

## Expression of Syntaxin 2 in Bovine Sperm

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### Abstract

Syntaxins are membrane integrated Q-SNARE proteins known to participate in exocytosis. Vesicle docking involves the binding of two plasma membrane proteins, syntaxin and SNAP-25, to the vesicle membrane protein VAMP to form a stable trimeric core complex; synaptophysin is thought to regulate the formation of this complex. Although the members of Q-SNARE proteins are characterized in somatic cells, it is not known whether related proteins function in the sperm acrosome reaction. The objective of the present study is to identify and localize syntaxin in bovine epididymal spermatozoa and to determine the fate of syntaxin 2 during the acrosome reaction. Western blots of caput and cauda sperm lysates and plasma membrane fractions, stained with anti-syntaxin 2, revealed the presence of a 31kDa band in both sperm lysates and plasma membrane fractions, respectively. Indirect immunofluorescence localized syntaxin 2 to the anterior but not the equatorial regions of the acrosomal segment. Several biochemical analyses demonstrated that syntaxin 2 is an integral component of bovine cauda sperm plasma membranes. Our immunoblot data reveals that syntaxin 2 of bovine cauda sperm is released after lysophosphatidylcholine (LPC)-induced acrosomal exocytosis. It is assumed that syntaxin 2 may be involved in triggering the acrosome reaction through a ligand-receptor mediated signal transduction pathway.

**Received Date: July 06, 2016**

**Accepted Date: July 30, 2016**

**Published Date: August 02, 2016**

**Citation:** Nagdas, S.K., et al. Expression of Syntaxin 2 in Bovine Sperm. (2016) Cell Immunol Serum Biol 2(2): 41- 47.

**DOI:** 10.15436/2471-5891.16.999

**Keywords:** Bovine sperm-syntaxin-acrosomal reaction

### Introduction

The successfulness of mammalian fertilization completely relies on the efficacy of the acrosome reaction. The mammalian sperm acrosome reaction entails extensive fusion between the periacrosomal plasma membrane and the outer acrosomal membrane, with the release of hydrolases which function in sperm penetration through the zona pellucida<sup>[1-3]</sup>. Following the acrosome reaction the fused hybrid membrane complex remains adherent to the zona surface,<sup>[4]</sup> and the newly exposed inner acrosomal membrane of the spermatozoon functions as the limiting membrane or plasma membrane equivalent<sup>[1]</sup>. Receptors on the inner acrosomal membrane maintain sperm adhesion to the zona pellucida by binding ZP2<sup>[5]</sup>, and although the inner acrosomal membrane makes first contact with the egg membrane<sup>[1,6]</sup>, the plasma membrane overlying the equatorial, or possibly the postacrosomal segment appears to function in the initial fusion with the egg plasma membrane<sup>[1,7,8]</sup>. These diverse functions performed by the acrosomal segment during fertilization reveal the importance of identifying the specific signaling events and protein constituents which initiate and regulate the membrane fusion process of the acrosome reaction and prepare spermatozoa to fuse with the egg.

The acrosome reaction is analogous to regulated exocytosis in somatic cells in that both are initiated by ligand acting at the plasma membrane, both require activation of signaling pathways and ion channels, both are Ca<sup>2+</sup>-dependent and both result in the fusion of the plasma membrane with the membrane of a docked secretory granule (acrosome)<sup>[2,9,10]</sup>. In contrast to the sperm acrosome reaction, the secretory pathway of somatic cells has received detailed characterization in recent years and a great deal has been learned of the processes regulating vesicle docking and fusion with the plasma membrane. Key interacting proteins of the plasma membrane, the cytosol and the vesicle membrane which function in the membrane fusion pathway, have been identified in a variety of cell types and characterized at the molecular level<sup>[10-14]</sup>. These include syntaxin and SNAP25 (synaptosome-associated

