Functional distribution of synapsin I in human sperm

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ABSTRACT

Proteins known to function during cell–cell communication and exocytosis in neurons and other secretory cells have recently been reported in human sperm. Synapsins are a group of proteins that have been very well characterized in neurons, but little is known about synapsin function in other cell types. Based upon previous findings and the known function of synapsin, we tested the hypothesis that synapsin I was present in human sperm. Washed, capacitated, and acrosome induced sperm preparations were used to evaluate the functional distribution of synapsin I using immunocytochemistry. Protein extracts from mouse brain, mouse testis/epididymis, and human semen were used for protein blotting techniques. Immunolocalization revealed synapsin I was enriched in the sperm equatorial segment. Protein extracts from mouse brain, mouse testis/epididymis, and human semen were positive for synapsin I using several different antibodies, and dot blot results were confirmed by Western blot analyses. Finally, treatment of capacitated and acrosome reaction induced samples with anti-synapsin antibodies significantly reduced sperm motility. Localization of synapsin I in human sperm is a novel finding. The association of synapsin I with the sperm equatorial segment and effects on motility are suggestive of a role associated with capacitation and/or acrosome reaction, processes that render sperm capable of fertilizing an oocyte.

1. Introduction

Successful fertilization involves species-specific communication between the sperm and oocyte. *In vivo*, as sperm travel through the female reproductive tract, a series of calcium dependent biochemical changes, known collectively as capacitation, occur. Changes include sterol oxidation and efflux from the membrane, movement of lipid rafts, increase in intracellular cAMP, tyrosine phosphorylation, and hyperactivated motility [1]. Upon interaction with the oocyte zona pellucida, sperm undergo acrosome reaction (AR); an exocytotic process whereby the contents of the acrosome are released. Previous studies have shown that both capacitation and acrosome reaction are necessary for sperm to acquire the ability to fertilize an oocyte [2,3]. Vesiculation of the sperm membrane and simultaneous release of the acrosomal contents could be likened to the release of signaling substances as a means of cell–cell communication in other cell types. This communication must be efficient, reliable, and carefully regulated. Exocytosis and

release of signaling substances in neurons have been studied in detail. One group of proteins implicated in these processes are synapsins. The synapsins arise from three separate genes in vertebrates, and multiple isoforms exist due to alternative splicing [4–6]. Synapsin I has two isoforms, Ia and Ib, with molecular weights of 86 and 80 kDa respectively [7]. Synapsin I was discovered as a phosphorylation target dependent on cAMP [7–9], and later as a phosphorylation target of Ca^{2+}-calmodulin dependent protein kinases [10–12]. Synapsin I is highly abundant in both peripheral and central nervous systems. It is associated with small synaptic vesicles within presynaptic terminals but dissociates upon calcium dependent phosphorylation [13–18]. Several studies have shown that synapsin I interacts with actin, which changes upon phosphorylation [19–23]. Synapsin I is thought to create a reserve pool of vesicles by binding to synaptic vesicles and actin. Upon phosphorylation following calcium influx, synapsin I dissociates from synaptic vesicles and actin, allowing vesicle mobilization and subsequent release. Additionally, synapsins play roles in neuronal growth, development, and synapse formation [24–28]. Early studies suggested synapsin proteins were neuron specific [7,9,29,30], and little is known about their function in non-neuronal cells. Synapsin I has been characterized in cell lines of non-neuronal origin, such as AtT-20 cells [31] and PC12 cells treated with NGF [32,33]. Synapsin I has been implicated in insulin

exocytosis, although whether it is associated with secretory granules or other vesicular structures is unclear [34–37]. Synapsin I has also been reported in cultured astrocytes [38], liver, NRK epithelial cells [39,40], and osteoblasts [41].

Rab3a and Munc13, whose respective functions in synaptic vesicle targeting and priming in neurons are well known [42,43] also regulate secretion in bovine chromaffin cells [44–46] and insulin release [47,48]. The interaction and functions of several key secretory regulators have been investigated in acrosomal exocytosis in human sperm [49–51]. Recently, Rab3a, Munc13, and RIM have been reported in human sperm with functions during acrosomal exocytosis [49,51]. These findings led to our hypothesis that synapsin proteins may be present in human sperm. The purpose of this study was to investigate the localization and functional distribution of synapsin I in washed, capacitated, and acrosome induced human sperm. Additionally, we evaluated sperm motility following capacitation and acrosome reaction in the presence and absence of anti-synapsin antibodies. Results reported herein revealed synapsin I enrichment in the sperm equatorial segment. Inclusion of anti-synapsin antibodies during capacitation and acrosome reaction significantly reduced sperm motility.

2. Materials and methods

2.1. Semen sample collection

Human semen samples were collected with written consent from volunteers 18 years of age and older. All experimental procedures involving human samples were approved by the Bloomsburg University Institutional Review Board (approved protocol # 2013-4). All samples were de-identified such that volunteer confidentiality was maintained, and samples were assigned a random study identification code. Whole semen was analyzed within 1–2 h of collection. Total sample volume, pH, total cell count, and percent motility were recorded.

