1 (residues 1-25) binds to the outer surface of Munc18a domain 1 and the Habc, linker, and H3 regions (25-262) interact with the concave surface formed by domains 1 and 3a of Munc18-1 (Han et al., 2010b). Moreover, it was previously shown that a 40 residue C-terminal truncation of syntaxin-1 resulting in residues 1-226 significantly abolished the Munc18-syntaxin-1 interaction (Burkhardt et al., 2008). Using yeast two hybrid analysis I attempted to create syntaxin-1 truncations to confirm these observations and compare it to the CAPS1 binding to syntaxin-1 (Figure 9A). Hence, mCAPS1-5 (residues 1003-1355) which was shown to strongly bind syntaxin-1 and full length Munc18 constructs were tested on their ability to interact with full length syntaxin-1 (lacking TMR) as well as syntaxin-1 C-terminal truncations consisting of residues 1-253, 1-242 and 1-220 (Figure 9A).

It was seen that indeed Munc18-1 does bind to the full length of syntaxin-1 (Figure 9B). Also, further small C-terminal syntaxin-1 truncations resulting in residues 1-253 and 1-242 also did not effect this interaction (Figure 9B). However, a larger truncation of 44 residues (amino acids 1-220) completely abolished Munc18s interaction confirming what was previously shown by Burkhardt et al (Figure 9B). CAPS1 was also tested on its ability to bind to these syntaxin-1 C-terminal truncations. CAPS1 exhibited completely different results from Munc18-1 such that only
full length syntaxin-1 was capable for the binding of CAPS1. Any C-terminal truncations to syntaxin-1 (i.e. Syntaxin-1 residues 1-254, 1-242 and 1-220) completely abolished its interaction with CAPS1 (Figure 9B).
Figure 9. A small deletion at the C-terminal end of syntaxin-1A prior to the transmembrane region abolishes the binding to CAPS1, but not to Munc18-1.

A) mCAPS1-5 and full length Munc18 constructs were tested on their ability to interact with full length syntaxin1 (lacking TMR) as well as syntaxin1 C-terminal truncations residues 1-253, 1-242 and 1-220

B) Munc18-1 binds to the full length syntaxin-1 (lacking TMR) as well as smaller 10 and 20 residue C-terminal truncations. However, there was no interaction with the larger C-terminal truncation. On the other hand, CAPS1 requires full length syntaxin-1 to bind as even a 10 residue truncation completely eliminates its interaction with syntaxin-1. β-galactosidase was used to measure the strength of this interaction. Error bars indicate ±S.E. (n = 12) (p < 0.001)
5.4 CAPS1 vs. Munc18: Binding to “Open” and “Closed” Conformations of Syntaxin-1

Syntaxin-1 is known to exist in two conformational states an “open” and “closed” state. The formation of the SNARE complex requires the conformational switch of syntaxin-1 from the “closed” to the “open” state. This change is thought to be regulated by two proteins; Munc18 and Munc13. It is known that Munc18-1 preferentially binds to the “closed” conformation of syntaxin-1 (Lin and Scheller, 2000; Rizo and Rosenmund, 2008b). Moreover, it was previously shown Munc18-1 binds tightly to, but does not block SNARE complex assembly of, the open syntaxin-1a variant (L165A/E166A:Syx1<sup>LE</sup>) (Figure 10A) (Burkhardt et al., 2008). Hence, I decided to test such interaction and compare it to CAPS1 such that mCAPS1-5 and full length Munc18-1 were tested on their ability to bind to the “open” and “closed” conformations of syntaxin-1 (Figure 10B). It was observed that Munc18 does preferentially bind to the closed conformation of syntaxin-1 but was still capable of binding the “open” conformation (L165A/E166A (Syx1<sup>LE</sup>) syntaxin-1 variant albeit to a significantly lesser degree (Figure 10C). CAPS1 on the other hand bound to the “open” conformation of syntaxin-1 with greater affinity (Figure 10C). Hence, CAPS1 and Munc18-1 have distinct binding modes to syntaxin-1.
Figure 10. CAPS1 preferentially binds to “open” conformation of syntaxin-1 whereas Munc18-1 preferentially binds to “closed” conformation of syntaxin-1.

A) Two different conformation states of syntaxin-1; “closed” and “open”. In the closed states the SNARE complex is unable to form and it is known that Munc18 preferentially binds to syntaxin-1. Munc13 is then thought to displace Munc18 facilitating the open conformation of syntaxin-1 and SNARE complex formation. Adapted from Carr (2001).

B) mCAPS1g5 and full length Munc18 were tested on their ability to bind to the open and closed conformations of syntaxin-1.

C) Munc18 preferentially binds to the closed conformation of syntaxin-1 as the β-galactosidase activity was significantly increased. On the other hand CAPS1 preferentially binds to the open conformation on syntaxin-1. Error bars indicate ±S.E. (n = 12) (p< 0.001)
5.5- Munc13-1 Syntaxin Interaction vs CAPS1-Syntaxin Interaction

Munc13-1 is a priming protein which is thought to promote the disassembly of Munc18-1 from the “closed” syntaxin-1. In essence, this allows syntaxin-1 to bind to the other members of the SNARE complex thereby facilitating membrane fusion. Therefore, to further verify my yeast two hybrid results I analyzed the known Munc13-1 interaction with syntaxin-1 and compared it to the CAPS1 syntaxin-1 interaction. Previously through yeast two hybrid analysis, it was shown that the C-terminal fragment of the Munc13-1 (residues 1181-1735), that includes both MHDs and the C2C domain preferentially bound to the N-terminus of syntaxin-1a (residues 1-80) and syntaxin-1b (residues 1-79) (Basu et al., 2005b; Betz et al., 1997b). To test this, the C-terminal region of Munc18 (residues 1181-1735) and mCAPS1-5 (residues 1003-1355) were tested on their abilities to bind to the N-terminus of synatxin-1a and 1b as well the “open” and “closed” conformations of syntaxin-1 (Figure 11A).

My results confirmed this finding as it was observed that indeed Munc13-1 (1181-1735) did interact with the N-terminus of syntaxin-1a/b (Figure 11B). Moreover, the interaction with syntaxin-1b was stronger than that with syntaxin-1a as previously reported (Betz et al., 1998; Betz et al., 1997b) (Figure 11B). Munc13-1 (1181-1735) did not interact with full length syntaxin-1 or the “open: conformation mutant L165A/E166A:Syx1 LE (Figure 11B). CAPS1 on the other hand did not bind to the N-terminus of syntaxin-1 but instead required full length syntaxin-1 to bind (Figure 11B). Moreover, this interaction is stronger for the open conformation of syntaxin-1 (as previously seen when comparing CAPS1 and Munc18’s interaction). This suggests that although CAPS1 and Munc13-1 have similar domains their mode of syntaxin-1 binding is distinct.
Figure 11. Munc13-1 C-terminal region binds to the N-terminus of syntaxin-1 whereas CAPS1 C-terminal region binds to the entire cytoplasmic domain of syntaxin-1.