protein of 25kDa) localized to the plasma membrane; VAMP/synaptobrevin, synaptophysin and synaptotagmin of the vesicle membrane; and the cytosolic proteins N-ethylmaleimide sensitive factor (NSF), a trimeric ATPase required for membrane fusion, and the  $\alpha$ - $\beta$ -Y-SNAPs (soluble NSF attachment proteins), which function in binding NSF to the membrane<sup>[10,14]</sup>. Although the members of these protein families are widely distributed among various types of somatic cells<sup>[10,11]</sup>, it is not known whether related proteins function in the acrosome reaction. Vesicle docking involves the binding of two plasma membrane proteins, syntaxin and SNAP-25, to the vesicle membrane protein VAMP to form a stable trimeric core complex<sup>[10]</sup>; synaptophysin is thought to regulate formation of this complex<sup>[15-17]</sup>. Exocytosis is triggered by an increase in cytosolic calcium levels, and the synaptotagmin, a Ca<sup>2+</sup>-binding protein to the vesicle membrane, is proposed to function as a sensor and molecular trigger in the final steps leading to membrane fusion<sup>[10,13,14,18,19]</sup>. These steps include the association of the soluble SNAPs and NSF with the core complex to form a 20S complex responsible for membrane fusion. Thus, a highly regulated and ordered set of protein-protein interactions leads to membrane fusion. What is the molecular machinery employed to generate the specific fusion of the plasma membrane and outer acrosomal membrane during the acrosome reaction? It has been shown that syntaxin 2 was present over the acrosomal region of rat sperm and during the acrosome reaction syntaxin 2 was shed from sperm heads<sup>[20]</sup>. Hutt et al<sup>[21]</sup> showed that mouse sperm syntaxin 2 binds to synaptotagmin I, VI and VIII isoforms in a calcium-dependent manner. The interaction of synaptotagmin VI and VIII isoforms to syntaxin 2 can potentially mediate calcium-stimulated secretion and exocytosis. All these studies strongly suggest that the mammalian sperm acrosome reaction employs proteins related to those involved in regulated exocytosis of somatic cells. The objective of the present study is to localize and biochemically characterize syntaxin 2 in bovine epididymal spermatozoa and to elucidate its role in the acrosome reaction.

## Materials and Methods

### Sperm preparation

Bovine epididymides were obtained from Randolph Packing Co., Inc., Asheboro, NC. Caput and cauda epididymides were minced and incubated for 5 minutes at 37°C in Hank's balanced saline, pH 7.4, to permit sperm release. The sperm suspensions were centrifuged at 5,000 rpm for 10 minutes. The pellets were washed by re-suspension in Hank's saline followed by centrifugation as above. The final pellets were re-suspended in a Tris-saline-protease inhibitor solution (TNI) composed of 150 mM NaCl, 25 mM Tris-HCl (pH 7.5), 2 mM benzamidine, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1 mM NaF, 1 mM sodium orthovanadate and 0.05% sodium azide, and centrifuged at 5,000 rpm for 10 minutes at 4°C. The pellets were washed one additional time with TNI as described above. Sperm pellets were used in extraction and fractionation protocols described below. An enriched plasma membrane fraction was isolated from cauda spermatozoa using nitrogen cavitation and sucrose density gradient centrifugation<sup>[22]</sup>. Protein was estimated by the procedure of Bradford<sup>[23]</sup>.

**Preparation of cell fractions for western blot analysis:** Cauda sperm plasma membrane was extracted in high salt (0.5M NaCl) in TNI for 1 hour at 4°C followed by centrifugation at 30,000 rpm for 35 minutes at 4°C in a Beckman SW40 rotor. The supernatant was collected, and the pellet was suspended in TNI. The volume of both pellet and supernatant fractions were made equal to that used in the initial high salt extraction step. To identify the integral membrane protein(s), phase separation analysis of cauda sperm plasma membranes was performed<sup>[24]</sup>. Both aqueous and detergent phases were analyzed on 12% SDS-PAGE and transferred to PVDF membranes for immunoblot analysis.

