

RE-LOCALIZATION OF SNARE PROTEINS IN MOUSE SPERM PRIOR TO THE
ACROSOME REACTION

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THESIS

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ABSTRACT

The principal role of SNARE proteins is to arbitrate vesicle fusion to a target membrane. Formation of tripartite SNARE protein complexes between SNARE proteins on opposing membranes is the minimal requirement for membrane fusion. The SNARE protein family is large, consisting of more than 60 members. A member of the SNARE family, syntaxin, is found on the sperm plasma membrane while synaptobrevin, is found on the outer acrosomal membrane. During the sperm acrosome reaction, the outer acrosomal membrane fuses at hundreds of points with the overlying plasma membrane, resulting in release of the acrosomal contents. We hypothesize that syntaxin and synaptobrevin re-localize within the sperm plasma membrane prior to the acrosome reaction to form SNARE complexes and promote membrane fusion at hundreds of specific points. Immunofluorescence was used to localize both syntaxin and synaptobrevin in mouse epididymal sperm before and after capacitation. Sperm were fixed and incubated with antibodies to syntaxin, synaptobrevin and then fluorescent secondary antibodies. Super resolution Structured Illumination Microscopy (SR-SIM) was used to examine samples collected at 0, 10, 30, 60, and 120 min of capacitation time, to obtain 3D images of SNARE localization. Quantification of the images was completed using western blotting and by image analysis, using IMARIS, which interpreted the total syntaxin and synaptobrevin positive volume. Results showed that syntaxin-positive volume and syntaxin content remained the same in capacitated and non-capacitated sperm but the location of syntaxin after capacitation was more

restricted to the apical ridge of the plasma membrane overlying the acrosome in more than 90% of the sperm observed. The effect of bicarbonate (HCO_3^-) and BSA, agents necessary in the medium for capacitation, was also investigated. Bicarbonate (HCO_3^-), which activates soluble adenylate cyclase, was not necessary for re-localization of syntaxin. On the other hand, BSA, which promotes cholesterol efflux, was required for syntaxin re-localization.

In the case of synaptobrevin, the volume of protein remained the same in both capacitated and non-capacitated sperm. Synaptobrevin was found at the apical ridge of the sperm head prior to and following capacitation in approximately 83% of the sperm observed. Our results demonstrate that, unlike syntaxin, synaptobrevin does not shift during the plasma membrane modifications that occur in sperm during capacitation. Syntaxin and synaptobrevin did not co-localize at any time during capacitating and non-capacitating conditions in sperm. Our results will help in identification of pathways that may regulate SNARE localization and function during capacitation, membrane fusion and the acrosome reaction.

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CHAPTER 1

INTRODUCTION

The beginning of a new life is one of the outcomes of successful fertilization. Females are not the sole contributors in this process; their male partners have equal involvement. Complications can be present at both male and female level with a similar impact.

Male factor infertility is responsible for approximately 30-35% of infertility among infertile couples (Ombelet, et al., 2008; WHO, 2014). There can be a number of reasons behind it ranging from physiological to behavioral or sometimes even social. About 30 percent of male factor infertility is known to be idiopathic, as the reasons underlying the defects remain unknown (Wu et al., 2010). Unfortunately no clear cause for subnormal semen parameters or pathological cause can be found for such cases (Kamischke & Nieschlag, 1999). Currently, rational approaches for treatment of such cases are absent. Upon deeper investigation, it has been found that the majority of patients dealing with idiopathic male infertility either have a problem with acrosomal exocytosis or sperm binding to the zona pellucida (Hamada et al., 2012; Irvine, 1998; Flynn et al., 2010). This notion is grounded on the observation of certain cases unexplained infertility in couples with poor success in in-vitro fertilization

(IVF) and intrauterine insemination (IUI) (Liu et al. , 2000). Oddities in sperm and zona pellucida binding and penetration cause low success rate during IVF or IUI procedures (Hamada et al., 2012; Liu et al., 2000). These defects are be detected by routine semen analysis, but are evident by using tests assessing sperm and zona pellucida interactions (Liu et al., 2003) . Unfortunately, no clear cause for subnormal semen parameters or can be found for such cases (Kamischke & Nieschlag, 1999). Therefore, reasonable methods or treatments for solving such infertility cases are not available.

Efficacious fertilization can only happen if the sperm entering the female tract goes through required physiological changes at the right place and at the right time. These changes come in to effect after sperm are in contact with specific factors present in the female reproductive tract (Suarez & Pacey, 2006). This process, known as capacitation, is a term coined by Austin and Chang (Austin, 1952; Chang, 1984) ; they concluded “ the sperm must undergo some form of physiological change or capacitation before it is capable of penetrating the egg” (Austin, 1952). Capacitation can be induced in vitro by using a medium containing bicarbonate (HCO_3^-), calcium (Ca^{2+}) and serum albumin (Visconti et al., 1998). The fresh ejaculated semen encounters a change in bicarbonate (HCO_3^-) concentration when it enters the female reproductive tract along with the change in pH and membrane potential (Rahman et al., 2013). Bicarbonate (HCO_3^-) regulates various cellular pathways in the presence of calcium (Ca^{2+}) (Abou-haila & Tulsiani, 2009).

Additionally, cholesterol efflux and its redistribution in the plasma membrane of sperm allow the destabilization of the plasma membrane and induction of capacitation (Lamirande, 1997). Removal of cholesterol and its movement in the plasma membrane of sperm is accomplished by albumin (Buschiazzo et al., 2013; Flesch et al., 2001; Nolan & Hammerstedt, 1997; Schuck et al., 2006). Cholesterol removal from a cell changes the fluidity of the cell membrane along with making it more permeable (Simons, 2000). These changes in cells happen due to cholesterol being an important part of cell plasma membrane micro-domains such as lipid rafts and caveolae, which are rich in sphingolipids and saturated phospholipids (Fang et al., 2006; Simons, 2000). Disruption of these micro-domains containing cholesterol causes physiological changes, along with activating a number of signaling pathways, which affect protein sorting, signal transduction, and membrane trafficking (Fang et al., 2006; Incardona & Eaton, 2000; Smart et al., 1999). Therefore, the cholesterol-binding agent, albumin plays a major role during capacitation of the sperm as it aids in removal of cholesterol from the sperm plasma membrane (Davis et al., 1980; Kabouridis et al., 2000).

All these changes during capacitation help sperm undergo the acrosome reaction (de Lamirande, 1997). The process of capacitation is also known to stimulate SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) complex formation, the foundational force behind most intracellular membrane fusion, including exocytosis. (Boerke et al. 2008; Duman & Forte, 2003; Tsai et al., 2009; Tsai et al. 2010; Ungar & Hughson, 2003)

As fertilization entails the fusion of a sperm and an oocyte, it requires the merging of two cells, such that they come to form a single cell, the zygote (Austin, 1975). After capacitation, which encompasses changes in the sperm plasma membrane, the sperm needs to undergo an exocytotic event, known as acrosomal exocytosis. The acrosome is a large secretory vesicle containing lytic enzymes present in the sperm head (Harrison, 1982). These enzymes are known to help sperm in its movement through the cumulus and zona pellucida of the oocyte so that the sperm can fuse with it (Meizel, 1985; Roldan & Shi, 2007; Stein et al., 2004).

Membrane fusion during acrosomal exocytosis is notably similar to neural exocytosis (Sørensen, 2005; Bogaart et al., 2010; Zhao et al., 2007). It has been found by various research groups that the proteins involved in exocytotic events of somatic cells are similar to those involved in acrosomal exocytosis (Buffone et al., 2014). In neurons, secretory vesicles translocate to the active zone and dock there. To some degree, this may happen in sperm as well. In this position, vesicles are then primed for calcium (Ca^{2+}) signaling. Trans-SNARE complexes form between the vesicle membrane and the target cell membrane. Ca^{2+} influx into the cell is likely the activator of membrane fusion (Gerber & Südhof, 2002). The contents of the vesicle are then released. In neurons, exocytosis is swift and vesicles are recycled after the release of their contents. Neither of these is true for acrosomal exocytosis (Bello et al., 2012; Meizel, 2004; Zhao et al., 2007).

The SNARE family includes a large number of proteins that play a major role in intracellular membrane fusion (Chen & Scheller, 2001). This role is

conserved from yeast to mammals and from somatic cells to germ cells (Fasshauer et al., 1998). In sperm, three proteins form the SNARE core complex, syntaxin, SNAP-25 and synaptobrevin, also known as vesicle associated membrane protein (VAMP). These three proteins are sufficient in artificial systems to promote membrane fusion. Assembly of the SNARE core complex in sperm is triggered by cues that are unknown. Once the tripartite core complex is assembled, it promotes fusion of the outer acrosomal membrane with plasma membranes to aid in exocytosis (De Blas et al., 2005; Zhao et al., 2007). Thus SNARE proteins are integral parts of the sperm acrosome reaction (Ikawa et al., 2010). Problems with SNARE complex formation could be one of the causes behind idiopathic infertility. There is evidence in stallions that semen samples with more sperm staining positively for SNAREs had higher fertility (Gamboa & Ramalho-Santos, 2005).

This thesis focused on determining where vesicle and target SNARE proteins localize in sperm during capacitation. The effects of bicarbonate (HCO_3^-) and albumin, supplied as bovine serum albumin, which can induce capacitation in-vitro, were investigated. Super-resolution structured illumination fluorescence microscopy (SR-SIM), a technique with twice the resolution of traditional confocal microscopy, was used to assess SNARE localization during sperm capacitation in-vitro. To be certain that a change in the amount of each SNARE did not complicate the analysis of localization before and after capacitation, immunoblots and 3D volume rendering software IMARIS were

used to assess the amount of the SNARE proteins (syntaxin and synaptobrevin).

Co-localization between SNAREs was also assessed.

FIGURES

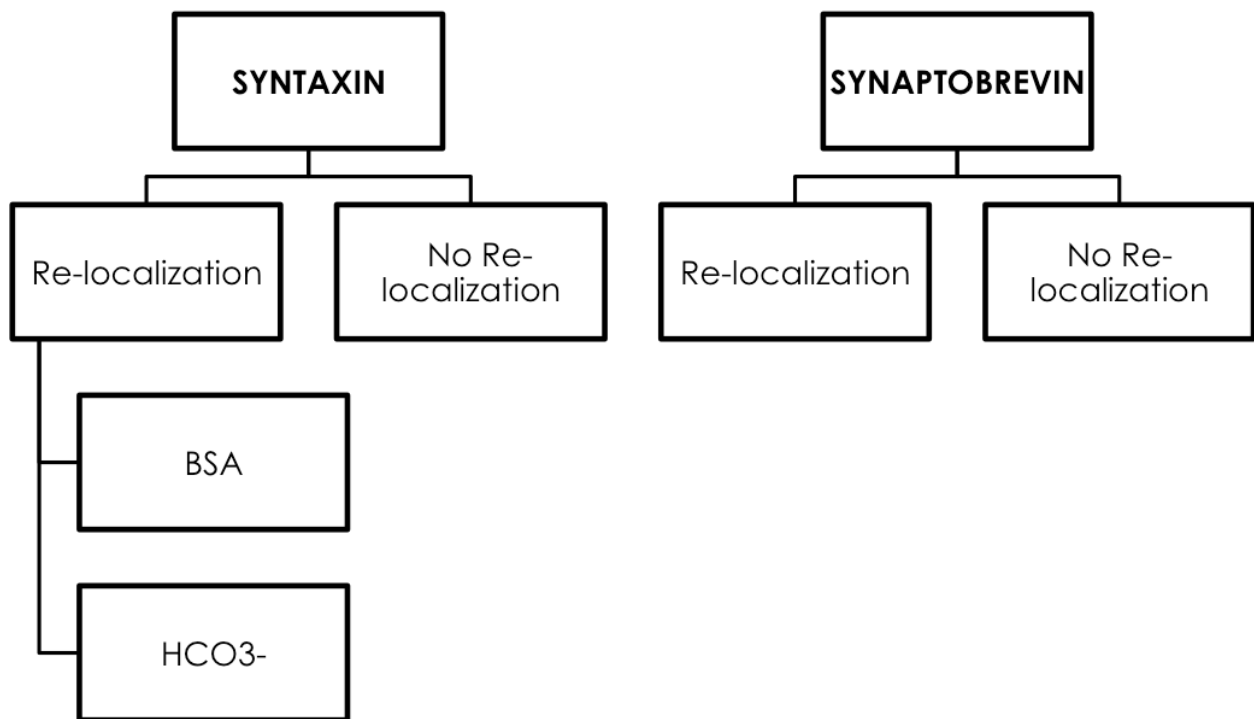


Figure 1.1. Road map of the project

CHAPTER 2

LITERATURE REVIEW

Male factor infertility

Infertility afflicts more than 30% of people all over the globe (Miyamoto et al., 2012; Thoma et al., 2013). Infertility has been declared a disease according to “The Americans with Disabilities Act”. A disease is defined as any form of change from the typical structure or function of the body (organs, tissues or systems), which might be due to a special set of indications or symptoms. Male infertility meets all these benchmarks of a disease and thus is now considered one (Kamischke & Nieschlag, 1999; Rowe et al., 1993). If a couple doesn't conceive after approximately 12 months of unprotected intercourse, they are considered infertile and can avail themselves of the treatments accessible to them (Niederberger, 2001).

A number of case studies suggest that male factor infertility is a major factor behind infertility (figure 2.1) (Irvine, 1998). Causes behind male infertility are multiple ranging from treatable to un-treatable. Significant reasons for infertility are chromosomal abnormalities, micro-deletions, cystic fibrosis trans-membrane conductance regulator (CFTR) mutations and other

genetic factors (Diemer & Desjardins, 1999; Egozcue et al., 2002; Irvine, 1998). The World Health Organization has projected a classification system, which helps in diagnosis of the male partners accountability in the infertile couple (Rowe et al., 1993).

In the WHO's laboratory manual for the examination of human semen and semen-cervical mucus interaction, idiopathicity is described as a condition for which no etiological factor can be found using common clinical, instrumental or laboratory techniques (WHO, 1999). Idiopathic infertility is a condition of couples who are unable to conceive, even with no abnormalities seen during recurrent investigations of both male and female reproductive systems or semen/oocyte quality (Seshagiri, 2001). As male infertility is a multifactorial syndrome, it encompasses a wide variety of disorders. Thus, nearly more than half of infertile men suffer with idiopathic infertility, which could be either congenital or acquired (Krausz, 2011).