2.2. Sperm sample preparation

Washed human sperm were prepared by placing 1.5 mL of whole, human semen into a 15 mL conical tube with 5 mL of human sperm medium (HSM) [52]. The sample was then centrifuged for 10 min, $500 \times g$ at room temperature (RT). The supernatant was carefully decanted and the sperm pellet was resuspended in 1.5 mL HSM. For capacitation, 1 mL of washed sperm was mixed with 1 mL of HSM with 7% BSA and incubated 3–4 h at 37 °C with 5% CO_2. Following capacitation, the sample was microscopically observed for the presence of hyperactivation. Only samples with at least 50% motility were utilized for acrosome reaction induction. Capacitated sperm were induced to undergo acrosome reaction by incubation with 10 µM bromo-A23187 ionophore (EMD Millipore Corporation) for 30 min at 37 °C with 5% CO_2.

2.3. Protein extract preparation

2.3.1. Extraction buffer

Protein extraction was conducted using chilled RIPA buffer. Briefly, RIPA buffer was prepared in sterile 1X PBS and contained 1% Nonidet-P40 (Fisher Scientific), 0.5% deoxycholic acid sodium salt (Fisher Scientific), 0.1% sodium dodecyl sulfate (SDS) (Thermo Scientific), and 1X protease inhibitor cocktail (Sigma–Aldrich).

2.3.2. Protein extracts from mouse samples

All experimental procedures involving animal samples were approved by the Bloomsburg University Institutional Animal Care and Use Committee (protocol # 106). All tissues were extracted post-mortem from male mice (Harlan Laboratories, Hsd:ICR, CD-1) that were rapidly euthanized with an overdose of the inhalant anesthetic halothane, in accordance with the IACUC protocol. Protein extracts for dot blot and Western blot analyses were prepared from mouse brain and mouse testis/epididymis (T/E). Tissue was harvested from six different mice on separate occasions. For each mouse, extracts were prepared individually and samples were not pooled. Brain and T/E extracts from three different mice were utilized for the experiments presented herein and representative results are shown. The entire brain or T/E dissected from each mouse was weighed and 10 µL of RIPA buffer were added per mg of tissue. Samples were homogenized using a Polytron tissue homogenizer for 10 s. Following homogenization, samples were centrifuged for 20 min, $16,000 \times g$ at 2 °C. The supernatant was decanted and placed into sterile cryovials and used for dot and Western blot analyses. Total protein was quantified by bicinchoninic acid (BCA) assay. Samples were diluted with 1X PBS. Ten microliters of each sample were placed into a well of a micro-plate, mixed with 200 µL Pierce™ BCA working reagent (Thermo Scientific), and incubated for 60 min at 37 °C with shaking. Samples were run in triplicate. Absorbance was quantified at 570 nm using a Tecan GENios Plus plate reader. Total protein was determined by comparison to bovine serum albumin (BSA) standard curve.

2.3.3. Protein extracts from human semen

Human sperm and seminal plasma extracts were prepared by pooling whole semen samples from the same donor collected on separate days. For each sample, 1.5 mL of whole semen (approximately 62,000,000 sperm cells) were placed into a sterile microfuge tube and centrifuged at $6000 \times g$ for 15 min. The top 500 µL of supernatant were decanted and placed into a new microfuge tube containing 500 mL of RIPA buffer with 1X protease inhibitor. This sample was labeled as seminal plasma and frozen at −80 °C. The remaining supernatant was discarded and the pellet was resuspended with 250 µL of RIPA buffer with 1X protease inhibitor. This sample was labeled as sperm pellet and frozen at −80 °C. The second semen sample from the same donor was prepared following the above protocol. The first two samples were thawed and added to either the previous seminal plasma or sperm pellet extracts. Samples were then referred to as pooled seminal plasma or pooled sperm cell. Each pooled sample was sonicated at 9 V for 45 s. The samples were centrifuged at $15,000 \times g$ for 10 min. The top 400–500 µL of supernatant were reserved, placed into a new cryovial, and used for subsequent dot and Western blot analysis. Total protein was quantified using BCA assay as previously described.