**SDS-PAGE and western blotting:** SDS-PAGE was performed on 12% acrylamide gels<sup>[25]</sup>. Protein bands were stained with Coomassie Brilliant blue dye<sup>[26]</sup>. For 2D-PAGE, Bio-Rad precast immobilized pH-gradient (pH 3 - 10) strips (7 cm) were used for isoelectric focusing (IEF) in a Bio-Rad Protean IEF cell according to the manufacturer's instructions. Western blots were prepared on polyvinylidene difluoride (PVDF) membranes<sup>[27]</sup>. For immunoblot analysis, nonspecific protein binding sites on blots were blocked with buffers containing BSA, goat serum, nonfat dry milk, and Tween-20. Blots were stained with anti-syntaxin 2 polyclonal antibody (Catalog# ab118829, abcam, Cambridge, MA, USA) followed by affinity-purified horseradish peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive bands were visualized using diaminobenzidine for color development or by ECL detection.

**Immunocytochemistry:** For immunofluorescence microscopy of intact cauda epididymal sperm, prepared as described above, were fixed at 4°C in 4% formaldehyde in 0.1 M sodium phosphate buffer, pH7.6, for at least 30 minutes, attached to poly-L-Lysine coated coverslips, washed with PBS, and permeabilized by incubation for 10 minutes in -20°C acetone. After three rinses in PBS, nonspecific protein binding sites were blocked in PBS containing 0.1% Tween-20 and 2.5% BSA (blocking solution). Coverslips were then incubated with anti-syntaxin 2 polyclonal antibody in blocking solution, washed and incubated with FITC-conjugated goat anti-rabbit IgG in blocking solution. Coverslips were washed with PBS and the cells were examined by phase contrast and epifluorescence microscopy.

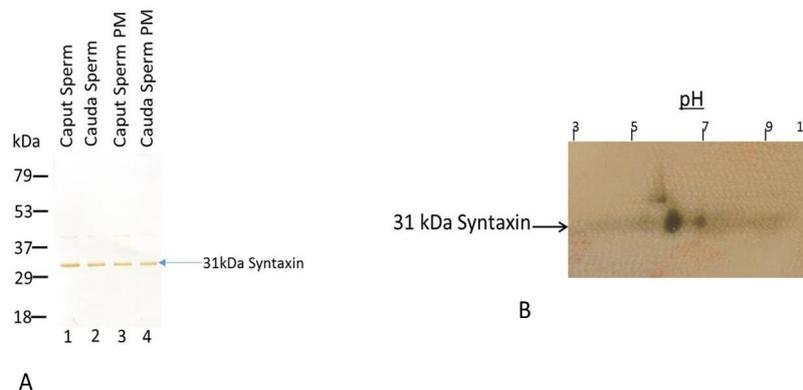
**Capacitation and acrosome reaction:** Sperm (4 - 6 X 10<sup>7</sup>/ml) were capacitated in the presence heparin (10 mg/ml) in a modified Tyrode's medium (pH 7.4) for 4 hours at 39°C with a 95% air: 5% CO<sub>2</sub> atmosphere<sup>[28]</sup>. To initiate the acrosome reaction, sperm were incubated with 100  $\mu$ g/mL lysophosphatidyl choline (LPC) for 15 minutes following the end of the 4 hour incubation. Samples were centrifuged at 4000 x g for 10 minutes at 4°C. The pellets and supernatants were adjusted to equal volumes and used for SDS-PAGE and acrosin determination. Morphologically, the fluorescent PSA lectin staining was performed to examine the efficacy of LPC-induced acrosome reaction of bovine cauda epididymal spermatozoa<sup>[29]</sup>.

**Acrosin assay:** Acrosin activity of the pellet and supernatant fractions of capacitated and acrosome reacted spermatozoa were assayed<sup>[30,31]</sup>. One unit of acrosin is defined as the quantity of enzyme required to hydrolyze 1  $\mu$ m of N-p-tosyl-gly-pro-arg-p-nitroanilide per minute.

