SNARE protein mediated secretory events in porcine fertilization

SNARE eiwitten en fertilisatie bij het varken

(met een samenvatting in het Nederlands)

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You make me want to be a better person

To my parents,

sister, Wen and Joshua

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Chapter 1

General Introduction:

Molecular kinetics of proteins at the surface of porcine sperm before and during fertilization

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Fertilization is a decisive moment in life and enables the combination of the two gametes to ultimately form a new organism. The sperm surface, especially on the head area has different subdomains that are involved in the distinct parts of the fertilization process. This sperm head surface is subject to continuous remodelling during epididymal maturation of sperm and sperm migration in the male and female genital tracts. Intriguingly however, the identity, origin and spatial ordering of proteins at the sperm surface that are involved in mammalian fertilization are essentially unknown. This review does focus on sperm surface protein modifications that are under somatic cell control. As soon as the sperm is released from the seminiferous tubules it is subject to such modifications. The surface reorganisation continues until the sperm resides in fallopian tube where it meets and may fertilize the oocyte. A selective process will favour functional mature and intact sperm to optimally interact and fertilize the oocyte [1]. Even the peri-vitelline fluid, between the zona pellucida (ZP) and the oolemma (the oocyte's plasma membrane), is involved in sperm surface remodelling and contains factors which could facilitate the first penetrating sperm to fertilize the oocyte. In this chapter, the kinetics of proteins at the sperm surface will be reviewed.

The general consensus is that only functionally matured sperm can fertilize the oocyte and that this maturation is especially accomplished at the surface of the sperm head. The sperm is a highly polarized cell with a minimum of cytosol and organelles [2]. The sperm head consists of a nucleus that houses the male haploid genome which is highly condensed with protamines in the late haploid phase of spermatogenesis, and a large secretory granule called the acrosome that is located over the anterior area of the sperm nucleus. The flagellum emanates at the distal part of the sperm head. In the mid-piece of this flagellum, mitochondria are spiralled around the microtubules of the flagellum. In the tail, specific cytoskeletal elements surround the microtubules of the flagellum. The surface of the sperm head, mid-piece and the tail of the sperm is heterogeneous [3;4] and reflects the polar distributed organelles underneath the surface. In particular, the sperm head surface is heterogeneous and at least three subdomains can be distinguished which have separate functions in the fertilization process. In general, the sperm has lost many somatic cell features such as endoplasmic reticulum, Golgi, lysosomes or peroxisomes. Moreover, the loss of ribosomes disabled the sperm's capability to activate and express genes (both transcription and translation processes are silenced [5]). Furthermore, due to the removal of almost the entire cytoplasm during

spermatogenesis [2] and epididymal maturation [6] the remaining organelles and cytoskeletal elements of the sperm are typically ordered and this is probably reflected by the lateral domain structure of the sperm's surface [7].

Function of sperm membrane domains at fertilization

The subdomains of the sperm head surface have diversified functions in the series of processes that are involved in fertilization. The apical ridge area of the sperm head specifically recognizes and binds to the zona pellucida (ZP) [8]; a larger part of the sperm head surface (the pre-equatorial domain) is involved in the acrosome reaction (AR) which results in the release of acrosomal components required for ZP-penetration [1;9]. The equatorial segment of the sperm head remains intact after the acrosome reaction and is the specific area that recognizes and fuses with the oolemma in order to fertilize the oocyte [10]. Although the sperm plasma membrane at the mid-piece and tail is also heterogeneous, the function of these surface specialisations is not yet understood [11] but could for example be involved in sperm motility characteristics. Thus the sperm surface protein organisation is rather complex and, especially in the sperm head, the surface is subjected to constant dynamical changes evoked by the series of environmental changes in the male and female genital tract or during sperm handling as is reviewed in the next section.

Sperm surface kinetics

The subdivision of the sperm surface is already apparent in testicular sperm [2]; however, the molecular dynamics, involved in the establishment of the surface specialisation during and upon spermatogenesis, are largely unknown. Generally, the polar organization of the extracellular matrix components, the cytoskeleton and the cell organelles of the sperm are involved in its heterogeneous surface. In mature spermatids, the amount of cytosol is minimal and indeed the observed surface domains mirror the organisation of the acrosome, the post-equatorial nucleus, the mitochondria and the fibrous sheath, respectively. Moreover, once liberated into the lumen of the seminiferous tubule, the sperm will start its travel through the male and female genital tract and will meet a sequence of different environments. During this voyage, surface remodelling takes place most likely at any site of the two genital tracts. These continuous changes start with stabilization of sperm in the male genital tract which is probably accomplished upon epididymal maturation [6;12] and by re- and decoating events induced by the accessory fluids added at ejaculation [13;14]. Beyond surface rearrangements, epididymal maturation also results in the removal of cytoplasmic droplets (the cytosolic remnants of the bridges between spermatids) and

the acquisition of sperm motility. In the pig, most of the seminal fluid originates from the seminal vesicles and the bulbo-urethral gland (see Fig. 1).

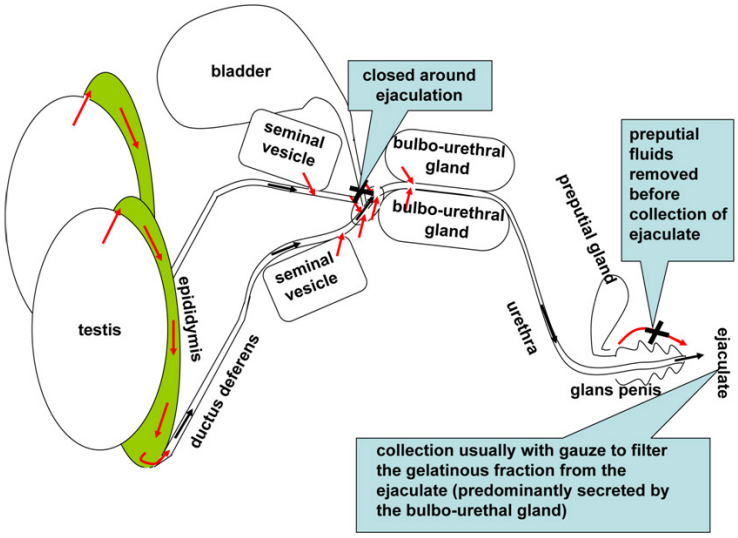

Figure 1 An overview of the reproductive organs of the boar. Sperm that leave the testis mature in the epididymis (green), then migrate via the deferent duct and are mixed with secretions of the accessory sex glands at ejaculation. Red arrows indicate where the secretions are added to the migrating sperm. The direction of transport of sperm is indicated with black arrows.

After the deposition of sperm in the female genital tract, the reorganization process continues to facilitate its further journey and prepare the sperm surface for fertilization. The removal of extracellular glycoprotein coating (release of decapacitation factors) and further remodelling by secretions from the female genital tract activate the sperm to meet the oocyte (*in vivo* capacitation) [15;16] (see Fig. 2). In fact, surface reorganizations are also induced by the interaction of the sperm with cumulus cells and remaining follicular fluid components that surround and impregnate the ZP [17;18] as well as with the peri-vitelline fluid [19;20]. All these changing environments cause surface remodelling of sperm and thus may influence its potential to fertilize the oocyte.

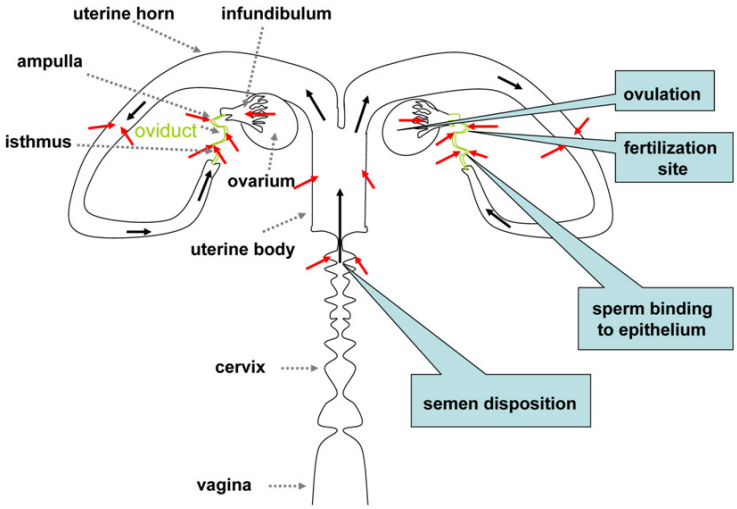

Figure 2 An overview of the sow reproductive organs. The area where sperm specifically interact with the epithelia is indicated in green (oviduct). Note that the entire female genital tract has secretory activity. The quantity and composition of the epithelial secretions varies at the different sites of the female genital tract and are influenced by the stage of the ovulatory cycle. Red arrows indicate where the secretions are added for interaction with migrating sperm. The direction of transport of sperm is indicated with black arrows.

The possible mechanisms whereby the sperm surface is altered have been reviewed earlier [21] and are summarized in Fig. 3. It is very difficult to study sperm surface alterations *in situ*. However, for many mammalian species, including human, specific sperm handling and incubation media have been optimised for efficient *in vitro* fertilization. In general, mammalian sperm are activated in a medium that reflects that of the oviduct as it contains several capacitation factors, such as high concentrations of bicarbonate, free calcium ions, and lipoproteins such as albumin [9]. In some species, specific glycoconjugates [22] or phosphodiesterase inhibitors are added for extra sperm activation [23]. All strategies are designed to evoke capacitation *in vitro*. This implies that the relevant sperm surface reorganisation primed under *in vitro* conditions for fertilization can be observed. The membrane composition as well as the ordering of membrane components can be compared with that under control conditions (media without capacitation factors) or at collection time. Sperm can be collected from boars where the preputial fluid is removed before collection and the gelatinous fraction is removed by filtering through gauze (see Fig. 1). The collected sperm are washed through a discontinuous density gradient to remove aberrant sperm, non-sperm particles, seminal plasma and factors delaying sperm capacitation [24].

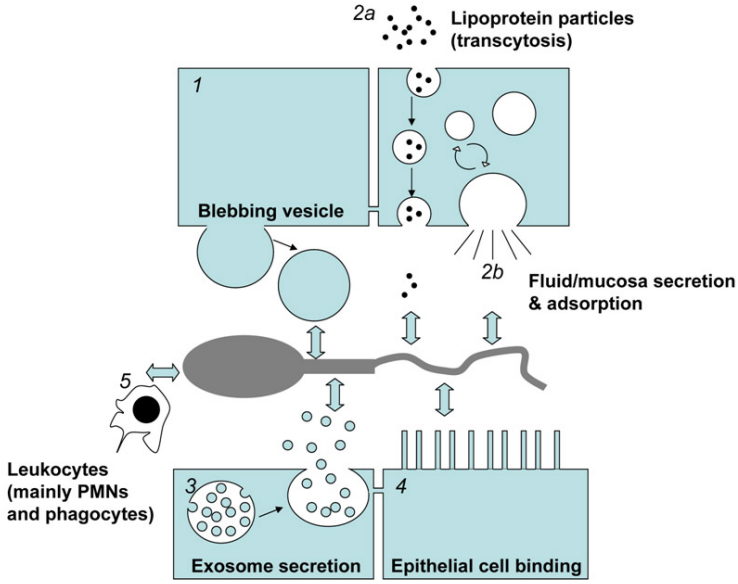

Figure 3 Hypothetical scheme of interactions of male and female genital tract components with the sperm surface. 1. From various epithelia of the male and female genital tract, blebbing vesicles containing cytosol are released into the genital fluids. Such vesicles interact and exchange surface components with sperm. It is unlikely that such vesicles fuse with the sperm as this would dramatically increase the volume of sperm (which has been reduced maximally in order to obtain an ergonomically designed cell optimally suited for fertilization). Blebbing of vesicles has been demonstrated in the epididymal duct and the epithelia of the vesicular gland and the prostate of the boar (Rodriguez-Martinez personal communication). 2a. Serum components are released into the genital fluids by transcytosis [68]. Interestingly, lipoprotein particles may invade the surroundings of sperm and facilitate exchange at the sperm surface. 2b. Fluid phase secretion and adsorption alter the extracellular matrix (ECM) of sperm. 3. Apocrine secretion of exosomes alters the sperm surface and function. Exosomes are secreted by the epididymis (epididymosomes), by the prostate (prostasomes) and by the uterus (uterosomes) [12;35;47]. Interestingly, exosomes may provide sperm with tetraspanins, a group of membrane proteins involved in tethering of proteins into protein complexes. Addition of CD9 onto the sperm surface by membrane particles occurs even when sperm reaches the perivitellin space [19;20]. 4. Sperm interacts with ciliated epithelial cells in the epididymis and the oviduct [12;55]. This last interaction has physiological role in *in situ* capacitation. The importance of sperm interactions with other ciliated epithelial cells of the female and male genital tract are not fully understood but could be crucial for sperm surface remodelling and for sperm function. 5. Semen entering the uterus evokes immunological responses [49] such as the migration of leukocytes into the uterine fluid [44], affecting the surface of sperm and cleaning the uterus from deteriorated sperm [50]. Sperm involved in fertilization colonize the lower parts of the oviduct (where no leukocyte infiltration takes place).

The pelletted cells have, after being re-suspended into *in vitro* capacitation media, been extensively studied for the surface reordering of membrane proteins and lipids in the sperm head (for reviews see [9;25]). Most relevant for fertilization is that sperm surface proteins that are entrapped into small lipid ordered domains (detergent

resistant membrane, also know as membrane rafts) are clustered into the area that is specifically involved in sperm-zona binding [26] as well as in the docking of the sperm plasma membrane to the outer acrosomal membrane [27]. It is necessary to stress the importance of sperm surface reordering and changes in composition of membrane components by diverse extracellular factors. The induced lateral redistribution of membrane components appear to also be instrumental for the assembly of a functional sperm protein complex involved in sperm-zona binding as well as for the zona-induction of the acrosome reaction [8;27;28] (Fig. 4).

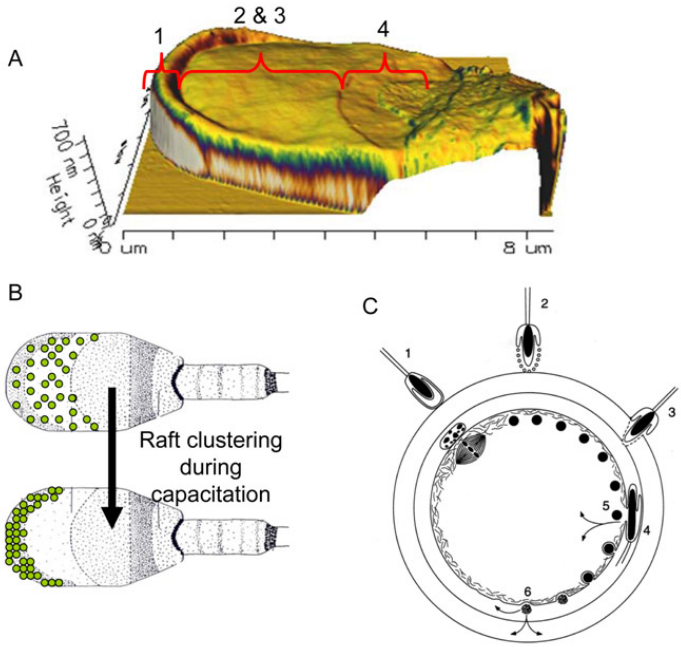

Figure 4 Protein kinetics at the sperm surface. A. An atomic force microscopic surface view of a porcine sperm head. B. Lipid ordered microdomains at the sperm surface cluster into the apical ridge area of the porcine sperm during in vitro capacitation. C. Sperm-zona pellucida and sperm-oocyte interaction leading to fertilization. 1. zona binding, 2. acrosome reaction, 3. zona drilling, 4. oolemma binding and fertilization, 5. activation of pronucleus formation and oocyte activation, 6. induction of a blockade for polyspermic fertilization. (Numbers in panel A refer to the specific sperm surface areas where these interactions take place)

Therefore, in this thesis, we investigate sperm surface proteins (involved in zona binding and in sperm plasma membrane docking with the acrosome) identified by using *in vitro* capacitated sperm, and further study how these proteins are organized

and form complexes for their functional role in fertilization (see Fig. 5). However, we have to bear in mind that the relatively simply defined *in vitro* capacitation methods do not provide all information on sperm surface reorganisations *in utero* and in the oviduct, where hormones and other bioactive non-protein components can modulate sperm function.

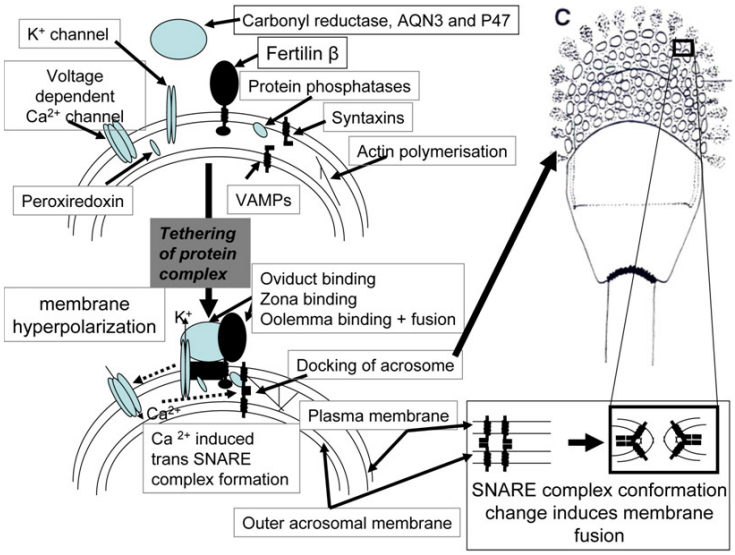

Figure 5 Left: Hypothetical model of a sperm zona pellucida-binding complex formed during sperm capacitation by raft-induced protein clustering. This results in a multifunctional protein complex that plays a role in the diverse processes leading to fertilization. For explanation and identification of proteins see text. This scheme is based on biochemical and proteomic investigations on epididymal and ejaculated sperm before and after IVF incubations (no sperm surface remodelling by the female genital tract in situ is taken into account in this model). **Upper right:** Multiple membrane fusions involved in the acrosome reaction exclusive for the anterior part of the sperm head surface. **Lower right:** *Trans* to *cis* re-configuration of SNARE complexes (Ca^{2+} dependent) for membrane fusions required to execute the acrosome reaction (from Tsai & Gadella, unpublished observations).