Diagnosis of infertility starts with semen analysis. Abnormalities in the semen can be innumerable and can be analyzed via seminograms. Analysis may show a patient to be azoospermic, oligozoospermic, teratozoospermic, asthenozoospermic, necrospermic or pyospermic (Sarrate et al., 2010). It is currently estimated that approximately 30 percent of male patients seeking treatment for fertility are either oligozoospermic or azoospermic of unknown etiology (Poongothai et al., 2009). Therefore, there is an ultimate need to find the causes of infertility, as it is only known in less than 50 percent of the cases. Introduction of new and advanced cell and molecular techniques along with

improved diagnostic tools have offered greater insight into the genetic and molecular basis of infertility. However, our understanding of the majority of the causes of male infertility remains limited.

Overview of Fertilization

Fertilization of the egg and its journey towards life is a complex and intricate process. This journey encompasses various phenomena happening at the right time and at the right place or else death of both sperm and egg is imminent. Egg and sperm are two totally different types of cells coming together from two different individuals (figure 2.2, A&B) (Evans et al., 1995). These cells fuse in the oviduct, the passageway from ovaries to the uterus, to create a new life (Wassarman et al., 2001). The oviduct is the place where fertilization occurs in almost all the vertebrates (Suarez & Pacey, 2006).

Over the course of evolution, the oviduct has been modified in many ways. The oviduct has moved from a simple mucus secreting gland in fishes and amphibians to an amnion secretor such as in reptiles, birds and egg laying mammals (Blackburn, 1998.; Holt & Fazeli, 2010; Rodriguez-Martinez, 2007; Wassarman et al., 2001). Further development in the structure is seen in several classes of mammals such as marsupials and placental mammals (Anderson, Dixon, & Dixon, 2006). In their case, the female reproductive tract becomes lined by endometrium that plays a major role in implantation of the fertilized

egg and gives support to the growing and developing embryo (Gualtieri & Talevi, 2003; Gualtieri, 2000). Likewise, sperm locomotion, lifespan and storage in the oviduct are extremely important (Hunter et al., 2014). The oviduct affects both the egg and sperm during the whole process of fertilization along with events proceeding and succeeding sperm-egg union.

In mammals, various cellular and molecular changes happen before, during and after fertilization (figure 2.2, C) (Wassarman et al., 2001). These changes influence both male and female gametes, affecting their developmental potential, physiology and cell surface (Rodríguez-Martínez et al., 2005; Tienthai et al. 2004; Wessel et al., 2009). Most of the critical changes happening in both male and female reproductive tracts can be easily mimicked under proper in-vitro conditions (Flesch & Gadella, 2000; Harrison & Gadella, 2005).

The detailed molecular processes a sperm goes through before fertilizing the oocyte are still unclear. Understanding fertilization is obviously an important area of research. What is unreservedly clear at this point is that only functionally intact and undamaged sperm can fertilize the oocyte (Yanagimachi, 2008).

Although morphologically mature, ejaculated sperm are not completely functional, they are unable to fertilize an oocyte (Abou-haila et al., 2009). To develop sperm fertilizing ability, surface molecules and proteins undergo re-localization and re-organization, a significant event that happens during sperm activation and maturation also known as capacitation (fig.2.2, 2.3) (Austin, 1975; Boerke et al., 2008). These surface changes are perhaps required to enable

the sperm to bind to the extracellular matrix of the oocyte, the zona pellucida (ZP) (figure 2.2, D) (Gadella et al., 2004). During sperm-egg interaction in the oviduct, the apical ridge of sperm containing various membrane protein complexes recognizes the oocyte and binds to the zona pellucida (Van Gestel et al., 2005; Gadella et al., 2007). This binding or some signal during recognition of oocyte induces the acrosome reaction (Wessel et al., 2009).

There are several controversies regarding the key issue of acrosomal exocytosis. During the process of fertilization, the site of acrosomal exocytosis is still controversial (Buffone et al., 2014). As the sperm undergoes final maturation in the female reproductive tract during the process of capacitation, it is primed to undergo acrosome reaction (figure 2.2, C&D) (Aitken & Nixon, 2013). What actually triggers the acrosome reaction and the release of hydrolytic enzymes is still not completely comprehensible (Buffone et al., 2014). Acrosomal exocytosis can occur in response to sperm interaction with the environment surrounding the cumulus mass, direct interaction with the cumulus matrix, within cumulus or when in contact with the zona pellucida (figure 2.3) (Aitken & Nixon, 2013; Buffone et al., 2014). Further research and experimentation in this area are needed to have a better understanding of what roles do cumulus and zona pellucida of the oocyte play in release of acrosomal content from the sperm.

The enzymatic machinery and protein complexes required for acrosomal exocytosis and the initiation of sperm fusion are present at the apical ridge of the sperm head (Tsai et al., 2009). As the sperm moves through the zona

pellucida it enters the peri-vitelline space and fuses with the egg plasma membrane (Stein et al., 2004). During this whole process of zona pellucida penetration and sperm fusion with the egg membrane and later pronuclear interaction, different sets of molecular factors are involved. All these factors come into effect at the right place and the right time leading to successful yet complicated completion of fertilization (Vjugina, 2008).

Capacitation

Sperm capacitation has been under study since early 1950's. Rabbits, rats and hamsters were frequent subjects of the early studies (Austin, 1975). Changes due to capacitation in sperm were found to vary from species to species (Chang, 1984;).

Ejaculated sperm in the uterus move through it towards the oviduct. They first lose extracellular coating proteins and then gain some of the factors from the oviductal environment leading to capacitation (figure 2.4) (Rodriguez-Martinez, 2007). Sperm in the oviduct meet with the fluid environment, which is rich in bicarbonate (HCO_3^-) and albumin (Aitken & Nixon, 2013; Flesch et al., 2001; Meizel, 1985). Capacitation can be mimicked in the laboratory. To do this, freshly ejaculated sperm or epididymal sperm are washed to remove seminal plasma or extracellular debris. The resulting washed cells are incubated in capacitating medium, which contains salts, carbohydrates, bicarbonate (HCO_3^-), albumin, calcium (Ca^{2+}) and in some cases, glycosaminoglycans. Glycosaminoglycans are believed to remodel the surface proteins of at least

bovine sperm, thus to enhance and promote capacitation (Chamberlain et al., 2001; Gadella, 2008).

Zona pellucida proteins play a major role between sperm and oocyte interaction (Primakoff & Myles, 2002). The mouse contains three of these glycoproteins and human's four, but of these, which protein binds and interacts with sperm remains controversial (Avella et al., 2014). Earlier it was thought that sperm need to be in contact with the zona pellucida protein (ZP3) to undergo acrosomal exocytosis (Wassarman et al. , 2001). More of the recent studies now suggest that it is not only zona pellucida (ZP3) but (ZP2) and a complex of some other proteins, which form a scaffold to perfunctorily promote acrosome reaction (figure 2.3) (Aitken & Nixon, 2013; Avella et al., 2014; Buffone et al., 2008; Buffone et al., 2014).

For a sperm to pass through the zona pellucida, the acrosomal provides the lytic enzymes (figure 2.5). The change in sperm during acrosomal exocytosis occurs along the outer acrosomal and plasma membranes except for the posterior region also know as the equatorial region (Barros et al., 1967). The fusion of sperm with oocyte occurs at the equatorial region of sperm head (Yanagimachi, 1988). The sperm-egg fusion site is specific on both gametes, which indicates the presence of unique protein or lipid populations with the distinct membrane morphology required for fusion (Stein et al., 2004).

It has been believed for quite a number of years that attachment of sperm to the oocyte and the process of fertilization works under multi-protein interaction system, but the direct interaction between specific molecules has

been found recently (Bianchi et al., 2014). IZUMO1 a membrane protein is found to be the most important sperm protein in fusion to the oocyte plasma membrane; knockouts are completely infertile due to lack of fusion (Ellerman et al., 2009; Klinovska et al., 2014). On the other side, the oocyte protein that interacts with IZUMO1 is a protein now named JUNO, also known as folate receptor 4 (Folr4) (Bianchi et al., 2014). As it is present on the inner acrosomal membrane of the sperm, IZUMO can only interact with the oocyte, after the acrosome reaction (Inoue et al., 2005; Sebkova et al., 2014; Vjugina, 2008).

Vesicle exocytosis

The acrosome reaction is a type of vesicle exocytosis in which an internal membrane is exposed externally. Exocytosis requires that the vesicle membrane fuse with the plasma membrane. It has been established in the last few decades that almost every eukaryotic cell depends on membrane fusion for its growth, development and differentiation (Jahn & Südhof, 1999; Jahn & Grubmüller, 2002). In fact, it is thought to be the only mechanism via which addition of membrane can happen in the plasmalemma (Jahn & Südhof, 1999). Looking at exocytosis from the molecular level shows that it involves specialized protein families and complexes. Most of these proteins are conserved from yeast to mammals. If membranes need to expand or neurotransmitters are to be released from the neurons, all these processes involve exocytotic events (Gerber et al., 2002).

Conserved membrane fusion proteins such as Rab proteins, SNAREs and SM proteins are involved in most of exocytotic cellular processes, which also encompasses fusion of intracellular trafficking vesicles and intracellular secretory pathways (Jahn & Grubmüller, 2002). These proteins not only give membranes their fusion specificity before fusion, but also build larger docking complexes. Mediation of membrane attachment, arrangement and fusion all happens via these conserved proteins (Chen & Scheller, 2001; Jahn & Grubmüller, 2002).

Exocytosis is both temporally and spatially specific. Typically the process includes fusion, generation of vesicles, their transport and their exocytosis continuously (fig 2.4). All these steps are subjected to either regulation or no regulation via regulatory proteins (Jahn, 2004). The primary contact between secretory vesicles and the target membrane happens via docking. Those vesicles then undergo priming. In this step, vesicles are allowed to move in the pool of vesicles that are either slow or readily releasable (Becherer et al., 2006). Primed vesicles are released immediately in response to the increase in cytosolic concentration of calcium (Jahn et al., 2003).

Membrane fusion in neurons has received considerable research attention (Becherer et al., 2006; Gerber et al., 2002; Meizel, 2004; Sørensen, 2005). There are a number of similarities along with differences in membrane fusion during neuronal secretion and acrosomal exocytosis (figure 2.6). Increase in cytosolic calcium and SNARE complex formation are required for both types of exocytosis (Hu et al., 2003; Jungnickel et al., 2001; Lopez et al., 2007; Tomes

et al., 2002). On the other hand the size of acrosome is larger as compared to a neuronal secretory vesicle, which makes the exocytosis process and its content dispersal slower (Kim et al., 2003; López-González et al., 2001; Rettig et al., 2002). Although the small neuronal secretory vesicle fuses only at one point, the acrosome fuses at hundreds of points during exocytosis (Nolan et al., 1997). Another difference in both acrosomal and neural exocytosis is their ability to recycle their vesicles, something that neurons are capable of but sperm are not. Because the acrosome is a single vesicle, the acrosome reaction is an all-or-nothing exocytosis event that occurs only once and membrane fusion at multiple points must be synchronized (Bello et al., 2012). Activation of specific proteins such as Rab3 and SNAREs and synaptotagmin are responsible for this acrosomal exocytosis (Bello et al., 2012; Bustos et al., 2014). Several SNARE proteins including different isoforms of syntaxin, synaptobrevin (VAMP-2) and SNAP-25 have been found in the sperm acrosomal region (Boerke et al., 2008; Tomes et al., 2002). An increase in cytosolic calcium is believed to promote formation of SNARE complexes. A number of proteins interact with individual SNAREs or SNARE complexes to help and participate in the membrane fusion (Buffone et al., 2014).

As acrosomal exocytosis is a significant event in fertilization, understanding its molecular mechanism along with what regulates this process is of paramount significance if we want to comprehend sperm physiology.

SNAREs

SNAREs are represented by a superfamily of mostly membrane-anchored proteins. They are small proteins with molecular weights ranging from 10-35 kDa. What defines them, as a family, is a conserved stretch of 60-70 amino acid sequence motif, common to all SNAREs, known as a SNARE motif (Chen & Scheller, 2001; Ungar & Hughson, 2003). SNARE proteins are found in both somatic and germ cells and are responsible for promoting membrane-fusion (Sørensen, 2005).

In a typical SNARE protein, the conserved SNARE motif is present at the C-terminus of the protein. The variation in SNARE proteins is usually at the N-terminal region, which consists of only a few amino acids or can have an independent folded domain connected to the SNARE motif by a linker region. N-terminal regions can have some shared tertiary structures, but their sequences are not conserved through out the SNARE protein superfamily (Jahn, 2004). Membrane fusion is thought to be initiated when N-terminal SNARE motifs pull the lipid bilayers together toward the C-terminal membrane anchors (Klopper et al., 2007). All the proteins in the SNAREs are classified on the basis of SNARE motif identity, which could either be R or Q. R-SNAREs contribute only one helix while Q-SNAREs contribute three. R-SNAREs are proteins that contribute an arginine (R) residue, whereas Q-SNAREs are proteins that contribute a glutamine (Q) residue in the formation and assembly of core SNARE complex (Fasshauer et al., 1998; Klopper et al., 2007). An example of one particular R-SNARE is synaptobrevin, which is located on the synaptic

vesicles. On the other hand Q-SNAREs including syntaxin and SNAP-25 are located on the target membrane.

This R and Q classification also helps in predicting the *cis* or *trans* configuration of the SNARE complex (Hanson et al., 1997). In a complex when both Q and R SNAREs reside on the same membrane, the complex is in *cis*-configuration, which does not let membrane fusion take place (Hanson et al., 1997; Xu et al., 1999). When Q and R SNAREs reside on opposite membranes, the complex is in *trans*-configuration, which encourages membrane fusion (De Blas et al., 2005; Bogaart et al., 2010). The Q- and R-SNAREs form tight heterotrimeric complexes during the fusion process, also known as SNARE core complexes (Tomes et al., 2002).

SNARE core complexes are the main drivers of membrane fusion during exocytosis. The core complex in neurons is composed of three SNARE proteins namely syntaxin, SNAP-25 and synaptobrevin (figure 2.7) (Brunger, 2005; Chen et al., 2002). Syntaxin and SNAP-25 are typically sited on the plasma membrane and synaptobrevin is present on the membrane of synaptic or neurosecretory vesicles (Klopper et al., 2007; Shi et al., 2012). Thus syntaxin and SNAP-25 are known as target SNAREs (t-SNAREs) and synaptobrevin 2/VAMP-2 is known as a vesicle SNARE (v-SNARE) (Pfeffer, 1996; Shi et al., 2012; Bogaart et al., 2010).