2.3.4. Dot blot

Protein extracts from mouse brain, mouse T/E, human sperm cells, and human seminal plasma were spotted three times onto a piece of 0.20 µm nitrocellulose membrane (Thermo Scientific) in 2 µL aliquots, with drying time between applications. Vertical pencil lines were hand-drawn on each blot to delineate the area in which each sample was applied. All samples were applied to a single, continuous blot. The membrane was wet in 1X PBST (1X PBS containing 0.1% Tween-20 (Fisher Scientific)), placed into a 10 cm petri dish, and blocked for 1 h with 10 mL of 5% milk in 1X PBST on a shaker at RT. Following blocking, milk was poured off and 10 mL of primary polyclonal goat anti-synapsin Ia/b A-15 antibody (Santa Cruz Biotechnology Cat# sc-55774), polyclonal rabbit anti-synapsin Ia/b H-170 antibody (Santa Cruz Biotechnology Cat# sc-20780), or monoclonal mouse anti-synapsin Ia/b B-11 antibody (Santa Cruz Biotechnology Cat# sc-376622) were added. A-15 was raised against a peptide near the C-terminus of human synapsin Ia, H-170 was raised against amino acids 491–660 at the C-terminus of human synapsin Ia/b, and B-11 was raised against amino acids 2–29 at the N-terminus of human

synapsin Ia/b. All primary antibodies were used at a final concentration of 1 μg/mL. The anti-synapsin A-15 and H-170 antibodies have been previously shown to be specific for synapsin Ia/b in immunoblotting [53,54].

Membranes were incubated for 2 h at RT with shaking. Primary antibodies were poured off and the membrane was washed three times, five minutes per wash, with 1X PBST. Ten milliliters of secondary antibody rabbit anti-goat IgG (H + L) conjugated to horseradish peroxidase (HRP) (KPL, 14-13-06, Lot # 121056), goat anti-rabbit IgG (H + L) conjugated to HRP (KPL, 04-15-06, Lot # 101132), or goat anti-mouse IgG (gamma) conjugated to HRP (KPL, 074-1802, Lot # 130639) were added (0.2 μg/mL final concentration). Membranes were incubated for 1 h at RT with shaking. Secondary antibody was poured off and membranes were washed twice with 1X PBST and once with 1X PBS, five minutes per wash. One milliliter of TMB Membrane Peroxidase Substrate (KPL) was pipetted onto each blot and incubated for 3 min in the dark. Sterile, distilled water was poured onto the blot to stop the reaction. Development of color was documented digitally using an Alpha Innotech gel imaging system. Densitometry was performed using FluorChem HD software to determine mean pixel intensity for each sample. Equal areas for each sample were analyzed. All samples within the same dot blot were normalized to RIPA (average pixel intensity of sample − average pixel intensity of RIPA). Microsoft Excel was used to generate graphs. Dot blot analysis with each antibody was performed in triplicate using samples from different donors. Representative dot blots are shown. All primary antibodies were diluted in 5% milk in 1X PBST and all secondary antibodies were diluted in 1X PBST.

2.3.5. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot

SDS–PAGE was performed using a vertical gel apparatus (Fisher Scientific) with pre-cast NuPAGE 4–12% Bis-Tris gels (Novex by Life Technologies). Protein extracts were prepared as described above for dot blot analysis. Synapsin Ia/b (m): 293T lysate (Santa Cruz Biotechnology) was loaded as a positive control. Each extract was diluted with NuPAGE LDS Sample Buffer (Novex by Life Technologies), NuPAGE Sample Reducing Agent (Invitrogen), and sterile distilled water according to the manufacturer's instructions, and heated at 70 °C for 10 min. Fifteen microliters of kaleidoscope pre-stained protein standard (BioRad) or 25 μL of each protein extract were loaded and subjected to electrophoresis in 1X NuPAGE MOPS SDS Running Buffer (Novex by Life Technologies) with NuPAGE antioxidant (Invitrogen) at 200 V for 25–30 min. Following separation, proteins were electrotransferred onto 0.2 μm nitrocellulose membrane in 1X NuPAGE Transfer Buffer (Novex by Life Technologies) at 30 V for 1 h. The ladder can sometimes fade during membrane processing; therefore, pencil lines were drawn on the nitrocellulose to the right of the bands after transferring to mark their position. Membranes were blocked with 5% milk powder in 1X PBST and incubated for 1 h at RT with shaking. Ten milliliters of primary polyclonal rabbit anti-synapsin Ia/b H-170 antibody (1 μg/mL) in 5% milk with 1X PBST were added to the membrane and incubated at 4 °C for 20 h with shaking. The primary antibody was poured off and the membrane was washed three times, five minutes per wash, with 1X PBST. Ten milliliters of secondary antibody goat anti-rabbit IgG (H + L) conjugated to HRP (0.2 μg/mL) in 1X PBST were added to the membrane and incubated for 1 h at RT with shaking. The secondary antibody was poured off and the membrane was washed twice with 1X PBST and once with 1X PBS, five minutes per wash. Two milliliters of TMB Membrane Peroxidase Substrate were pipetted onto the membrane and incubated for 5 min in the dark. Sterile, distilled water was poured onto the membrane to stop the reaction. Development of color was documented digitally and the figure was assembled using Adobe

Photoshop CC. Western blot analysis was performed multiple times on separate occasions using samples from different donors to confirm results.