Origin and identification of sperm proteins involved in the cascade leading to *in vitro* fertilization

Testicular sperm, just released from the Sertoli cells into the lumen of the seminiferous tubules, are equipped with a number of proteins reportedly involved in ZP-binding. At its surface, the sperm has transmembrane proteins belonging to the ADAMs (a disintegrin and metalloprotease) family, initially considered to be involved in the fertilization process and now reported to be involved in sperm-ZP binding. ADAM-2,

also named fertilin β, has such a function on boar sperm [8]. Other testicular sperm proteins like sperm lysosomal like protein (SLLP1) [29], and sperm acrosomal membrane proteins (SAMP14 and 32) [10] and Sp56 [30] are involved in secondary ZP-binding as they are localized in the acrosome and only become exposed to the ZP after the induction of the acrosome reaction. Some secretory proteins like CRISPs (sperm-associated cysteine-rich secretory proteins) are also involved in sperm-ZP adhesion, sperm-oolemma binding or the fertilization fusion [31-33]. Interestingly, CRISP 2 is of testicular origin but CRISP 1 and 4 originate from the epididymis [34]. The exact way in which CRISPs are associated with the sperm surface is not yet known, although CRISP 1 is one of the abundant proteins in epididymosomes [35]. Epididymosomes are also reported to influence the lipid composition of the sperm surface [36]. Other proteins that have been shown to be added to the sperm surface in the epididymis are P47 or SED1, known to have a role in oviduct and ZP-binding [37]. In porcine, AQN-3 (a spermadhesin) and carbonyl reductase are also added to the sperm surface in the epididymis [38]. Inhibition of hamster carbonyl reductase activity caused a decreased affinity for the ZP while the sperm remained motile and intact [39]. AQN-3 was able to bind to the ZP even under very stringent detergent conditions [8]. Proteomics analyses identified proteins that do not have a direct function in sperm ZP-binding but that are associated with a ZP-binding protein complex [8]. Some of these proteins are involved in sperm signalling (such as protein phosphatases), while others are involved in the redox balance (peroxiredoxin 5). The latter include a potassium channel which might induce membrane hyperpolarization by K^+ efflux. This hyperpolarization may in turn open voltage dependent Ca^{2+} channels that enable Ca^{2+} dependent processes in the capacitating sperm. An interesting observation was that the major ZP-binding proteins mentioned above tend to aggregate in capacitating sperm (under IVF conditions) at the surface area involved in ZP-binding i.e. the apical head area [8;26] and that this coincides with the attraction of SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) involved in the acrosome reaction [27]. The fact that both outer acrosomal SNAREs and plasma membrane SNAREs were identified after capacitation led to the assumption that the lipid ordered membrane aggregation is a preparative step for the acrosome reaction (as proposed in Fig. 4). The ZP-interacting protein complex is thus not only involved in sperm-ZP binding but may link this event with preparative steps for the acrosome reaction.

Surface modifications in the female genital tract

As mentioned above, far less is known about the contribution of the female genital tract to sperm remodelling related to the sperm-ZP binding processes in mammals (Fig. 2). Sperm resides for hours to days in the cervix, uterus and eventually the

isthmic part of the oviduct (depending on species for time and deposition place). In pigs, the semen is deposited into the cervix. Although there are no data on the role of cervix epithelia and secretory products, it is well established that small numbers of sperm can be inseminated when directly deposited into the uterine body [40;41]. Moreover, a very low dose of sperm can be used for deep intrauterine insemination (for review see Vazquez *et al.* [42;43]). A recent *ex vivo* study using a lectin competition binding assay in uterine segments established that binding of sperm to the uterine epithelium was carbohydrate-dependent [44]. However, the proteins involved in this binding are not yet identified and it is also not clear whether this binding is selective, thus allows only small numbers of sperm to migrate deeper into the uterus or whether this binding modifies the sperm surface needed for subsequent processes leading to fertilization. The composition of uterine fluid and its effects on the sperm surface or on sperm functioning has been completely neglected by most researchers. The only study concerning the effects of porcine uterine fluids on sperm reports about lipid modifications in sperm membranes [45]. Porcine sperm contain non-genomic progesterone receptors at the plasma membrane [46] and most likely progesterone binding to its sperm surface receptor is part of the *in vivo* sperm capacitation process. Murine uterine fluids have been shown to contain exosomes. Interestingly these particles called uterosomes contain the sperm adhesion molecule SPAM-1 and other GPI-linked proteins that can be exchanged with cauda epididymal sperm [47]. It is possible that this exchange improves the sperm's capacity to fertilize the oocyte. However, this possibility as well as the presence of uterosomes in the pig species need to be confirmed. When semen enters the uterus, it elicits immunological responses as reflected by a migration of leukocytes (predominantly polymorphnuclear neutrophils; PMNs) into the uterine fluid [44;48;49]. It is not clear whether this infiltration will affect sperm that later enter the oviduct but leukocytes clearly reduce the amount of sperm that will enter the oviduct by phagocytosis [50]. It is also uncertain whether phagocytosis is selective (for aberrant sperm) or only unselectively depletes the amount of sperm that migrate further towards the oviduct. The isthmus itself is free of leukocytes and is the specific site where sperm are capacitated *in vivo* in order to fertilize the oocyte [51]. In pigs, the role of leukocyte infiltration in fertilization is questionable as the vanguard cohort of sperm that occupy the oviduct have been shown to reside and bind to the oviduct epithelia of the isthmic region within 30 minutes after insemination [52] which is long before the invasion of PMNs into the uterus.

Fluids from the oviduct stimulate sperm capacitation and induce hyperactivated sperm motility. One of the factors involved in this sperm activation is bicarbonate, which is also commonly used in IVF protocols [15]. Oviduct-specific glycoproteins (OSG) as well as osteopontin do support fertilization in the cow and are secreted by the oviduct [53]. A sperm binding glycoprotein from the oviductal fluid has recently

been shown to induce porcine sperm capacitation [54]. The lower part of the oviduct functions as the sperm activation site, making sperm capable to meet and fertilize the oocyte. In the isthmus, small numbers of sperm are bound and become capacitated *in vivo.* There, sperm await to be released at ovulation, to migrate to the upper part of the oviduct (the ampulla) and to fertilize the passing oocyte(s) (for review see Suarez [51]). To this end, the oviduct epithelia and fluids contain sperm binding factors as well as sperm releasing factors that facilitate sperm adhesion and release at the correct time around ovulation (for sperm binding and release characteristics in the cow oviduct see also Sostaric *et al*. [55]). Most likely, spermadhesins such as AQN-1 are involved in the formation of the oviductal sperm reservoir as they are involved in sperm binding to this specific epithelium [38]. Note that some spermadhesins (DQH [56], AQN-3, AWN, P47 [25]) are added to the sperm during epididymal sperm maturation, others (like PSP-I /PSP-II subunits) are already added in part in the testis [57]. Interestingly, oviduct-specific glycoproteins modulate sperm-ZP interaction and reduce polyspermic fertilization rates in pigs [58;59]. Polyspermy is a well recognised problem in pig IVF and prevention of this unwanted phenomenon may be accomplished by addition of oviductal fluid components to the IVF media. Oviduct epithelial annexins have been suggested to immobilize bovine sperm (by binding bovine sperm proteins BSP [60]). Annexin A2 has also been proposed to be involved in sperm-oviduct binding in the sow [61]. In the bovine species this binding is reversed by oviductal fluid factors such as catalase [62]. Catalases, secreted by the oviduct may protect sperm from peroxidation damage as is demonstrated in the cow [63]. The interplay of various glycoproteins at the surface of sperm and of oviduct epithelia or in oviductal fluid as well as the varying amounts and composition of glycosidases probably orchestrate proper sperm activation just around ovulation in the pig [64;65]. However, the effect of oviduct and uterine proteins on sperm-ZP binding as well as their putative association to the sperm surface is not yet established. Of course it is possible that products secreted by the female genital tract enhance sperm-ZP binding and that more specific proteins from the female genital tract could be added to the surface of the ZP-interacting sperm. In this respect, also the ZP itself, besides being a binding target, may add proteins to the sperm surface. The cumulus cells and the ZP were impregnated with follicular fluid and remnants of this fluid will remain attached to the cumulus oocyte complex. For instance, different growth factors and extracellular matrix components have been involved in interactions of sperm with the cumulus oocyte complex (for review see Einspanier *et al.* [66]). Sperm that interact with these structures may respond to these fluid components like they do to the extracted follicular fluid through displaying hyperactivated motility [17;18].

Finally, membrane remodelling occurs after the acrosome reaction when sperm reaches the peri-vitelline space but before the fertilization fusion. Membrane fragments containing CD9 are added to the sperm surface within the peri-vitelline

space [19;20]. If correct, this would demonstrate the “bestowment principle” that may exist in mammalian reproduction as the oocyte facilitates the first incoming sperm in the peri-vitelline space to fertilize by transferring functional tethering proteins to the surface of sperm cells. It remains to be established whether such a process also enables oviductal sperm to bind to the ZP. It may also be mentioned that sperm proteins involved in oolemmal binding and the fertilization fusion are reported for mouse and human sperm [10;67] but data for boar sperm are scarce.

Scope of the thesis

The continuous sperm surface remodelling during sperm transit from the rete testis towards the oviduct and possibly even within the peri-vitelline space after acrosome reaction and the physiological role of this surface kinetics is, to a large extent, *terra incognita*. As intracellular fusion processes (the acrosome reaction of the sperm cells and the cortical reaction of the oocyte) play a crucial role in successful fertilization, the concepts of SNARE protein involvement in membrane fusion events in general and execution of exocytosis in mammals in particular need to be investigated. Hence, the current knowledge on SNARE dependent membrane interactions and on SNARE dependent processes in mammalian fertilization is reviewed (see chapter 2). The scope of this thesis is to further elucidate SNARE-mediated membrane interactions involved in mammalian fertilization. The focus is on the acrosome reaction in sperm (chapters 3-5) and on the cortical reaction in the oocyte (chapter 6).

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Chapter 2

Membrane Fusion:

SNARE mediated protein interactions in gametes

In preparation

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Introduction

Membrane fusion takes place in many biological processes, from endo- or exocytosis at the surface of somatic cells [1], to intracellular membrane traffic (like vesicle budding off or vesicle mediated transport of proteins and lipids) [1-3] neurotransmission [3-6] and hormone secretion [7;8]. Likewise membrane fusion is also involved in processes indirectly or directly relevant to reproduction e.g. in sperm-oocyte fusion and subsequent secretion of cortical granulae to ensure monospermic fertilization. In this chapter we provide an overview on the localization and function of specific SNARE (soluble *N*-ethylmaleimide-sensitive factor {NSF} attachment protein receptor) proteins that reside at the cytoplasmic site of two interacting membranes and their role in membrane fusion in gametes

1. Membrane fusion

Membrane fusion involves the merging of hydrophobic cores of two initially distinct lipid bilayers (Fig. 1). Complex sequential processes take place prior to membrane fusion, these include (1) the tethering of two opposing membranes by specific tethering factors, (2) the rearrangement of fusion proteins and lipids at the site of membrane fusion (in this process lipid-ordered microdomains of membranes are considered to play an important role), (3) the docking and subsequent priming of loosely tethered membranes into a tightly appositioned bilamellar structure via the formation of specific sets of protein complexes (4) the merging of the two outer leaflets into a shared monolayer which keeps inner leaflet components peripheral from the membrane fusion area [9-14] (5) completion of this hemi-fusion structure in which the two interacting membranes become continuous and the cargo of the vesicle is released. Depending on the cell type, membrane fusion can take micro seconds (100 µs for instance in synaptic vesicle exocytosis [15]) or up to minutes (acrosome exocytosis [16]). Therefore, membrane fusion processes require precise and efficient regulation for the two interacting membranes to overcome energy barriers and for adequate rearrangement of functional (fusion) components at the area where membrane fusion will take place [13;14;17].

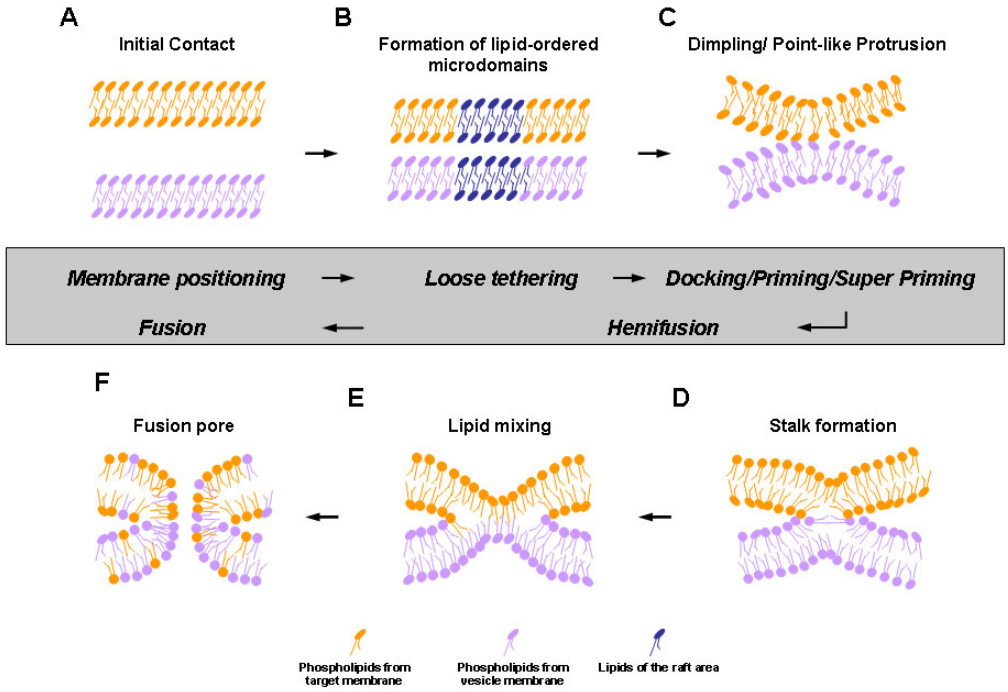

Figure 1 Schematic illustration of lipids arrangement during membrane fusion. In the resting, non-activated stage (A), both phospholipid (in orange and light purple) and glycosphingolipids (dark blue) are homogeneously distributed throughout the membrane. (B) The formation of lipid-ordered microdomains (membrane rafts; enriched with glycosphingolipid, sphingomyelin and saturated glycerophospholipids) occurs in response to intra- or extracellular stimuli and serves as the functional platform for protein-protein or protein-lipid interactions resulting in the activation of signaling cascades. (C) Point-like membrane protrusion minimizes the resistance from the repulsion of two membranes that come into immediate contact. (D) The formation of a lipid stalk is the initial step in hemifusion. This hemifusion stalk is formed by the inner leaflets of two interacting membranes while the outer leaflets remain distinct. (E) By the expansion of the hemifusion stalk, the two original distinct outer leaflets become attached and further mixing of lipids from the outer leaflets leads to a transient intermediate stage (hemifusion). (F) A fusion pore is formed when both inner and outer leaflets of the two interacting membranes merge. This model is partially based on the concepts from refs [6;9-15;22;36;60;105;129].

2. Vesicle trafficking

The eukaryotic cell contains many membrane-enclosed organelles and compartments, hence the re-location of components from one place to another requires highly organized transport (likely regulated by Rab proteins; small GTPases that interact with SNARE proteins [18;19]). One way to move cellular components is by vesicle-mediated membrane transport in which membrane and molecular segregation

processes enable specific delivery of required components to their destination. This process takes place intracellularly as well as inter-cellularly. Each process begins with budding of vesicles and ends by membrane fusion [20]. First, vesicles that carry the cargos are budded and pinched off from the “factory/donor” compartment (such as the endoplasmic reticulum or pre-synaptic terminal); in mammals, several additional intermediate trafficking steps involving intracellular organelles (e.g. Golgi network) are required to ensure that functional components are directed to the correct cellular locations. Once these vesicles reach their target compartment, they are tethered, docked and fused with the target membrane (e.g. the plasma membrane or the post-synaptic terminal) and SNARE proteins play a crucial role in these final steps [21-23].

3. SNARE proteins: the fusion machinery

“SNAREs” were first characterized in the late 1980s, elucidating their involvement in yeast secretory pathways [24;25]. Although in 1989, an integral membrane protein called synaptobrevin was described in small synaptic vesicles of the rat brain [26], the earliest indications for SNARE proteins to be involved in intracellular vesicle-mediated transport and membrane fusion were denoted by Wilson et al. and Clary et al. [27;28]. Subsequently, many novel SNARE proteins have been identified and characterized [29-31]. In 1993-1994, the term “SNARE” as well as the working hypothesis for SNARE interaction were introduced when protein complexes present in synaptic vesicles appeared to consist of VAMP, SNAP 25 and syntaxin [32-34] (Fig. 2).

The SNARE superfamily consists of more than 60 members (for an overview of identified SNARE proteins, see [21;35], for SNARE proteins investigated in this thesis and their nomenclature, see Table 1). This large SNARE protein superfamily is sub-divided into three major families: syntaxin, SNAP (synaptosome-associated proteins) and VAMP (vesicle-associated membrane proteins) [22;36;37]. The primary role of SNARE proteins is to mediate vesicle fusion, the exocytosis of cellular transport vesicles to the target compartment. The first SNARE hypothesis proposed by Söllner et al. [34] was that SNAREs isolated from fusion particles originate from either transport vesicles (v-SNAREs) or from the target membrane (t-SNAREs), and that these fusion particles contain NSF and SNAP (soluble NSF attachment proteins) that interact with either one or both SNARE proteins on the membrane. Furthermore it predicted that this interaction would lead to the attachment of the vesicle to its target membrane [32]. The understanding of which SNARE proteins are involved in intracellular membrane fusions has led to a number of revisions and adaptations of the original SNARE concept, and as a result, the hypothetical working model has also been modified and gradually expanded throughout the years. Initially, SNARE proteins were functionally classified into v-SNAREs which are incorporated into the

membranes of transport vesicles during budding, and t-SNAREs, which are located in the membranes of target compartments.