A) The C-terminal region of Munc18 (residues 1181-1735) and mCAPS1-5 were tested on their abilities to bind to the N-terminus of syntaxin-1a and 1b as well the open and closed conformations of syntaxin1.

B) Munc18 preferentially binds to the N-terminus of Syntaxin 1a/1b. CAPS1 on the other hand requires the full length syntaxin to bind and preferentially binds to the open conformation of syntaxin. β-galactosidase activity was used to measure the strength of the interactions between Munc13-1 and CAPS1 with syntaxin-1 respectively. Error bars indicate ±S.E. (n = 12) (p <0.001)
5.6- MH Domain Point Mutations did not Effect CAPS1-Syntaxin Interaction

Through yeast two hybrid analysis it was shown that the minimal region of interaction for CAPS1 consists of residues 1003-1355 (mCAPS1-5). This region consists of only a partial region of the Munc homology (MH) domain. Moreover, the yeast two hybrid analysis was confirmed with further comparison of CAPS1 to Munc 18 and Munc13 which yielded similar results to previous studies. Hence, I made five point mutations in the MH domain of CAPS1 that was observed to be required for interaction with syntaxin-1.(Figure 12). These point mutations exist in the underlined region of Figure 12. I had previously made six other point mutations in the MHD but this region was outside the minimal interaction domain I observed (Figure 12). The point mutations in the minimal interacting domain were termed Mutants G-K respectively. These point mutations were made based on the sequence homology between mouse CAPS1 and CAPS2, Drosophila CAPS, C. elegans CAPS (Unc31) and rat Munc 13-1/2/3. Hence, all mutated residues were conserved in all orthologues. Mutant G (D1068R) converts the negatively charged aspartic acid to the positively charged arginine. Mutant H (F1070E) converts the hydrophobic phenylalanine side chain to the acidic negatively charged glutamic acid. Mutant I (L1073A) and J (L1083A) converts the larger hydrophobic leucine to smaller alanine therefore reducing any hydrophobic interaction. Lastly, mutant K (R1097D) converts the positively charged arginine to the acidic negatively charged aspartic acid. Furthermore, mCAPS1-5 wild type and six point mutants. G-H were tested on their ability to bind syntaxin open and closed conformations (Figure 13A) All of these point mutants however did not effect CAPS1 interaction with syntaxin but, there still was the trend of CAPS1 preferentially binding to the open syntaxin (L165A/E166A (Syx1LE) construct (Figure 13B)
Figure 12. Sequence alignment of MH and DCVB domains of CAPS homologues and Munc13 isoforms.

Underline indicates the residues of mouse CAPS1 that are required for binding to syntaxin-1A (mCAPS1g5 construct). The highlight indicates the residue that was mutated and its substituted residue is shown above. Six residues were mutated outside the binding region whereas five residues were mutated inside the binding region. The five residues mutated inside the region of interaction are termed Mutants G-K respectively.

Mutant G (D1068R), Mutant H (F1070E), Mutant I (L1073A), Mutant J (L1083A) and Mutant K (R1097D).
Figure 13: CAPS1 Point Mutants Do Not Significantly Effect Syntaxin-1 Binding

A) mCAPS1-5 wild type and six point mutants Mutant G-H were tested on their ability to bind syntaxin open and closed conformations

B) None of the point mutants significantly reduced the CAPS1 syntaxin-1 interaction as measured with the β-galactosidase activity. However, the trend of CAPS1 preferentially binds to the open conformation of syntaxin was still observed. *Error bars indicate ±S.E. (n = 12)*
5.7- Narrowing Down the CASP1- Syntaxin-1 Interaction: Munc Homology (MH) Domain

Up until now I have shown that residues 1003-1355 (mCAPS1-5) consisting of a partial MH domain are critical for CAPS1 interaction with syntaxin-1 (Figure 8A&B). However, none of the point mutations of the conserved residues in this area significantly reduced the binding to syntaxin-1 (Figure 13A&B). Moreover, complete removal of the MH domain 1114-1355 (mCAPS1-6) completely abolished this interaction (Figure 8&AB). Hence, to narrow down the minimal region of interaction further truncations were made. The strategy employed here was to use the construct with no interaction mCAPS1-6 (1114-1355) and keep adding by a multiple to 10 residues each time hence the following constructs mCAPS1-9 (1064-1113), mCAPS1-110 (1074-1113), mCAPS1-11 (1084-1113), mCAPS1-12 (1094-1113) and mCAPS1-15 (1104-1113) were made (Figure 14A). These constructs were then tested on their ability to bind “open” and “closed” syntaxin-1. Employing yeast two hybrid analysis these truncations all interacted with syntaxin-1 but as hypothesized more strongly with the “open” conformation (Figure 14B). Hence, the new minimum region for CAPS1 interaction with syntaxin-1 now consists of residues 1104-1335.
Figure 14: CAPS1 Minimum Interacting Domain With Syntaxin Excludes the Majority of the Munc13 Homology Domain.

A) Numerous truncations of the Munc13 Homology Domain between mCAPS1-5 and mCAPS1-6 were generated and tested for their ability to interact with syntaxin open and closed conformation

B) The minimum region of CAPS1 required to interact with syntaxin consists of residues 1104-1355 (mCAPS1-15). Complete removal of the Munc13 homology domain (mCAPS1-6) however completely abolishes this interaction as measured with β-galactosidase activity. Error bars indicate ±S.E. (n = 12)
5.8- Narrowing Down the CAP1- Syntaxin Interaction: Dense Core Vesicle Binding Domain

Previously, it was shown that the C-terminal region of CAPS1 was critical for its localization with dense core vesicles as well as its role in the release of norepinephrine from cracked PC12 cells (Grishanin et al., 2002). It was shown that a 135 amino acid truncation of the C-terminus completely abolished the CAPS1 function in secretion as well as DCV localization. In my study, initially it was observed that large C-terminal DCVB domain truncations mCAPS1-3 (Δ70: 919-1286) and mCAPS1-4 (Δ135: 919-1221) completely abolished the interaction between CAPS1 and syntaxin-1 (Figure 8A&B). Hence, to further narrow down the critical region of this DCVB domain smaller C-terminal DCVB domain truncations were generated. Firstly, a 15 amino acid C-terminal truncation termed mCAPS1-13 (Δ15: 1003-1340) was generated. Secondly, a 25 amino acid C-terminal truncation termed mCAPS1-14 (Δ25: 1003-1330) was made. Moreover, these mutants were tested on their ability to interact with full length open and closed syntaxin-1 (Figure 15A). Unlike the larger C-terminal DCVD domain truncations (mCAPS1-3 and mCAPS1-4) which completely abolished the syntaxin-1 interaction the smaller C-terminal DCVB domain truncations mCAPS1-13 and mCAPS1-14 severely but not completely reduced the interaction with syntaxin-1.
Figure 15: CAPS1 DCVB Domain is Critical for its Interaction with Syntaxin

A) Smaller C-terminal truncations were generated mCAPS1-13 (∆15 1003-1340) and mCAPS1-14 (∆25 1003-1330) and tested on their ability to interact with full length open and closed syntaxin-1. These constructs were also compared with the previous C-terminal truncations mCAPS1-3 (∆70 919-1286) and mCAPS1-4 (∆135 919-1221) that demonstrated a complete loss of syntaxin 1 interaction.