2.4. Immunocytochemistry

Eight microliters of washed (approximately 320,000 cells), capacitated (approximately 240,000 cells), or acrosome induced (approximately 208,000 cells) human sperm were applied to Fluorescent Antibody Rite-On Microslides (Gold Seal), allowed to air dry, and fixed with 100% methanol for 10 min. All staining procedures were performed at RT in a humidified slide box. Slides were moistened with 1X PBS and covered with a blocking solution containing either 5% normal goat serum (Invitrogen) or 5% milk. One hundred microliters of primary polyclonal rabbit anti-synapsin Ia/b H-170 antibody (20 μg/mL) were applied to each slide. The anti-synapsin H-170 antibody has been previously shown to be specific for synapsin Ia/b in immunostaining [55,56].

Slides were incubated for 1–2 h then rinsed three times with 1X PBS. Fluorescently tagged secondary goat anti-rabbit IgG (H + L) conjugated to Alexa Fluor 488 (Molecular Probes/Life Technologies) was applied to each slide at a final concentration of 4 μg/mL in PBS and incubated for 1 h. Control slides were treated with 1X PBS alone during the primary antibody step. For some experiments, slides were doubly labeled with RITC conjugated lectin from *Pisum sativum* (RITC-PSA) (Vector Laboratories) to evaluate membrane status. Thirty-five microliters of RITC-PSA (20 μg/mL) were added to the slides and incubated for 30 min at RT. Following several rinses, slides were covered with Mowiol and a No. 1 coverslip (Fisher Scientific). Slides were analyzed using a Zeiss LSM 510 Meta confocal microscope equipped with a 63X oil objective. Images were acquired in multi-track mode using Zen 2008 software. Figures were assembled using Adobe Photoshop CC. Immunocytochemistry experiments were performed using samples from multiple donors and representative results are shown.

2.5. Sperm motility assay

For some experiments, sperm were capacitated and acrosome induced in the presence of the polyclonal goat anti-synapsin Ia/b A-15 antibody or the polyclonal rabbit anti-synapsin Ia/b H-170 antibody (20 μg/mL). Following each treatment, percent motility was microscopically quantified using a Cell Vu® counting chamber and gridded coverslip. The number of motile and non-motile sperm was recorded from representative fields in each sample. Sperm making forward progress were classified as motile. Sperm motility was documented by placing 10 μL of each treatment into a Cell Vu® counting chamber with non-gridded coverslip. Video micrographs were captured using a Nikon microscope equipped with a Sony Exwave HAD DSP 3CCD color video camera. Percent motility was calculated by dividing the number of motile sperm by the total number of sperm counted. Average cell concentration for motility assays was 23–30 million cells/mL for A-15 and 20–25 million cells/mL for H-170 experiments. Descriptive statistics and paired *t*-tests were performed using SigmaPlot 12.5 and Microsoft Excel. Significant differences were at or below $p \leqslant 0.05$.

3. Results

By visual interpretation, dot blot analysis using polyclonal antibodies H-170 (Fig. 1A) and A-15 (Fig. 1C) and monoclonal antibody B-11 (Fig. 1E) revealed synapsin I was present in mouse brain, mouse T/E, sperm cell, and seminal plasma fractions. Fig. 1 shows single, continuous dot blots probed with each antibody. As expected in all blots, synapsin I was present in mouse brain (positive control) and absent in RIPA buffer with 1X protease inhibitor
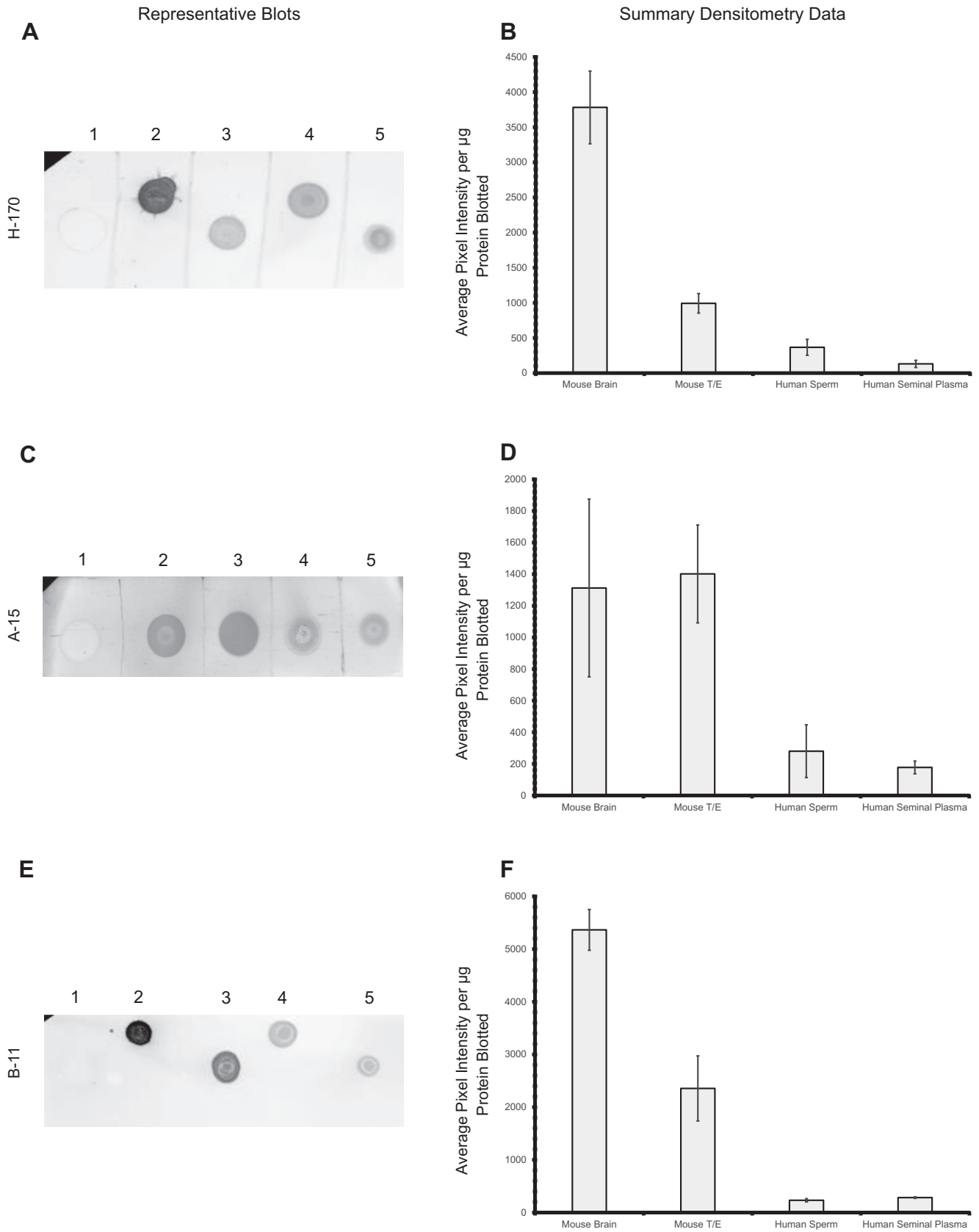
Representative Blots