There has been an increase in research related to sperm SNARE protein regulation and working mechanism after their identification in rat and porcine sperm (Ramalho-Santos et al., 2000; Tsai et al., 2009). SNARE core complexes

are also required for exocytosis in human sperm (Hu et al., 2003; Katafuchi et al., 2000; Ramalho-Santos et al., 2000; Tomes et al., 2002). Syntaxin (t-SNARE) is localized at the apical ridge of the sperm, the area that binds to zona pellucida (Gamboa et al., 2005; Tomes et al., 2002; Tsai et al., 2009; Weninger et al., 2008). The binding partner of syntaxin, synaptobrevin/VAMP (v-SNARE) is localized to the same region of sperm (Tsai et al., 2007) but is expected to be on the acrosomal membrane.

Capacitation and sperm activation are believed to be required for SNARE complex formation, which is a prerequisite for membrane fusion (Boerke et al., 2008; Tsai et al., 2012). During capacitation, sperm go through multiple physiological changes in the female reproductive tract (Abou-haila & Tulsiani, 2009; Chang, 1984; Tulsiani & Abou-Haila, 2004). These changes modify and reorient the sperm plasma membrane for membrane fusion with the outer acrosomal membrane (fig 2.6) (Tulsiani & Abou-Haila, 2004). Final maturation, priming and acrosome reaction in the sperm requires the presence of calcium (Ca^{2+}), bicarbonate (HCO_3) and albumin (Abou-haila & Tulsiani, 2009; DasGupta et al., 1993; Florman et al., 1992; Fraser et al., 1995; Tsai et al., 2012). Research suggests that the process of capacitation is analogous to early stages of calcium (Ca^{2+}) triggered membrane fusion in somatic cells and viruses (Tulsiani & Abou-Haila, 2004; Xu et al., 1999). The sperm plasma membrane and outer acrosomal membrane ultimately fuse together in response to a signal, and the preparation for this fusion happens during capacitation (Russell et al., 1979; Tulsiani & Abou-Haila, 2004; Yanagimachi, 1988). Even after years of

research the regulation formation and function of the SNARE complex is still not completely clear. What ultimately triggers the acrosome reaction and membrane fusion in sperm is still undetermined (Buffone, Hirohashi, et al., 2014).

MATERIALS AND METHODS

Reagents and animals

Mice used were CD-1, purchased from Harlan Sprague-Dawley. Syntaxin-2 (Cat# 110123). Synaptobrevin-2 (Cat# 104211-C3) antibodies were purchased from Synaptic Systems. Secondary antibodies coupled to Alexa Fluor-488 (Cat# A-11008) and Alexa Fluor-568 (Cat# A-11031) were purchased from Life Technologies. Prolong Gold anti-fade mount (Cat# P-36930) was purchased from Life Technologies. Actin antibodies made in rabbit (Cat# A2066) and protease inhibitor cocktail (Cat# S8820) were purchased from Sigma-Aldrich. HRP conjugated anti-rabbit antibodies made in a goat (Cat# 32260) were purchased from Pierce Thermo Scientific. Protein markers and 4%-20% pre-cast gradient gels were purchased from Bio-Rad. The BCA protein assay kit (Cat# 23227) was purchased from Pierce Thermo Scientific. BSA was used as a standard for the BCA assays.

Preparation of mouse sperm for immunofluorescence assay

To obtain mouse sperm for immunofluorescence assay, 3-5 male mature mice were euthanized. For euthanasia, carbon dioxide asphyxiation was used, according to the guidelines given by the Institutional Animal Care and Use Committee. Cauda epididymides were collected from the mice using an approved experimental protocol. Epididymides were washed in PBS for 30 seconds to remove the extracellular debris. Piercing the cauda with a 23-gauge needle after washing released sperm. The pierced cauda epididymis was incubated for 10 minutes at 37°C, allowing the sperm to swim out. This was done in a petri dish containing 1 ml of medium (dmKRBT) per cauda. Sperm concentration and motility were measured using a haemocytometer. Only samples with at least 65% motility were used for the experiments. The sperm were incubated in either capacitating medium (dmKRBT; 120 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 10 mM NaHCO₃, 1.2 mM MgSO₄, 0.36 mM NaH₂PO₄, 5.6 mM glucose, 1.1 mM pyruvic acid, 25 mM TAPSO, 18.5 mM sucrose, 0.6% BSA, 10 units/ml penicillin, 10 units/ml streptomycin (pH 7.3)) or non-capacitating medium (dmKRBT in which bicarbonate (HCO₃⁻) was replaced with an equimolar amount of TAPSO, dmKRBT in which BSA was replaced with an equal mass of PVP and dmKRBT in which both BSA and bicarbonate (HCO₃⁻) were replaced with TAPSO AND PVP respectively). Sperm were collected at 0, 30, 60 and 120 min of incubation and a drop of 50 ul was spread on a cover slip to air dry. Air-dried sperm were fixed with 4% paraformaldehyde for 20 minutes. Permeabilization was done using 0.05% Triton X-100 for 10 minutes. Later, sperm on the cover slips were blocked with 3% BSA for 30 minutes before incubation with primary antibodies.

Cover slips were incubated with primary antibody (1:200) overnight at 4°C. Secondary antibody (1:150) incubation was done at 37°C for 1 hr. The cover slips were washed with PBS 3 times between each step and 5 times between primary and secondary antibody incubation. Cover slips were mounted on slides with a drop of Prolong Gold anti-fade (Invitrogen, Cat# P36930). Slides were cured for at least 12 hours at 4°C before imaging.

Super resolution structure illumination Immunofluorescence microscopy (SR-SIM)

Microscopy was done using a 4-laser system of SR-SIM (Zeiss Elyra S1) with 405nm, 488nm, 561nm, and 633nm lasers. The laser used was appropriate for the secondary antibody. For co-localization of both syntaxin and synaptobrevin, two lasers were used simultaneously. Slides were focused using a 100x oil objective. All the images obtained were of intact, morphologically normal looking sperm. Z-stack was adjusted with reference to the amount of fluorescence present. All the Z-sections with fluorescence were captured. The final image was cropped in a way that only the sperm head remained a part of the image.

Data analysis

Multiple images per Z-stack, collected from SR-SIM microscopy were combined and processed to form a 3-D picture using ZEN-2011's Structured Illumination tool. Later, IMARIS was used to create 3D surfaces on the SR-SIM

images. The “Surfaces” tool was used, which renders 3D volume to fluorescent spots. This predicted the volume of the fluorescence along with the protein’s 3D location in the sperm head.

Images collected using SR-SIM microscopy were analyzed on the basis of fluorescence pattern. This analysis was done without knowledge of the sample being evaluated. Data were collected in the form of percentages representing the number of sperm showing re-localization or no re-localization.

For images with both syntaxin and synaptobrevin co-localized, the “COLOC” tool in IMARIS was used to analyze the area in which both proteins were co-localizing. The data analysis tool built in to COLOC was used for Pearson correlation coefficient test to assess the relationship between syntaxin and synaptobrevin localization.

Confocal microscopy

Confocal microscopy was done using a 7-laser system (Zeiss LSM 710 NLO) with 405nm, 458nm, 488nm, 514nm, 561nm, 594nm and 633nm lasers. The appropriate laser was used to identify the location of syntaxin on mouse sperm. Slides were focused using a 100x oil objective. All the images obtained were of intact, morphologically normal looking sperm. Z-stack was adjusted with reference to the amount of fluorescence present. All the Z-sections with fluorescence were captured and then overlaid on the bright field image to have a better understanding of where the fluorescence is localized. For

consistency, the final image was cropped in a way that only the sperm head remained a part of the image.

Protein collection and quantification

To obtain mouse sperm for immunofluorescence assays, 6-8 male mature mice were euthanized by the procedure described above. Sperm were capacitated and aliquots removed at 0, 30, 60 and 120 min. Sperm samples were then centrifuged at 12,000 x g for 10 min and supernatant was removed before washing with PBS. The pellet was gently mixed before centrifuging again for 5 min at 8000 x g. After removal of supernatant, the pellet was suspended in RIPA buffer (50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors, pH 7.3) and vortexed for 30 seconds before placing on ice for 20 minutes. Centrifugation was done at 8000xg for 5 min; supernatant was collected. The BCA assay was used to quantify the protein using BSA as a standard.

Western blot analysis

To verify the amount of syntaxin at different treatments and time points, protein collected from the mouse sperm was diluted with Laemmli sample buffer (Cleaveland et al., 1977) and denatured at 100°C for 5 min. Sperm protein (20ug) was loaded in precast linear gradient 4%-20% gels (Bio-Rad cat# 456-1094). Proteins in the gel were transferred to a nitrocellulose membrane for western blotting. The membrane was blocked with 5% non-fat milk in TBS for 1

hr at room temperature or overnight at -4°C with gentle agitation. The membrane was incubated with syntaxin primary antibody (1:5000 dilution) for 1 hr at 37° or overnight at 4°C with gentle agitation. After this, the membrane was incubated with HRP conjugated secondary antibodies (1:5000 dilution) for 1 hr at 37°C . Actin was used as a loading control (1:200 dilution) and the membrane was then incubated with the HRP conjugated secondary antibody (1:5000 dilution). HRP activity of the secondary antibodies was detected using ECL chemiluminescence substrate (Thermo Fisher Scientific Inc., IL) and an ImageQuant 4010 imaging system (GE Healthcare). Membranes were washed 3 times for 10 min between every step. Experiments conducted were all with pooled semen samples at least from 5 mice. Each experiment had 3 biological replicates. Band intensities of the western blots were measured using ImageQuant TL software (GE healthcare) and normalized to actin. Statistical significance was determined using (ANOVA). Specific difference were tested by $p < 0.10$.

RESULTS

Syntaxin re-localizes during capacitation in mouse sperm

Previous reports using confocal microscopy have suggested that the distribution of syntaxin in porcine sperm changes slightly during capacitation, presumably in preparation for the acrosome reaction (Tsai et al., 2009). To examine this with higher resolution, we used immunofluorescence staining and super resolution structured microscopy (SR-SIM) to detect syntaxin. SR-SIM provides twice the resolution of confocal microscopy (Allen et al., 2014).

After collection, mouse sperm were immediately fixed, permeabilized, and syntaxin was labeled with an antibody. Syntaxin was localized in a punctate pattern in the sperm head in non-capacitated sperm (figure 2.8). Syntaxin was also observed in the tail and cytoplasmic droplet of the sperm (figure 2.9). Negative control experiments in which primary antibody was not included showed no fluorescence.

To assess the potential migration of syntaxin during capacitation, sperm were fixed at various time points during the incubation to promote sperm capacitation. After 30 min of capacitation, some syntaxin shifted towards the apical ridge of the sperm head in approximately 66% of the sperm observed. At 60 min of capacitation, syntaxin was localized in puncta that were mostly restricted to the apical ridge of the plasma membrane overlying the acrosome in over 90% of sperm. Similarly at 120 min of incubation, 83% of sperm observed

syntaxin was found in the apical ridge of the sperm plasma membrane (figure 2.12).

Syntaxin re-localizes during capacitation in mouse sperm in the absence of bicarbonate (HCO_3^-)

Bicarbonate (HCO_3^-) is an ion that activates sperm soluble adenylyate cyclase and the protein kinase A (PKA) pathway and leads to hyper-activated motility and capacitation of the sperm (Hess et al., 2005; Visconti et al., 1998). Because syntaxin was re-localized during capacitation, its re-distribution might be regulated by HCO_3^- . To address this possibility, sperm were incubated in medium in which HCO_3^- was replaced by an equimolar amount of TAPSO.

Syntaxin was localized in the same punctate pattern in the apical ridge of the sperm head region at 0 and 60minutes of incubation in HCO_3^- -free medium. Therefore, withdrawal of HCO_3^- did not affect syntaxin re-localization (figure 2.10 & figure 2.12).

Syntaxin does not re-localize during capacitation in mouse sperm in the absence of bovine serum albumin (BSA)

Removal of cholesterol from the sperm plasma membrane modulates the process of capacitation, which is a requirement for mammalian fertilization to occur (Flesch et al., 2001; Travis & Kopf, 2002). Bovine serum albumin (BSA) in the capacitating medium acts as a sink to remove cholesterol and, perhaps, change the function of membrane rafts (Kabouridis et al., 2000; Tsai et al.,

2009; Gestel et al., 2005). In addition to making the membranes more fusogenic, ultimately albumin may activate serine/tyrosine phosphorylation (Boerke et al., 2008; Flesch et al., 2001; Hess et al., 2005). This activation leads sperm to be prepared to release acrosomal content in response to the right signal (Buffone et al., 2014; Nolan et al., 1997). Sperm for immunofluorescence were incubated in medium in which BSA was replaced with PVP. The location of syntaxin at 0 min incubation was consistent with the previous experiments done with capacitating media and media with no HCO_3^- . However at 60 minutes of incubation in the absence of albumin, less than 10% of sperm had re-localized syntaxin. In these cells, syntaxin was found in the same punctate pattern scattered over the sperm head as it was at the very beginning of the incubation (figure 2.11). These results showed that albumin is necessary for syntaxin re-localization during capacitation (figure 2.12).

Re-localization of syntaxin during capacitation does not change the amount of syntaxin

Although sperm are not active in protein synthesis (Gur & Breitbart, 2006), it was important to distinguish between simple movement of existing syntaxin in sperm or if some syntaxin is lost or some new syntaxin is synthesized. If some syntaxin is lost and some new syntaxin is synthesized, we would expect there to be change in amount of syntaxin before and after capacitation. So we determined if the amount of syntaxin increased or decreased during capacitation. For this purpose, we measured the volume of syntaxin-

positive fluorescent area on sperm via IMARIS, which estimates the approximate amount of protein present in the sperm head. The results showed that the volume of syntaxin-positive area in sperm remains constant during the process of capacitation in spite of its re-localization (figure 2.13). To further confirm these results, we collected sperm protein at different time points (0, 30, 60, or 120 min) of capacitation and performed western blots with syntaxin antibody (figure 2.14). There was also no change in the amount of syntaxin by Western blotting, indicating that it was very unlikely that the re-localization of syntaxin was due to new synthesis.

Along with normal capacitating medium we also tested the increase or decrease in the amount of syntaxin under non-capacitating conditions and medium without either BSA or HCO_3^- . These treatments were done at 0, 10, 30, 60 and 120 min of incubation time. The amount of syntaxin did not change regardless of whether it was quantitated using IMARIS or western blots at ($p>0.10$).