B) Unlike the larger C-terminal DCVD domain truncations mCAPS1-3 and mCAPS1-4 which completely abolished the syntaxin-1 interaction the smaller C-terminal DCVB domain truncations mCAPS1-13 and mCAPS1-14 significantly but not completely reduced the interaction with syntaxin-1 as measured with β-galactosidase activity. Error bars indicate ±S.E. (n = 12) (p < 0.0001)
5.9-Rescue of CAPS1 Stable Knock Down Cells

To examine the function of the CAPS1 truncations generated I used stable PC12 lines where the expression of CAPS1 was strongly downregulated by RNA interference and then rescued by the various CAPS1 constructs. These constructs were expressed into secretion deficient CAPS1 knockdown PC12 cells (KD4) via lentivirus mediated infection and selected using blasticidin as CAPS1 knockdown cells were previously generated via a plasmid conferring puromycin resistance. Previously, independent colonies of these CAPS1 knock down cells were successfully isolated and examined for CAPS1 expression (Fujita et al., 2007). It was observed that CAPS1 was successfully downregulated by ~ 90% (Fujita et al., 2007). Moreover, the expression level of other proteins involved in neurosecretion such as synaptotagmin I (a major calcium sensor for secretion), and neuronal SNARE proteins (syntaxin-1, SNAP25 and VAMP2) were tested. There were no changes in the expression levels of these proteins suggesting that the PC12 cells generated were specific for CAPS1 down regulation (Fujita et al., 2007). Moreover, norepinephrine secretion was significantly diminished in these clones in response to stimulation by 70mM KCl (high K⁺).

In order to rescue these CAPS1 knockdown clones CAPS1 was reexpressed using the pLVX plasmid conferring blasticidin resistance and the subsequent cells were grown in a puromycin-blasticidin medium. I found that to get the best expression of CAPS1 the CMV (Cytomegalovirus early enhancer element) promoter was replaced by a CAG (combination of the cytomegalovirus (CMV) early enhancer element and chicken beta-actin promoter) promoter. CAPS1 knockdown clone number 4 (KD4) cells were initially rescued with a GFP control, CAPS1 wild type (WT-2) and a CAPS1 Wild type fused with a C-terminal GFP (WT-2 GFP) (Figure 16). Moreover, the small C-terminal truncations of 15 and 25 residues wild type -13 (WT-13) and wild type -14 (WT-14) respectively were also generated (Figure 16). These truncations severely reduced (but not completely) CAPS1 interaction with syntaxin in the yeast two hybrid analysis (Figure 15A&B). Western blot
analysis of these constructs revealed no change in the expression of the SNARE complex proteins syntaxin1, SNAP25 and VAMP (Figure 16).

Moreover, norepinephrine secretion was analyzed using 70mM KCl (high K\(^+\)) mediated stimulation. The KD4 clones exhibit a secretion defect secreting only 10-15% of total NE upon stimulation (Figure 19). However, upon CAPS1 re-introduction these cells secrete ~30% of total NE resulting a 200% increase in secretion (Figure 19). Hence, CAPS1 wild type (WT-2) and wild type GFP (WT-2 GFP) significantly rescued the secretion defect seen in the CAPS1 knockdown clone 4 (KD4). Additionally, the smaller C-terminal truncations (WT-13 and WT-14) also were able to rescue secretion albeit to a lesser effect of about 25% of total NE. (Figure 20).
**Figure 16: Wild Type and C-Terminal Mutant CAPS1 Rescue Western Blot**

CAPS1 knock down cells (KD4) were rescued with GFP control, wild type CAPS1 (WT-2), wild type CAPS1 GFP (WT-2-GFP), a 15 amino acid C-terminal DCVB domain truncation (WT-13) and a 25 amino acid C-terminal DCVB domain truncation (WT-14). The expression of the neuronal SNARE proteins syntaxin1, SNAP25 and VAMP2 were also tested with the expression being similar to that of wild type PC12 cells. Moreover a GAPDH loading control was also used to test for even loading of the SDS PAGE gel.
5.10- CAPS1 is a Cytosolic Protein

To ensure that the CAPS1 protein is indeed expressing and is cytosolic in nature immunofluorescence experiments were preformed. CAPS1 knockdown (KD4) PC12 cells transfected with GFP control or mCAPS1 WT-GFP were subjected to nerve growth factor (NGF) differentiation for three days and immunofluorescence was preformed with GFP antibody as well as the dense core vesicle marker secretogranin 2 (sg2) antibody. Firstly, control CAPS1 knockdown (KD4) cells transfected with GFP were analyzed. The GFP expression was seen throughout the growth factor (NGF) differentiated PC12 cells including the nucleus (Figure 17A). Subsequently, these cells were stained with the dense core vesicle marker secretogranin 2 (Sg2). Sg2 was expectedly localized at the plasma membrane of these differentiated cells (Figure 17B). Additionally, CAPS1 knockdown PC12 cells (KD4) rescued with mCAPS1 protein tagged with GFP (mCAPS1-GFP) was analyzed. Using GFP antibody it was observed that the CAPS1 protein was cytosolic in nature as previously described in the literature (Figure 18A). Additionally, sg2 was indeed confirmed to be localized to the plasma membrane in these mCAPS1-WT2 GFP cells (Figure 18B).
Figure 17: Immunofluorescence CAPS1 KD4 Cells Transfected with GFP control

A) KD4-GFP control cells were immunostained with GFP antibody. GFP is highly expressed through the whole cell including the nucleus

B) KD4-GFP control cells were immunostained with SGII antibody. Sg2 is highly expressed at the plasma membrane of these cells

C) Merged GFP and SgII image from (B). Sg2 is localized at the plasma membrane of these cells indicating its affinity to DCVs while the GFP is expressed everywhere
Figure 18: Immunofluorescence CAPS1 KD4 Cells Transfected with mCAPS1-WT-GFP

A) KD4 –mCAPS1WT-GFP cells were immunostained with GFP antibody. mCAPS1 is highly expressed in the cytosol

B) KD4 –mCAPS1WT-GFP cells were immunostained with the dense core vesicle marker secretogranin2 (Sg2). Sg2 is highly expressed at the plasma membrane of these cells

C) Merged GFP and SgII image from (B). While CAPS1-GFP is naturally expressed throughout the whole cell excluding the nucleus. Sg2 is localized at the plasma membrane of these cells indicating its affinity to DCVs.
Figure 19: CAPS1 Rescue into KD4 Cells Restores $[^3]H$NE Catecholamine Secretion
CAPS1 knock down cells (KD4) exhibit a neurosecretion defect of catecholamines upon high K$^{+}$ induced stimulation but are rescued by wild type CAPS1/CAPS1-GFP and the C-terminal truncations. There was no significant difference in wild type rescue versus C-terminal truncation rescue (p>0.05). Error bars indicate ±S.E. (n = 9)
6.0- Discussion

The CAPS1 protein was initially discovered as a cytosolic soluble 145kDa protein which was necessary to restore calcium dependent norepinephrine secretion in cracked PC12 cells (Walent et al., 1992). Furthermore, early studies into the function of this protein yielded it as a key regulator in the priming stage of dense core vesicle exocytosis (Ann et al., 1997; Jockusch et al., 2007) (Liu et al., 2008). However, more recent findings suggest that CAPS may also play a role in synaptic vesicle exocytosis as well as in the loading of monoamine neurotransmitters (Brunk et al., 2009; Jockusch et al., 2007).