A

Summary Densitometry Data

B

C

D

E

F

Fig. 1. Dot blot analysis revealed the presence of synapsin I in human sperm. RIPA (1), mouse brain (2), mouse T/E (3), human sperm (4), and human seminal plasma (5) samples were blotted onto nitrocellulose and probed with anti-synapsin Ia/b antibody H-170 (A), A-15 (C), or B-11 (E). Data were normalized to RIPA and presented as average pixel intensity per µg protein blotted (B, D and F). Error bars denote standard error.

(negative control). Polyclonal antibodies A-15 and H-170 recognize the C-terminus of synapsin Ia/b, whereas monoclonal antibody B-11 recognizes the N-terminus. Densitometry was performed to quantify average pixel intensity and normalized to RIPA. Total protein was determined using BCA and calculated by comparison to BSA standard curve ($R^2 = 0.98$). Average pixel intensity per µg protein blotted is shown (Fig. 1B, D and F) for blots probed with H-170, A-15, and B-11 respectively. This analysis was consistent with visual interpretation of the blots. Results from dot blot analysis were consistent over multiple replicates using samples from different donors. Taken together, antibodies raised against the C-terminus and N-terminus support the presence of synapsin I in human sperm.

Western blots were performed to confirm dot blot results. Results from a typical Western blot using H-170 antibody are shown (Fig. 2). As expected, an intense band was present in the lysate and the mouse brain lanes (positive controls). These bands were within the expected molecular weight range (75–100 kDa), as synapsin Ia/b is 80/86 kDa in size. Although these bands appear to be closer to the 75 kDa marker on the gel, this could be due to experimental variation during electrophoresis and electrotransfer. These positive control bands were consistently present for all Western blots performed. The bands present in the mouse T/E, human pellet, and seminal plasma lanes (Fig. 2, brackets) closely correspond to those in the lysate and mouse brain lanes (positive controls). Although the band from the pellet sample was faint, it was consistently present across multiple replicates and donors. Bands of lower molecular weight (37–75 kDa) were also present in all samples except the lysate. These lower molecular weight bands were not unexpected, as they are reported on the H-170 antibody data sheet from the manufacturer. These lower molecular weight bands could represent degradation products of synapsin I, or different isoforms specific to these samples. Additional experiments would be required to confirm the presence of a novel isoform in sperm; however, this was beyond the scope of the present study. Results from Western blot analyses were consistent over multiple replicates using samples from different donors. Similar results were obtained by probing nitrocellulose membranes with anti-synapsin antibody A-15 (data not shown). Although bands in the correct molecular weight range were noted, increased background was apparent with this antibody. Data from these experiments provided a second line of evidence confirming the presence of synapsin I in human sperm.