Synaptobrevin does not re-localize during capacitation in mouse sperm

To form a SNARE complex, synaptobrevin in the outer acrosomal membrane has to localize at fusion points apposing syntaxin on the plasma membrane (Shi et al., 2012; Wickner & Schekman, 2008). So we examined whether synaptobrevin would, like syntaxin, re-localize during capacitation. The results of this experiment would provide insight into whether syntaxin and synaptobrevin localization is regulated in the same fashion at the same time. We

performed the same type of immunofluorescence staining using a synaptobrevin antibody and permeabilized sperm. Synaptobrevin was localized at the apical ridge of the sperm in the head region in both capacitating and non-capacitating conditions at 60 min incubation time (figure 2.15, 2.16 & 2.17). Negative control experiments were also conducted in which primary antibody was not included; without primary antibody no fluorescence was detected. Thus, no change in synaptobrevin localization during capacitation was observed.

Synaptobrevin does not change its volume during capacitation

To determine if the amount of synaptobrevin changes during capacitation, we used IMARIS, which provides the volume of sperm stained with the synaptobrevin antibody. The results showed that the amount of synaptobrevin in the sperm remained constant during a 60 min capacitation time ($p > 0.10$, figure 2.17).

Synaptobrevin and syntaxin do not co-localize during capacitation in mouse sperm

To have a better understanding of when SNARE complexes form during fertilization, syntaxin (t-SNARE) and synaptobrevin (v-SNARE) co-localization was assessed during capacitation. Both proteins were localized using immunofluorescence. Two different secondary antibodies were used to clearly distinguish synaptobrevin and syntaxin. Negative control experiments were also conducted in which primary antibody was not included; no fluorescence was

detected in controls. But in samples in which syntaxin and synaptobrevin were both labeled, there was no apparent co-localization at 0 or 60 min (figure 2.18).

To test formally whether syntaxin and synaptobrevin were co-localized, the COLOC tool in IMARIS and Pearson's correlation were used. No significant co-localization was found at ($p > 0.10$) (figure 2.19)

DISCUSSION

SNARE complex formation is known to be regulated during sperm capacitation, thus leading to acrosomal exocytosis (Buffone, Hirohashi, et al., 2014). How and when this regulation occurs, promoting complex formation between v-SNARE and t-SNARE is still unclear (Buffone, et al., 2014). Using super-resolution microscopy (SR-SIM), we have found that syntaxin, a t-SNARE, re-localizes to form a punctate pattern on the sperm plasma membrane overlying the acrosomal ridge, the region that fuses with the outer acrosomal membrane, in response to capacitation. A clear shift was observed between syntaxin in non-capacitated versus capacitated sperm at different time points. This protein shift confirms the hypothesis that protein and lipids present in the sperm membrane shift and re-model in response to capacitation (Boerke et al., 2008). Protein and lipids collectively make up membranes, which are dynamic and respond to environmental signals (Anantharam et al., 2010; Kabouridis et al., 2000; Russell et al., 1979). Capacitation not only re-fashions the plasma membrane

components, but also affects the way they respond to external stimuli (Kabouridis et al., 2000; Schuck et al., 2006). An external stimulus changes the membrane stability and shifts specific protein and molecules to particular places (Yeagle, 1989). Thus, changes in the function of the sperm apical plasma membrane are the result of lipid, cholesterol and protein changes, which happen during capacitation (Flesch et al., 2001; Tomes et al., 2002; Gestel et al., 2005; Gestel et al., 2007; Visconti et al., 1998).

As mentioned above, sperm must undergo capacitation to undergo acrosomal exocytosis, which is necessary for fertilization of an oocyte (Aitken & Nixon, 2013; Austin, 1975; Chang, 1984). The process of capacitation is contingent upon the action of bicarbonate (HCO_3^-), albumin and calcium (Ca^{2+}) (Abou-haila & Tulsiani, 2009; DasGupta et al., 1993; Florman et al., 1992; Fraser et al., 1995; Tsai et al., 2012). These capacitating components each cause changes in physiology of sperm, leading to surface molecule removal, migration or re-arrangement (Visconti et al., 1998). After observing syntaxin re-localization during capacitation I wanted to investigate which capacitation component (bicarbonate (HCO_3^-), albumin or both) is playing a role in syntaxin movement as it is during capacitation that sperm develop changes in motility and reorganize their apical plasma membrane and its components (Tsai et al., 2009). These changes make sperm capable of zona binding and undergoing the acrosomal reaction, thus leading to successful fertilization (Boerke et al., 2008; Breitbart et al., 1997; Tulsiani & Abou-Haila, 2004).

Syntaxin re-localization was observed before the acrosome reaction occurred. Under incubation without albumin in the capacitating medium, syntaxin did not re-localize to the apical plasma membrane of the sperm. Rather, it stayed in the same punctate pattern over the entire head as it was in conditions that did not promote capacitation. On the other hand, syntaxin was still re-localized in the absence of bicarbonate (HCO_3^-) from the capacitating medium. These results coincide with the previous knowledge that albumin can sequester cholesterol from the sperm plasma membrane resulting in reorganization of lipid rafts, which may, in turn, affect membrane fusion and exocytosis (Tsai et al., 2009). Loss of cholesterol leads to lipid rafts re-arrangements (Buschiazzo et al., 2013; Kabouridis et al., 2000). The SNARE proteins involved in the acrosomal exocytosis are recruited in the aggregating raft area upon cholesterol depletion, regulated via albumin (Buffone et al. 2014). Lipid rafts act as a platform for proteins involved in zona binding and membrane trafficking to interact and work together (Gestel et al., 2005). Lipid rafts also play a role in re-localization of exocytosis machinery which includes SNARE proteins, the proteins that provide the basic force behind membrane fusion (Tsai et al., 2009). This was observed by a research group who used boar sperm to study re-localization of SNARE proteins in response to capacitation (Tsai et al., 2009). Consistent with this interpretation, western blot analysis revealed that during re-localization, the amount of syntaxin remained constant, regardless of incubation in capacitating or non-capacitating conditions.

Synaptobrevin is a vesicle associated SNARE protein (v-SNARE), which is present on the outer acrosomal membrane rather than on the plasma membrane like syntaxin (t-SNARE) (De Blas et al., 2005; Rossetto, 1995; Shi et al., 2012; Tsai et al., 2009). After observing syntaxin shift in sperm during capacitation, I also observed how synaptobrevin responds to the process of capacitation. I did not observe protein re-localization in response to capacitation with the v-SNARE, synaptobrevin. These results were found to be at odds with research conducted on boar sperm, which suggested that the synaptobrevin shift in response to capacitation (Tsai et al., 2009).

One of the reasons synaptobrevin's location was unchanged during capacitation could be that synaptobrevin is on the outer acrosomal membrane, which is within the plasma membrane, which is impermeable to albumin. In the case of syntaxin (t-SNARE), albumin was found to be required for the re-localization of the protein, but albumin, being larger in size and hydrophilic cannot pass through the plasma membrane to reorder cholesterol in an inner membrane, leaving synaptobrevin (v-SNARE) unaffected (Davis et al., 1980; Flesch et al., 2001; Hissa et al., 2013; Meizel, 1985). Thus synaptobrevin was found localized to the same area in mouse sperm head in both capacitating and non-capacitation conditions. Because no shift in location of the vesicle associated SNARE protein was seen, this suggests that the trigger for membrane exocytosis protein complex formation is something other than final maturation of sperm in the oviduct (Buffone et al., 2014).

In another set of experiments we studied syntaxin and synaptobrevin co-localization during capacitation. These two proteins need to form a complex while being apposed on membranes before acrosomal contents can be released via vesicles (Chen & Scheller, 2001; De Blas et al., 2005; Shi et al., 2012; Bogaart et al., 2010). Our data indicate very little co-localization between syntaxin and synaptobrevin during capacitation. These results suggest that synaptobrevin and syntaxin do not co-localize until just prior to the acrosome reaction. These two SNARE's may co-localize immediately before membrane fusion in what would be considered part of the acrosome reaction. The distinction between the end of capacitation and the beginning of the acrosome reaction is not clear and some researchers include the acrosome reaction as part of capacitation while others define capacitation as changes prior to the acrosome reaction (Austin, 1975; Chang, 1984).

Distinct timing of syntaxin and synaptobrevin re-localization is consistent with the hypothesis that they are separately regulated. Final acrosomal exocytosis occurs in response to a stimulus which is calcium (Ca^{2+}) dependent (Abou-haila & Tulsiani, 2009; Ramalho-Santos et al., 2000). However what leads to calcium (Ca^{2+}) surge and what happens after this surge is still under investigation by various research groups. Calcium (Ca^{2+}) is known to be the main trigger for the fusion of sperm's plasma membrane with the outer acrosomal membrane so that the hydrolytic enzymes can be released to aid in fusion of sperm with the oocyte during fertilization (Blas et al., 2002). Although there is much evidence that Ca^{2+} affects synaptotagmin, there is little evidence

Ca²⁺ affects syntaxin or synaptobrevin function directly (Castillo et al., 2010; Sugita et al., 2001).

The importance of clarifying the exact mechanism behind acrosomal exocytosis cannot be emphasized enough, as it is the key to the successful fertilization. During our research we found that factors such as albumin, that are required for capacitation also affect a SNARE protein present on the target membrane (syntaxin), but not a vesicle associated SNARE (synaptobrevin). On the other hand, regulation of SNARE complex formation is not identical to regulation of capacitation because syntaxin shifted even in the absence of bicarbonate (HCO₃⁻) and synaptobrevin did not migrate during capacitation. These experimental results further strengthen the hypothesis that SNARE complex formation is not completed during capacitation (Buffone et al., 2014, 2012, 2008). How and why SNAREs are regulated independently is an intriguing and important question, to understand how this unusual membrane fusion in sperm is regulated.

FIGURES AND TABLES

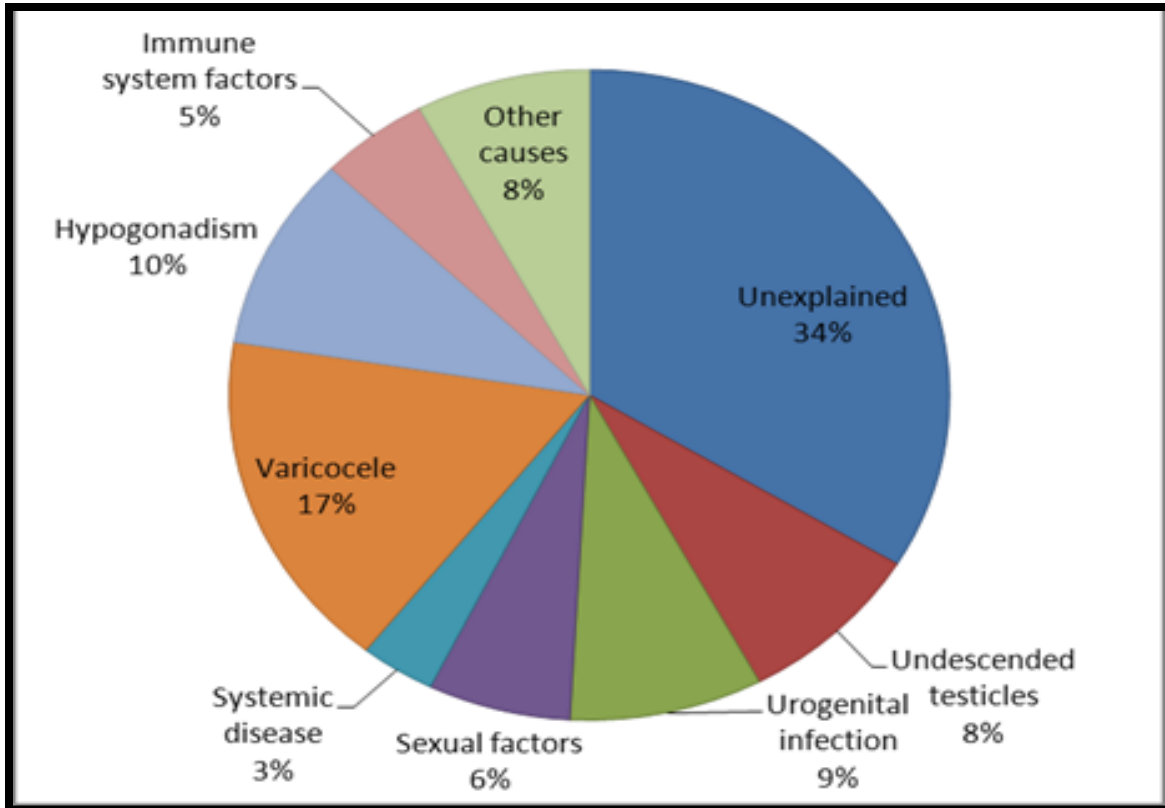


Figure 2.1. Causes of male infertility.

Out of all the different causes of male infertility, more than 30% are due to unexplainable reasons (Data source Dohle et al., 2010) .