## Results

### Identification of syntaxin 2 in caput and cauda epididymal spermatozoa

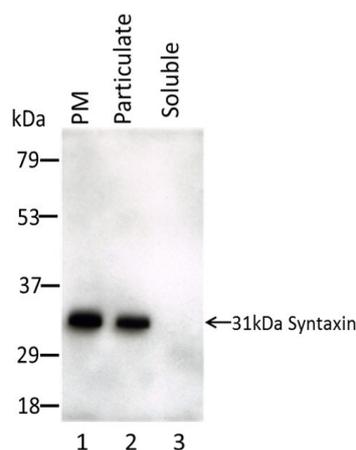
To identify syntaxin 2 in bovine epididymal spermatozoa, Western blots of total lysate of caput sperm (Figure. 1A, lane 1) and cauda sperm (Figure. 1A, lane 2) were stained with anti-syntaxin 2 antibody. Bovine caput (Figure. 1A, lane 3) sperm revealed the presence of a 31kDa syntaxin 2 immunoreactive band. We then examined the presence of syntaxin 2 in bovine caput and cauda plasma membrane fractions. Immunoblots of the enriched plasma membrane fractions of caput (Figure. 1A, lane 3) and cauda (Figure. 1A, lane 4) spermatozoa stained with anti-syntaxin 2 antibody showed the presence of a 31kDa syntaxin 2 band. An identical blot stained with preimmune IgG did not exhibit any immunoreactive band (data not shown). This study showed the presence of a 31kDa syntaxin 2 both in bovine caput and cauda epididymal spermatozoa. Two-dimensional PAGE was performed to examine charge variant isoforms of syntaxin 2 polypeptide. The cauda sperm plasma membrane fraction was fractionated by two-dimensional PAGE and transferred to PVDF membranes for immunoblot analysis. Blot stained with anti-syntaxin 2 exhibited the presence of a major isoform of 31kDa syntaxin 2 polypeptide (pI~5.9) (Figure. 1B). A duplicate blot stained with preimmune serum was used as the control and exhibited no positive spots (data not shown).



**Figure. 1A:** A 31kDa syntaxin 2 was present both in bovine caput and cauda epididymal spermatozoa. Immunoblot analysis of syntaxin 2 in total caput (lane 1) and cauda (lane 2) sperm lysates and in plasma membranes isolated from caput (lane 3) and cauda (lane 4) epididymal spermatozoa. **B:** 2D PAGE: The cauda sperm plasma membrane fraction reveals the presence of a major isoform of a 31kDa syntaxin 2 polypeptide having pI~5.9. Immunoblot, stained with anti-syntaxin 2, of cauda sperm plasma fraction, exhibiting the presence of a major isoform of a 31kDa syntaxin 2 polypeptide (pI~5.9).

### Membrane anchoring of syntaxin 2 in spermatozoa

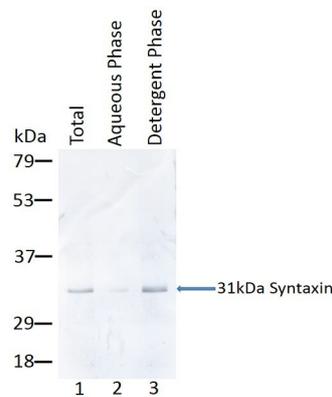
We next examined the solubility pattern of syntaxin 2 of cauda sperm plasma membrane fraction in high salt (0.5M NaCl). Western blot of the total (Figure. 2, lane 1), the particulate (Figure. 2, lane 2), and the high salt soluble fraction (Figure. 2, lane 3) stained with anti-syntaxin showed the presence of a 31kDa immunoreactive syntaxin 2 band both in the total (lane 1) and in the high salt extracted particulate fraction (lane 2). This study suggests that the syntaxin 2 is an integral component of bovine cauda sperm plasma membranes.