When sequenced the CAPS protein was found to be similar to the Unc-31 protein of *C. elegans* and it was further confirmed that *Drosophila* express one CAPS protein termed *d*CAPS while mammalian cells have two isoforms CAPS1 and CAPS2 (Ann et al., 1997; Renden et al., 2001). CAPS1 is distributed over much of the brain and neuroendocrine cells while CAPS2 is found in restricted areas of the brain such as the cerebellular granule cells and in other organs such as the lungs, liver, and kidneys (Speidel et al., 2003).

Structural analysis onto the structure of CAPS1 has yielded a protein consisting of almost 1400 amino acids and at least five domains (*Figure 3A*). Firstly, there is a N-terminal dynactin binding domain which as shown to be critical for the proper localization of CAPS2 in the brain (Stevens and Rettig, 2009). Moreover, losses of this domain lead to mice expressing autistic characteristics. Residues 397-492 contain a C2 domain which is thought to function as a calcium dependent phospholipids binding domain similar to those existing in the synaptotagmin family of proteins (Stevens and Rettig, 2009). Additionally, there is a PH domain (residues 520-624) which is known to play a role in the binding of acidic phospholipids with specific selectivity for phosphatidylinositol (4,5) bisphosphate (PIP2) (Stevens and Rettig, 2009). There also exists a Munc
homology domain (MHD) similar to that existing in Munc13. Encompassing the MHD is a MUN region which also includes a domain of unknown function. Munc13 has two MUN domains which CAPS1 contains only one (Figure 7). Munc13 is a large 200kDa protein consisting of three C2 domains, a C1 domain and a MUN region with two MHDs (Ashery et al., 2000). Munc13 is thought to play a role in the priming stage of exocytosis facilitating the opening of syntaxin-1 from its previously closed (Munc18 bound) conformation. Hence, it has been suggested that CAPS1 and Munc13 may play the same or a similar role in dense core vesicle exocytosis. Interesting CAPS DKO chromaffin cells overexpressed with Munc13 failed to restore secretion, suggesting that CAPS and Munc13 may act independently of each other (Liu et al., 2010). Differences also exist in, *Drosophila* dCAPS mutants which display an accumulation of both synaptic vesicles and dense core vesicles per active zone, while dUNC13 mutants only show an accumulation of synaptic vesicles (Renden et al., 2001). Lastly, CAPS1 contains a C-terminal dense core vesicle binding domain which was shown to be critical for CAPS1 in its localization to DCVs as well as its ability to reconstitute calcium dependent secretion of NE in cracked PC12 cells (Grishanin et al., 2002).

Recently, liposome fusion assays have implicated CAPS1 in the binding to syntaxin-1 (James et al., 2009a). CAPS1 was found in the bound fraction of syntaxin-1 containing vesicles. Moreover, they have implicated CAPS1 binding to syntaxin-1 through syntaxin-1’s SNARE/H3 domain and linker region (residues 196-266) (Daily et al., 2010). However, these assays take place in a non-physiological environment hence, they can pick up interactions that may not actually exist or vice versa. Thus, a more physiological way to pick up such interactions is to use the Yeast Two hybrid assay. The yeast host bears a high resemblance to the eukaryotic system and can detect weak transient interactions more often than non-physiological assays. Additionally, protein expression in yeast allows tertiary protein structures to form, provides a neutral internal pH, and produces the disulfide bonds and other post-translational modifications common to other eukaryotes. These
features are important for maintaining native protein structures to promote authentic protein-protein interactions. Moreover, the strength of such interaction can easily be quantified using β-galactosidase activity assay and most importantly it allows for the modification of important residues in testing known interactions.

6.1- CAPS1-Syntaxin-1 Interaction : MHD Domain

CAPS1 contains a MUN region containing a MHD and a C2 domain like that of Munc13-1 (Stevens and Rettig, 2009). Using Yeast two hybrid assay CAPS1 truncations were generated and tested for their ability to interact with the full length syntaxin (1-264) lacking the transmembrane region. B-galactosidase activity was measured as an indication of the strength of interaction. Initially, it was hypothesized that CAPS1 may interact with syntaxin-1 through its MHD like that of Munc13. Indeed, it is seen that CAPS1 interacts with syntaxin-1 through a region consisting of the MHD and full DCVB domain (mCAPS1-1) (Figure 8A&B). However, complete removal of the MHD (mCAPS1-6) abolished the interaction with syntaxin-1 (no B-galactosidase activity like that of the empty vector control) (Figure 8A&B). Interestingly however, removal of the first 68 residues in the MHD (mCAPS1-5) increase the interaction with syntaxin-1, indicating that the first half of this region is not critical for CAPS1 binding to syntaxin-1 independent of the spliced or un-spliced version of CAPS1 (Figure 8A&B). Additionally, the N-terminal C2 region of CAPS1 (mCAPS1-2) does not play any role in this interaction (Figure 8A&B).

To further narrow the region of interaction in the MHD required for interaction further truncations were made in-between amino acids 1003-1114. Unexpectedly, it was determined that the majority of the MHD can be removed before there is no interaction (Figure 14A&B). More importantly, the minimal residues required for interaction was determined to be 1104-1355 which excludes all but the final 10 amino acids in the MHD. Hence, the very distal end of the MH domain
seems to be critical for this interaction as complete removal (mCAPS1-6; residues 1114-1355) results in a complete loss of any interaction (Figure 8A&B). Thus, unlike Munc13 which requires its own MH domains for interaction with syntaxin-1 this region in CAPS1 does not seem to be as essential. This indicates that CAPS1 and Munc13-1 have distinct roles in the secretion process. This hypothesis is further verified by previous studies which show that Munc13 is unable to rescue secretion in CAPS DKD chromaffin cells, thus indicating distinct functions.