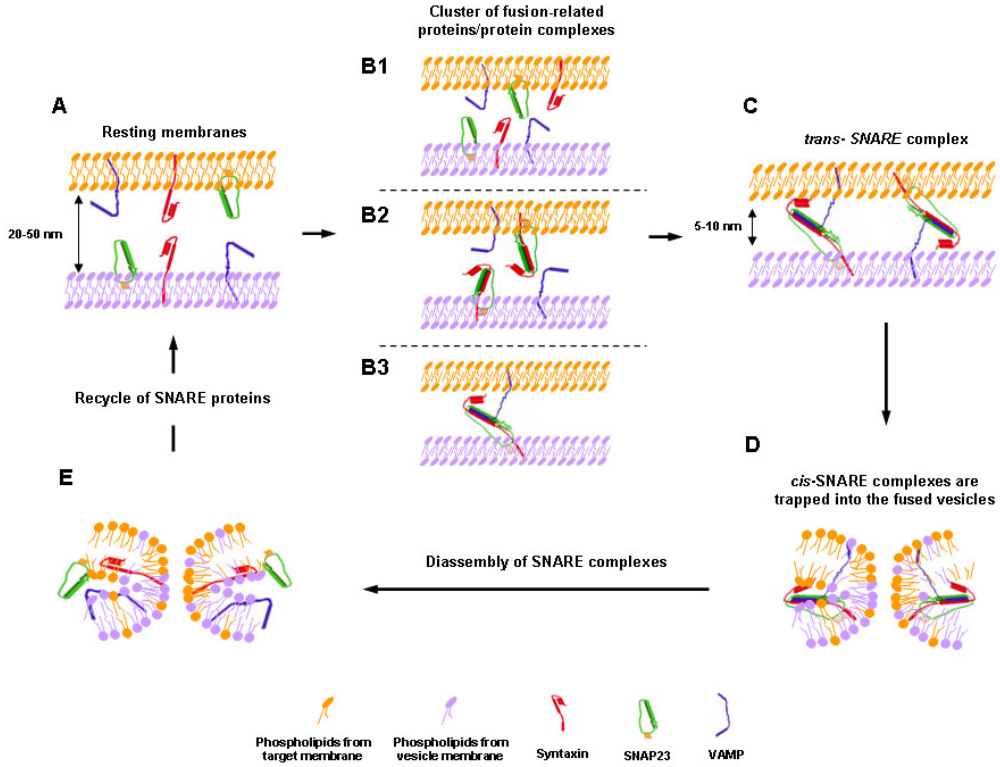

Figure 2 Schematic illustration of SNARE proteins arrangement during membrane fusion. (A) The average distance between resting, non-activated membranes can vary from 20-50nm. At this stage, Q-SNARE proteins syntaxin (red) and SNAP (green) are located on the same side of a membrane while R-SNARE VAMP (blue) is located on the opposite membrane. (B) Upon cell activation (i.e. capacitation in sperm cells), SNARE proteins are recruited into lipid-ordered microdomains. Three hypothetical action modes are proposed. SNARE proteins can be recruited individually (B1), as a protein complex (B2, an example of the Q-SNARE complex consisting of syntaxin/SNAP) or after the formation of a loose *trans*-SNARE complex (B3). (C) By formation of a loose *trans*-SNARE complex, opposing membranes are brought into close arrangement and subsequently primed and docked. By the tight interaction of a stable SNARE complex, loosely primed and docked membranes become firmly attached (also called super priming) at a later stage. (D) In response to extracellular stimulation (i.e. calcium), the fusion process further proceeds and SNARE complexes are trapped into fused vesicles. (E) SNARE complexes can be disassembled into individual proteins by the SNARE interacting/regulating proteins (see Fig. 3) and can be subsequently recycled via endocytotic pathways. This model is partially based on the concepts from refs [6;9-15;22;36;60;105;129;158].

Table 1 Information for SNARE proteins investigated in current thesis.

Symbol Name	***Nomenclature***[a]	***Synonyms***	***Predicted and (found)*** [b] ***Cellular Location***
Syntaxin 1	Qa (t-SNARE)	HPC-1	PM (PM/OAM; NF)
Syntaxin 2	Qa (t-SNARE)	Epimorphin	PM (PM; PM)
Syntaxin 3	Qa (t-SNARE)		PM (PM; PM)
SNAP 23	Qbc (t-SNARE)	Syndet; synaptosome-associated protein of 23 kDa	PM (OAM, CP)
SNAP 25	Qbc (t-SNARE)	synaptosome-associated protein of 25 kDa	PM (NF; NF)
VAMP 1	R (v-SNARE)	Synaptobrevin 1	SV (OAM; PM)
VAMP 2	R (v-SNARE)	Synaptobrevin 2	SV (OAM; PM)
VAMP 3	R (v-SNARE)	Cellubrevin	SV (PM; NF)

a: Nomenclature for t- and v-SNAREs was according to their function and localization found of these proteins. Other nomenclature for Q- and R-SANREs was based on structural contribution of amino acid to the central residue of SNARE motif (zero layer).

b: Found locations indicated in this table were based on the studies carried out in current thesis of porcine sperm cells (chapter 3-5) and porcine oocytes (chapter 6). Cellular localizations of particular SNARE protein observed in gametes were separated by semicolon (in sperm; in oocyte).

PM: plasma membrane; NF: not found; OAM: outer acrosome membrane; CP: cytoplasm; SV: synaptic vesicle.

The comparison of amino sequences of SNARE proteins led to the new division into R-SNAREs and Q-SNAREs which takes certain structural features of SNARE motifs into account [35]. Secondly, the concept of SNARE-mediated membrane fusion was refined from interacting SNAREs at the opposing membranes to assemble and dissemble of *"cis-"*(all essential complexed proteins reside at one membrane) and "*trans-"*(all essential complexed proteins reside at the two interacting membranes) SNARE complexes that bridge the opposing lipid bilayers, bringing them in close proximity for fusion. Thirdly, formation of a SNARE complex alone is not enough for the initiation of the fusion process but further requires calcium [38-41]. Moreover, additional proteins stabilize or de-stabilize the SNARE complex prior to and after the fusion. These SNARE- associated molecules (called SNARE regulators) will be discussed in the next section (section 3).

In most cell types, SNARE proteins are reused after membrane fusion and this process is orchestrated in a defined recycling mechanism. Over the years, several pathways/models have been proposed for SNARE protein recycling. The general concept is that exocytosis membrane fusion is followed by an endocytosis driven membrane recycling. The endocytotic mechanisms can be divided into clathrin-dependent or clathrin-independent pathways (for review, see [42]). Structural and functional evidence suggest that for the clathrin-dependent recycling pathway, a cargo-selective clathrin adaptor AP-2 plays an important role in the sorting of transmembrane components into the overlaying clathrin-coat [43;44], the internalized components are sorted in the early endosome and can later be budded into the late endosome for reuse (for review, see [45]). Recently, clathrin-independent recycling pathways have drawn increased attention from cell biologists (for reviews, see [46;47]). These include membrane raft-involved flotillin-associated pathways [48], low capacity but highly regulated caveolae/caveolin-associated pathways [49] and even non-protein mediated endocytosis pathways like GEECs (GPI-anchored protein-enriched early endosomal compartments) [50]. These endocytotic pathways thus ensure the efficient recycling of released components in variety of cell types.

The SNARE recycle system is necessary in all somatic cell types and this recycling machinery is best studied in neuronal synapses where vesicle exocytosis takes place in micro- to milli-seconds after excitation and involves hundreds or thousands of vesicles from the nerve- terminal. The compensatory endocytotic machinery enables the synapse to recover within ~115 milliseconds in order to release a new set of vesicles containing neurotransmitters after a new excitatory event [41]. In addition, the fast recycling of SNARE proteins by endocytosis guards the fusion competence in the secretory pathway without the need of *de novo* synthesis (slow) of SNARE proteins [51;52].

SNARE proteins are believed to be the central players in membrane fusion over the cytosolic sides of two adjacent membranes. Nevertheless, this type of membrane

fusion has also been reported to occur in an NSF independent way, for instance in homotypic endoplasmic reticulum fusion [53]. The SNARE superfamily consists of numerous isoforms (e.g. syntaxin 1a/b, VAMP 1/2/3) and functional homologues (such as SNAP 23/25) that are probably partially overlapping or redundant in functionality [54-58]. Therefore, a possible explanation for some “SNARE-independent” fusion processes is that the execution of fusion is compensated or replaced by homologues or isoforms originally considered to be “functional redundant”.

4. SNARE interacting proteins and regulators

A number of proteins have been described to interact with SNARE proteins and have a function either up- or downstream of the formation of a SNARE core complex or are involved in SNARE protein organization (including recycling of SNARE proteins) by stabilizing or de-stabilizing the SNARE complex [21;35;59;60]. While in general SNARE proteins are involved in almost any intracellular membrane fusion event, specific SNARE interacting proteins may modulate this event in specific cell types or between specific membranes. In this section, we review some important SNARE interacting proteins and regulators according to their presence and role in the cycle of SNARE- mediated membrane fusion (Fig. 3).

4.1 Rab 3 (Fig. 3 A – B)
When SNARE proteins are synthesized, they need to be relocated to other cellular compartments or areas where they can become functionally active. The Rab family of GTPases, are cytosolic proteins of the Ras superfamily of monomeric G proteins that regulate specific trafficking of SNARE-containing membrane vesicles by binding to SNARE proteins and leading the vesicles to the appropriate subcellular localization [61-66]. In addition, the Rab proteins are involved in the initial attachment and priming (preparation for fusion) of membranes by recruiting and binding tethering factors or cytoskeleton proteins [61;67]

4.2 SM (Sec/Munc) (Fig. 3 B – C)
Munc proteins (Sec), another family of cytosolic SNARE interacting proteins, function in a variety of membrane trafficking steps by modulating SNARE complex formation [68-70]. Various members of the Munc 18 family can bind to SNARE proteins at the plasma membrane in a cell specific manner. The best studied isoform is Munc 18a which is primarily localized in brain tissue [70]. Munc 18a is involved in synaptic vesicle fusion by interacting with either the closed conformation of syntaxin 1 and therefore inhibiting the formation of a SNARE core complex [15;71;72] or by interacting with the open conformation of syntaxin 1 and then further stabilizing the

syntaxin 1/SNAP 25 complex [73]. Munc 18a may also restrict fusion to specific sites of the plasma membrane by preventing the formation of intracellular exocytotic SNARE complexes before syntaxin is delivered to the plasma membrane [74]. Moreover, Munc 18 promotes the stability of syntaxin 1 but is not essential for SNARE complex formation since Munc 18a knockout mice can still form SDS-resistant SNARE complexes [75]. Compared to Munc 18a, the function of Munc 18b is less transparent. In contrast to the more specific expression of Munc 18a in neuronal tissues, Munc 18b is expressed ubiquitously in most non-neuronal tissues and it can strongly bind to syntaxin 1-3 [76;77]. Munc 18b is known to regulate the apical membrane transport by interacting with syntaxin 3 and thus may control the association of apical SNAREs [78]. The less specific binding properties of Munc 18b to different syntaxin isoforms as well as its ubiquitous expression in non-neuronal tissues indicates a function distinct from that of Munc 18a and may also indicate that Munc 18b is involved in different types of exocytosis in various tissues.

4.3 Synaptotagmin: (Fig. 3 C – D)
Synaptotagmin has an affinity for Ca^{2+} and by binding of Ca^{2+} on its two Ca^{2+} binding domains it will regulate the final step of exocytosis namely the execution of the fusion process of the secretory vesicle with its target membrane [79-82]. Elevated cytosolic Ca^{2+} levels lead to the binding of calcium to synaptotagmin and due to the subsequent conformational change, the inhibitory effect caused by complexin can be overcome [83]. Furthermore, two calcium binding domains (C2A or C2B) react at different concentrations of cytosolic calcium and sequentially contribute to the fusion process. Dimerization is necessary for synaptotagmin to interact with syntaxin 1, and the formation of a synaptotagmin dimer occurs via the C2B domain that responds at low concentrations (3-10 mM) of cytosolic calcium. As Ca^{2+} concentrations rise to a level that may trigger exocytosis (20-200 mM), synaptotagmin dimers bind to syntaxin via the synergistic action of both C2 domains [84-86].

4.4 Complexin (Fig. 3 D – E)
The complexins are another group of cytosolic SNARE binding proteins. Complexin binds to the SNARE core complex with its central alpha-helix [87-89] and can stimulate as well as inhibit membrane fusion [90-93]. This may either be attributed to the different complexin isoforms (I-IV) with different putative interactions and regulatory functions towards SNARE proteins or to cell specific regulation of complexin-SNARE interactions. Complexin stabilizes a *trans*-SNARE complex [94;95] by acting as a pre-fusion clamp that arrests SNARE complexes and thus prevents fusion. At a later stage complexin can be removed from the *trans*-SNARE complex in a calcium-dependent manner. When synaptotagmin-1 detects calcium, it subsequently promotes the conformational change of the assembled SNAREs-complexin-

synaptotagmin complex and displaces complexin from the SNARE complex. This is instrumental for the execution of membrane fusion [83;94-100]. In contrast to the locking of the *trans*-SNARE complex described above, complexin can also stimulate membrane fusion [98;101].

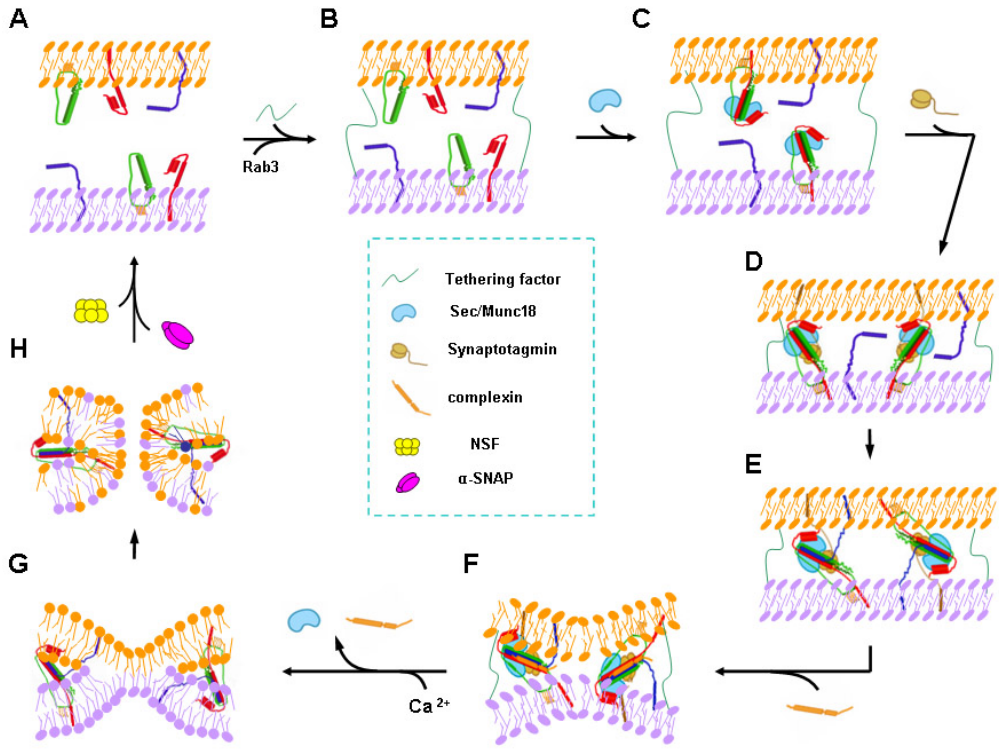

Figure 3 The involvement of SNARE interacting/regulating proteins and molecules during membrane fusion. (A-B) A tethering factor is involved in the initial apposition of two membranes and facilitates the membrane interaction. Rab 3 can regulate the intracellular trafficking of these SNARE proteins to their target location and/or is involved in the initial attachment of membranes. (B-C) Munc 18 can bind to either the syntaxin alone to prevent the formation of a SNARE complex or associate with syntaxin/SNAP23 complex and further stabilize the complex. (C-D) Synaptotagmin mediates the docking step of two membranes and functions as calcium sensor to respond to an eventual calcium influx. (D-E) Complexin is involved in the stabilization of the *trans*-SNARE complex on the docked and primed membranes prior to the fusion. (F) Extracellular calcium promotes the fusion reaction. (G) NSF and alph-SNAP are involved in the disassembly of the SNARE complex after completion of the fusion. This step is crucial for recycling of SNARE proteins for reuse. For detailed description of each protein and their functions, see section 4 in the text. This model is partially based on the concepts from refs [6;9-15;22;36;60;105;129;158].

4.5 NSF and α-SNAP (Fig. 3 G)
The proteins NSF and α-SNAP regulate the disassembly and recycling of SNARE complexes after membrane fusion. They operate on the *cis*-SNARE complex that emerges after the fusion of the vesicle and the target membrane, the monomeric SNARE proteins then formed ensure appropriate membrane and SNARE recycling through an endocytotic pathway [102;103]. NSF has also been described to activate Rab 3A which promotes not only the association of tethering proteins but also of NSFs. NSFs are protected in a macromolecular complex and would be ready to activate SNARE proteins for the formation of the *trans*-complexes and thus may have a role in promoting SNARE mediated membrane fusion [104-106].

5. SNARE- mediated membrane fusion in fertilization
The aim of this thesis is to study the role of SNARE-mediated intracellular fusion processes prior to and after the fertilization of the oocyte in both sperm cells as well as in the oocyte itself. Two specific secretory events are investigated namely the acrosome reaction that is initiated when the sperm cell interacts with the zona pellucida (chapters 3-5) and the cortical reaction which is creating the block for polyspermic fertilization once the oocyte is fertilized (chapter 6). In this section, the involvement of SNARE proteins in these processes is reviewed with special emphasis on the studies described in chapters 3-6.

5.1 SNARE proteins and the acrosome reaction of sperm cells
The acrosome reaction, the fusion of the plasma membrane with the outer acrosome membrane thus releasing the acrosomal content, is an extraordinary exocytotic process that enables the sperm cell to penetrate the zona pellucida of the oocyte. A tightly regulated mechanism is required to prevent premature or belated release of acrosomal enzymes as that will result in a failure of fertilization [107]. The sperm acrosome is a cap-shaped membrane embedded Golgi-derived organelle that covers the anterior half of the nucleus [108-110]. During its formation in early spermatids, the orientation of the *cis*- and *trans*- face of the Golgi apparatus is reversed by scrolling of the Golgi complex to the growing acrosomal vesicle that is attached to the sperm nuclear envelope [111]. At that time vesicles bud off from the *trans*-Golgi network and migrate towards the acrosome granule, different from the process in somatic cells where such vesicles migrate to the plasma membrane for direct secretion. This spermatid specific phenomenon allows the creation of one extraordinary large secretory vesicle, the acrosome, and prevents its migration towards the spermatid plasma membrane (the Golgi network at that stage covers the acrosome granulum [112]). Later when the Golgi network is relocated to a more distal position; the acrosome acidifies and then contains active cleaving enzymes such as acrosin and

hyaluronidase [109;113]. At the time the sperm cells are to be released from the seminiferous epithelium, a substantial reduction or complete removal of the cytosol, Golgi and other intracellular structures has taken place. This renders the acrosome being framed between the plasma membrane and the nuclear envelope at the apical area of the sperm head. Despite of this tight conjunction of the acrosome with the sperm plasma membrane, it is of crucial importance that the acrosome remains intact until the sperm has reached the vicinity of the oocyte (see chapter 1). When acrosome-intact sperm arrive at the cumulus-oocyte complex, they may finally bind to the zona pellucida. At that moment an instantaneous induction of the acrosome reaction is required, not only for the individual sperm cell to be able to digest and penetrate the zona pellucida but also for it to be the first to fertilize the oocyte after completed zona penetration. Both the prevention of a premature acrosome reaction and the zona-mediated induction of the acrosome reaction are considered to be regulated by SNARE proteins and SNARE interacting proteins.