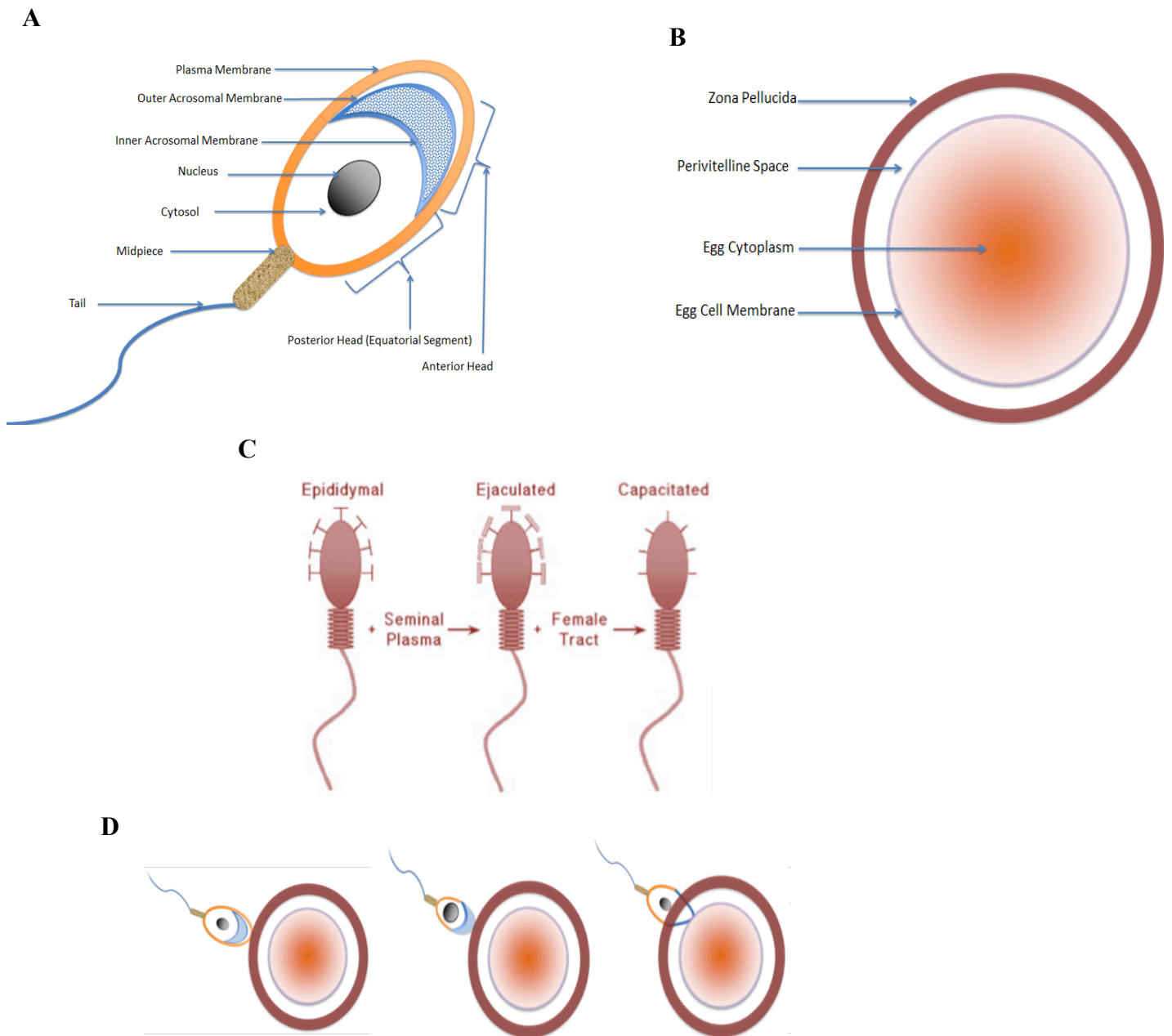


Figure 2.2. Overview of mammalian capacitation and fertilization.

A. Mammalian sperm; **B.** Mammalian egg; **C.** Final maturation and capacitation of ejaculated sperm; **D.** (from left to right) Sperm adhesion to zona pellucida, occurrence of the acrosome reaction and sperm–egg membrane fusion (Swanson et al., 2002).

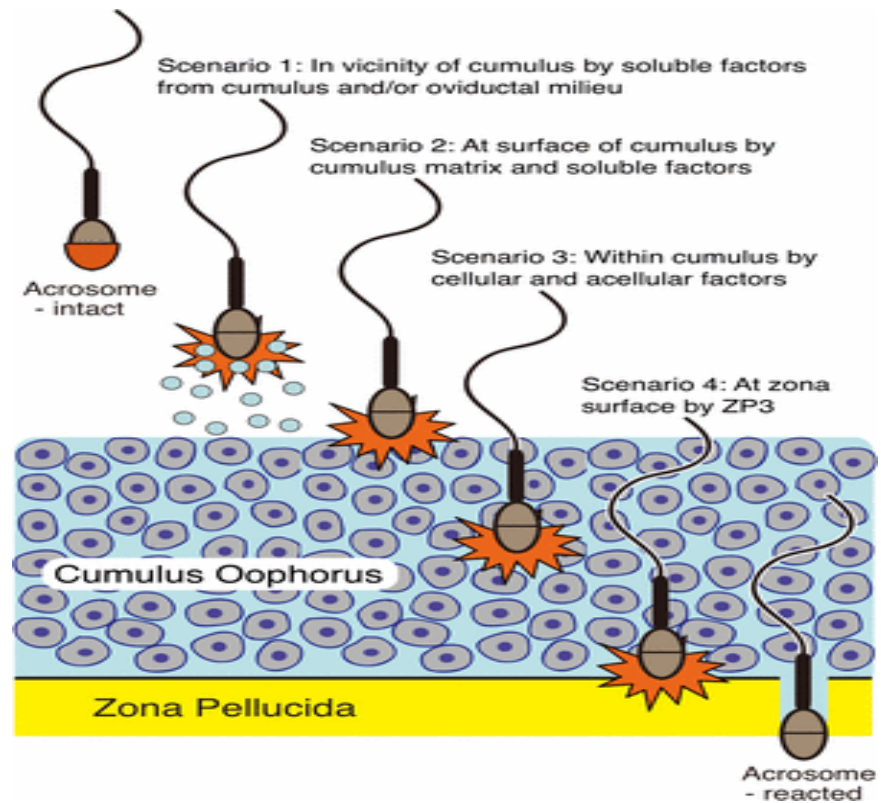


Figure 2.3. Controversies about the acrosome reaction.

Scenarios 1 to 4 exemplify where sperm may undergo acrosomal exocytosis while moving towards the oocyte membrane for gamete fusion (Buffone et al., 2014).

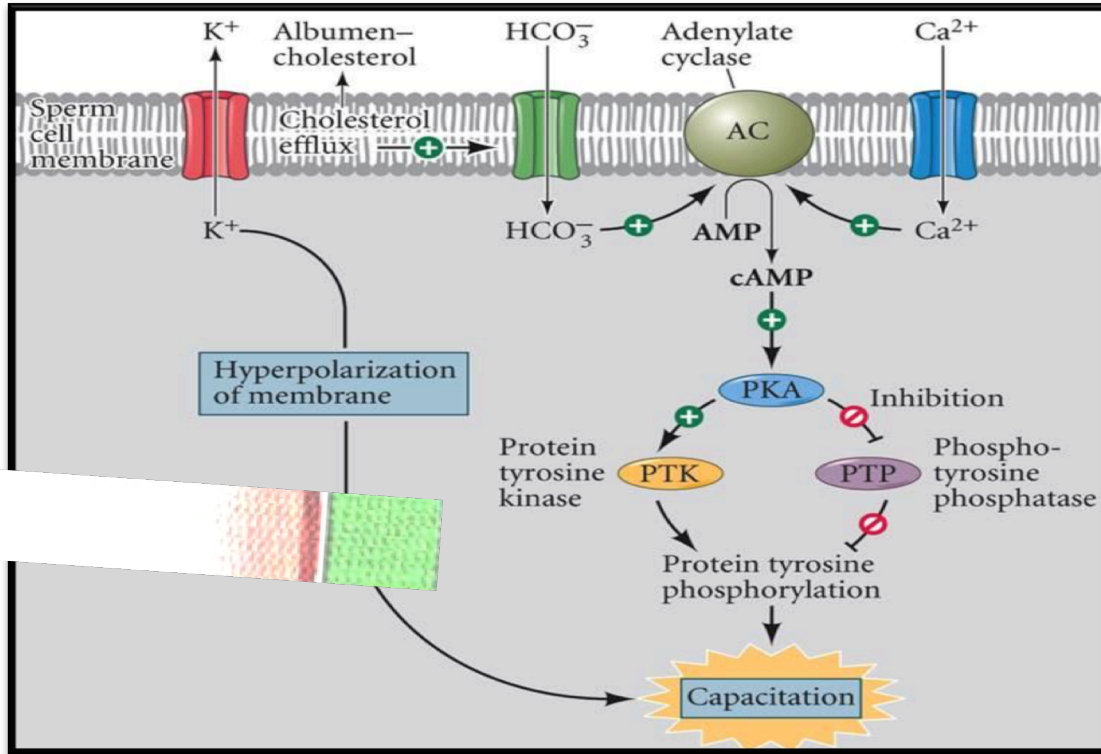


Figure 2.4. Series of molecular events happening in a sperm during capacitation.

Capacitation is a complex signaling cascade, which involves interaction between different pathways. Removal of cholesterol via albumin during initial phases of capacitation increases plasma membrane fluidity of the sperm. This leads to the entry of HCO₃⁻ and Ca²⁺ into the sperm through specific membrane channels. These changes lead to soluble adenylate cyclase (SACY) activation, leading to the production cAMP. cAMP, in turn, activates PKA, which then simultaneously inhibits PTP and activates PTK. PTK leads to protein tyrosine phosphorylation, which in turn acts as an on and off switch for multiple cellular processes during sperm capacitation.

(Adapted from, <http://www.dls.ym.edu.tw/lesson3/fert.htm>)

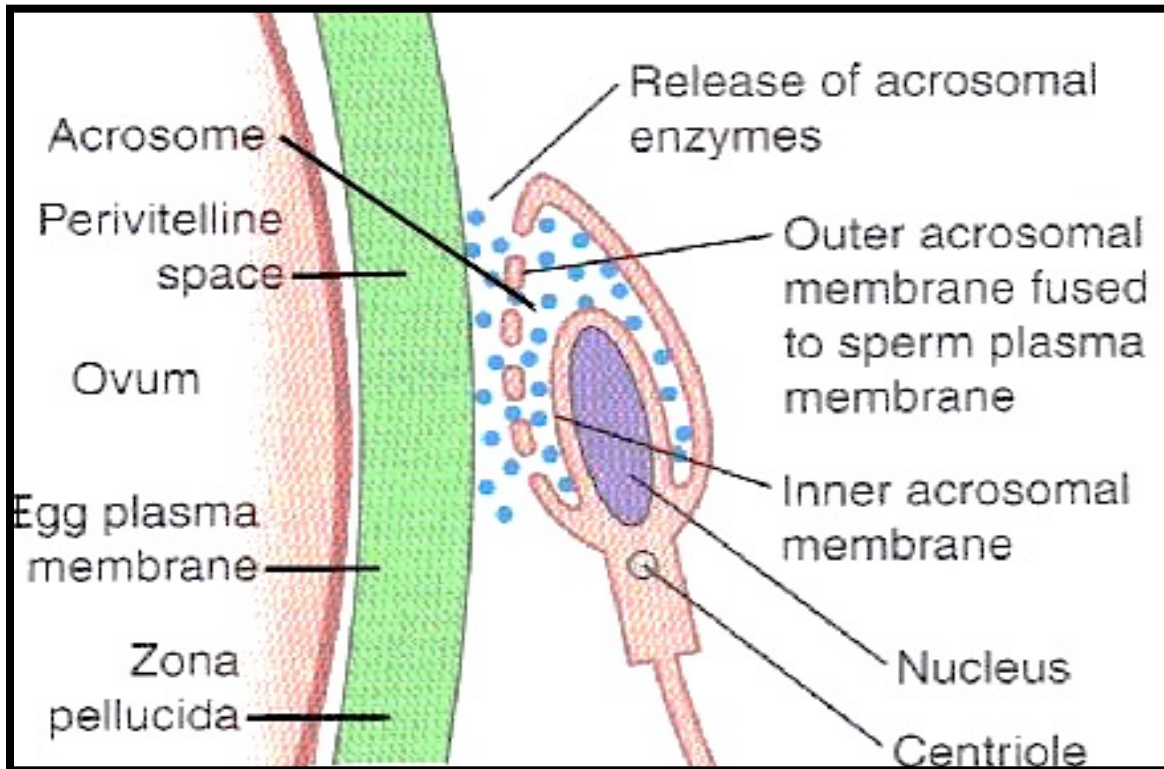


Figure 2.5. Overview of the acrosome reaction.

Ability of the sperm to undergo acrosome reaction makes it able to pass through the zona pellucida and fuse with the plasma membrane of the oocyte. During the acrosome reaction a number of hydrolytic enzymes are released which helps in zona pellucida penetration, making fertilization possible. Acrosomal enzymes are released when sperm plasma membrane and outer acrosomal membrane fuse together.

(Adapted from, <http://lithopedionbaby.wordpress.com/physiology>)

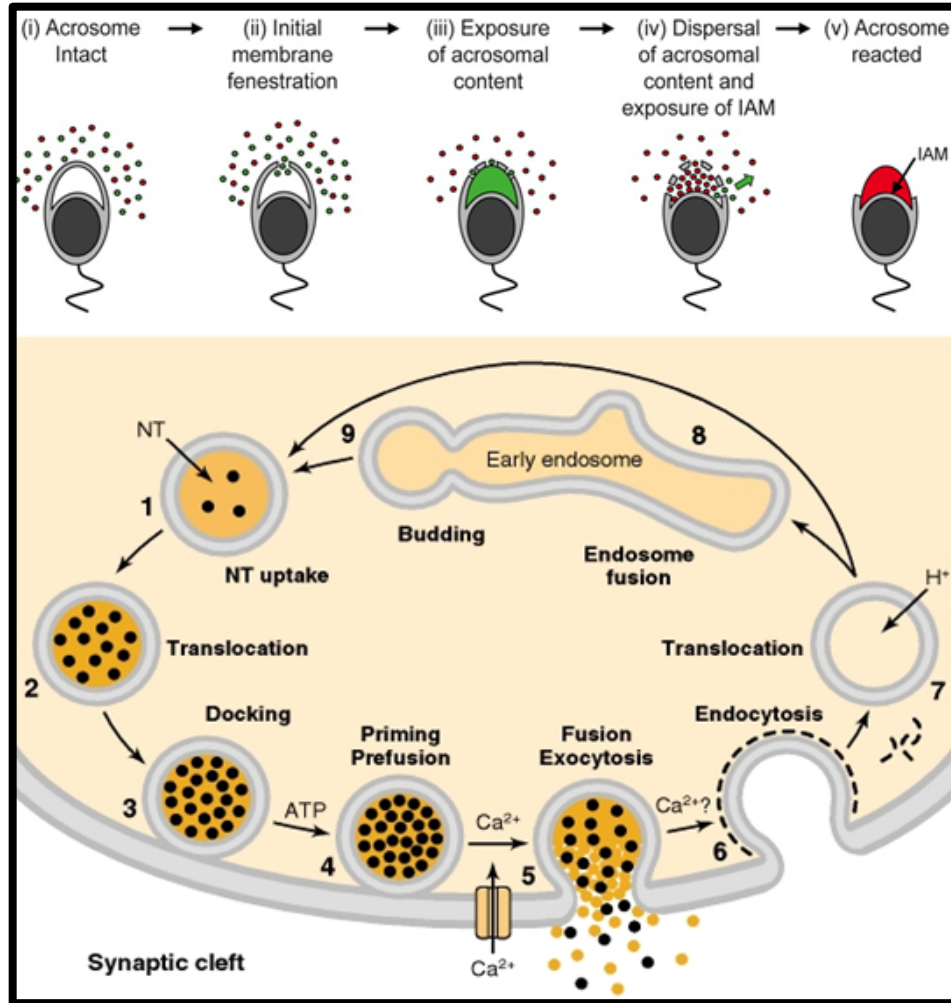


Figure 2.6. Differences and similarities between neuronal and acrosomal exocytosis.