6.2- CAPS1 Point Mutations did not Effect Syntaxin-1 Interaction

Initially, the residues 1003-1355 were shown to be critical for CAPS1 interaction with syntaxin-1 (Figure 8A&B). Moreover, this region still contained a small portion of the MH domain. Thus using sequence analysis a homology map of the MH domain between mouse CAPS1/CAPS2, dCAPS, C. elegans (unc31) CAPS, and rat Munc13-1,2 and 3 was generated (Figure 12). Five amino acids were seen to be conserved between all organisms; D1068, F1070, L1073, L1083 and R1079. Subsequently, five individual point mutations were made D1068R, F1070E, L1073A, L1083A and R1097D modifying charged residues to oppositely charged ones such as the conversion of aspartic acid (D) to arginine (R) and vice versa or adding a charged reside in the case of phenylalanine (F) to glutamic acid (E). Leucine (L) was also converted to alanine (A) reducing the hydrophobic bonding ability. These mutants termed G-K respectively all interacted with full length syntaxin-1 (Figure 13A&B). However, there is still a trend of open syntaxin-1 proving to have a stronger interaction than that of the closed version of syntaxin-1. It is not surprising that these point mutations did not affect CAPS1 interaction with syntaxin-1 since further analysis revealed that the minimal region required for CAPS syntaxin-1 interaction lies in-between residues 1104-1355 (Figure 14A&B).

6.3 -CAPS1-Syntaxin-1 Interaction : DCVB Domain
The C-terminal DCVB domain was previously shown to be critical for CAPS localization with DCV and rescue of DCV secretion (Grishanin et al., 2002). A 135 amino acid C-terminal CAPS1 truncation abolish CAPS1 ability to localize with synaptotagmin-1 on DCVs (Grishanin et al., 2002). Moreover, this truncation mutant was unable to reconstitute calcium dependent NE secretion in PC12 cells. To add to such results, C-terminal truncations of 70 amino acids (mCAPS1-3) and 135 (mCAPS1-4) amino acids were respectively generated (Figure 8A&B). These truncations completely abolished the CAPS1 interaction with syntaxin-1. Additionally, to further narrow down the critical residues in the DCVB domain smaller C-terminal truncations of 15 (mCAPS1-13) and 25 (mCAPS1-14) amino acids were respectively made. Interestingly, these truncations severely but not completely abolished the interaction between CAPS1 and syntaxin-1 (Figure 15A&B). This indicates that the DCVD domain is indeed critical for CAPS1 interaction with syntaxin-1. The physiological mechanism of this interaction was partially addressed in the NE secretion assay conducted on the wild type and small C-terminal truncations. It was seen that there is not a significant decrease in the high potassium medicated secretion compared to wild type CAPS1/CAPS1-EmGFP. However, this may be due to the fact that there is still an interaction with syntaxin-1 that albeit weak is still sufficient to promote dense core vesicle secretion. To address this further, the larger C-terminal truncations (mCAPS1-3 and mCAPS1-4) need to be expressed in the KD4 PC12 cells and then tested on their ability to rescue high potassium mediated secretion. Since CAPS1 is thought to exist as a dimer in physiological conditions is it possible that one CAPS1 protein may indeed bind to the dense core vesicle using its C-terminal dense core vesicle binding domain, while the other CAPS1 protein may bind to syntaxin-1 through a similar C-terminal domain thus serving as a bridge-like structure (Fig 20) which may facilitate dense core vesicle fusion.

6.4- Munc13-1 and CAPS1 interaction with Syntaxin-1
The Munc13 protein family consists of four members: Munc13-1, -2, -3 and -4. Munc13-1, -2 and -3 are expressed primarily in neurons in the brain (Augustin et al., 1999a; Koch et al., 2000). Munc13-1 is a relatively large protein about ~200kDa in size that contains three C2 domains (C2A, C2B and C2C), a CI domain and a MUN domain. The N-terminal C2 domain is thought to be important for Munc13-1 homodimerization and binding to the Rab Interacting Molecule (RIM) family of proteins (Lu et al., 2006). It is thought that that homodimerization of Munc13 inhibits its priming function, and RIMs activate priming by disrupting Munc13 homodimerization (Lu et al., 2006). The CI domain is known to bind diacylglycerol and phorbol esters with high affinity as mutations in this domain impair neurotransmitter secretion (Betz et al., 1998). Work on the C. elegans orthologue, unc-13, using yeast two-hybrid studies revealed an interaction between the N-terminus of syntaxin-1 (1-80 Syx1a/1-79 Syx1b) and a C-terminal fragment of the Munc13-1 (l181-1735), that includes both MHDs and the C2C domain (Betz et al., 1997). This C-terminal construct of Munc13-1 (l181-1735) was analyzed along with CAPS1 (residues 1103-1355) on their ability to bind N terminal syntaxin (1-80 Syx1a/1-79 Syx1b) or full length “open” and “closed: syntaxin-1. Munc13 (through an N-terminal interaction) is thought to displace Munc18 from syntaxin-1 causing it to “open” up and facilitate SNARE complex formation. Previously, it was shown that a constitutively "open" syntaxin-1 mutant was able to rescue vesicle exocytosis in C. elegans lacking the Munc13 homologue UNC-13 (Richmond et al., 2001). This suggested that Unc13/Munc13 may "open" syntaxin-1 for SNARE complex assembly. This open conformation can be generated by a L165A/E166A (LE) double mutant which removes the critical hydrophobic contact between the Habc and H3 domain yet maintaining proper stability of the protein. (Burkhardt et al., 2008)

The results show that Munc13 indeed does favour the binding to the N-terminus of syntaxin-1a/1b as previously shown (Figure 11A&B). Moreover, Munc13 does not interact with the full length open or closed versions of syntaxin-1 (Figure 11A&B). Perhaps, the folding of the full
syntaxin protein blocks the N-terminal binding site for Munc13-1. However, when syntaxin-1 is bound to Munc18-1 this site may become available allowing Munc13-1 to bind and promote the open conformation of syntaxin-1 and subsequent SNARE mediates vesicle fusion. On the other hand, CAPS1 requires the full length of syntaxin-1 to bind and is unable to bind to the N-terminal truncations’ of syntaxin-1a/1b. CAPS1 was shown to require residues 1-264 of syntaxin-1 to bind and even a 10 residue deletion (1-254) completely abolished this interaction, hence it is not surprising that CAPS1 is unable to bind to the N-terminus of syntaxin-1 alone. The fact that CAPS1 preferentially binds to the open conformation of syntaxin-1 suggest that it functions downstream of Munc13-1 binding possibly stabilizing the DCV- plasma membrane SNARE protein complex.

Hence, CAPS1 and Munc13 have distinct binding modes to syntaxin-1. I have confirmed the N-terminal binding mode of Munc13 but have added that CAPS1 requires full length syntaxin-1 and cannot bind to the N-terminus alone. Moreover, CAPS1 preferentially binds to the “open” conformation of syntaxin-1 as quantitatively seen in the increased β-galactosidase activity while Munc13 is unable to bind to either full length “open” or “closed” syntaxin-1 (Figure 11A&B).