In 2000, Kierszenbaum [114] hypothesized that the Rothman model for SNARE-mediated exocytosis [3] is involved in the release of the acrosomal content during the acrosome reaction. At that time several proteins that are players in the Rothman model had already been identified in rodent sperm [115-117]. Kierszenbaum predicted, from the Rothman model, that plasma membrane-associated SNAREs (target membrane SNAREs or t-SNAREs) are interacting with SNAREs on the outer acrosomal membrane (the vesicle membrane SNAREs or v-SNAREs) and that regulatory proteins like Rab, a small GTPase and NSF-SNAP complexes are involved in this process [114]. Apart from the proteins described by the Rothman model additional SNARE interacting proteins have been identified in sperm including complexin [118] and dynamin 2 [119]. These proteins regulate SNARE complex formation and dissociation thereof [41;83;89;91].

Most of the investigations concerning the role of SNAREs in the acrosome reaction have been performed on rodent or human sperm and have made use of either streptolysin-O and/or calcium ionophore treated sperm samples. In both treatments, the acrosome/plasma-membrane fusions are induced artificially and prevention of such fusion could be achieved by using intervening components [120]. However, the SNARE protein organization and restructuring has not been investigated under more physiological conditions, a primary goal of this thesis (chapters 3-5).

The lateral and sub-cellular localization of SNARE proteins were investigated on sperm that was incubated in control media or activated in an *in vitro* fertilization medium (in which sperm are capacitated; see chapter 1 for explanation of that process). It is of considerable interest that the sperm surface has a defined structure with dynamic lateral domains [121]. Recent studies have shown that lipid ordered microdomains (also known as membrane rafts) are at least in part responsible for the dynamics in lateral reorientation of sperm surface molecules: capacitation of sperm

causes an aggregation of small lipid-ordered microdomains at the apical ridge of the sperm head [122]. Even with the still existing uncertainties and discrepancies in nomenclature and in methods for isolation of microdomains [122-126], it is obvious that these microdomains are involved in the spatial regulation and the dynamics of proteins and lipids during sperm capacitation. As these domains are difficult to investigate in biological membranes, one of the most commonly used techniques to study putative raft-associated proteins is by extracting these microdomains with Triton X-100 or similar detergents. By this approach, insoluble membrane fragments (also called detergent resistant membrane fragments) float over a discontinuous sucrose gradient and can be separated for further study [127-129].

This resulting detergent resistant membrane fraction (DRM, considered to represent the pre-existing membrane rafts in live cells) contains small insoluble membrane particles that are enriched in sterols and sphingolipids. DRMs are not only involved in membrane traffic and cellular signal transduction [12;130;131], but also in the regulation of exocytosis in many cell types including the sperm cell [132-134]. Studies in various species revealed a dynamic redistribution and reorganization of marker components of these microdomains upon sperm capacitation [123;126;135;136]. Of considerable interest is the notion that SNARE mediated exocytosis is linked to membrane rafts [130;137-139] as recently described for mouse sperm DRMs [140]. At the start of this thesis however, it still was not known how SNARE interactions were functionally linked to capacitation-induced sperm surface dynamics. Sleight et al. suggested that capacitation alters these lipid-ordered domains and causes the dissociation of DRMs resulting in a reduction of DRM-associated proteins in these microdomains [140]; however, other data suggest that capacitation-dependent depletion of cholesterol occurs only in non-DRM areas which subsequently changes the membrane fluidity and facilitates the aggregation of these microdomains [141]. Moreover, Nixon et al. hypothesized that partition of chaperones (e.g. heat shock proteins) into DRM fractions upon capacitation acts as the sorting machinery of required proteins from the non-DRM fraction into DRM fractions [142]. This is in line with the observation of Ackermann et al. who proposed a multi-PDZ domain (a common structural protein subdomain found in many types of signaling proteins in variety cell types) to be crucial to facilitate the sorting of required proteins into DRMs [132].

In this thesis, the ultrastructural localization of SNARE proteins upon capacitation as well as their association with membrane rafts (*chapter 3*) was investigated. The presence of SNARE proteins is reported for the apical plasma membrane and the outer acrosomal membrane. Their capacitation-dependent migration to the area of the sperm surface where rafts are clustering and where zona pellucida binding is also initiated. This area of the sperm surface is also the specific site where the fusions for the acrosome reaction are initiated after zona binding. In *chapter 4*, focuses on the

process of docking and priming of acrosome to the plasma membrane via the formation of a 80 kDa *trans*-SNARE protein complex at the apical surface of the sperm head after the induction of capacitation, applying a combination of biochemical, immunochemical and ultra-structural approaches. The acrosome reaction does not take place under such specific conditions and the two docked membranes could even be isolated as a stable bilamellar structure. Clearly this demonstrates that after membrane docking and priming the process of exocytosis is arrested and does not yet result in membrane fusion, which is logical as the acrosome reaction should only be triggered once the sperm is bound to the zona pellucida. It is well-established that SNARE-mediated membrane fusion is calcium sensitive [143] and that zona binding results in increased free Ca^{2+} levels in the sperm's cytosol. This process can be mimicked by incubating sperm in presence of Ca^{2+} ionophores. In *chapter 5*, such conditions were applied both in control sperm and in capacitated sperm. Multiple fusions of the sperm plasma membrane with the outer acrosomal membrane was evoked and the subsequent formation of mixed vesicles of these two membranes (see chapter 1). Interestingly, the primed *trans*-SNARE complex present in capacitated sperm (see chapter 4) interact with complexin forming a 118 kDa protein complex that can be dissociated either by 100 ºC boiling in Laemmli sample buffer (heat-sensitive) or by Ca^{2+} (addition of ionophore). Remarkably, the 118 kDa complex is unique for the mixed vesicles and for the DRM fraction isolated from capacitated and acrosome reacted sperm, and is not formed in control sperm. These results are summarized in a model in which the bicarbonate-dependent docking/priming and protection for membrane fusion occur during sperm capacitation in the area where sperm rafts cluster. A prediction is made that the zona binding-induced Ca^{2+} influx subsequently causes dissociation of complexin from the primed SNARE complex and thus causes the *trans* to *cis* conformational changes that execute the acrosome membrane fusions which is probably regulated by a Ca^{2+} induced conformational change of synatotagmin-4, a component which recently been identified by proteomics analysis of the isolated fused membranes.

5.2 SNAREs in oocytes

While the involvement of SNARE proteins in the regulation of sperm acrosome reaction has attracted the attention of reproductive biologists, little is known about the function of SNAREs in the oocyte. In mammals, the first part of oogenesis starts in the germinal epithelium with the formation of ovarian follicles. In the primary oocytes, an enlarged fluid-filled nucleus, named the germinal vesicle (GV), is formed by the interplay of Golgi and ER [144]. At the periphery of this GV the DNA chromatin organization remains arrested in the prophase of meiosis-I [145;146]. Oocytes are arrested at this GV stage until they are recruited for final maturation. The resumption of meiosis starts with GV breakdown and continues until the metaphase stage of

meiosis-II (M-II arrested oocyte) [147]. Once mammalian sperm meet the oocyte, these cells are resident in the oviduct and the oocyte is still in its M-II arrested stage. During the development of the oocyte from the GV to the M-II stage a number of secretory vesicles relocalize towards a position just underneath the oolemma. These secretory granules (also known as cortical granules, CG) originate from typical, hypertrophic Golgi complexes and are formed during early maturation [148]. Bovine cortical granules cluster as large aggregates throughout the ooplasm at the GV stage, while in the later M-I stage smaller aggregates as well as individual particles can be observed more peripherally, closer to plasma membrane. When maturing unfertilized oocytes reach the M-II stage, all CG are located in very close proximity to the oolemma [149]. In rodents [150;151] as well as in swine [152], the microfilament network plays a role in this CG relocalization. Once the CGs are positioned just underneath the oolemma they become detached from microfilaments [152].

The rationale for the redistribution of these secretory granules is to prepare the oocyte for the cortical reaction. This reaction is induced by fertilization, the fusion of the oolemma with the plasma membrane of a sperm cell. The fertilization immediately activates the oocyte by a Ca^{2+} mobilization (induced by PLC-zeta, a soluble protein from sperm mobilizing intracellular Ca^{2+} in the oocyte) [153]. The subsequent enhanced cytosolic Ca^{2+} levels rapidly induce the fusion of the cortical granulae with the oolemma [154]. The secretion of these granulae results - depending on the species - either in the surface coating of the oolemma and/or in zona hardening and thereby rapidly prevents additional sperm to fuse or bind to the oolemma and/or to bind or penetrate the zona pellucida [153]. This serves as an efficient blockage to polyspermic fertilization [155].

Obviously, the cortical reaction might - in analogy to the acrosome reaction - be under control of SNARE protein interactions. This has been the primary research topic in *chapter 6*. The cortical reaction is an exocytotic event and all studied exocytotic events are considered to be controlled by SNARE protein interactions. However, only sparse data are available on SNARE proteins in oocytes: Sea urchin oocytes do contain SNARE or SNARE like proteins that may be involved in the fertilization-induced exocytotic event that is involved in the formation of a polyspermic fertilization block [156]. Scarce information on mouse oocytes indicates that SNAP 25 plays a role in the cortical reaction [157]. However, the organization of SNARE proteins in the oolemma and the cortical granules has not been studied nor the temporal organization of SNARE proteins and their interacting entities. This is described in *chapter 6* where we investigated the localization of SNARE proteins during oocyte development from the GV towards the M-II stage and up to 8 hrs post-fertilization. The identified SNARE proteins are considered to form functional complexes to orchestrate membrane fusion between the oolemma and the cortical granules. These SNARE proteins typically showed a pronounced concentration in the area just underneath the oolemma at the

M-II stage. Moreover, complexin (the key molecule that arrest the *trans*-SNARE complex for spontaneous fusion) and clathrin (a protein that is involved in endocytotic pathways) are also highly concentrated in the same sharply defined cortical area of the oocyte at the M-II stage. Taken together these findings have brought us to the following explanation: In the oocyte the exocytotic and endocytotic processes are arrested at the M-II stage and the recycling of vesicles is started by the fertilization-mediated release of Ca^{2+} from intracellular stores into the cytosol. The arrest of vesicle recycling at the oolemma is probably instrumental for the cortical reaction and thus for the polyspermic fertilization blockage.

6. Bridging SNARE interactions with fusion events in gametes

In summary, this chapter reviews membrane fusion mechanisms in general and specifically the cellular presence of SNARE proteins and their regulators as well as their functions. In chapters 3-6, we investigated the molecular mechanism related to the role of SNARE proteins in exocytosis in mammalian gametes. The principal findings are summarized and discussed in *chapter 7* where we also present modified models for the acrosome and cortical reaction. Furthermore, the importance of the silencing of exocytotic processes in the sperm cell and the oocyte prior to their final interaction to achieve mammalian fertilization is discussed.

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Chapter 3

Syntaxin and VAMP association with lipid rafts depends on cholesterol depletion in capacitating sperm cells

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Abstract

Sperm cells represent a special exocytotic system since mature sperm cells contain only one large secretory vesicle, the acrosome, which fuses with the overlying plasma membrane during the fertilization process. Acrosomal exocytosis is believed to be regulated by activation of SNARE proteins. In this paper, we identified specific members of the SNARE protein family, i.e. the t-SNAREs syntaxin1 and 2, and the v-SNARE VAMP, present in boar sperm cells. Both syntaxins were predominantly found in the plasma membrane whereas v-SNAREs are mainly located in the outer acrosomal membrane of these cells. Under non-capacitating conditions both syntaxins and VAMP are scattered in well defined punctate structures over the entire sperm head. Bicarbonate-induced in vitro activation in the presence of BSA causes a relocalization of these SNAREs to a more homogeneous distribution restricted to the apical ridge area of the sperm head, exactly matching the site of sperm zona binding and subsequent induced acrosomal exocytosis. This redistribution of syntaxin and VAMP depends on cholesterol depletion and closely resembles the previously reported redistribution of lipid raft marker proteins. Detergent-resistant membrane isolation and subsequent analysis shows that a significant proportion of syntaxin emerges in the detergent-resistant membrane (membrane raft) fraction under such conditions, which is not the case under those conditions where cholesterol depletion is blocked. The v-SNARE VAMP displays a similar cholesterol depletion-dependent lateral and raft redistribution. Taken together, our results indicate that redistribution of syntaxin and VAMP during capacitation depends on association of these SNAREs with lipid rafts and that such a SNARE-raft association may be essential for spatial control of exocytosis and/or regulation of SNARE functioning.

Keywords: Sperm, syntaxin, cholesterol depletion, lipid rafts, activation

Introduction

Mammalian sperm cells ejaculated into the female genital tract are initially unable to fertilize the oocyte. During migration in the female genital tract, sperm cells are activated and acquire fertilizing properties. As a result, sperm cells are hypermotile and are able to bind to the zona pellucida of the oocyte, which in turn triggers the acrosome reaction [1]. This activation process (henceforth called capacitation) is partly induced by bicarbonate present in the female genital tract, and thus can be mimicked *in vitro* by incubating sperm cells in media containing bicarbonate/CO_2 and albumin [2;3].

High bicarbonate concentrations in combination with extracellular calcium and albumin have been shown to activate intracellular signaling pathways, such as sperm-specific adenylyl cyclases, cAMP-dependent protein kinase A (PKA) and protein tyrosine phosphorylation. Important downstream events of these bicarbonate-induced signaling pathways include rearrangements in the lipid architecture of the sperm plasma membrane, i.e. redistribution of glycolipids, phospholipids and cholesterol [2;4-9], and efflux of cholesterol in the presence of a cholesterol-acceptor [10].

Cholesterol is an important component of lipid bilayers since it can intercalate with sphingolipids and gangliosides to form lipid microdomains (rafts) [11-13]. These so-called lipid rafts represent small liquid-ordered lipid domains in the further liquid disordered plasma membrane. One of the most compelling characteristics of these lipid rafts is their capacity to sequester specific proteins in a specific membrane location, while excluding others in response to intra- and intercellular stimuli [14;15]. It has been postulated that the concentration of proteins in rafts facilitates specific protein-protein interactions [15] and contributes to spatial control of protein interactions [14;16;17].

Lipid rafts have recently been identified in sperm of several species [12;18;19], but their precise role in sperm-specific processes like zona pellucida binding and acrosome reaction is unclear. Using lipidomic analysis, we have previously shown that capacitation in porcine sperm involves lipid raft aggregation in the apical plasma membrane rather than dissociation or disruption of lipid rafts [18]. Proteomic analysis indicates that this redistribution of lipid rafts corresponds with a reordering of specific raft adhering proteins to the apical plasma membrane [18]. Proteins that have thus far been identified in sperm lipid rafts include i) proteins involved in zona binding [17-19], ii) proteins involved in redox balance [18], and iii) proteins necessary for the acrosome reaction [19].

During the acrosome reaction, the apical plasma membrane fuses at multiple sites with the underlying outer acrosomal membrane resulting in the release of hydrolytic enzymes accumulated in the large Golgi-derived acrosome. These enzymes assist sperm-cell penetration of the zona and enable subsequent membrane fusion which is

necessary for fertilization of the oocyte (for a concise review see [20]). Proteins of the soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor (SNARE) family are involved in many Ca^{2+} mediated membrane fusion processes in a wide range of cell types. SNARE-protein mediated exocytosis involves interactions of complementary SNARE proteins (t-SNAREs in the plasma membrane and v-SNAREs in the vesicle membrane) to form 7S or 20S complexes which enable membranes to fuse [21-24]. SNARE protein interactions are aided by many additional proteins, such as small GTPases Rab's, NSF and α SNAP [24;25]. In sperm, some SNARE proteins have been identified, but their role in fertilization is not completely understood [22;26-28].

Although lipid rafts have been shown to play a key role in the sorting of proteins necessary for zona binding [18], no information is available on the role of these lipid microdomains in the spatial and functional (re)organization of the exocytotic machinery in sperm cells during activation. In the present paper, we have investigated whether lipid raft redistribution or depletion upon capacitation relocates specific SNARE proteins to those areas on the apical plasma membrane where acrosomal exocytosis is bound to happen. We have identified SNARE proteins present in boar sperm and assessed the distribution of specific t-SNAREs syntaxin 1, 2 and its complementary v-SNARE VAMP before and after capacitation. Additionally, we have investigated whether these SNAREs associate with, or dissociate from, lipid rafts during capacitation. The relevance of these processes in the acrosome reaction responsiveness of zona-bound sperm is discussed.

Materials and Methods

Chemicals

All chemicals were obtained from Merck (Darmstadt, Germany), unless otherwise stated. Rabbit polyclonal antibodies raised against syntaxin 1A/B (batch I378) were a gift from Matthijs Verhage, (CNCR, Amsterdam, the Netherlands). Rabbit polyclonal syntaxin 2 antibodies were obtained from Synaptic Systems (Göttingen, Germany) and from Sigma (St. Louis, MI, USA). Mouse monoclonal antibodies against VAMP (recognize both VAMP1 and 2 isoforms) were from Synaptic Systems, whereas polyclonal antibodies against VAMP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against raft specific protein flotillin 1 were purchased from Santa Cruz. Normal goat and normal rabbit serum were from Vector (Burlingame, CA, USA). Alexa 488 conjugated goat-anti-rabbit IgG (H+L) and propidium iodide (PI) were obtained from Molecular Probes-Invitrogen (Eugene, OR, USA). Ca^{2+} ionophore (A23187) and FITC-conjugated Arachis hypogaea (peanut) agglutinin were from Sigma.

Collection and preparation of spermatozoa

Sperm of highly fertile boars (*Sus scrofa domestica*), used for artificial insemination, was obtained from a commercial breeder (Cooperative Center for Artificial Insemination in Pigs, 'Utrecht en den Hollanden', Bunnik, the Netherlands). Freshly ejaculated sperm was filtered through gauze to remove gelatinous material and diluted in saline (137 mM NaCl, 2.5 mM KCl, and 20 mM HEPES, HBS, pH 7.4) to obtain a concentration of $1.5x10^8$ motile cells/ml. Sperm was subsequently washed through a discontinuous Percoll (GE Healthcare, Diegem, Belgium) gradient (70% v/v and 35% v/v, respectively), as previously described [29]. All solutions were iso-osmotic (290-310 mOsm/kg) and at room temperature.