A- Temporal characteristics of acrosome reaction in mammalian sperm.

B. The synaptic vesicle cycle.

(Harper et al., 2008, Siegel et al.,1999)

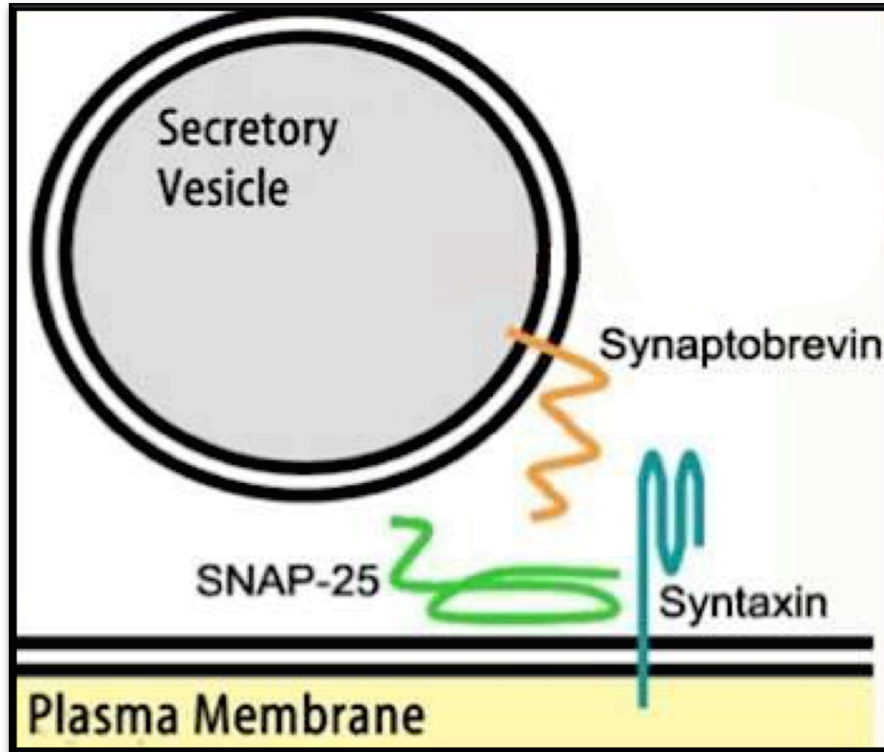


Figure 2.7. SNARE proteins, the basic fusion machinery.

SNARES are the proteins known to be as basic force behind most of the cellular exocytosis events. Formation of tripartite SNARE complex between syntaxin, synaptobrevin and SNAP-25 on opposing membranes is considered to be the minimal requirement for membrane fusion. Synaptobrevin is present on the vesicle end is known as v-SNARE, while syntaxin and SNAP-25 are present on the plasma membrane and are known as t-SNAREs.

(Adapted from: <http://neuromuscular.wustl.edu/pathol/snare.htm>)

A

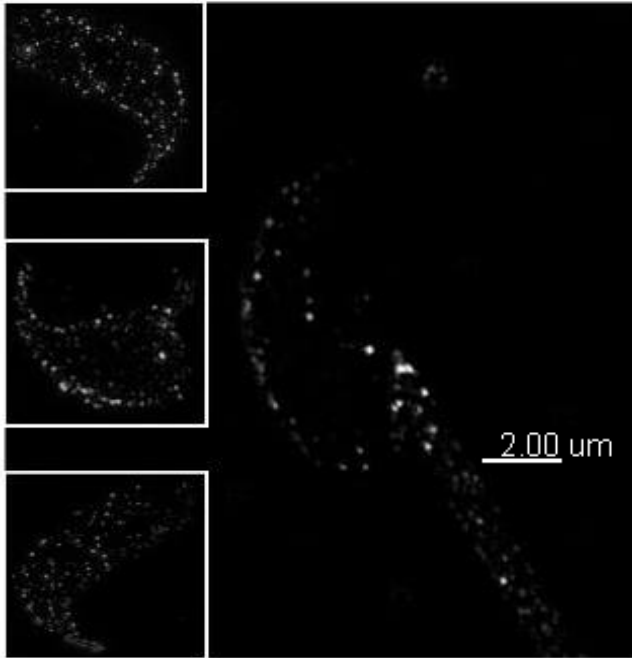


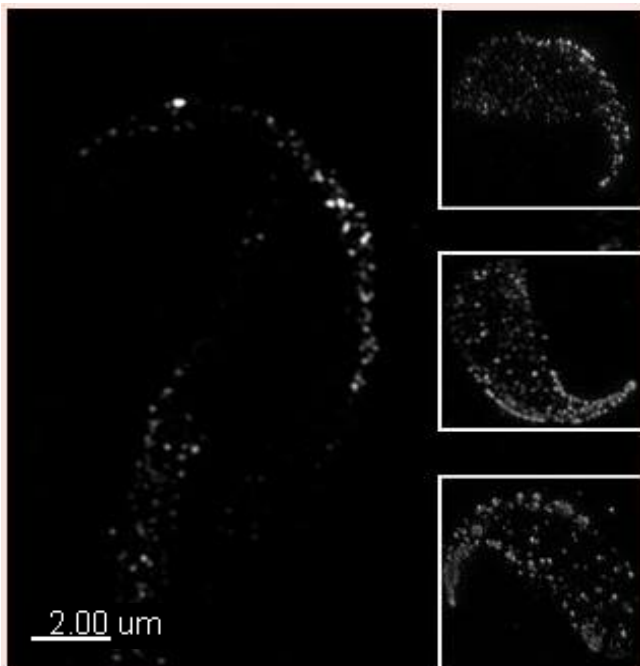
Figure 2.8. Syntaxin re-localizes in sperm during capacitation.

Super resolution images collected using SR-SIM microscopy.

A. Images of sperm incubated in non-capacitating medium for 60 min. A punctate pattern of syntaxin can be observed scattered over the sperm head in larger as well as three small images on **(right)**.

B. Images of sperm incubated in capacitating media for 60 min. Localization of syntaxin can be observed at the apical ridge of the sperm head when compared with **Figure 2.8(A)**. Larger picture and three small images on **(left)** show the similar trend.

B



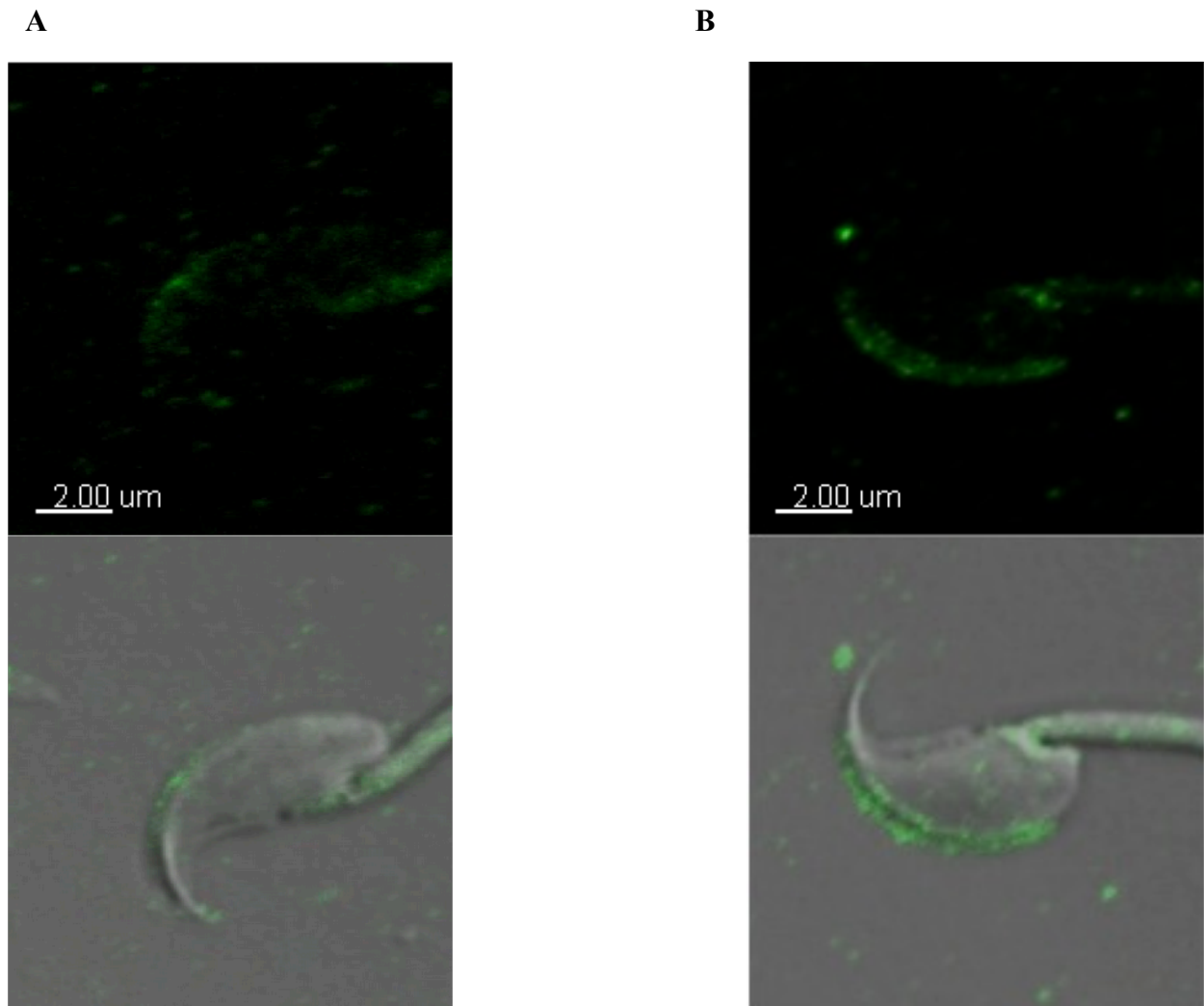


Figure 2.9. Location of syntaxin in capacitated and non-capacitated sperm.

Fluorescent and bright field images were collected using confocal microscopy.

A. Sperm incubated in non-capacitating medium for 60 min. Syntaxin was found in a punctate pattern all over the sperm head.

B. Sperm incubated in capacitating medium for 60 min. Syntaxin was found to be more abundant at the apical ridge of the sperm head when compared with **Figure A**.

A

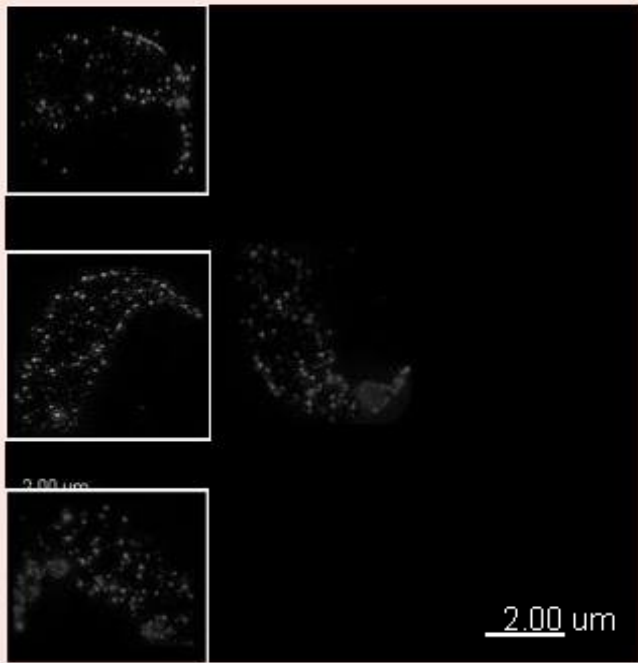
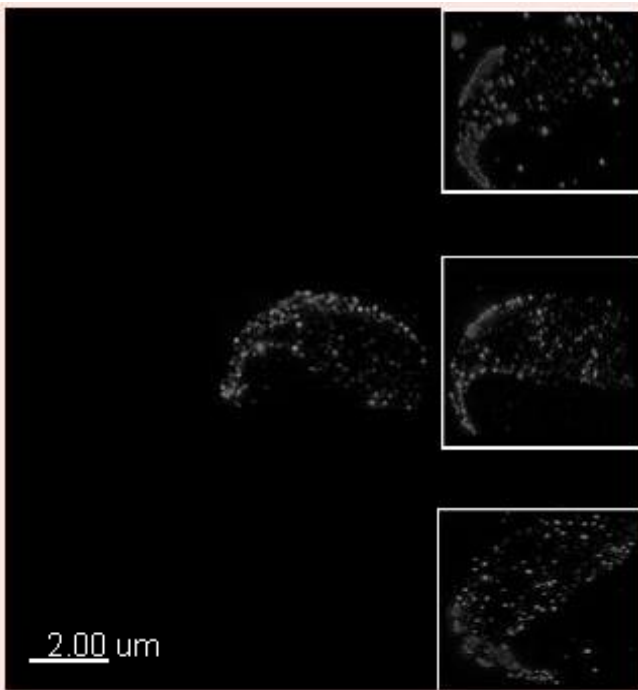


Figure 2.10. HCO_3^- is Not Necessary for Syntaxin Re-localization.

Super resolution images collected using SR-SIM microscopy.

A. Images of sperm incubated in medium with no HCO_3^- for 0 min. A punctate pattern of syntaxin can be observed scattered over the sperm head in larger as well as three small images on **(left)**.

B



B. Images of sperm incubated in media with no HCO_3^- for 60 min. Saturation of syntaxin can be observed at the apical ridge of the sperm head when compared with **Figure 2.10(A)**. Larger picture and three small images on **(right)** show the similar trend.