6.5- Munc18 and CAPS1 Interaction with Syntaxin-1

Munc18 contains three domains (1,2a/2b,3a) characterized my α-helices and β-sheets (Figure 4) (Rizo and Rosenmund, 2008a). It has been shown that all conserved regions of syntaxin-1 interact with Munc18-1 (Figure 4) (Burkhardt et al., 2008). It was also shown that a C-terminal deletion of 36 amino acids from syntaxin-1 (1-226) significantly weakens the syntaxin-Munc18 interaction (Burkhardt et al., 2008). Interestingly, Munc18 has been shown to bind to the open conformation (LE mutant) version of syntaxin-1 however it preferentially binds to the closed conformation of syntaxin-1 (Han et al., 2010a). Munc18 is thought to act as a chaperone binding to the closed conformation of syntaxin-1 and guiding it to the plasma membrane preventing ectopic SNARE
conformations (Medine et al., 2007). Moreover, studies indicate that Munc18-1 is a neuron-specific member of a Sec-1/Munc18 (SM) family of proteins which play essential roles in priming reactions for neuronal and endocrine vesicle exocytosis (Rizo and Rosenmund, 2008; Toonen and Verhage, 2007; Wojcik and Brose, 2007). Munc18-1 strongly binds to full length syntaxin-1 as well as smaller C-terminal truncations (1-252 and 1-242) however a 44 amino acid truncation (1-220) completely abolishes this interaction (Figure 10A&B). This agrees with the previous findings in that a C-terminal deletion of 36 amino acids from syntaxin-1 (1-226) significantly weakens the syntaxin-1 Munc18 interaction. More importantly, CAPS1 requires full length syntaxin-1 to bind and any such C-terminal truncations completely abolishes the binding (Figure 10A&B). Hence, these finding indicate that CAPS1 and Munc18 also have different binding modes in that Munc18 preferentially binds to the “closed” conformation of syntaxin-1 while CAPS1 preferentially binds to the “open” conformation. Additionally, Munc18-1 does not require full length syntaxin-1 (residues 1-264) to bind like CAPS1 does instead resides 1-242 are sufficient for such an interaction to take place (Figure 10A&B). The fact that CAPS1 requires full length syntaxin-1 to bind is an important point however, there still is the possibility that the binding ends somewhere between the residues 254-264. Hence, further analysis into where the exact binding occurs needs to be elucidated. Additionally, it is unclear at what stage of the exocytotic CAPS1 may bind syntaxin-1, this binding may occur after Munc13-1 has displaced Munc18-1 from syntaxin-1 thus promoting the “open” conformational state. CAPS1 may then act downstream of Munc13 by binding to the “open” syntaxin-1 and facilitating DCV exocytosis. Recently, it has been shown that the “open” conformation of syntaxin-1 is able to out-compete closed syntaxin-1 for CAPS1 binding through liposome fusion assays. To further study the effect of such a relation I tested all the regions of CAPS1 known to interact with syntaxin-1 on their ability to bind “closed” or “open” syntaxin-1. In every case the interaction between open syntaxin-1 and CAPS1 was approximately two times
stronger as measure by the B-galactosidase activity (Figures 10/11/13). This suggests that indeed there is a preference for CAPS1 to interact with the open conformation of syntaxin-1 possible downstream of Munc13 and facilitating the formation of the SNARE machinery and vesicular release.

The question now arises whether CAPS1 regulates the assembly of the SNARE complex, as it has been shown to favour the open conformation of syntaxin-1 for binding. CAPS1 may promote the syntaxin-1 SNARE complex formation or possibly bind syntaxin-1 and inhibit SNARE complex formation. Preliminary studies using GST pulldown assay with brain homogenate and mCAPS1-1 (residues 919-1355) were conducted to test this. If CAPS1 is only able to pull down syntaxin-1 alone this suggests it may inhibit SNARE complex formation. On the other hand, if all members of the SNARE complex are pulled-down it suggests that CAPS1 does not interfere with SNARE complex assembly. Essentially, CAPS1-1 was shown to pull down all members of the SNARE complex (syntaxin-1, VAMP-2 and SNAP25) (Data not shown). This indicates that CAPS1 may bind to these proteins as well, or that it does not interfere with SNARE complex formation. Hence, CAPS1 may regulate the membrane fusion process after the SNARE complex assembly.

6.6- CAPS1 Wild Type and C-terminal Truncation Rescue in KD4 PC12 Cells

To examine the function of these CAPS1 mutants, stable PC12 cell lines in which the expression of CAPS1 was strongly downregulated by RNA interference was used. These cells were co-transfected with a short hairpin RNA against rat CAPS1 and a plasmid conferring puromycin resistance (Fujita et al., 2007). Subsequently, using these previously generated cells rescue experiments were preformed such that wild type CAPS1 (WT-2) or CAPS1 mutants plasmids conferring blasticidin resistance were transfected and then selected with puromycin-blasticidin medium. These cells were selected and western blot was preformed testing for CAPS1 expression
(Figure 16). During the selection process it was observed that the cytomegalovirus (CMV) promoter in the pLVX vector system did not yield satisfactory expression of CAPS1. Hence, a CAG (combination of the cytomegalovirus (CMV) early enhancer element and chicken beta-actin promoter) promoter version of these constructs was generated. The CAG promoter has been shown to promote stable expression in progenitor embryonic stem cells (Alexopoulou et al., 2008). Moreover, the CAG promoter is a very strong and ubiquitous promoter as comparison analysis has shown that the CAG promoter is more efficient than the CMV promoter (Alexopoulou, Couchman et al. 2008). Using the CAG promoter yielded better expression levels which allowed for other data to be collected.

Norepinephrine secretion was performed using physiological saline solution (PSS) and high potassium (70mM KCl) stimulated secretion. It was determine that both CAPS1 and CAPS1-GFP was able to rescue secretion in these knockdown cells. Upon CAPS1 re-introduction these cells secrete ~30% of total NE resulting a 200% increase in secretion compared to the knockdown cells transfected with GFP alone. The C-terminal truncations of Δ15 (mCAPS1WT-13) and Δ25 (mCAPS1WT-14) residues respectively were also tested (Figure 16). These cells were also able to rescue the secretion defect to levels almost on par with wild type CAPS1 secreting ~25% of total NE (Figure 19). This may be due to the fact that their interaction with syntaxin-1 although severely reduced is still there facilitating the promotion of the fusion machinery and vesicle release (Figure 15A&B). The fact that small C-terminal CAPS1 truncations were still able to rescue while their ability to bind syntaxin-1 significantly decreased leaves some unanswered questions. In order to fully address this issue the larger C-terminal truncations that completely abolish the syntaxin-1 interaction must be transfected and stably isolated in these CAPS1 KD4 cells. Then it would be possible to test if secretion is indeed rescued when the CAPS1-syntaxin-1 interaction is completely abolished.
6.7-Possible Model of CAPS1 Function