Next, sperm was subjected to different treatments according to the following protocols: (G1) sperm cell suspensions were stimulated in HEPES-buffered Tyrode's medium (HBT: 90 mM NaCl, 21.7 mM lactate, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 1.0 mM pyruvate, 0.4 mM $MgsO_4$, 0.3 mM NaH_2PO_4, 2 mM $CaCl_2$, 100 µg/ml kanamycine; 300 mOsm/kg, pH 7), 15 mM $NaHCO_3$ in open vials for 2 hrs at 38.5 °C in humidified atmosphere with 5% (v/v) CO_2, and the acrosome reaction was subsequently induced by addition of 5 µM Ca^{2+} ionophore A21387 (Sigma) to the medium and additional incubation for 1 hr at 38.5 °C; (G2) sperm cells were stimulated only by incubation in HBT supplemented with 15 mM $NaHCO_3$ in open vials for 2 hrs at 38.5 °C in humidified atmosphere with 5% CO_2; (G3) sperm was incubated in HBT medium with the omission of bicarbonate in air-tight vials for 2 hrs at 38.5 °C in a tube rack placed in a pre-warmed water bath and the acrosome reaction was subsequently induced as described above; (G4) sperm cells were incubated in HBT medium with the omission of bicarbonate in air-tight vials for 2 hrs at 38.5 °C in a tube rack place pre-warmed water bath (control). All 4 conditions were prepared either in the presence of the cholesterol acceptor bovine serum albumin (0.3% w/v BSA in HBT: delipidated fraction V; Boehringer, Mannheim, Germany) or in the absence of a cholesterol acceptor (replaced by 0.15 % w/v polyvinyl alcohol and 0.15 % w/v polyvinylpyrrolidone to avoid sperm agglutination, see [3]. Those conditions in which sperm cells where incubated in the presence of both bicarbonate and BSA are considered to mimick normal capacitation conditions and those conditions are henceforth called "capacitated". Those conditions in which sperm cells are incubated in bicarbonate only are henceforth called "activated".

FACS-analysis was used to assess the efficiency of the ionophore- induced acrosome reaction. For this, sperm cells of G1, G2, G3 and G4 conditions (with 0.3% BSA) were washed in HBT and subsequently incubated for 5 min in 1 µg/ml FITC conjugated PNA (PNA-FITC) to distinguish acrosome-reacted cells from non-reacted cells. The membrane impermeable vital stain propidium iodide (PI) was used to counter-stain deteriorated cells (final concentration 25 nM). The sperm suspensions were analyzed in a FACScalibur flow cytometer equipped with a 100 mW argon laser

(Becton Dickinson, San Jose, CA, USA). Cell fluorescence was excited at 488 nm and the FITC and the PI-emission intensity per cell was detected in the logarithmic mode of FL-1 (530/30 nm band pass filter) and FL-3 (620 nm long pass filter), respectively. Forward and sideways scatter (FSC and SSC) data were collected in the linear mode and sperm specific events were gated for further analysis. Two-dimensional dot plots representing 10,000-gated events were made with FL-1 data expressed on the x-axis and FL-3 data on the Y-axis. The proportion of cells positively stained with one or both fluorescent dyes were scored using quadrant analysis in Win MDI software (version 2.8, J.Trotter, free-ware).

Isolation of apical plasma membranes and protein extraction

Apical plasma membranes from fresh Percoll washed sperm were isolated according to a standard protocol [29]. In short, $4x10^8$ sperm cells/ml in PM-buffer (5 mM Tris, pH 7.4, 0.25 M sucrose and protease inhibitors; containing complete protease inhibitor cocktail, EDTA free, Roche, Mannheim, Germany) were subjected to nitrogen cavitation (10 min, 45 bar) in a cell disruption device (Parr Instruments, Moline, IL, USA). The cavitate was slowly extracted and centrifuged for 10 min at 1000g at room temperature. The supernatant was collected and the pellet was washed with PM-buffer and again centrifuged for 10 min at 1000g. Both supernatants were combined and centrifuged for 10 min at 6000g. Apical plasma membranes were pelleted from these supernatants by centrifugation for 70 min at 285,000g at 4 ºC. The pellet was washed once more in HEPES-buffered saline (HBS: 5 mM HEPES, 2.7 mM KCl, 146 mM NaCl and protease inhibitors, pH 7.4) and centrifuged for 40 min at 285 000g at 4 ºC. Next, plasma membranes pellets were resuspended in HBS, flash-frozen in liquid nitrogen and stored at –20 ºC for later use. Purity of the isolated plasma membrane fraction was determined by measuring the alkaline phosphatase and acrosin activity as described before [29;30].

Protein extraction was performed by incubation of sperm cell samples or boar and rat brain tissue samples (positive controls) in solubilization buffer (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM EGTA, 1 mM EDTA, 1% w/v sodium dodecyl sulphate, 1% w/v Triton-X-100, and protease inhibitor cocktail (Roche). The sperm cell suspension was sonicated (MSE Ultrasone, Crawley, UK) and subsequently incubated at 4 ºC for 14 hrs under continuous rotation. After centrifugation at 14,000g for 30 min at 4 ºC, the supernatant was flash-frozen in liquid nitrogen and stored at –20 ºC for later use.

Isolation of Detergent-Resistant Membrane (DRM) fraction (lipid rafts)

DRMs were isolated according to [18]. In short, sperm cells were washed in HBS and resuspended (~10^9 cells/ml) in 2-(N-morpholino)ethanesulfonic acid (Mes) buffer (25 mM Mes, 150 mM NaCl, 1 mM EGTA, protease inhibitors (Roche), 1% v/v Triton-X-

100; pH 6.5) and kept on ice for 30 min. The suspension was subsequently mixed with an equal volume of 80% w/v buffered sucrose solution. The mixture was overlaid with 8 ml 30% w/v sucrose followed by 4 ml 5% w/v sucrose in Mes buffer and centrifuged for 18 hrs at 200,000g at 4 ºC. The DRM's appeared as an opalescent band in the low-density fraction of the gradient. Starting from the top of the gradient 1 ml fractions were collected (fractions 1-13 and pellet fraction 14) and used for both Western blotting and dot-blotting experiments.

Western blotting

Protein concentrations in boar sperm samples, boar and rat brain samples were determined according to the Lowry method [31]. Of both sperm and control brain samples, 100 µg of total protein extract was resuspended in a small amount of loading buffer (final concentrations 66 mM Tris/HCl, pH 6.8, 3% w/v SDS, 5% v/v glycerol, 2% v/v β-mercaptoethanol, and 0.001% w/v bromophenol blue) and boiled for 10 min prior to Western blotting [32]. In case of the DRM fractions, 12 µl of sample was mixed with loading buffer. Proteins were separated in an 11% SDS-PAGE gel with a 4% stacking gel and wet-blotted onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). After blocking for 1 hr in 5% w/v dry milk powder, 1% v/v normal serum and 0.05% v/v Tween-20 in TBS (20 mM Tris/HCl and 137 mM NaCl), blots were incubated with primary antibodies, diluted in blocking buffer for 12 hrs at 4 ºC. After washing the blots in TBS with 0.05% v/v Tween-20, blots were subsequently incubated with secondary antibodies for 30 min. After washing the blots in TBS with 0.05% Tween, protein staining was visualized either by using enhanced chemifluorescence (ECF-detection kit;GE Healthcare) detected with a Storm analyzer (Molecular Dynamics, Sunnyvale, CA, USA) or by chemiluminescence (ECL-detection kit; Supersignal West Pico, Pierce, Rockford IL, USA).

Dot-blotting

Dot blotting was performed by using the Easy-Titer™ ELIFA dot blot system (Pierce). Nitrocellulose membranes (Protran BA 85, Whatman, Dassel, Germany) were rinsed in MilliQ-water and 6 µg of total protein was subsequently pipetted into each well. Membranes and adhering proteins were dried using a vacuum system (flow rate 100 µl/ 1.5min/well). After blocking in 5% w/v BSA and 0.05% v/v Tween-20 in TBS (overnight at 4 °C), blots were incubated with primary antibodies, diluted in blocking buffer, for 1 hr at room temperature. After washing in TBS with 0.05% v/v Tween-20, blots were subsequently incubated with secondary antibodies for 30 min at room temperature. After washing in TBS with 0.05% v/v Tween-20, protein staining was visualized either by using enhanced chemifluorescence (ECF-detection kit; GE Healthcare) detected with a Storm analyzer (Molecular Dynamics) or by chemiluminescence (ECL-detection kit; Supersignal West Pico, Pierce). Quantitative

analysis of dot blot labeling was performed by scanning the blots with a GS-700 densitometer (Bio-Rad Laboratories, Hercules, CA, USA) using Quantity One acquisition software (version 4.3, Biorad). Densitometric quantitation was carried out with the use of *Gel-Pro Analyzer* software (version 3.0, MediaCybernetics, Silver Spring, MD, USA). Statistical analyses were performed with the use of SPSS 12.0 statistical software (SPSS Inc., Chicago, IL, USA) and/or SigmaPlot 9.0 (Systat Software Inc. Richmond, CA, USA).

Immunofluorescence microscopy

Percoll-washed sperm cells were rinsed twice in PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and subsequently fixed according to either of two fixation protocols: i.e. 1) fixation in 2% v/v paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS with 0.4% w/v polyvinyl pyrrolidone (PVP) for 1 hr at room temperature. (free floating) or 2) fixation on slides in 2% v/v paraformaldehyde in PBS for 30 min at room temperature, followed by incubation in ice-cold methanol for 1 min. Sperm cells fixed according to the free floating technique were immunostained by means of free floating incubation, whereas in case of fixation of sperm cells on slides, all immunostainings were carried out on slides.

For immunostaining, sperm cells were washed in PBS supplemented with 0.05% v/v Tween-20 and subsequently incubated in blocking buffer (PBS, 0.05% v/v Tween-20, 1% w/v BSA, and 5% v/v normal goat serum) for 2 hrs. Next, cells were incubated in primary antibodies, diluted 1:100 in blocking buffer for 2 hrs at room temperature. After extensive rinsing, cells were incubated with Alexa 488-conjugated secondary antibodies (1:100) in blocking buffer for 2 hrs at room temperature. Finally, cells were rinsed in PBS, mounted in FluorSave (Calbiochem, San Diego, CA, USA) and coverslipped. As a control for the specificity of the secondary antibodies, samples were immunostained as described above; however, the primary antibodies were replaced by normal serum. All samples were imaged with a Leica TCS-SP inverted confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) (488 nm argon excitation line, detection of emission wavelength between 510-540 nm). All images were made in the extended focus mode using a pinhole of 1 airy disk (for 60x magnification objective used to capture the presented images the thickness of the optical section is 0.7 um).

Results

SNARE proteins are present in sperm cells

In a first series of experiment, we set out to determine the presence of important representatives of the postulated SNARE protein family in boar sperm cells, i.e. the t-

SNAREs syntaxin and the v-SNARE VAMP. Western blotting of crude protein extracts demonstrated the presence of syntaxin 1A/B, syntaxin 2, and VAMP in sperm homogenates (Figure 1).

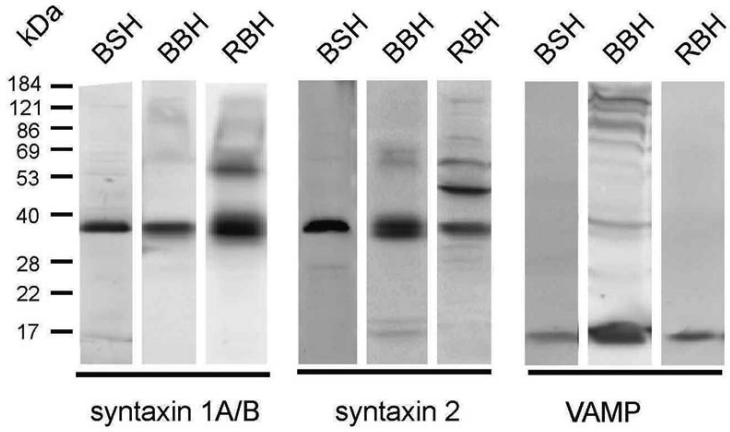

Figure 1.
Compilation of Western blot data from different experiments showing the presence of the t-SNAREs, syntaxin 1 and 2, and the v-SNARE, VAMP, in protein extracts (100 µg total protein/lane) from boar sperm cells and control tissues. BSH: total boar sperm homogenate; BBH: total boar brain homogenate (control); RBH: total rat brain homogenate (control).

Since all antibodies used in this study were raised against rat or mouse brain derived proteins, we included rat and boar brain protein extracts in the Western blot experiments to serve as positive controls. Our Western blot analysis indicated that the apparent molecular weights of the boar sperm syntaxin isoforms, as well as VAMP were comparable to those found in rat and boar brain samples, 37 and 18 kDa, respectively.

Relative distribution of SNARE proteins in sperm cells

SNARE-proteins can be subdivided into t-SNARE-proteins, which are present in target membranes, and v-SNARE proteins, which are present in vesicle membranes. We determined their presence in whole sperm cell plasma membranes and in isolated plasma membranes. The apical plasma membrane was released by nitrogen cavitation and collected by differential centrifugation [29]. Acrosin and alkaline phosphatase activity measurements (markers for the acrosome and plasma membrane, respectively), showed that the plasma membrane was 10-12 times enriched in the membrane fraction, when compared with the whole sperm cavitate (Figure 2a).

Quantification studies on Western blots showed that both t-SNAREs syntaxin 1A/B and syntaxin 2 are predominantly enriched in the apical plasma membrane fraction (Figure 2b). Likewise, the v-SNARE VAMP could be specifically demonstrated in the whole sperm fraction and was almost absent in the isolated plasma membrane fraction indicating that this protein most likely resides in the outer acrosomal membrane (Figure 2b) which is confirmed by immunolocalization of VAMP as described in the next section.

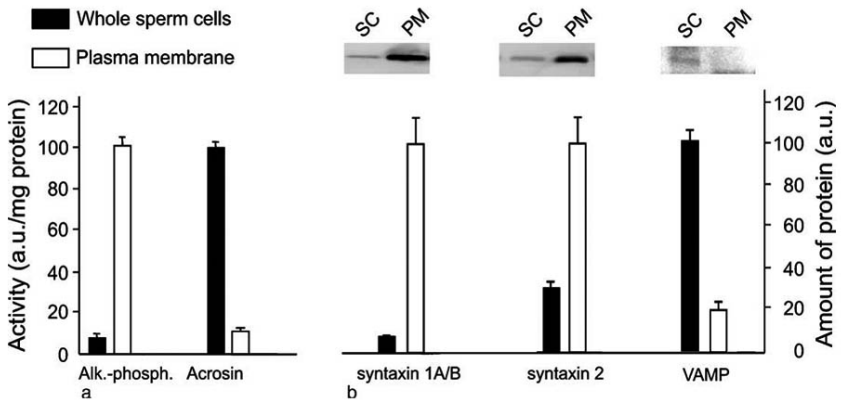

Figure 2.
a. Relative purification of the isolated sperm plasma membrane fraction after nitrogen cavitation and subsequent differential centrifugation.
Measurements of alkaline phosphatase (a plasma membrane marker) and acrosin (an acrosomal membrane marker) activity in equal amounts of isolated sperm plasma membranes (normalized to 50 µg total protein; open bars) and of whole sperm cells (normalized to 50 µg total protein; black bars) indicate that the plasma membrane is 10 -12 times enriched in the membrane fraction.
Values are expressed as arbitrary units (a.u.) per mg protein and are means ± SEM of three different protein concentrations and three independent experiments.
b. Relative distribution of syntaxin 1 and 2, and VAMP as extracted from isolated sperm plasma membranes (normalized to 100 µg total protein; open bars) and whole sperm cells (100 µg total protein; black bars). Western blot data are shown as insets. Values are expressed as arbitrary units per mg protein and are means ± SEM of three different protein concentrations and three independent experiments.

Immunolocalization of t-SNARE proteins

In order to determine whether lipid raft redistribution during capacitation provides a platform for exocytotic protein reorganization in sperm, we studied the distribution of both t-SNARE and v-SNARE proteins in the sperm cell during the capacitation process. We applied two different fixation protocols for the immunostainings. Both fixation protocols yielded the same results. In all control experiments, no fluorescence was detected when primary antibodies were omitted. We used different specific antibodies against syntaxin 1, 2 and VAMP on capacitated (condition G2+BSA) and

non-capacitated (condition G4+BSA; control) sperm cells. Under control conditions, both syntaxins were localized in a punctate fashion in the head plasma membrane with a preference for the more apical areas of the plasma membrane (Figures 3a, b, e, f). Syntaxin 2 was also found in the membrane covering the tail of the sperm cell (Figure 3h).

After incubation of sperm in capacitation medium, we observed that the distribution of both syntaxins changed from a dispersed punctate pattern over the entire sperm head to a more clustered and restricted area of the apical ridge (Figures 3c, d, g, h). The observed change in distribution of the V-SNARE VAMP was rather similar to that of the syntaxins and which implies that VAMP must be localized in the acrosomal membrane. Under control conditions, punctate VAMP-immunostaining was mainly present on the pre-equatorial and apical parts of the sperm head (Figure 3i), whereas after capacitation, the distribution changed into a more clustered pattern in the apical region (Figure 3j). Syntaxin- and VAMP-immunostaining of sperm cells subjected to Ca^{2+} ionophore-induced acrosome reaction with and without prior capacitation (conditions G1+BSAand G3+BSA, respectively) did not show a specific clustering of these SNARE proteins in the apical region. Only a residual punctate staining pattern could be observed on the pre-equatorial and apical regions of the sperm cell (T. van Haeften, M. de Boer-Brouwer, P.S. Tsai, unpublished work).

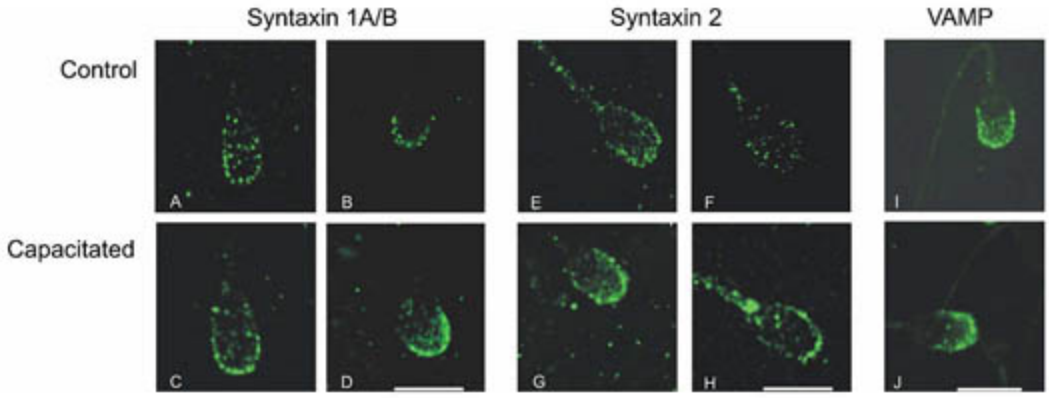

Figure 3.
Confocal-microscopic images of immunostained sperm cells illustrating the subcellular localization of :syntaxin 1A/B in control boar sperm cells (a, b) and in capacitated sperm cells (c, d), of syntaxin 2 in control (e, f) and capacitated sperm cells (g, h), and of VAMP in control (i) and capacitated sperm cells (j). Note the change from punctate dispersed to an apical ridge concentrated distribution. Bars represent 10 µm.