Immunolocalization using washed, capacitated, and acrosome induced human sperm preparations was performed and results shown in Fig. 3. Slide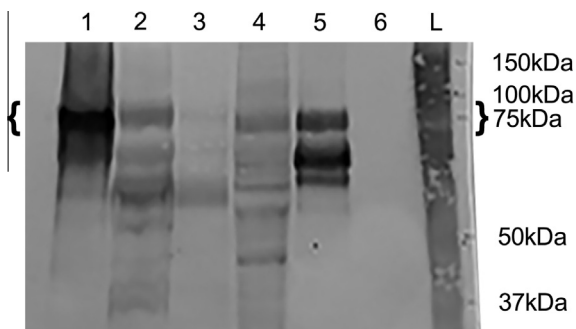s were doubly labeled with RITC-PSA to evaluate the status of the acrosomal membrane. Previous studies have shown that labeling sperm with lectin from *Arachis hypogea* (PNA) and *P. sativum* (PSA) are useful markers to evaluate the status of acrosomal membranes [57,58]. Synapsin I staining varied slightly depending upon the functional state of the sperm. Washed human sperm probed with H-170 antibody revealed staining in the sperm equatorial segment (Fig. 3A). The intensity of the stain varied depending upon the status of the acrosomal membranes. Cells in which the entire head was brightly and evenly stained with RITC-PSA showed light synapsin I staining. Sperm with diffuse RITC-PSA staining, denoting changes in the sperm membranes, showed a brighter band in the equatorial segment. These results were not unexpected, as previous studies have shown that in a given population of motile sperm, not all sperm undergo acrosome reaction and some spontaneously react [59]. Each sample analyzed will therefore contain sperm at various functional states. Capacitated and acrosome induced preparations revealed bright synapsin I staining in the equatorial segment (Fig. 3B and C). RITC-PSA staining was diffuse, with some cells unstained and some with equatorial bands. These staining patterns were expected, as the acrosomal membranes change during capacitation and acrosome reaction induction. Slides incubated with secondary antibody alone (control) revealed some background staining that was not associated with sperm (this non-specific background staining was noted for all experiments). Representative control fields selected at random for washed, capacitated, and acrosome induced preparations are shown in Fig. 3 as an overlay of the fluorescence and DIC channels. Similar results were obtained from immunolocalization experiments using the A-15 antibody (results not shown). As the sperm membrane status changes and the equatorial segment becomes more accessible, the intensity of the synapsin staining increased. Results from these experiments clearly document the localization of synapsin I in the human sperm equatorial segment.

Data evaluating the functional role of synapsin in human sperm are shown in Fig. 4. Sperm were capacitated and induced to undergo acrosome reaction in the absence and presence of anti-synapsin A-15 (Fig. 4A) or H-170 (Fig. 4B) antibody. Following each treatment, percent motility was microscopically quantified using a Cell Vu® counting chamber and gridded coverslip. The number of motile and non-motile sperm was recorded from representative fields in each sample. Fig. 4 shows a significant decrease in average percent motility for preparations capacitated and acrosome induced in the presence of A-15 and H-170. Capacitation in the presence of A-15 antibody decreased motility 23% ($p = 0.0002$) and the presence of H-170 decreased motility 27% ($p = 0.004$) compared to the control. A significant decrease in sperm motility was also noted during acrosome reaction induction. In the presence of A-15 antibody, motility decreased 16% ($p = 0.003$), and 27% in the presence of H-170 antibody ($p = 0.002$) compared to the untreated control. A classic sign of successful capacitation is sperm hyperactivation, in which the flagellar motion becomes asymmetrical with high amplitude [2]. In both the control and antibody treated samples, hyperactivated sperm were present; however, the sperm in the antibody treated preparations appeared sluggish upon microscopic observation. Video micrographs documenting motility for each treatment can be found in Supplementary files (S1–S6). We hypothesize that treatment with synapsin I antibody may have reduced sperm motility by disrupting actin polymerization. Previous studies have shown a relationship between actin polymerization and hyperactive sperm motility during capacitation.