CAPS1 (~145 kDa) is thought to exist as a dimer as it was shown through gel filtration and sucrose gradient centrifugation that this protein exists in ~300kDa conformation under native conditions (Walent, Porter et al. 1992). Additionally, through mass spectrometry analysis, protein Kinase CK2 was also shown to phosphorylate CAPS in vitro (Nojiri, Loyet et al. 2009). This phosphorylation is thought to promote the formation of the dimer (~300kDa) (Nojiri et al., 2009). Interestingly, CAPS1 de-phosphorylation was shown to promote a tetramer formation, additionally de-phosphorylated CAPS1 is unable to rescue catecholamine secretion in cracked PC12 cells possibly due to this tetramer formation (Nojiri et al., 2009). Hence, CAPS1 may function such that in its dimer state one CAPS1 protein binds to the dense core vesicle via its DCVB domain while the other CAPS1 facilitates an interaction with syntaxin-1 such that CAPS1 may function as a bridge between secretory vesicles and the plasma membrane, which may facilitate exocytotic membrane fusion.
Figure 20: Possible Model of CAPS1 Function
CAPS1 binds to secretory vesicles via DCVB-domain. CAPS1 also binds to syntaxin-1 at the plasma membrane via MH- and DCVB-domains. The dimerization of the CAPS1 at the secretory vesicles and CAPS1 near plasma membrane may function as a bridge between secretory vesicles and the plasma membrane, which facilitates exocytotic membrane fusion.

6.8- Summary
The minimal region of CAPS1 required for strong syntaxin-1 interaction consists of amino acids 1104-1355 which includes only very small portion of the MHD (last 10 residues) but requires the majority of the DCVB domain. Therefore, it can be seen that the full MHD is not necessary for CAPS1 and syntaxin-1 interaction. This indicates that CAPS1 and Munc13-1 having a similar function due to their sequence homology is not the case. CAPS1 and Munc13-1 have different binding modes to syntaxin-1 and likewise may have independent functions. This is clearly evident as both proteins fail to cross-rescue each other in their respective knock out chromaffin cells (Jockusch et al., 2007). That is in CAPS1 knock out chromaffin cells, over-expressing Munc13-1 fails to rescue and likewise, vice versa (Jockusch et al., 2007). Additionally, Munc18-1 and CAPS1 also have different binding modes. Unlike Munc18, CAPS1 preferentially binds to the open conformation of syntaxin-1. Hence, CAPS1 may possibly act downstream of Munc13 after it has converted syntaxin-1 to the open conformation. Moreover, the C-terminal DCVB domain does indeed seem to be critical for both CAPS1-syntaxin interaction as well as CAPS1’s ability to promote NE secretion. Hence, CAPS1 may function as a bridge between the secretory vesicle and the SNARE complex and thus promoting exocytotic membrane fusion (Figure 20).

6.9- Relationship with Previous Studies

CAPS1 has been shown to interact with the SNARE complex in particular to syntaxin-1. It was shown through liposome fusion assays that CAPS facilitates the formation of the trans-SNARE complex through its interaction with syntaxin-1 (James et al., 2009a). Moreover, when the fused liposomes were subjected to buoyant density gradient separation CAPS1 was retained on the liposomes that contained syntaxin-1/SNAP 25 but not the ones that contained VAMP2 (James, Kowalchyk et al. 2009). Also, liposomes containing only syntaxin-1 were also shown to retain CAPS1 indicating that CAPS is a syntaxin-1 binding protein (James, Kowalchyk et al. 2009). More recently, CAPS was shown to bind to syntaxin-1 and SNAP25 with high affinity and VAMP2 with
lower affinity (Daily et al., 2010). The interaction with syntaxin-1 was shown to strongly be
mediated through syntaxin-1 SNARE (H3) and linker domain (amino acids 191-266) (Daily,
Boswell et al. 2010). Liposomes containing syntaxin residues 1-254 and 1-177 only weakly
interacted with CAPS1. Additionally, CAPS failed to interact with the closed full length syntaxin-1
and with the N-terminus region up to the SNARE domain (amino acids 1-177) (Daily, Boswell et al.
2010). Moreover, it was also isoform specific as syntaxin isoforms 1, 2 and 4 interacted with CAPS
while isoforms 3 and 6 did not. These previous studies mainly used liposome fusion assays which in
itself are not physiological in nature.

Just this month a high impact factor paper published in the journal Cell Metabolism suggests
that the CAPS1 Munc13 homology domain (MHD) is indeed the site that mediates SNARE binding
and priming of vesicle exocytosis. It was suggested that the corresponding amino acids ~972-1040 in
the MHD of CAPS1 is required for t-SNARE binding as well as VAMP2 binding (Khodthong et al.,
2011). Through GST pull down assays this region was shown to bind to syntaxin-1, SNAP 25 and
VAMP2. Moreover, a deletion mutant CAPS (∆972-1040) abolished CAPS1 rescue in permeabilized
PC12 cells. In addition, the number of exocytotic events as measured through TIRF microscopy was
significantly reduced in CAPS1 KD cells but could be rescued by wild type CAPS1 but not the
deletion mutant CAPS (∆972-1040) (Khodthong et al., 2011). Hence, CAPS1 binding is similar to
Munc13-1 as both their MHD’s are thought to be important for syntaxin-1 interaction. It is unclear
however; if CAPS1 has the similar function to Munc13-1 since their binding modes to syntaxin-1
seem to be located in their respective MH domains. This new finding differs from my results as I
demonstrate that the CAPS1 binding to syntaxin-1 is largely independent of its MH domain.
Moreover, I show that the binding to syntaxin-1 is largely dependent on the C-terminal DCVB
domain.

6.10- Strengths & Limitations
The basis of my work lays in the use of the yeast two hybrid technique to study protein-protein interaction. This technique allowed me to further characterize CAPS1-syntaxin binding in terms of the critical residues of CAPS1 involved. This technique has numerous strong points in that yeast host can be considered an advantage since it has a greater resemblance to higher eukaryotic systems and is physiological in nature unlike liposome fusion assays (Sobhanifar S, 2003). Moreover, classical biochemical approaches tend to require high quantities of purified proteins or good quality antibodies, however in this technique only the cDNA of the gene of interest is needed (Sobhanifar S, 2003). Also, this system can detect weak interactions like that of CAPS1 and syntaxin-1 since there is amplification of the reporter gene response which in itself is a slippery slope as false positives may occur (Sobhanifar S, 2003). However, the biggest advantage with this system lies in the fact that it is useful for analysis of known interactions, which then can be tested by the modification important residues and observing this effect on binding leading to discoveries of important domains of interaction (Sobhanifar S, 2003). This was specifically applied in my study as the critical domain of CAPS1 and syntaxin-1 were analyzed in order to determine the region of interaction.

The main problem with the yeast two hybrid assay is the chance of false positives. This is why appropriate controls (empty vector) were done for each assay as well as numerous repetitions. Additionally, the transmembrane regions of proteins were removed to allow for greater translocation into the nucleus which in itself is another drawback since large proteins may not be expressed well. Also, I tested the interaction of proteins known to bind in literature and confirmed their binding modes to be similar to what has been published before. However, negative results in these assays may not always be the case, such that protein expression level may come into play or even post-transcriptional modifications which may or may not occur in yeast however may be critical in
mammalian systems for protein interaction. Additionally, to verify the yeast two hybrid assay further biochemical techniques should be done in order to confirm the CAPS-1 syntaxin-1 interaction.