SNARE protein association with lipid rafts

Since syntaxins (Figure 3), VAMP (Figure 3), and lipid raft marker proteins [18] redistribute in a similar fashion during capacitation, we investigated whether this capacitation-induced lipid raft redistribution underlies the observed changes in the staining pattern of the t-SNARE syntaxin and the most prominent v-SNARE VAMP. In addition, all fractions were screened for the presence of the raft specific protein flotillin 1. Sperm cells were either control treated, activated or acrosome reacted either with or without BSA as cholesterol acceptor. FACS-analysis of sperm showed that Ca^{2+} ionophore-induced acrosome reaction was very efficient since 95% of the sperm cells were acrosome-reacted (Figure 4).

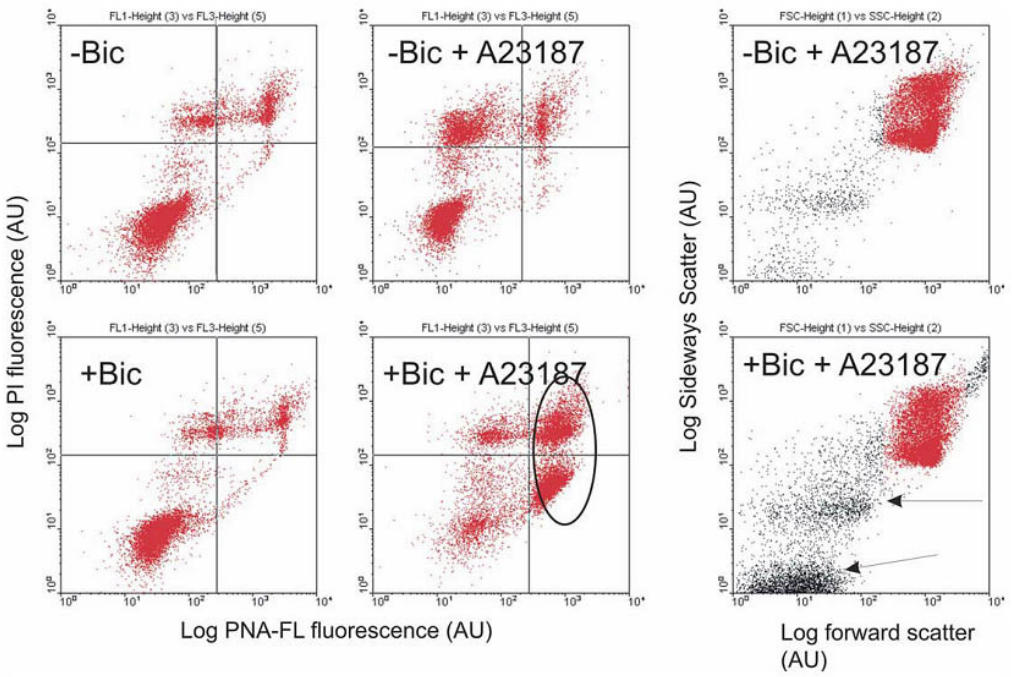

Figure 4.
Flow cytometric analysis of PNA-FITC (to distinguish acrosome reacted cells from non-reacted cells) and propidium iodide labeling (to distinguish dead cells from viable cells) of sperm cells after incubation in control medium (-BIC) and medium with bicarbonate and BSA (capacitated:+BIC). Additionally, PNA-FITC and propidium iodide labeling was determined in capacitated and subsequently acrosome reacted sperm cells (+BIC + A23187) or acrosome reacted sperm cells without preceding capacitation (-BIC + A23187). Total population of acrosome-reacted sperm as induced by Ca^{2+} ionophore A23187 is indicated with the ellipse. Right panel: arrows indicate the population of mixed vesicles which are a result of multiple fusions of the plasma membrane with the outer acrosomal membrane and which are formed during the acrosome reaction.

The incubated sperm cells were subjected to ice-cold 1% Triton-X-100 extraction and centrifuged over a discontinuous sucrose gradient to isolate the detergent-resistant membrane fraction which represents liquid-ordered membrane microdomains (or lipid rafts) [18]. Fourteen fractions were collected from top to bottom (fraction 1 through 13 and the pellet fraction 14) and were screened for the presence of syntaxin 2, VAMP and flotillin by means of immuno- or dot-blotting and subsequent densitometric quantitation.

In all experimental conditions, both syntaxins were present in the high-density fractions 10-13, demonstrating that most of the syntaxins are present in the non-raft fraction of the sperm plasma membrane. However, under those conditions where capacitation was induced in the presence of BSA, the distribution of syntaxin 2 and to a lesser extent of syntaxin 1 shifted more towards the raft containing fractions (8-3) (Figure 5a and 6b, c). A similar behavior was observed for syntaxin when the acrosome reaction was induced in the presence of BSA (Figure 5a). However, a syntaxin accumulation did not appear in fractions 8-4 in the absence of bicarbonate (Figure 5a and 6 b, c) or in the absence of BSA (Figure 5b).

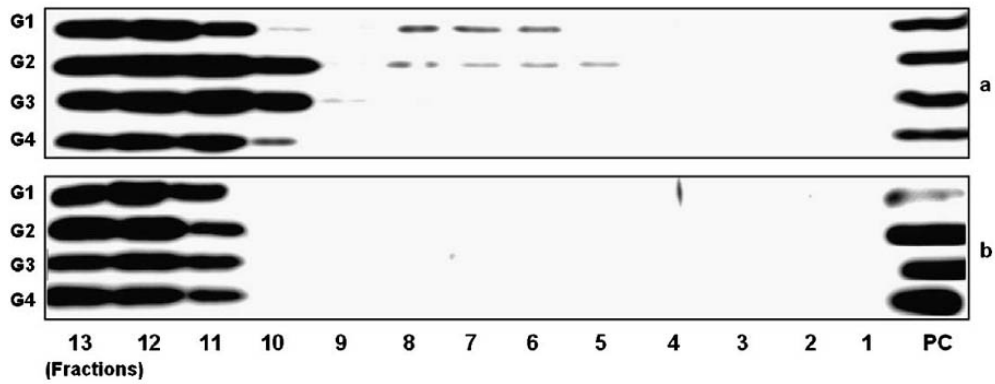

Figure 5.
a) Western blot profile showing the dynamics of t-SNARE protein syntaxin 2 association with sucrose gradient isolated membrane fractions under different conditions in the **presence** of a cholesterol acceptor (BSA). G1: capacitated and acrosome-reacted; G2: capacitated only; G3: acrosome-reacted without capacitation; G4: no capacitation and no acrosome-reaction. PC: pig brain sample as a positive control.
b) Western blot profile showing the dynamics of t-SNARE protein syntaxin 2 association with sucrose gradient isolated membrane fractions under different conditions in the **absence** of a cholesterol acceptor. G1: capacitated and acrosome-reacted; G2: capacitated only; G3: acrosome-reacted without capacitation; G4: no capacitation and no acrosome-reaction. PC: pig brain sample as a positive control.

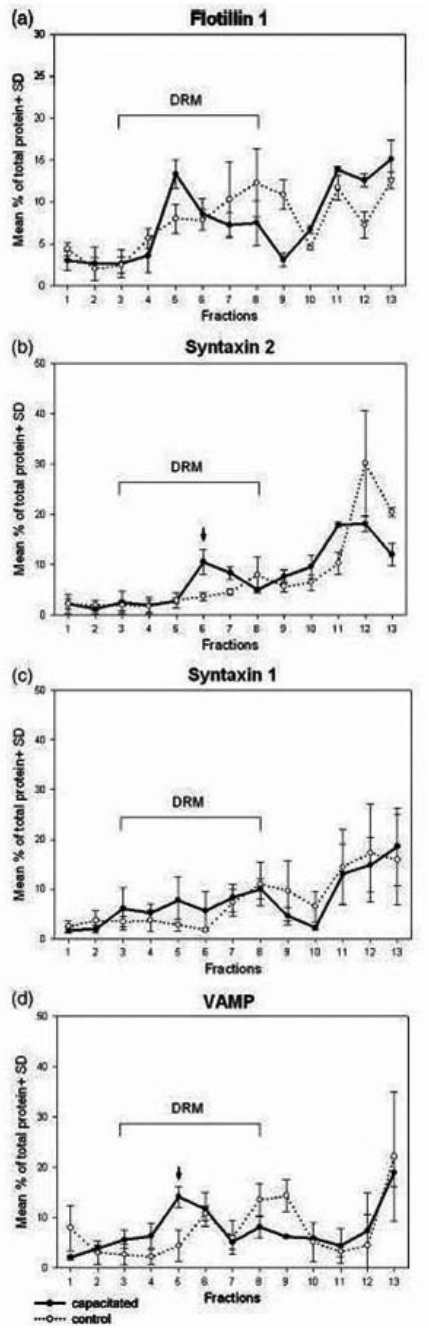

Figure 6.
Changes in distribution of the raft marker flotillin 1, and t-SNAREs syntaxin 1,2, as well as v-SNARE VAMP of lipid raft fractions isolated by sucrose gradient centrifugation. Fractions were prepared by ice-cold Triton-X-100 extraction of control (G4: dotted line) and capacitated (G2: solid line) sperm cells. Data are shown as the mean ±SD (n=3-5). The raft fractions (3-8) are indicated.
a. Distribution of raft specific protein flotillin 1; **b.** the t-SNARE syntaxin 2; **c.** the t-SNARE syntaxin 1A/B; **d.** the v-SNARE VAMP

Since Western-blotting under denaturating conditions appeared to be detrimental to the preservation of VAMP- (see Figure 1) and flotillin-immunoreactivity on blots, we performed immunodot-blotting under non-denaturing conditions to show the distribution of VAMP and flotillin in all sucrose-gradient fractions of conditions G2 (capacitated) and G4 (control). In both control and capacitated conditions, VAMP was present in both raft and non-raft fractions. After bicarbonate-induced capacitation in the presence of BSA, VAMP distribution specifically shifted towards the raft containing fractions (8-3) (Figure 6d). However, a similar change in distribution was not observed when sperm cells were incubated in the absence of bicarbonate (Fig 6d).

The distribution of the raft marker flotillin 1 did not change during bicarbonate-induced capacitation in the presence of BSA. In both control and capacitated conditions, flotillin 1 was specifically found in the raft fractions (8-3) and was less present in all other fractions (Figure 6a).

Discussion

For successful mammalian fertilization and subsequent embryonic development a well-defined interplay between the male and female gametes is essential. First, in order to attach to the zona pellucida of the oocyte, sperm cells must be capacitated under the influence of factors originating from the female genital tract. This process is dependent on activation factors such as bicarbonate, BSA and extracellular Ca^{2+}. As a result sperm cells become hypermotile, and their apical plasma membrane organization changes. These changes enable sperm cells to penetrate the cumulus and bind to the zona of the oocyte which triggers the acrosome reaction. The acrosome reaction results in the formation of hybrid membrane vesicles between the sperm plasma membrane and the outer acrosomal membrane, as well as in the release of hydrolytic acrosomal enzymes that enable the sperm cell to penetrate the zona and fuse with the oolemma.

It has been clearly documented that capacitation and subsequent acrosome reaction are preceded by changes in lipid, cholesterol and protein composition of the apical sperm plasma membrane [7;8;10;18;33;34]. One of the most striking changes in plasma membrane composition during bicarbonate-induced activation in the presence of BSA (=capacitation) is the concentration of lipid microdomains (rafts) in the apical ridge of the sperm head [18]. In general, lipid rafts have been proposed to perform a key role in the sorting of membrane proteins [14;16;17] and hence sperm rafts may constitute an interacting platform for proteins involved in zona binding, signal transduction, membrane trafficking and exocytosis in sperm [18;35]. In the present study, we have investigated whether activation-induced changes in lipid raft reorganization (re)allocates specific SNARE proteins to those areas in the sperm plasma membrane where acrosomal exocytosis will take place.

In first instance, we have determined which components of the membrane fusion machinery are present in sperm using the framework of the SNARE-hypothesis as a model [22;23;36]. Previous studies have identified SNARE proteins in sperm cells of a few mammalian species; i.e. human [28;37], horse [38], cattle [27], and rat [39]. We have extended these findings to a porcine model and report for the first time that specific SNARE proteins are present in porcine sperm cells. Western blot analysis shows that syntaxin 1A/B, syntaxin 2, and VAMP are present in sperm protein extracts. Other members of the SNARE 20S complex, i.e. SNAP-25, NSF and α-SNAP have recently also been demonstrated by us in porcine sperm (K.J. de Vries; M. de Boer-Brouwer; B.M. Gadella and T. van Haeften, unpublished work). Isolation of sperm plasma membranes and subsequent protein analysis shows a different localization of t- and v-SNARE proteins. Syntaxin isoforms, representing t-SNARE proteins, are predominantly present in the plasma membrane fraction, whereas the v-SNARE VAMP is almost absent in the plasma membrane. Although we did not directly

demonstrate the presence of VAMP in the outer acrosomal membrane, the combination of the immunolocalization of VAMP exclusively in the acrosomal head of the sperm, together with its exclusion from the purified apical plasma membranes, leads us to conclude that VAMP is indeed specifically localized in the acrosomal membrane. Interestingly, capacitation induces a lateral as well as DRM redistribution of SNARE members present in both vesicles as well as target membranes (the outer acrosomal and plasma membrane respectively). This not only supports the hypothesis that complementary t- and v-SNAREs are localize both in opposing sperm membranes as suggested by Kierszenbaum (2000) but also demonstrate that the albumin- and bicarbonate-dependent redistribution of raft proteins and t- and v-SNAREs are similarly orchestrated in both opposing sperm membranes. At the moment we cannot distinguish between the possibility that v-SNAREs become associated to the DRM fraction by interacting (docking) with t-SNAREs or alternatively that the acrosome contains a proportion of the DRM fraction that behaves similar to plasma membrane DRM. We consider the first possibility more likely as only the combination of bicarbonate + albumin caused the v-SNARE redistribution: Albumin as a large soluble protein cannot directly influence the organization of an intracellular membrane. Thus our findings may implicate that membrane docking of v- and t-SNAREs is involved in the redistribution of VAMP. Functional evidence has emerged that SNAREs modulate secretion during the acrosome reaction [22;28]. Similarly, preliminary data from our lab shows, that anti-t-SNARE antibody inhibited the Ca^{2+} ionophore-induced acrosome reaction in porcine sperm (K. J. de Vries, T. van Haeften, unpublished work) in porcine sperm.

The localization of SNARE proteins involved in acrosomal exocytosis is expected to be strictly organized. The need for such a structural organization of the sperm exocytotic machinery is underlined by the observation that at least some of the SNARE proteins are present throughout the entire sperm surface, whereas only the apical region is involved in exocytosis [22;37;38]. In this study, immunofluorescent labeling experiments revealed that in vitro capacitation influenced the distribution patterns of both the t-SNAREs syntaxin 1, 2 and the v-SNARE VAMP. The more widespread punctate distribution of these proteins on the entire sperm head changed to a more restricted and aggregated distribution pattern on the apical ridge of the sperm head. Although our findings show that during capacitation SNARE proteins may be specifically recruited to those areas of the sperm head where the acrosome reaction occurs, it is not clear whether this redistribution of SNARE proteins is essential for the acrosome reaction. Interestingly, Ca^{2+}-ionophore-induced acrosome reaction in mouse sperm results in a clear reduction of SNARE proteins from the acrosomal surface domains [40]. On the other hand, capacitation preceding the acrosome reaction allows concentration of both syntaxin and VAMP in the apical ridge surface area of the acrosome and coincides with higher percentages of acrosome-

reacted cells emerging after the Ca^{2+}-ionophore challenge. These observations indicate that capacitation-induced priming of specific areas of the plasma and outer acrosomal membrane for acrosomal exocytosis is a plausible explanation for the observed redistribution of SNARE proteins.

Since capacitation-induced redistribution of lipid raft markers in the apical plasma membrane of the sperm cell resembles the observed syntaxin and VAMP redistribution [18], we have investigated whether association of syntaxin and VAMP with lipid rafts during capacitation underlies the observed redistribution. Our experiments show that most of the syntaxin resides in the non-raft fraction of the sperm plasma membrane in absence of bicarbonate and/or BSA. This observation is consistent with earlier reports showing that some SNARE proteins prefer the liquid disordered plasma membrane over the raft domains [41]. In presence of both bicarbonate and BSA (the latter as cholesterol acceptor), part of the syntaxin appears in the detergent-resistant membrane fraction (representing the lipid rafts 8-3 as indicated by flotillin staining). Contrastingly, VAMP distribution does not show a clear preference for non-raft fractions since it is present in both raft and non-raft fractions. However, in the presence of bicarbonate and BSA, VAMP distribution also shifts towards the raft fractions. This evidence strongly indicates that capacitation-induced raft reorganization may underlie the observed clustering of t-SNARE and v-SNARE in the apical ridge of the sperm cells, as observed by immunocytochemistry. Earlier studies in our lab have demonstrated that the total cholesterol content in the DRM fraction does not change upon activation and that albumin-mediated extraction of cholesterol from the sperm cell exclusively occurs in the non-raft surface area of the sperm cell [18]. This phenomenon coincides with the aggregation of raft marker proteins to the apical ridge area of the sperm head and may indicate that sperm rafts aggregate in this area [12;18] as has been hypothesized previously by Flesch et al. [10]. These findings and the observation that the distribution and relative quantities of the lipid raft marker flotillin 1 do not significantly change during capacitation contradict the alternative explanation for this phenomenon, namely that cholesterol depletion causes raft dissociation as has been proposed by Sleight et al. [42]. Interestingly, syntaxin is only detected in the detergent-resistant membrane after incubations in presence of bicarbonate and the cholesterol acceptor BSA. In other words, only incubation conditions that enable cholesterol efflux from the sperm surface cause syntaxin inclusion in the lipid rafts. One explanation can be that syntaxin becomes trapped into the clustering rafts another could be that syntaxin is modified in such way that it becomes incorporated in lipid rafts. Similarly, cholesterol efflux from the non-raft surface of the sperm cell enables a VAMP reshuffling from the non-raft to the lipid raft fraction.