**Figure. 2:** Syntaxin 2 is only associated with the particulate fraction. Western blot immunostained with syntaxin 2 antibody showing the total cauda plasma membrane (PM) fraction (lane 1), the particulate (lane 2) and soluble (lane 3) fractions obtained after high salt (0.5M NaCl) extraction of cauda plasma membrane fraction.

### Phase separation analysis of syntaxin 2

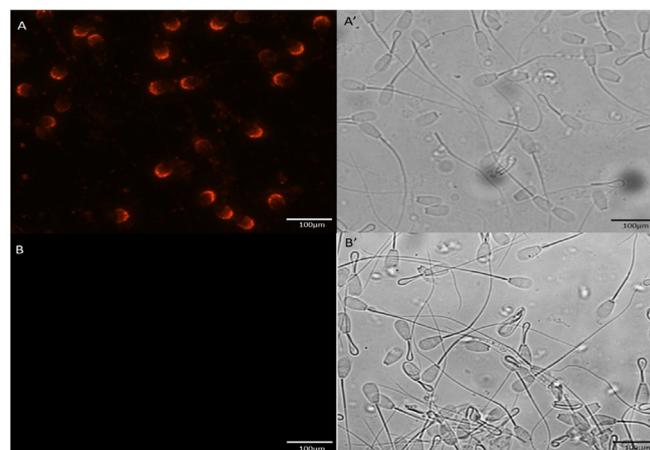
To determine if the syntaxin 2 is an integral or peripheral membrane protein, cauda plasma membrane fractions (3.4 µg protein/µl) were extracted in 1% Triton X-114. Resulting aqueous and detergent phases were collected and SDS-PAGE was performed on 12% gels. Western blot analysis with anti-syntaxin 2 revealed the presence of a significant portion of a 31kDa syntaxin 2 in the detergent phase (Figure. 3, lane 3); whereas, the remaining syntaxin 2 was present in the aqueous phase (Figure. 3, lane 2). Since the major portion of the 31kDa syntaxin 2 was existent in the detergent phase (Figure. 3, lane 3) by phase separation analysis, it strongly confirms that the syntaxin 2 is an integral membrane protein. This study has revealed that the bovine sperm syntaxin 2 polypeptide is embedded within the lipid bilayer of cauda sperm plasma membrane.



**Figure 3:** Syntaxin 2 is an integral membrane protein. Triton X-114 Phase Separation Analysis. Lane 1: Total bovine cauda plasma membrane protein prior to phase separation analysis. Lane 2: Aqueous phase. Lane 3: Detergent phase containing a significant portion of syntaxin 2 polypeptide.

### Immunolocalization of the syntaxin 2

Light microscopic immunocytochemistry were utilized to define the localization of syntaxin 2 in bovine cauda sperm. Acetone permeabilized sperm exhibited intense staining on sperm head with anti-syntaxin 2 (Figure. 4A). No staining of the equatorial segment or of the postacrosomal segment of the head was noted. Control specimens of acetone-permeabilized spermatozoa that were immunostained with preimmune IgG were completely negative (Figure. 4B).