A

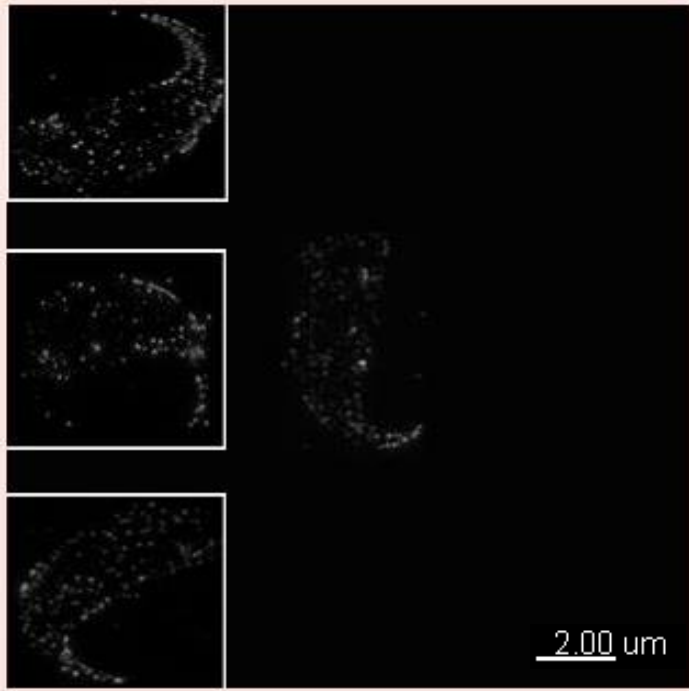
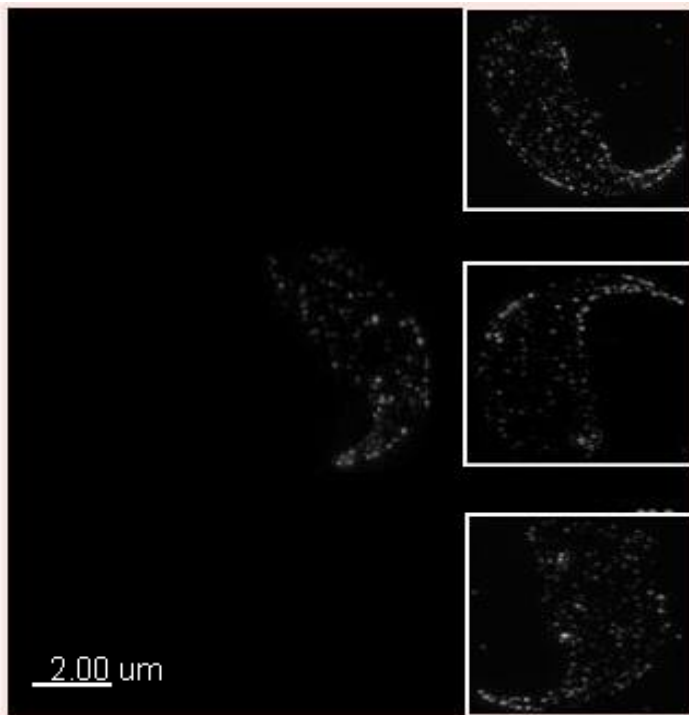


Figure 2.11. Albumin is necessary for syntaxin re-localization.

Super resolution images collected using SR-SIM microscopy.

A. Images of sperm incubated in medium with no BSA for 0 minutes. A punctate pattern of syntaxin can be observed scattered over the sperm head in the larger as well as three small images on **(right)**.

B



B. Images of sperm incubated in media with no BSA for 60 minutes. A punctate pattern of syntaxin can be observed scattered over the sperm head similar to **Figure 2.11(A)**. Larger picture and three small images on **(left)** show the similar trend.

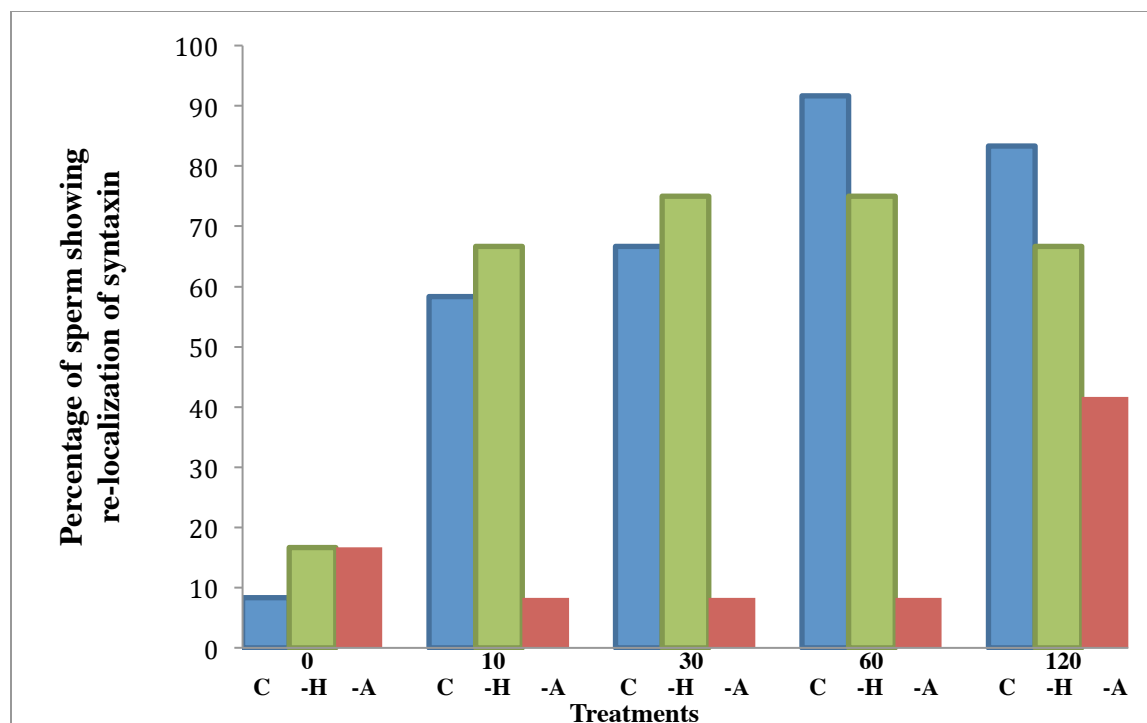


Figure 2.12. Percentage of sperm showing re-localization of syntaxin.

After the collecting images using SR-SIM microscopy via and their quantification via IMARIS. Images were collected at different time points (0, 30, 60, or 120 min) from sperm incubated in capacitating medium (C) media without BSA (-A) and medium without HCO_3^- (-H). They were divided on the basis of how many of them show re-localization of syntaxin.

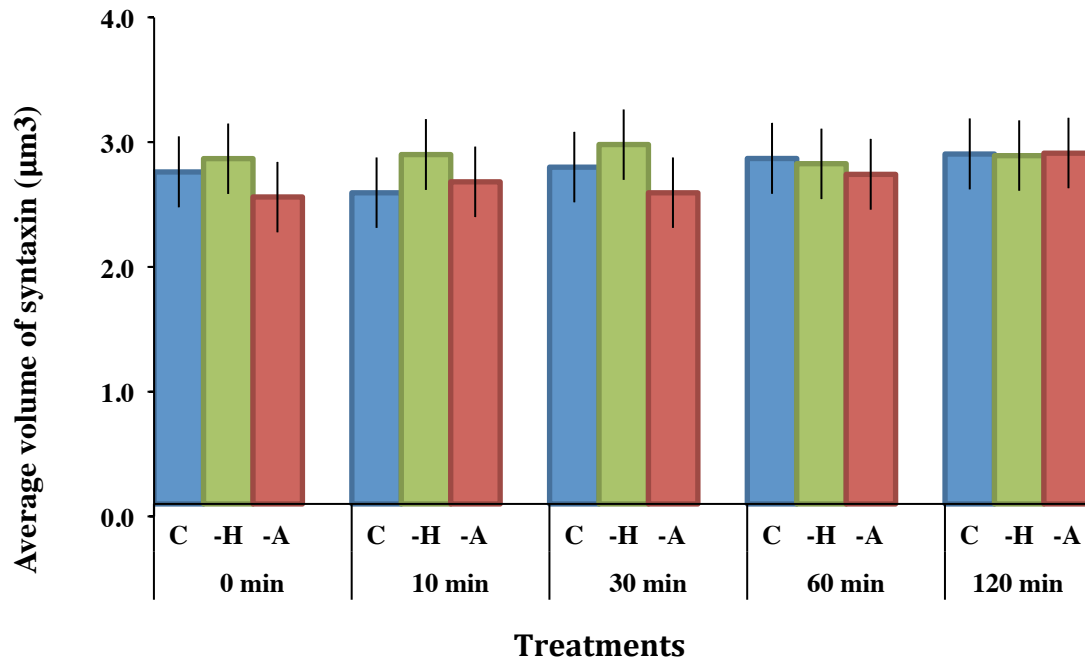


Figure 2.13. Re-localization of syntaxin during capacitation does not change the amount of syntaxin.

Quantification of images collected using SR-SIM microscopy via IMARIS. The volume of syntaxin-positive fluorescent area on sperm head was calculated. Images were collected at different time points (0, 30, 60, or 120 min) from sperm incubated in capacitating medium (C) media without BSA (-A) and medium without HCO_3^- (-H). No statistical difference was found ($p > 0.10$).

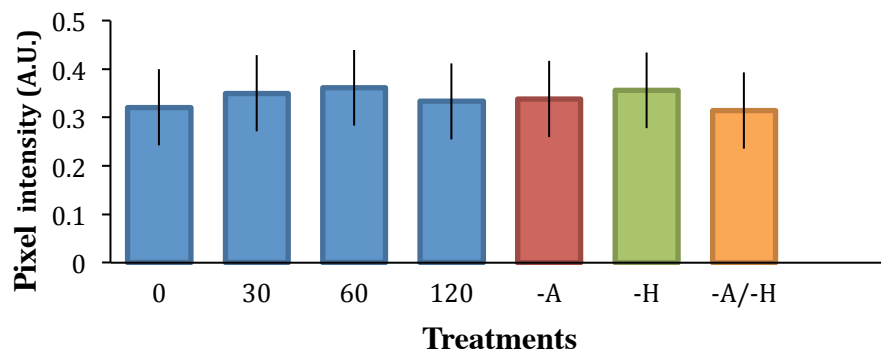
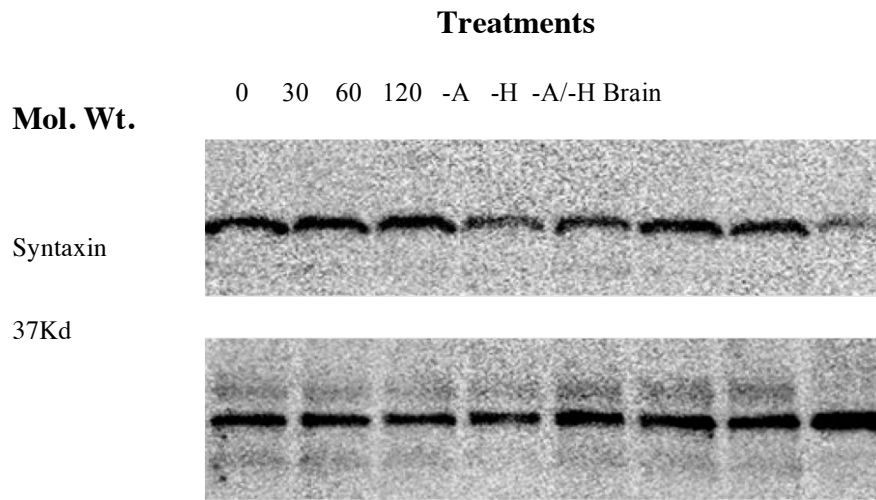


Figure 2.14. Re-localization of syntaxin during capacitation does not change the amount of syntaxin.

A- Western blot images showing no change in the amount of syntaxin collected from mouse sperm protein incubating at different time points (0, 30, 60, or 120 min) in capacitating media, incubation for 60 min in medium without BSA (-A), without bicarbonate HCO_3^- (-H) and without both bicarbonate HCO_3^- and BSA (-A/-H). Protein collected from mouse brain was used as a positive control. Proteins were separated by SDS-PAGE and immunoblotted for syntaxin and actin. Actin was used as a loading control. Mouse brain was used as a positive control.

B- Graphical representation of the data collected using western blot. Syntaxin was normalized against actin band intensities to account for any error in protein loading in gel. No significance was found ($p > 0.10$).

A

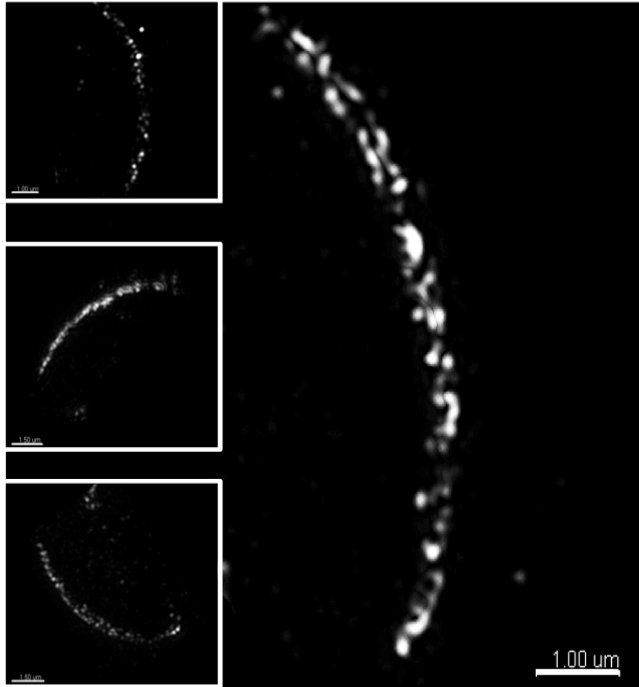
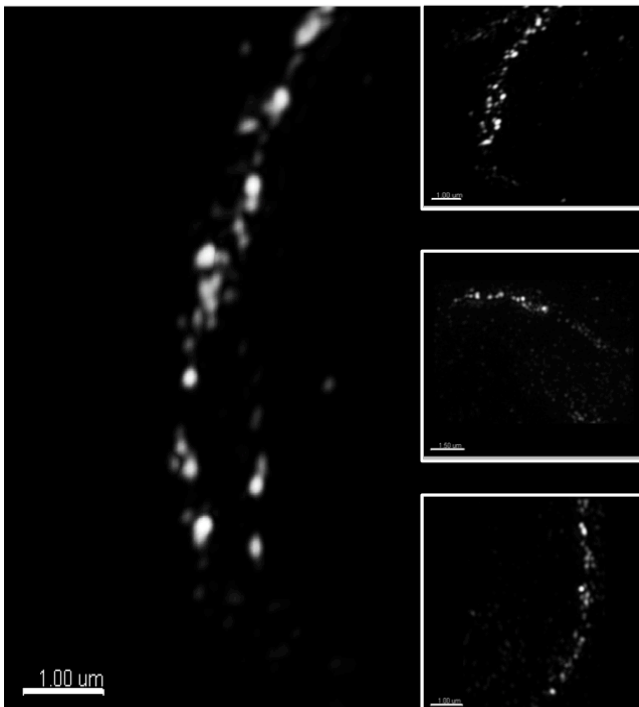


Figure 2.15. Synaptobrevin does not re-localize in sperm during capacitation.