SNARE proteins are to some extent associated with detergent-resistant raft domains in several cell types [14;43-45] and have been implicated in exocytotic processes in

these cells. For instance, disruption of rafts by MBCD in PC-12 cells has a detrimental effect on exocytosis indicating that rafts in the plasma membrane are important for fusion and exocytosis [14]. Recent studies, however, provide evidence that lipid rafts not merely provide a spatial platform for SNARE interactions but actively regulate SNARE function by controlling the sequestration of specific SNARE proteins in lipid rafts [46;47]. These findings are in line with an earlier proposition that individual rafts may be important in maintaining raft born signaling proteins in the 'off-state'. Clustering of individual rafts into larger aggregates may cause aggregation of proteins into functional complexes, the 'on-state' [48].

In conclusion, our study shows that SNARE proteins are present in porcine sperm and that the distribution of the most important t-SNARE protein syntaxins as well as that of the complementary v-SNARE VAMP changes during capacitation. In addition, we provide evidence that this redistribution depends on association of both SNAREs with lipid rafts which cluster into large aggregates in the apical ridge sperm membrane during activation in the presence of BSA. This clustering is due to aggregation of lipid rafts rather than to a dissociation or disruption of lipid rafts in capacitating sperm and could either facilitate spatial control of exocytosis and/or actively regulate SNARE function necessary for acrosomal exocytosis. Further investigation should reveal the physiological consequence of activation-induced redistribution of SNARE proteins into lipid rafts and its role in docking the two membranes involved in the acrosomal reaction.

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Chapter 4

How pig sperm prepares to fertilize: acrosome docking and priming to the plasma membrane.

Submitted

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Abstract

Background: Mammalian sperms are activated in the oviduct. This process, which involves extensive sperm surface remodelling, is required for fertilization and can be mimicked under *in vitro* fertilization conditions (IVF).
Methodology/Principal Findings: Here we demonstrate that such treatments caused stable docking and priming of the acrosome membrane to the apical sperm head surface without the emergence of exocytotic membrane fusion. The interacting membranes could be isolated as bilamellar membrane structures after cell disruption. These membrane structures as well as whole capacitated sperm contained stable ternary *trans*-SNARE complexes that were composed of VAMP 3 and syntaxin 1B from the plasma membrane and SNAP 23 from the acrosomal membrane. This *trans*-SNARE complex was not observed in control sperm.
Conclusions/Significance: We propose that this capacitation driven membrane docking and priming is a preparative step prior to the multipoint membrane fusions characteristic for the acrosome reaction induced by sperm-zona binding. Thus, sperm can be considered a valuable model for studying exocytosis.

Keywords: sperm, acrosome reaction, capacitation, membrane docking, SNARE

Introduction

Mammalian sperm cells are activated during migration in the female genital tract. This activation process (henceforth called capacitation) enables sperm cells to interact with the zona pellucida (ZP) of the oocyte which in turn triggers the acrosome reaction (AR) [1]. *In vitro*, this process can be mimicked by incubation of sperm cells in media containing bicarbonate/CO_2 and albumin [2,3]. Only capacitated sperm cells are able to undergo the zona-triggered AR and this process characteristically involves multipoint fusions of the sperm head plasma membrane (PM) with the outer acrosome membrane (OAM) [2].

Proteins from the Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment protein Receptor (SNARE) family are widely regarded as key players in Ca^{2+}-mediated membrane fusion processes [4-6]. Fusion in different cell types requires specific sets of SNAREs. SNARE-mediated exocytosis involves interactions of complementary SNARE proteins (typically Q-SNAREs in the plasma membrane and R-SNAREs in the vesicle membrane) to form 7S or 20S *trans*-SNARE protein complexes which after a calcium-dependent conformational change are believed to execute the membrane fusions [7-10]. The assemblage of the SNARE complex will not lead to an instant fusion, instead, the preformed complexes are stalled awaiting the appropriate stimulus (i.e. extracellular calcium influx) and additional factors (e.g. Rab 3A, NSF and complexin) which are involved in either the stabilization of the SNARE complex or the initiation of the actual fusion of two membranes [11-13]. It is clear that membrane docking (the first stage of membrane interactions in which complementary sets of SNARE proteins are brought and mixed together to form the SNARE complex) [4,14], priming (the stage after the docking step that the formed *trans*-SNARE complex pulls the interacting membranes into a close and tight apposition) [4,6], and fusion are distinct processes which are mediated by specific sets of proteins [14-16]. Multiple regulatory pathways and up/down stream cascade for SNARE complex formation have also been described [6]. Likewise in sperm, SNARE proteins have been identified and structural information regarding the ternary SNARE complex formation has been revealed recently, albeit that these analyses were carried out under calcium ionophore condition and/or under streptolysin-O permeabilization conditions [17,18]. These treatments, however, do not represent physiological conditions and as such do not only cause the formation of a *trans*-SNARE complex but also induce the fusion of interacting membranes (resulting in the formation of a *cis*-complex). These treatments also induce the loss of cell integrity. Until now it is not clear where and how SNARE proteins are physiologically involved in the regulation of acrosome docking, priming and fusion in sperm cells. Therefore, we studied for the first time how *trans*-SNARE complexes are formed and how the docking and priming of the acrosome membrane with the plasma membrane are regulated in living sperm cells upon capacitation. We

mimicked physiological sperm capacitation by activating sperm with *in vitro* fertilization media and we assessed whether this treatment affected the docking and priming of the acrosome by formation of *trans*-SNARE complexes.

Results

Capacitation induces close apposition between the apical sperm head surface and the outer acrosomal membrane

Ultrastructural examination revealed that about 90% (supplementary data 1) of the uncavitated control sperm have a loose but continuous PM on the entire sperm head surface (Fig. 1A-B). This is due to the osmotic effects which occur during processing of the specimen for electron microscopy (EM) and indicates that the sperm acrosome is probably not docked with the PM in control sperm. Interestingly, sperm activation in an *in vitro* fertilization medium (capacitation) resulted in a close parallel arrangement of the apical PM with the underlying OAM in 73% of the uncavitated sperm cells (supplementary data 1). The PM in the non-apical area, on the other hand, showed the same loose arrangement (Fig. 1C) as was observed in control spermatozoa. This close apposition of the apical sperm head PM with its underlying acrosomal membrane (the two membranes are hardly distinguishable in that area) suggests that the two membranes are interacting with each other.

To test this possibility, we have subjected control and capacitated sperm to nitrogen cavitation which enables the separation of membranes at the apical area of the sperm head [19]. Control cavitated sperm showed that the 1000g pellet consisted mainly of remaining sperm heads with intact acrosomes whereas the PM was released (Fig. 2A). The released apical PM (henceforth called cavitate membrane fraction), resealed into unilamellar membrane vesicles that were recovered in purified form in the 285000g pellet (Fig. 2B). In contrast, capacitated spermatozoa showed after cavitation and differential centrifugation, remaining sperm heads (in the 1000g pellet fraction) with disrupted acrosomes at the apical sperm head area (i.e. where the two membranes were more closely attached in Fig. 1C). At that area, the cavitation procedure not only resulted in the release of the PM but also of the OAM (Fig. 2C). This may reflect a stronger interaction of the two membranes. This possibility is strengthened by the ultrastructural properties of the cavitate membrane fraction released from capacitated sperm. These membranes characteristically showed a bilamellar (two bilayer membranes) morphology (Fig. 2D, supplementary data 2) indicating a stronger interactions which remained intact after the cell disruption procedure and subsequent differential centrifugation steps during their isolation. In line with this, the more distal sperm head area, where the interaction was not observed, did not show the stripping of the OAM (Fig. 2C). Furthermore, as observed in control

sperm [19], the PM of the sperm tail and mid-piece remained attached to these structures after the cavitation treatment in capacitated sperm (Fig. 2C). Therefore, the isolated bilamellar membranes appeared to be specific for the sperm head area which is involved in the acrosome reaction and which was reported previously to be enriched in SNARE proteins after sperm capacitation [20]. We should note here that the degree of spontaneous acrosome reaction during the incubation period for control and capacitated sperm was less then 3%, as has been reported previously (for review see [21] and see supplementary figure 3).

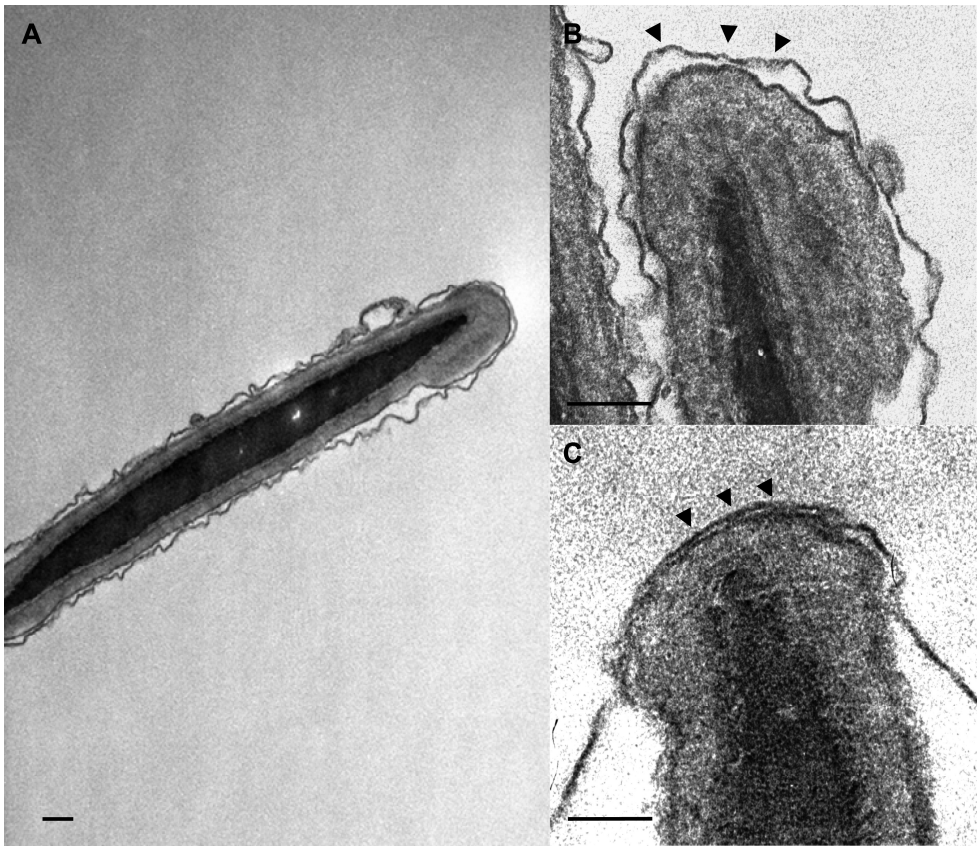

Figure 1. Capacitation alters the ultrastructure of the apical head and the acrosome of boar sperm (A): About 90% (supplementary data 1) of the uncavitated control non-capacitated boar sperm have a plasma membrane that is not tightly associated to the outer acrosomal membrane. The loose plasma membrane at the entire sperm head is due to processing of the specimen for TEM. **(B):** Higher magnification of the apical tip of the control non-capacitated sperm head. **(C):** The apical tip of a capacitated sperm head showing tight association of the plasma membrane with the outer acrosomal membrane whereas at a more distal area the two membranes do display a loose apposition (similar to control sperm). Arrow heads indicate the apical area of the sperm head. Bar represents 50 nm.

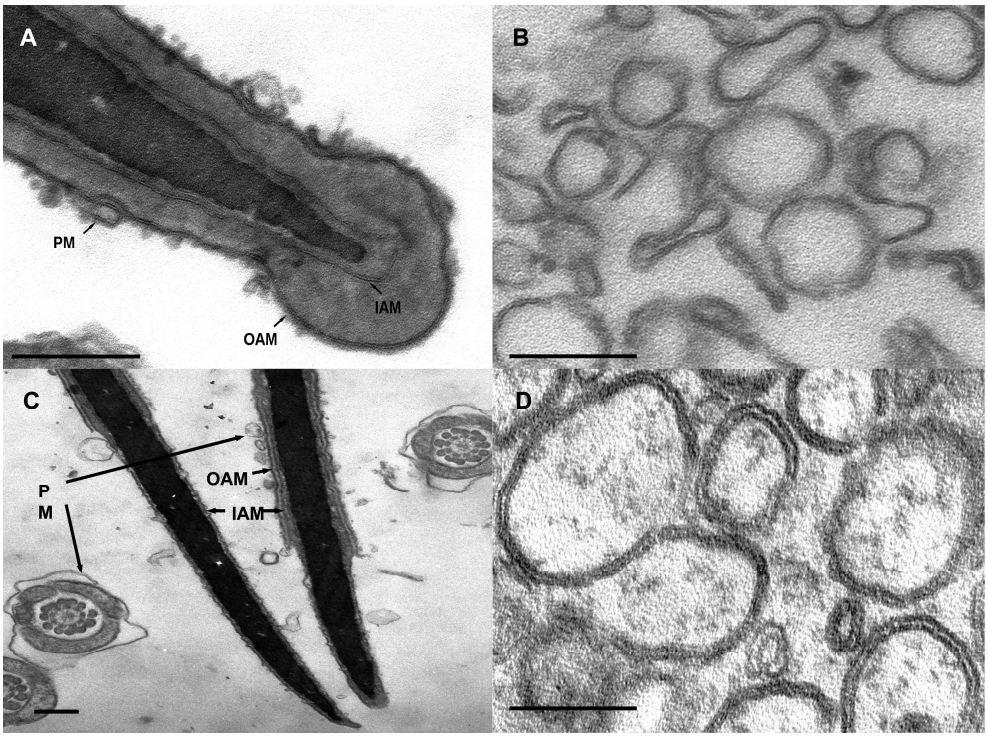

Figure 2. Capacitation promotes cavitation-induced release of membranes from the apical head and acrosome. (A): 85% (supplementary data 2-1) of the cavitated control boar sperm heads show only the removal of apical plasma membrane after nitrogen cavitation. **(B):** Unilamellar membrane vesicles recovered from cavitate membrane fraction from control spermatozoa. **(C):** 95% (supplementary data 2-1) of the capacitated boar spermatozoa show the removal of both plasma membrane and outer acrosomal membrane at the apical area of sperm head while the plasma membrane overlying the area containing mitochondria remains intact and the outer acrosome membrane remains attached to the spermatozoa at the non-apical area of sperm head. **(D):** Significant increase in the number of (from 20% in control to 89% in capacitated group, supplementary data 2-2) bilamellar membranes structure present in cavitate membrane fraction from capacitated spermatozoa. PM: plasma membrane; OAM: outer acrosomal membrane; IAM: inner acrosomal membrane. Bar represents 200 nm.

Bilamellar membranes contain acrosomal components

In order to quantify the interactions between the OAM and the PM, we assessed the level of purity of the cavitate membrane fractions by measuring alkaline phosphatase activity (a marker enzyme for plasma membrane for boar spermatozoa) and of acrosin (a marker enzyme for acrosomal content for boar spermatozoa) [19].

Alkaline phosphatase activity in cavitate membrane fraction was respectively 10.6 and 11.7 times higher than in whole cell lysates for the capacitation or the control group which indicates that the purification of PM material was similar for both sperm

treatment groups (Table 1). Under the control condition, only trace amounts of acrosin was detectable in cavitate membrane fraction (4% compared with whole cell lysate, Table 1), whereas a significant increase up to 10% of acrosin activity was measured in the bilamellar membrane fraction from capacitated cells (Table1). These results indicate that acrosomal material was co-purified due to disruption of the acrosome. Similarly, an OAM-specific lectin PNA (peanut agglutinin) binding site [19,22] was enriched in the cavitate membrane fraction from capacitated sperm when compared to control sperm. The relative PNA-binding was significantly increased (approximately 74%) after capacitation treatment, indicating an increased amount of OAM in the cavitate bilamellar membrane fraction (Table 1). The cavitate fractions were not contaminated with inner acrosomal membrane (IAM) since this membrane remained attached to the remaining sperm head (Fig. 2C) after cavitation and was completely separated from the vesicles during the differential centrifugation steps. Moreover, the cavitate membrane fraction was almost devoid of acrosin (an indicator of the presence of inner acrosome membrane [19], Table 1).

Capacitation induces the formation of a ternary trans-SNARE protein complex

Western-blotting showed that the Q-SNARE protein SNAP 23 is present at a molecular weight (MW) position of 23-25 kDa in remaining sperm heads in both capacitated and control sperm (Fig. 3A, lane 5, 10). SNAP 23 was not present in the cavitate membrane fraction of control sperm (Fig. 3A, lane 6-8); however, a substantial signal for SNAP 23 emerged in the cavitated membrane fraction of capacitated sperm (Fig. 3A, lane 11-13) at a 80 kDa band under the non-reducing condition (lane 11). These SNAP 23-containing protein complexes partially dissociated after a heat treatment at 90°C (lane 12) and fully dissociated into monomers after heat treatment at 100°C (lane 13). The abundance in the higher MW SNAP 23 found in the boar sperm (when compare with boar brain) might due to the tissue specific phenomenon. Since the presence of SNAP 23 in the cavitate membrane fraction of capacitated sperm as well as its emergence in a high MW band was unexpected, we performed a series of experiments to determine which SNARE proteins (e.g. syntaxin and VAMP) likely interact with SNAP 23 upon capacitation. Among the SNARE proteins tested, the R-SNARE protein VAMP 3 appeared as a 16 kDa band in the control cavitate membrane fraction (Fig. 3B, lane 6-8). Note that there is a low (16kDa) molecular weight form predominant in sperm and a high (18 kDa) molecular weight form predominant in brain (Fig. 3B). The differences in low and high molecular weight forms are probably cell specific expression variability [23]. After capacitation, VAMP 3 appeared in a similar capacitation-dependent 80 kDa band as was seen for SNAP 23 under the non-reducing condition (Fig. 3B, lane 11). Heating these samples at 90 or 100°C caused a large fraction of VAMP 3 to dissociate from the 80 kDa protein complex into the 16 kDa monomer band (Fig. 3B, lane 12-13).

Table 1. Enrichment of acrosomal membranes in cavitation-released fractions from capacitated sperm. Alkaline phosphatase as a specific plasma membrane indicator for the purity of membrane isolates and acrosin as a marker for the level of acrosome contamination. Both markers show a 10-12 times enrichment of plasma membrane material over the other material with minimal contamination (4%) of acrosomal material. Peanut Agglutinin (PNA) was used as marker lectin for the outer acrosome membrane material in the 285000g fraction of the membrane isolates.