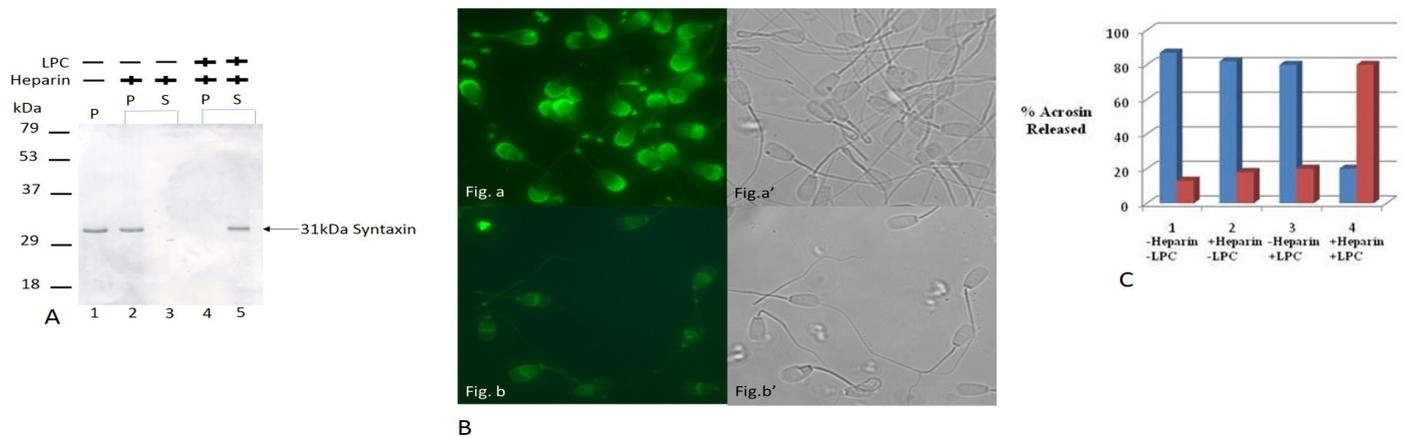


**Figure 4:** Syntaxin 2 is localized on bovine sperm head. Paired phase-contrast (A' and B') and fluorescence images (A and B) of bovine cauda sperm immunostained with anti-syntaxin 2 (A and A') and preimmune serum (B and B'). Cauda spermatozoa were permeabilized with acetone and then immunostained with antibodies. They show intense fluorescence of syntaxin 2 to the anterior but not equatorial regions of the acrosomal segment of bovine cauda sperm (A). No staining was observed when sperm stained with preimmune serum (B).

### Fate of syntaxin 2 during capacitation and acrosome reaction

To determine if syntaxin 2 is retained or released following capacitation and the acrosome reaction, Western blots of 12% SDS-PAGE were stained with anti-syntaxin 2. As demonstrated in Figure. 5A (lanes 1, and 2), syntaxin 2 was retained in the pellet before (lane 1) and after capacitation (lane 2), and the entire syntaxin 2 polypeptide was released after LPC-induced acrosome reaction (lane 5). Both morphological (stained with FITC-conjugated PSA-Figure. 5B) and biochemical (acrosin assay-Figure. 5C) assays were performed to confirm the occurrence of an LPC-induced acrosome reaction. Matched phase contrast (a' & b') and fluorescence (a & b) images of acrosome-intact (Figure. a & a') and acrosome-reacted sperm induced by LPC (Figure. b & b') stained with FITC-conjugated PSA (Pisum Sativum agglutinin) (Figure. 5B). Acrosome-intact sperm exhibited a homogenous signal over the entire acrosomal region (a), and the acrosome-reacted sperm displayed a signal over the equatorial region (b). As illustrated in Figure. 5C, fractions incubated in the presence of both heparin and LPC (Set 4) demonstrated a lower percentage (approximately 20%) of acrosin in pelleted fractions and a higher percentage (approximately 80%) of acrosin in supernatant fractions. This study

revealed that most of the acrosin present in cauda sperm was released in the supernatant fraction. All results support the proficiency of our acrosome reaction model. Previously, comparable results were also reported in bovine ejaculated sperm<sup>[32]</sup>. This data further suggests that the syntaxin 2 of bovine cauda sperm is released after LPC-induced acrosomal exocytosis.



**Figure 5A:** Syntaxin 2 is released after LPC-induced acrosome reaction of bovine spermatozoa. Immunoblot analysis of the pattern of syntaxin 2 in the particulate (P) and supernatant (S) fractions of spermatozoa after a 4 hr incubation in the presence or absence of heparin and with or without a final 15 min exposure to LPC. As demonstrated in figure A (lanes 1 and 2), the entire syntaxin 2 polypeptide was retained in the pellet of noncapacitated and capacitated sperm pellet fractions and was released after LPC-induced acrosome reaction (lane 5).