4. Discussion

The purpose of this study was to investigate the localization and functional distribution of synapsin I in human sperm. Data presented herein supported our hypothesis that synapsin I is present
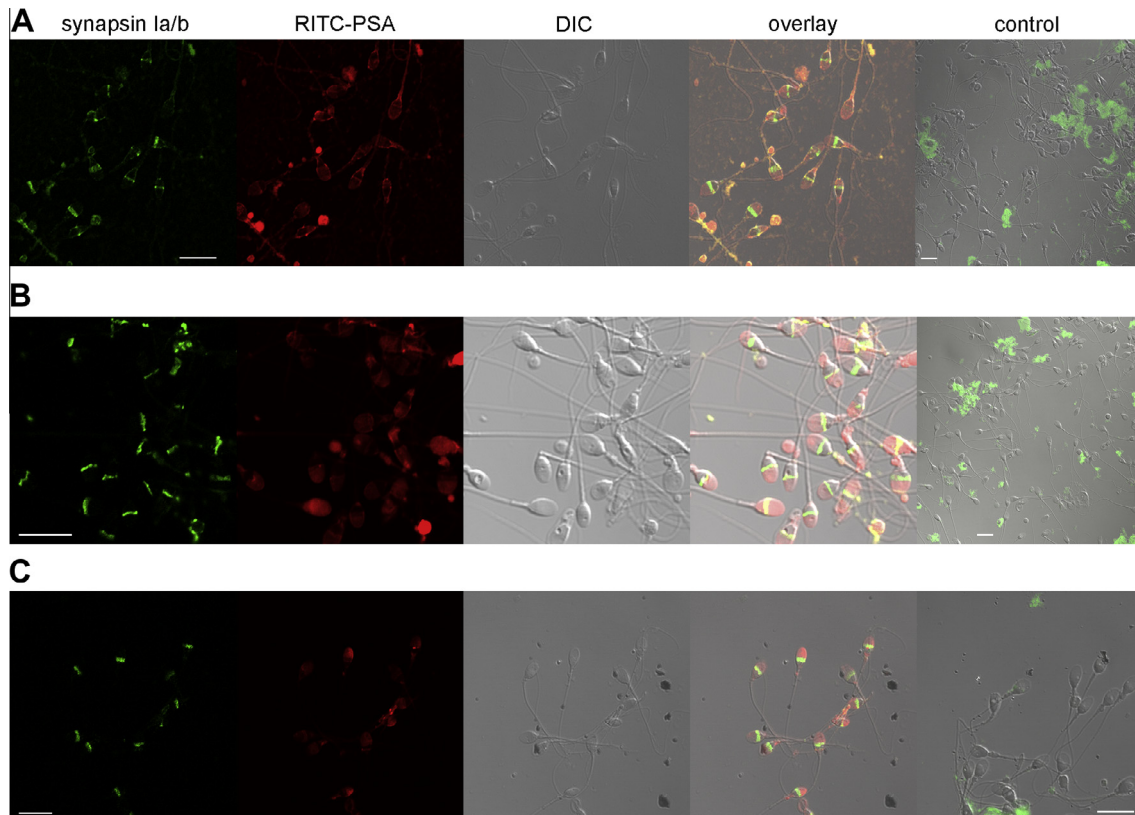

Fig. 2. Western blot analysis confirmed the presence of synapsin I in human sperm. Membranes probed with anti-synapsin Ia/b H-170 antibody showed bands of the expected size (75–100 kDa) in synapsin Ia/b transfected lysate (1), human seminal plasma (2), human pellet (3), mouse T/E (4), and mouse brain (5) lanes. No bands were present in RIPA buffer (6, negative control). Lanes 1 and 5 were positive controls. L = protein standard ladder.

Fig. 3. Immunolocalization revealed enrichment of synapsin I in the equatorial segment of human sperm. Washed human sperm labeled with anti-synapsin Ia/b antibody H-170 and RITC-PSA (A) showed localization to the sperm equatorial segment. Similarly, capacitated and acrosome induced sperm preparations showed bright staining in the sperm equatorial segment (B and C). In each series, the first four panels represent the same confocal field. The fifth panel shows a representative field from a secondary antibody only (control) slide. Note the background staining is not associated with sperm. Scale bar = 10 μm.

in human sperm. Using immunocytochemistry, synapsin I was localized to the sperm equatorial segment; with the brightest staining in capacitated and acrosome induced cell preparations. Dot blot techniques using antibodies against the C-terminus and N-terminus demonstrated positive results in human sperm cell, human seminal plasma, mouse T/E, and mouse brain extracts. Densitometry data supported the visual interpretation of the dot blots indicating the presence of synapsin I in all samples except RIPA (negative control). Western blotting techniques revealed a band between 75 and 100 kDa for mouse T/E, human pellet, and human seminal plasma extracts. These bands closely corresponded to the intense band present in the lysate and mouse brain lanes, confirming that the detected protein was synapsin I. The presence of synapsin I in the seminal plasma shown by both dot blot and Western blot techniques was likely protein from the sperm cells and not a soluble form of synapsin I. When preparing the protein extracts, it is possible that some sperm cells could have contaminated the seminal plasma fraction, even though microscopic inspection of a small aliquot showed no sperm present. Additionally, sperm cells may have been damaged during centrifugation, thereby causing the release of synapsin I into the supernatant. Studies investigating the source of the synapsin I in the seminal plasma extracts were outside the scope of this study.