PC12 cells also are a critical part of my work. The advantage of PC12 cell lines is that is has been used extensively in the study of neurosecretion. Moreover, they serve as a good model for primary neuronal cells. PC12 cells have the ability to reversibly differentiate into neuronal like cells in response to nerve growth factor (NGF) (Huff and Guroff, 1979). Undifferentiated PC12 cells are like that of immature adrenal chromaffin cells in many ways more specifically in their ability to secrete neurotransmitters. Additionally, neurotransmitter release from DCVs in these cells can be regulated by calcium and GTP dependent exocytotic pathways hence, PC12 cells serve as a good model for studying regulated neurotransmitter release.

6.11 Application and Significance of CAPS1

These results shed new light onto the function of the CAPS1 protein in its binding to syntaxin-1. The exact function of CAPS1 is still highly debatable with studies showing an importance in stages such as priming and fusion in dense core vesicle release (Liu et al., 2008). Recent studies have implicated CAPS1 in the release of synaptic vesicles as well as vesicle loading (Brunk et al., 2009). Hence, there is a need to clarify the actions of CAPS1 and the mechanisms behind it. The release of vesicles containing neurotransmitters and modulators are essential for our survival as they allows us to adapt to our environment, hence it is important to understand how this actually occurs. CAPS1 knockout mice die within minutes after birth indicating the importance of this protein for survival (Speidel et al., 2005). Since CAPS1 has been shown to be essential for calcium dependent exocytosis there are countless of areas where it may be important. The release of neurotransmitters serves as the basis for cell to cell communication. CAPS1 has been shown to be critical for the release of catecholamines which are largely involved in our response to stress.
Additionally, neuromodulators such as epinephrine have been used to treat a variety of conditions. Patients suffering from cardiac arrest may be administered epinephrine to increase peripheral resistance and to increase cardiac output (Brown et al., 1992; Floras et al., 1988). Additionally, as a result of its vasoconstrictive effects patients suffering from allergic reactions may also be given epinephrine to reduce any immune reaction to a specific allergen (Omar et al., 2011). The neurotransmitter dopamine is also secreted by dense core vesicles. The release of dopamine has been implicated in Parkinson’s disease as it is required for normal bodily movement (Heisters, 2011). Additionally, the CAPS1 protein has been shown to play a modulatory role in the release of insulin. Mice lacking CAPS1 were shown to be glucose intolerant as a result of a reduction of glucose induced insulin secretion (Speidel et al., 2008). In the next 15 years, it is anticipated that the global incidence of type 2 diabetes in children will increase by up to 50% not to mention the 1 in 17 adults who already suffer from this condition (Canadian Diabetes Association, 2011). Hence, it is important to determine the specific role that CAPS1 plays in the exocytotic process. Interestingly, since CAPS1 is found throughout the brain, the pancreas and at low levels in the heart it is possible that it is playing a conserved role in these organs.

The CAPS2 isoform is thought to have the same function as CAPS1 as they share 95% sequence similarity, with CAPS2 being expressed in areas such as the lungs, liver, testis and regional brain areas (Speidel et al., 2003). Interestingly, it is thought that deletion of exon 3 from the CAPS2 gene may cause susceptibility to autism. In one study where 16 autistic patients from which mRNA was isolated, four were missing exon 3 of CAPS2 (Sadakata et al., 2007c). Moreover, of the 24 control subjects in the experiment, none were missing exon 3 of CAPS2. Additionally, CAPS-2 knockout mice exhibit autistic-like phenotypes and fail to secrete BDNF from granule cells in the cerebellum (Sadakata et al., 2007a; Sadakata et al, 2007b; Sadakata et al., 2007c). CAPS2 is also a dense-core vesicle-associated protein that has been implicated in the release of brain-derived
neurotrophic factor (BDNF) (Shinoda, Sadakata et al. 2011). BDNF has a critical role in neuronal survival and development and is also associated with the development of inhibitory neurons and their circuits (Shinoda, Sadakata et al. 2011). Hence, CAPS2 has been shown to be critical for the development of inhibitory hippocampal GABAergic networks (Shinoda et al., 2011). Therefore, it is easy to see that the CAPS family of proteins are indeed essential for a variety of functions in the body and understanding how this protein functions will not only serve to increase our understanding of disease mechanisms but it may also lead to the development of treatments of such conditions as well.

6.12- Future Directions

In order to strengthen my work, further analysis into confirming the yeast two hybrid results must be done. Yeast two-hybrid assay is an unbiased method that can examine many interactions simultaneously and provides more in vivo like environment for binding interaction compared with the biochemical binding experiments. Nonetheless, this assay has a weakness in that it cannot confirm the expression of the introduced proteins inside the yeast or their transport to the yeast nucleus. Thus, negative interaction results could be due to the poor expression of the protein or its transport to the nucleus. Thus, the results have to be confirmed by the biochemical binding experiments. More specifically, using GST pull down assays of the various truncations is a top priority. In this assay, recombinant CAPS1 proteins will be made consisting of full length mCAPS1-1 (residues 919-1355) and mCAPS1-12 (residues 1094-1355) as well as the Δ25 (residues 919-1330/1094-1330), Δ70 (residues 919-1340/1094-1340) and Δ135 (residues 919-1221/1094-1221) C-terminal truncations (that were shown to decrease syntaxin-1 interaction) of these constructs will be generated. These recombinant proteins will then be tested on their ability to interact with the SNARE family of proteins. This will then be compared to yeast two hybrid assay where it was seen that the
smaller C-terminal truncation (Δ25) significantly but not completely abolished the CAPS1-syntaxin-1 interaction while the larger truncations Δ70 and Δ135 completely abolished this interaction.

Moreover, the questions of whether these truncation mutants are folded correctly also comes into play. Hence, there is a need to confirmation that this in indeed the case. In order to address this, biophysical protein folding assays must be carried out or collaborated with groups specializing in this field. Briefly, Circular Dichroism (CD) Spectroscopy is a technique often used to analyze the secondary structure of proteins (Whitmore and Wallace, 2008).

Additionally, further immunofluorescence studies will be carried out on the CAPS1 truncation mutants. These studies will focus on how the CAPS1 dense core vesicle binding (DCVB) domain truncations effect the localization of CAPS1. Once the larger C-terminal truncations are expressed in the KD4 PC12 cells immunofluorescence can be preformed into the localization of these mutants compared to wild type CAPS1. More specifically, the co-localization with the dense core vesicle marker Sg2 will be analyzed to see if indeed there is a reduction in the co-localization. Lastly, more physiological analysis on the functional roles of these truncation mutants will be conducted. These studies will mainly center on the PSS vs. High K⁺ secretion assay to test if there is indeed a physiological significance to the complex abolishment of the CAPS1-syntaxin-1 interaction. These studies will no doubt shed more light on the exact function of CAPS1 in the secretion of catecholamines from dense core vesicles.
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