**B:** FITC-conjugated PSA staining displays the efficacy of the acrosome reaction. Matched fluorescence (a & b) and phase contrast (a' & b') images of acrosome-intact (Fig. a & a') and acrosome-reacted sperm induced by LPC (Fig. b & b') and stained with FITC-conjugated PSA (*Pisum Sativum* agglutinin). Acrosome-intact sperm exhibited a homogenous signal over the entire acrosomal region (a), and the acrosome-reacted sperm displayed a signal over the equatorial region (b).

**C:** Release of Acrosin Following Acrosome Reaction. Set 1: Pellet and supernatant fractions incubated in the absence of heparin and LPC. Set 2: Pellet and supernatant fractions incubated in the presence of heparin and the absence of LPC. Set 3: Pellet and supernatant fractions incubated in the presence of LPC and the absence of heparin. Set 4: Pellet and supernatant fractions incubated in the presence of both heparin and LPC. The percent of acrosin released in Set 4 was significantly higher than that of Sets 1-3 resulting in a successful execution of the acrosome reaction. The data are representative of three experiments. Blue Bar-Pellet, Red Bar-Supernatant.

## Discussion

Soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins are present in mammalian sperm and could be involved in the acrosome reaction. Vesicle docking involves the binding of two plasma membrane proteins, syntaxin and SNAP-25, to the vesicle-associated membrane protein/synaptobrevin VAMP to form a stable trimeric core complex<sup>[10]</sup>; synaptophysin is thought to regulate formation of this complex<sup>[15-17]</sup>. Vesicle-associated membrane protein/synaptobrevin, a SNARE on the membrane of a vesicular carrier, and syntaxin I, a SNARE on the target membrane as well as the calcium sensor synaptotagmin I, are present in the acrosome of mammalian sperm<sup>[33]</sup>. Syntaxin 2 is one of the SNARE family members<sup>[34]</sup>. In the present study, we have demonstrated the localization of syntaxin 2 to the anterior but not the equatorial regions of the acrosomal segment. Katafuchi et al<sup>[20]</sup> showed that syntaxin 2 is localized to the acrosomal region of rat spermatozoa.

The post-translational modification(s) of proteins during epididymal transit could be an important biochemical event leading to the production of functionally mature spermatozoa<sup>[35-42]</sup>. In the present study, no change in the molecular form of syntaxin 2 was observed in total sperm lysates and in the isolated sperm plasma membrane fractions of both caput and cauda epididymal spermatozoa. Several lines of our biochemical studies reveal that bovine sperm syntaxin 2 is an integral membrane protein. Our 2D-PAGE reveals a major form of 31kDa syntaxin 2 (~pI 5.9) and a minor form. Tian et al<sup>[43]</sup> reported that syntaxin-1 is a substrate of DAP (death-associated protein) kinase, a calcium/calmodulin-dependent protein kinase. Other investigators showed that syntaxin-1 was phosphorylated by casein kinase II on both serine and threonine residues<sup>[44,45]</sup>. Risinger and Bennett<sup>[44]</sup> reported an enhanced interaction between phosphorylated syntaxin-1 and the synaptic vesicle protein synaptotagmin 1, a potential calcium sensor in triggering synaptic vesicle exocytosis. It is assumed that the syntaxin 2 of the bovine sperm plasma membrane fraction undergo phosphorylation during capacitation that may lead to the generation of different syntaxin 2 isoforms generated during capacitation. Future studies will address this issue.