Since their initial discovery, synapsins have been extensively studied in neurons. The localization of synapsin I in human sperm is a novel finding. Enrichment within the sperm equatorial segment is provocative, as this part of the sperm remains intact following AR and is the site of sperm-oocyte membrane fusion [60]. The precise function of synapsin I in sperm is unknown. Based upon its localization to the equatorial segment in capacitated and

acrosome induced preparations, as well as previous findings in other secretory cells, we hypothesize two functions for synapsin I. In its dephosphorylated form, synapsin I has been shown to bundle F-actin [19,20,61]. Calcium dependent phosphorylation of synapsin I results in the dissociation of synapsin from actin [19]. It has been reported that actin polymerization occurs during capacitation, and prior to AR, there is a rapid depolymerization of F-actin [62]. It is possible that synapsin I functions by stabilizing and/or bundling F-actin. Upon calcium influx, synapsin I could become phosphorylated by calcium-dependent kinases, acting as a trigger for F-actin depolymerization, allowing the AR to occur. Data reported herein documented a significant decrease in sperm motility following capacitation and induction of acrosome reaction in the presence of anti-synapsin antibodies. This further supports the notion that synapsin could be involved in regulating cytoskeletal dynamics during these processes.

The AR is an exocytotic event involving fusion of sperm membranes and formation of vesicles [2,63]. Another possible function of synapsin I could be associated with sperm membrane dynamics. Synapsin I, in a dephosphorylated state, could stabilize the sperm membranes. Calcium influx during capacitation could lead to the phosphorylation of synapsin I, thereby causing a re-distribution of the protein, as observed in neurons [16,17,64]. This may cause the sperm membranes to become unstable, promoting fusion of membranes and subsequent vesiculation. Synapsin I is not only a target of calcium/calmodulin dependent kinases, but it can also be phosphorylated by PKA, MAPK, cyclin dependent kinase 5 (cdk5) [65] and by tyrosine kinases [66]. The latter may be of particular interest, as tyrosine kinase phosphorylation has been strongly implicated in sperm capacitation [1].
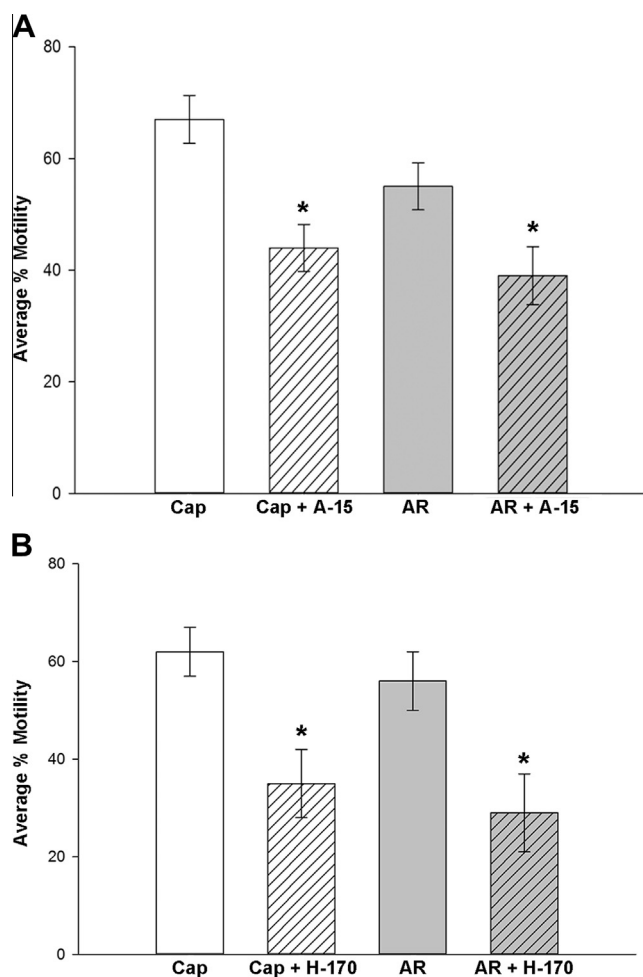
Fig. 4. Capacitation and induction of acrosome reaction in the presence of anti-synapsin antibodies A-15 and H-170 significantly reduced sperm motility. Human sperm were capacitated (Cap) and induced to undergo acrosome reaction (AR) in the absence (solid bars) and presence (striped bars) of A-15 and H-170. An * indicates a significant difference between treated and untreated samples using a paired *t*-test ((A-15: Cap $p = 0.0002$, AR $p = 0.003$); (H-170: Cap $p = 0.0004$, AR $p = 0.002$)). Seven samples from five different donors were analyzed for A-15 and six samples from three different donors were analyzed for H-170. Error bars represent standard error.

5. Conclusions

Multiple lines of evidence clearly support the presence of synapsin I in the human sperm equatorial segment. Functional studies suggest a potential role during capacitation and/or acrosome reaction. These novel findings add to our current understanding of sperm morphology and architecture. Understanding gamete structure is key to understanding the molecular events of fertilization. Unraveling the specific function of synapsin I during capacitation and/or acrosome reaction is the focus of future studies.

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J.J.V. and W.L.C. conceived and designed project, acquired, analyzed, interpreted data, prepared manuscript. A.C.K. acquired and analyzed data, reviewed manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.09.006.

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