Super resolution images collected using SR-SIM microscopy.

A. Images of sperm incubated in non-capacitating medium for 60 minutes. A punctate pattern of synaptobrevin can be observed at the apical ridge of the sperm in larger as well as three small images on **(right)**.

B



B. Images of sperm incubated in capacitating medium for 60 minutes. A similar pattern of synaptobrevin can be observed at the apical ridge of the sperm head when compared with **Figure 2.15(A)**. Larger picture and three small images on **(left)** show the similar trend.

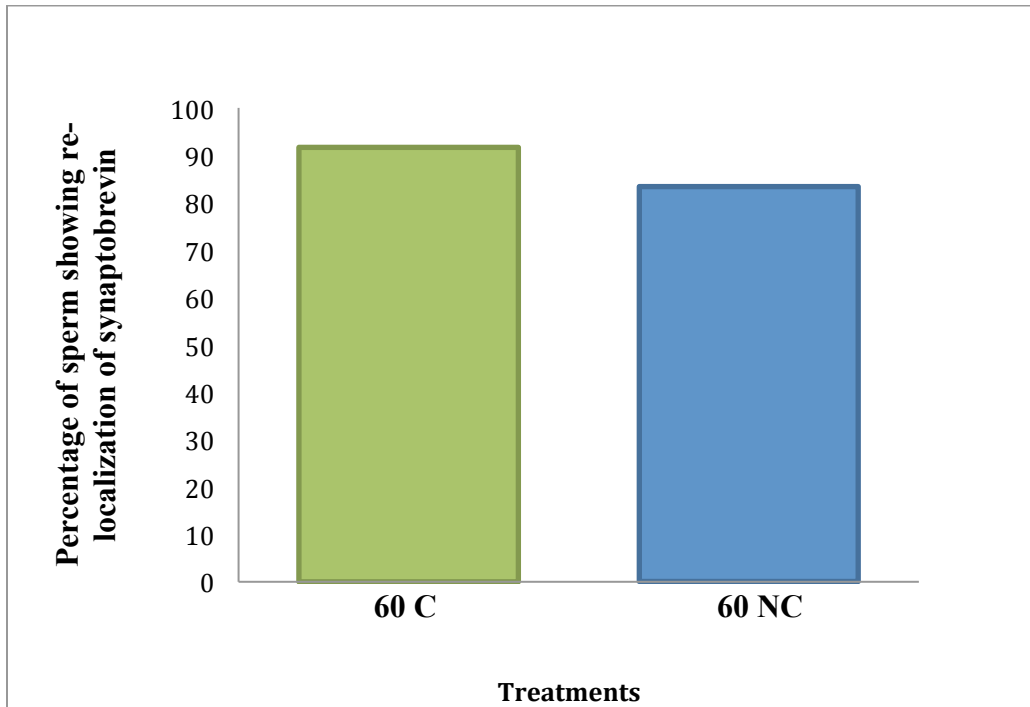


Figure 2.16. The localization of synaptobrevin does not change during capacitation.

Images collected using SR-SIM microscopy were quantified via IMARIS. Images were collected at 60 min in capacitating medium (C) and non-capacitating (NC) conditions. They were divided on the basis of how many of them show re-localization of syntaxin

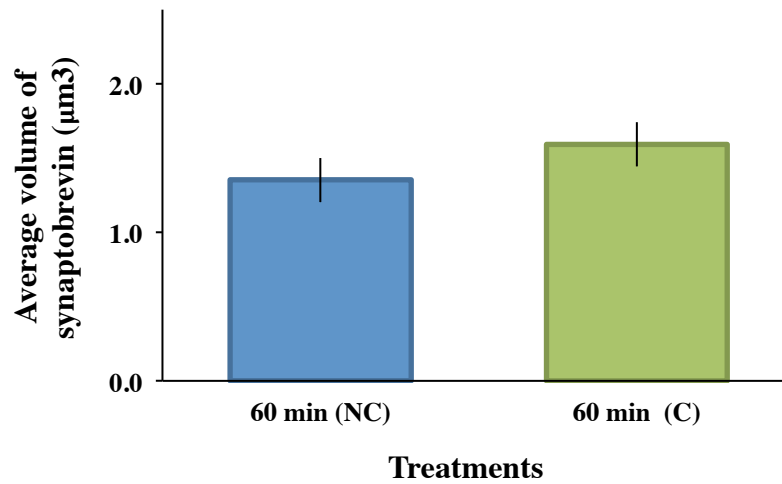


Figure 2.17. The volume of synaptobrevin does not change during capacitation.

Quantification of images collected using SR-SIM microscopy via IMARIS. The volume of synaptobrevin-positive fluorescent area on sperm head was calculated. Images were collected at 60 min from sperm incubated in capacitating (C) and non-capacitating (NC) medium. No statistical difference was found ($p > 0.10$).

A

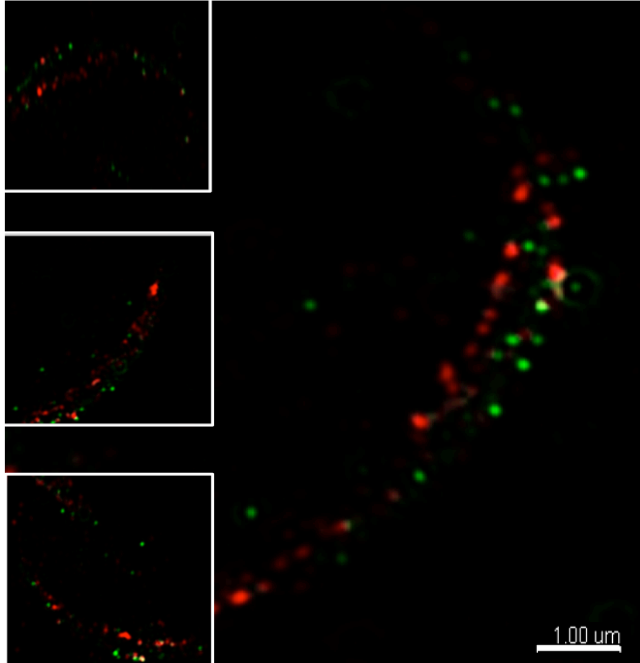
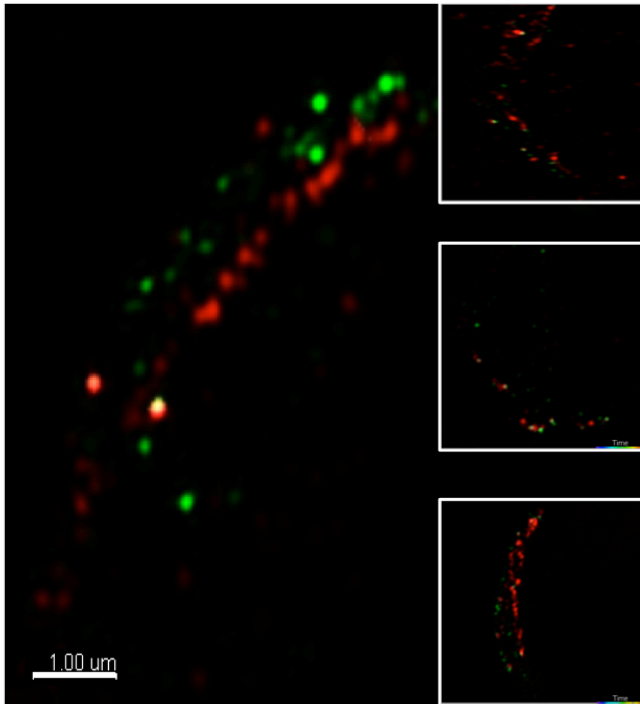


Figure 2.18. Synaptobrevin and syntaxin do not co-localize during capacitation.

Super resolution images collected using SR-SIM microscopy.

A. Images of sperm incubated in capacitating media for 0 min. The large picture and three small images on **(right)** depict the similar treatment.

B



B. Images of sperm incubated in capacitating media for 60 minutes. The larger picture and three small images on **(left)** show the similar trend. No co-localization was observed between syntaxin (green) and synaptobrevin (red) when images collected at both 0 and 60 min incubation in capacitating media were compared **Figure 2.9(A)**.

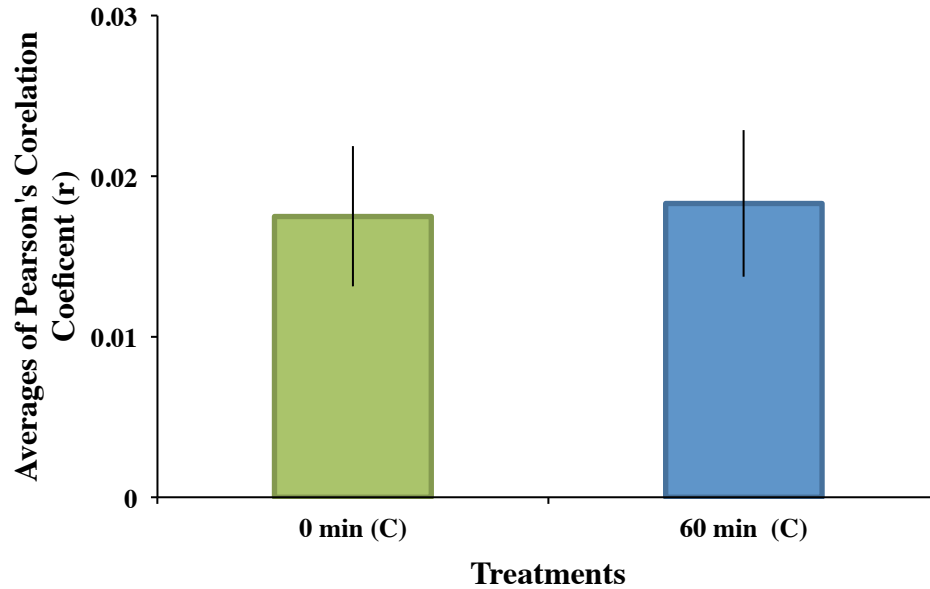


Figure 2.19. Synaptobrevin and syntaxin do not co-localizes during capacitation in mouse sperm.

Graphical representation of co-localization data obtained from SR-SIM images. The co-localization of syntaxin and synaptobrevin was calculated using the IMARIS, COLOC tool. Pearson's correlation analysis and student's T test was used to statistically analyze the data. No significant correlation was seen between both 0 min and 60 min treatment of sperm in capacitating medium ($p > 0.10$).

CHAPTER 3

CONCLUSION

I observed the re-localization of SNARE protein with a high-resolution microscopy technique (SR-SIM), which revealed that the t-SNARE syntaxin re-localizes during capacitation but the v-SNARE synaptobrevin does not. Thus, the movement of t and v-SNAREs in sperm during capacitation does not occur synchronously. SNARE proteins t and v-SNAREs are responsible for forming complexes with each other when at points on apposing membranes. They work together in coupling two membranes together and ultimately initiating membrane fusion.

SNARE core complex includes synaptobrevin (v-SNARE) residing on the vesicle membrane, and SNAP-25 and syntaxin (t-SNAREs) residing on the plasma membrane (Jahn & Südhof, 1999). Formation of the SNARE core complex is a stepwise process that requires triggering at different levels before membrane fusion can happen (Blas et al., 2005). Foundations for core complex are already laid in the ejaculated sperm but for it to come together and work in synchrony the ejaculated sperm needs to undergo capacitation and acrosome reaction (Baibakov et al., 2007; de Lamirande, 1997; Meizel, 1985). As mentioned before, what elicits the acrosome reaction is still controversial (Buffone, et al., 2014, 2012).

During sperm capacitation in the female reproductive tract, sperm undergo major changes in their physiology (Wassarman et al., 2001). One change is removal of cholesterol, which alters the fluidity of the plasma membrane (Flesch et al., 2001). Looking at the results collected from my experiments, it can be concluded that removal of cholesterol from the plasma membrane of the sperm by albumin likely plays a major role in syntaxin (t-SNARE) re-localization to the apical ridge of the sperm head. On the other hand, the location of synaptobrevin (v-SNARE) in the sperm outer acrosomal membrane is not effected by the removal of cholesterol from the plasma membrane. Bicarbonate (HCO_3^-), another very significant factor in the sperm capacitation process (Aitken & Nixon, 2013) does not seem to play a role in re-localization of SNARE proteins, at least during capacitation. Withdrawal of bicarbonate (HCO_3^-) from incubating medium did not affect syntaxin re-localization to the apical ridge of the sperm. Because withdrawing bicarbonate (HCO_3^-) prevents capacitation (Visconti et al., 1998), it is apparent that movement of SNARE proteins completely linked to the process of capacitation.

Other results showed that synaptobrevin (v-SNARE) and syntaxin (t-SNARE) are not synchronous in their movement during capacitation. This was concluded because syntaxin was re-localized during capacitation but synaptobrevin was not. These results indicate that along with SNARE proteins, membrane exocytosis requires other regulatory mechanisms (Buffone, Hirohashi, et al., 2014; Park et al., 2014) that might control the temporal and spatial arrangement of (v and t SNAREs) during capacitation and membrane fusion.

Sperm surface re-arrangements during fertilization are extremely complex and intricate (Gadella & Luna, 2014). It can be seen that even after years of research, the understanding of membrane fusion in sperm is far from complete. Further insights into the acrosomal exocytosis process will not only help in diagnosis and treatment of infertility but will also aid the development of new contraceptives.

FUTURE DIRECTIONS

This project addressed questions about the location of SNARE proteins namely syntaxin and synaptobrevin in mouse sperm prior to acrosome reaction in capacitating and non-capacitation conditions, in-vitro. This improved our understanding about the role of capacitation in acrosomal exocytosis. Figuring out the molecular mechanisms and events during capacitation, upstream and downstream to the Calcium (Ca^{2+}) influx would be one of the future goals of our lab.

Another imperative question, which needs to be answered along with these experiments, is looking at how the intermediates for acrosomal exocytosis are managed and what proteins are involved in this regulation. Looking at re-localization of another t-SNARE SNAP-25 will also aid in understating the spatial organization of SNARE protein before and after capacitation.

As removal of cholesterol during capacitation leads to re-ordering of lipid rafts, they are considered responsible for movement of transmembrane proteins in the plasma membrane (Boerke et al., 2008; Buschiazzo et al., 2013; Chamberlain et al., 2001; Kabouridis et al., 2000; Tsai et al., 2009; Gestel et al., 2005). It will be worthwhile to study the re-localization of lipid rafts during capacitation using various lipid raft markers. Co-localization assays can also be done between lipid rafts and t-SNAREs to follow their re-localization during capacitation.

CHAPTER 4

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