In the current study, we have shown that syntaxin 2 is released during LPC induced acrosome reaction. Rat sperm syntaxin 2 is released upon the acrosome reaction induced by calcium ionophore *in vitro*<sup>[20]</sup>. Other investigators also reported that bovine and rhesus sperm SNAREs are sloughed off during the acrosome reaction<sup>[33]</sup>. SNARE antibodies inhibit both the acrosome reaction and fertilization, without inhibiting sperm-egg binding<sup>[33]</sup>. In sea urchin-the involvement of syntaxin in the acrosome reaction, possibly through the interaction with VAMP and SNAP-25 during membrane fusion process, as proposed for synaptic vesicle exocytosis<sup>[46]</sup>. SNARE fusion machinery for neuron and endocrine tissues has something in common with that operating during the acrosome reaction in both invertebrates and rodents<sup>[20]</sup>. Rab3A, a small GTPase that has a regulatory role in many exocytotic fusion events, was also found in mammalian sperm<sup>[47,48]</sup>. The localization of syntaxin 2 to the acrosomal region suggest the involvement of SNARE and Rab GTPase in the membrane fusion phase of the mammalian acrosome reaction<sup>[20]</sup>. However, how the rab3A is involved in the acti-

vation or assembly of SNARE fusion machinery in mammalian spermatozoa is yet to be determined. It has been shown that syntaxin binds directly to a voltage-dependent calcium channel in the presynaptic membrane thereby directly linking regulated ion fluxes to the fusion machinery<sup>[49,50]</sup>. Whether syntaxin 2 in spermatozoa could associate with an acrosome-specific voltage-dependent calcium channel during the final steps preceding acrosomal exocytosis needs to be elucidated. Previously, Tsai et al<sup>[51]</sup> demonstrated the capacitation-dependent formation of a trimeric trans-SNARE complex (syntaxin 1B/SNAP23/VAMP3) that served to dock the outer acrosomal membrane with the apical porcine sperm plasma membrane. Recently, same investigators showed that calcium ionophore-induced acrosome reaction results in the formation of unilamellar hybrid membrane vesicles that contained syntaxin 3, SNAP 23 and VAMP2, with an additional SNARE interacting protein, complexin 2<sup>[52]</sup>.

Collectively, several components are involved in mammalian sperm acrosomal exocytosis that include Rab3A activation,  $\alpha$ -SNAP/NSF, synaptotagmin VI, complexin, and SNAREs (toxin-sensitive members of the synaptobrevin, syntaxin, and SNAP-25 families). In addition, it also requires cAMP and Epac<sup>[53]</sup>. Our current study reveals that bovine sperm syntaxin 2 is released during the acrosome reaction. Recently, we have also shown that LPC-induced heparin-capacitated sperm exhibit the release of a significant portion of SPACA3<sup>[54]</sup>. We proposed that acrosin participates in the release of SPACA3 during acrosome exocytosis<sup>[54]</sup>. Presently, we do not know whether syntaxin 2 is assembled into a pre-fusion core complex in epididymal spermatozoa or whether the core complex is assembled during capacitation. Alternatively, it is also possible that syntaxin 2 functions in the assembly of a membrane fusion complex during the lysophosphatidylcholine (LPC)-induced acrosome reaction. These possibilities will be addressed in future studies. The molecular machinery engaged to generate the specific fusion of the plasma membrane and outer acrosomal membrane during the acrosome reaction is yet to be elucidated. Our hypothesis is that germ cell syntaxin 2 functions in the regulated fusion of the periacrosomal plasma membrane with the acrosomal membrane during the acrosome reaction. Permeabilized<sup>[55]</sup> bovine spermatozoa can be utilized to examine the potential function of syntaxin 2 in acrosomal exocytosis in the presence of anti-syntaxin 2 IgG or Fab fragments of syntaxin 2 IgG. Future studies will address this issue.

**Acknowledgement:** Supported by NIH/NIGMS/ 1SC3GM096875, FSU NC-LSAMP Grant, FSU RISE NIH Grant # 2R25GM064508-13

**Conflict of Interest:** The authors declare that they have no conflict of interest.

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