Enzyme		Specific activity			Purification[a]
		whole sperm lysate	1000 g fraction	285000 g fraction	
Plasma membrane marker[b]					
alkaline phosphatase	capacitated	7.5±0.6	8.8±0.7	79.2±7.3	10.6
	control	6.7±0.5	9.0±3.1	78.3±10.1	11.7
Acrosomal marker					
Acrosin[c]	capacitated	337.8±66.6	251.9±11.8	45.5±2.9[e]	0.1
	control	323.7±38.6	289.9±34.1	14.1±1.8[f]	0.04
Outer acrosomal membrane marker					
PNA-binding[d]	capacitated	509.2±64.9	279.0±13.5	126.6±14.1[g]	0.3
	control	504.4±58.3	228.4±30.1	72.6±16.9[h]	0.1

a: ratio of the specific marker activities between UCF and whole sperm lysate.
b: nmol • mg protein^{-1} • min^{-1}
c: µIU: quantity of acrosin • µg protein^{-1} hydrolyzes 1 µmol BAPNA• min^{-1} at 22ºC.
d: Normalized OD_{450} • µg protein^{-1} • 10^9 cell^{-1}

• 1000 g fraction contains sperm head
• 285000 g pellet contains membrane vesicles
• e-f, g-h: significant different for 2-tailed tests between IVF incubations (actual p values for tests between e-f and g-h are both <0.01)
• n ≥ 5, ±s.d.

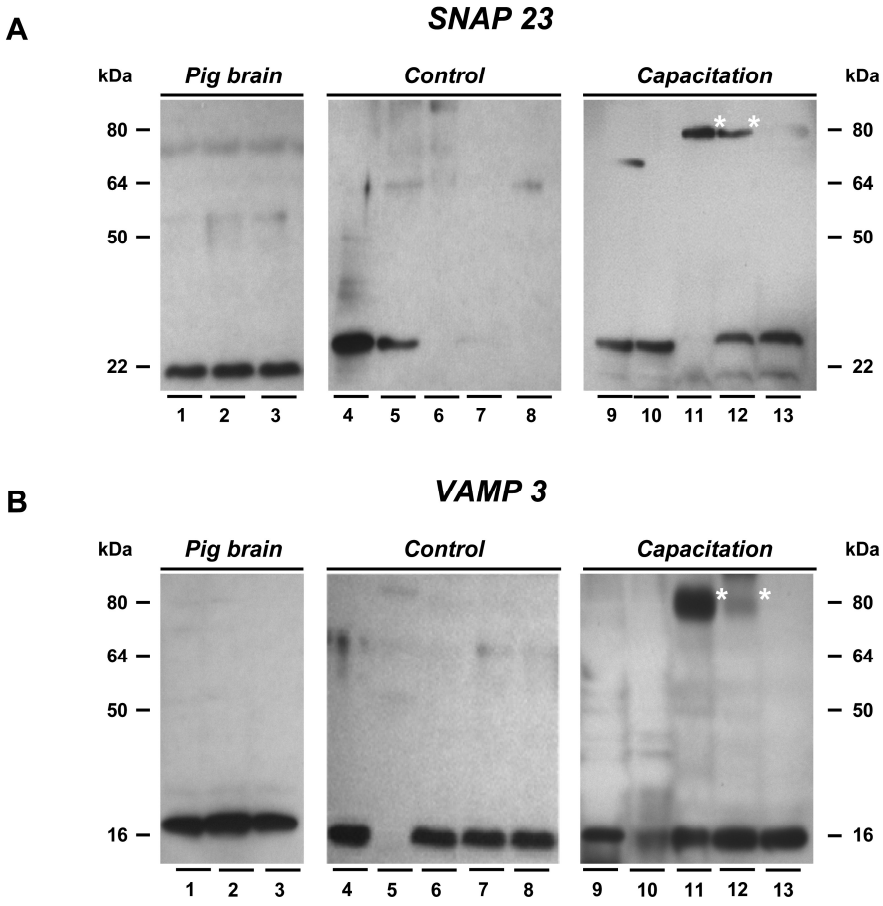

Figure 3. Capacitation-dependent formation of a ternary *trans*- SNARE protein complex.
Cavitate membrane fractions of control (lane 6-8) and *in vitro* capacitated (lane 11-13) sperms were isolated via nitrogen cavitation in combination with differential ultracentrifugation, the remaining sperm head (without the apical membrane; lane 5, 10) and the whole sperm lysate (lane 4, 9) under the same treatment were used for the comparisons for the presence of SNARE proteins/protein complexes. Samples including positive controls from pig brain were loaded under either non-reducing condition (lane 1, 6, 11) or treated with reducing agent (0.1M DTT), these samples were further heated at 90ºC for 5 minutes (lane 2, 7, 12) or at 100ºC for 10 minutes (lane 3, 8, 13). **(A)**: SNAP 23 appears in the free 23 kDa form in control whole sperm lysate and remaining head fraction but not in the cavitate membrane fraction; however, a 80 kDa SDS-resistant SNAP 23-containing protein complex emerges in the cavitate membrane fraction at upon capacitation (marked with asterisks) indicating its capacitation-dependent interaction with other proteins; this 80kDa protein complexes is partially resistant to heat treatment up to 90ºC and is fully dissociates into the monomer SNAP 23 under 100ºC boil condition. **(B):** VAMP 3 shows a plasma membrane-specific localization in the free 16 kDa form in control sperm; SDS and partially thermal resistant VAMP 3-containing SNARE complexes can be detected at 75-80 kDa after sperm capacitation (marked with asterisks). This protein complex is fully dissociated into monomeric VAMP 3 at 16kDa under reducing conditions with 100 ºC boiling treatment. 10 µg of total protein extract was used for all samples. Pig brain homogenate was used as positive control with comparable experimental setups (lane 1-3).

Whole sperm lysates and cavitate membrane fractions contained syntaxin 2 and 3, which were detected as a monomeric 36 kDa band in both capacitated and control sperm. Both syntaxins were enriched in the PM when compared to the acrosomal membrane (the remaining head fraction Fig. 4A-B, lane 5, 10). Similar to SNAP 23 and VAMP 3, both syntaxins also appeared in a heteromeric 80 kDa protein band after capacitation. The capacitation treatment resulted in an increase of syntaxin2/3-containing 80kDa heteromeric complex (Fig. 4A-B, long exposure).

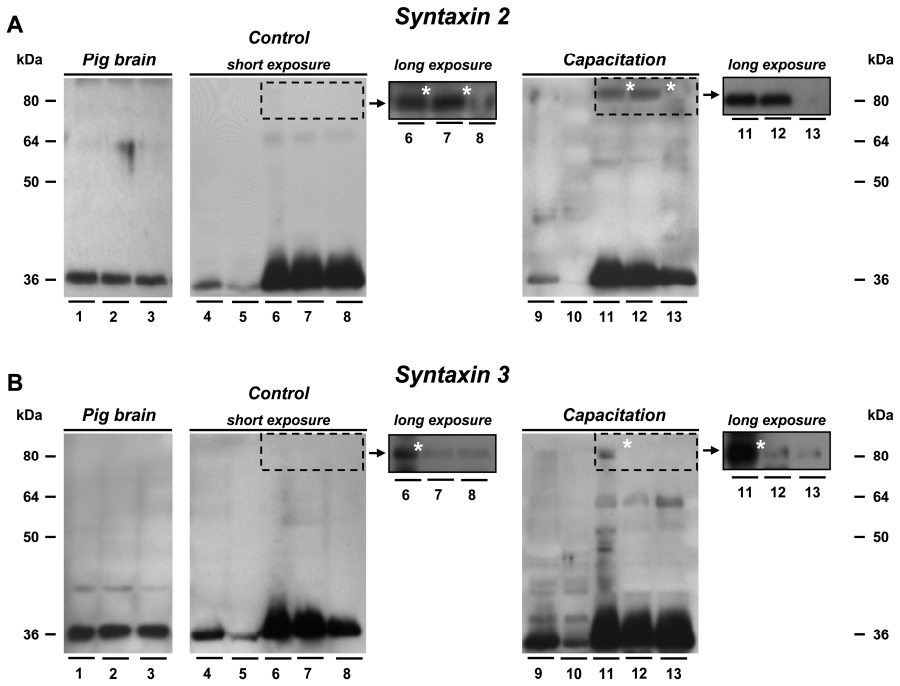

Figure 4. Syntaxin 2 and 3 interact with additional proteins independently from capacitation. (A): Syntaxin 2 is enriched in the cavitate membrane fraction in the free 36 kDa form in both control and capacitated sperm cells. A weak signal at 80 kDa can already be observed in control cavitate membrane fraction (long exposure blot, marked with asterisks); this 80kDa protein band is enhanced upon capacitation indicating a capacitation-enhanced but not capacitation-dependent interaction of syntaxin 2 with other proteins. **(B):** Similar to syntaxin 2, syntaxin 3 showed capacitation-independent formation of a 80kDa SNARE-containing complex and this interaction (formation) is promoted by capacitation. However, due to the enrichment of both syntaxins in the cavitate membrane fractions and the relative small amount of complexed syntaxins (~15%, see [20]) compared to the uncomplexed monomeric syntaxin, the dissociation of the syntaxins can not be observed clearly in these samples. 10 µg of total protein extract was used for all samples. Lane 1-3: pig brain as positive controls; lane 4-8: control cavitate membrane fractions; lane 9-13: cavitate membrane fractions from capacitated sperm cells. Non-reducing condition: lane 1, 6, 11; reducing conditions: (1) 0.1M DTT+ 5 min, 90ºC boil: 7, 12; (2) 0.1M DTT+ 10 min, 100ºC boil: 8, 13. Whole sperm lysate: 4, 9; remaining head fraction (without apical membrane): 5, 10.

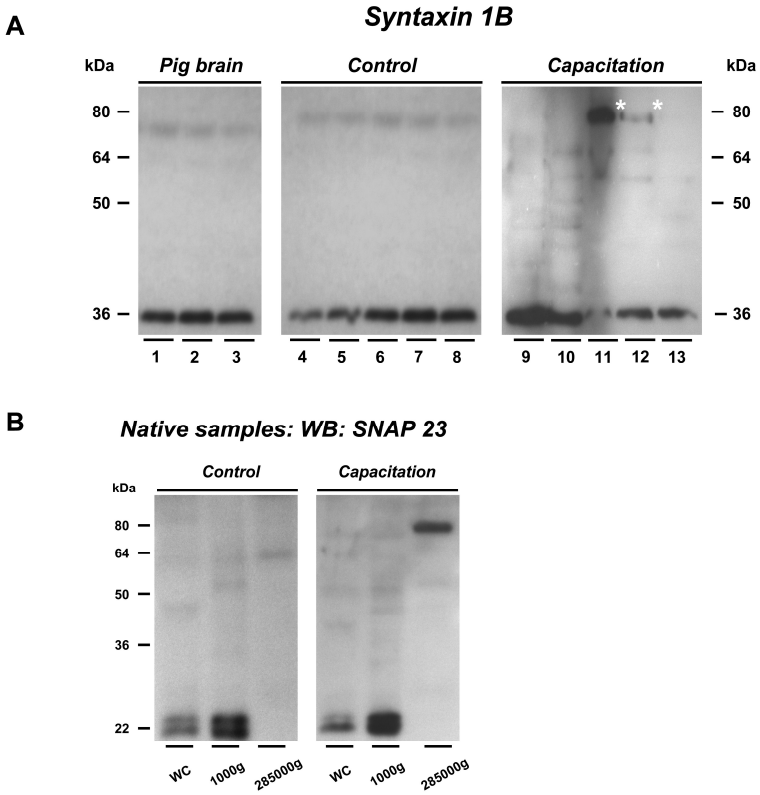

Figure 5. Syntaxin 1B is the interacting Q-SNARE partner of SNAP 23 and VAMP 3 upon capacitation. **(A)**: Syntaxin 1B appears not only in the remaining sperm head (lane 5, 10) but also in the cavitate membrane fraction (6-8, 11-13) in the free 36 kDa form; however, a 80 kDa SDS-resistant syntaxin 1B-containing protein complex emerges in the non-reducing cavitate membrane fraction of capacitated sperm cells (lane 11) indicating its capacitation-dependent interaction with other proteins; this 80kDa protein complex shows the same thermal stability (up to 90ºC) as SNAP 23 and VAMP 3-containing protein complexes (Fig. 3). Experimental setups are identical as in figure 3 and 4. **(B):** Western blot data of non-reducing samples reveal the presence of 80kDa protein complex is exclusively in the cavitate membrane isolate of capacitated sperm (285000g fraction) WC: whole cell lysate; 1000g: remaining head fraction; 285000g: cavitate membrane fraction. 10 µg of total protein extract was used for all samples. Pig brain homogenate was used as positive control.

Due to the relative abundance of syntaxin 2/3, only a sub-fraction of the proteins was participating in the formation of this 80kDa band while the remaining sub-fraction appeared as 36kDa monomers. Heat treatment of the cavitate membrane fractions caused a disruption of the 80kDa syntaxin 2/3-containing complex into the monomers. We should note that 90ºC heat treatments also resulted in incomplete dissociation of the 80kDa band into a 65kDa band that was positive for syntaxin 2/3 (Fig. 4B, lane).

Syntaxin 1B is the cognate Q-SNARE with SNAP 23 and VAMP 3

Participation of syntaxin 2 and 3 in a 80kDa SNARE complex in control sperm was in contrast to that of reported for VAMP 3 and SNAP 23 (see above section). Moreover, the syntaxin 2 and 3-containing 80kDa complex showed different thermal stability when compared to VAMP 3 and SNAP 23-containing 80kDa complex. We therefore investigated the presence of another syntaxin isoform (syntaxin 1B) in sperm, and whether this syntaxin was responsible for the capacitation-specific formation of the VAMP 3/SNAP 23-containing 80kDa SNARE complex. The rationale for this is that we previously showed that syntaxins 1-3 display a similar redistribution behavior to the apical ridge of the sperm head upon capacitation [20], and that syntaxin 1 has been reported to interact with SNAP 23 [24] and VAMP 3 [25,26]. Our data showed that syntaxin 1B was present in sperm and it only appeared in the monomeric form (36kDa, Fig. 5A, lane 4-8) in control sperm. Almost all syntaxin 1B participated in the capacitation-dependent formation of the 80kDa heteromeric SNARE complex. This complex was only recovered in the cavitate membrane fraction (Fig. 5A, lane 11). The complex was stable under non-reducing condition, but completely dissociated into monomeric syntaxin 1B (36kDa) after 100 °C heat treatment (Fig. 5A, lane 13). The OAM-specific SNAP 23 only participated in the 80kDa SNARE complex recovered from cavitate bilamellar membrane fraction of capacitated sperm. The SNAP 23 was not present in this band in other sperm sub-fractions (Fig. 5B), which is indicative for the capacitation-dependent formation of a *trans*-SNARE complex between OAM and PM.

Co-immunoprecipitation reveals SNARE protein interactions upon capacitation

Co-immunoprecipitation (IP) experiments were performed to reveal the possible capacitation-dependent interactions of SNARE complex partner proteins (i.e. syntaxin 1B, SNAP 23, VAMP 3) during acrosome docking and priming. When control cavitate membrane fractions were used, only a 36kDa signal was detected after IP with syntaxin 1B (Fig. 6B). Western-blot experiments using anti-syntaxin 1B antibodies indicate that both SNAP 23 and VAMP 3 do not interact with syntaxin 1B in control sperm. In contrast, after performing IP with anti-SNAP 23 and anti-VAMP 3 antibodies with cavitate membrane fractions recovered from capacitated sperm, a SDS-resistant protein complex was found containing SNAP 23/VAMP 3/ syntaxin 1B under non-reducing condition at 80kDa and this protein complex dissociated into the monomeric SNARE proteins after 100°C heat treatment and was detected at 36kDa when WB with anti-syntaxin 1B antibodies (Fig. 6C). The presence of the 80kDa SNARE-containing protein complex demonstrates the trimeric interactions between syntaxin 1B/ SNAP 23/ VAMP 3 upon capacitation. The detected 80kDa SNARE protein complex further confirmed the formation of the *trans*-SNARE complexes upon capacitation (Fig. 3-5). The specificity of the detected signals in the cavitate

membrane fractions was showed by adding additional negative control experiments using purified rabbit IgG (the same host species of which the antibodies were derived from, Fig. 6A) and anti-syntaxin 2 antibodies (with no affinity with syntaxin1B).

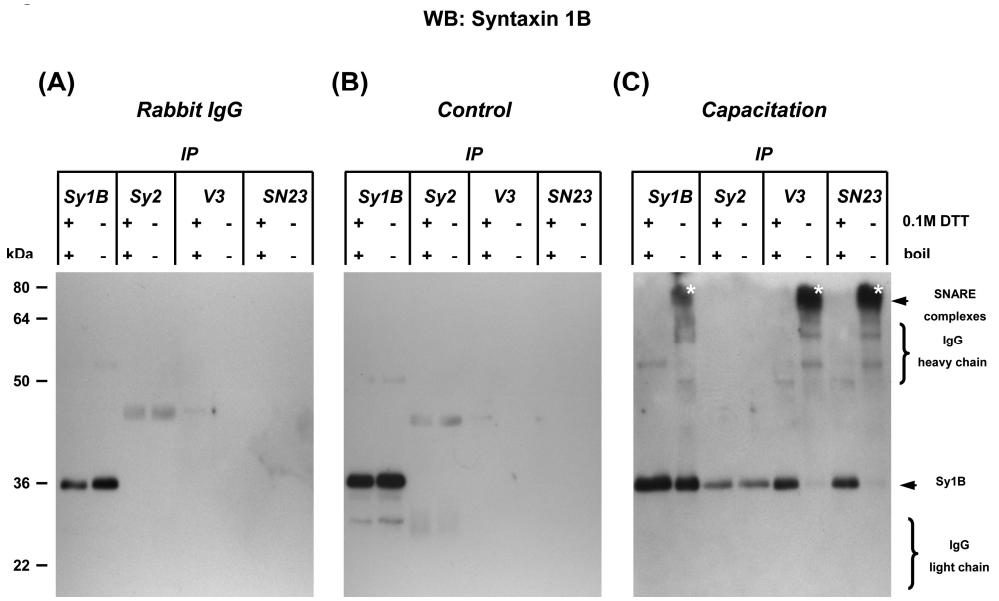

Figure 6. Detection of capacitation-dependent SNARE interactions by co-immunoprecipitation. Cavitate membrane fractions from boar sperm were used. Samples were treated in non-reduced condition or in full-reduced condition that were heated at 100ºC for 10 minutes. **(A):** Purified rabbit IgG serves as a negative control for IP experiments where no interactions between VAMP 3/syntaxin 1B, SNAP 23/syntaxin 1B was detected. When an IP was performed with anti-syntaxin 1B antibody, the signal was recovered as expected in this positive control; this validates the specificity of the signals detected after IP. Syntaxin 2 is not expected to interact with syntaxin 1B and is therefore used as additional negative control to demonstrate the specificity of the detected signals. **(B)** No binary SNARE interactions can be observed in the control cavitated membrane fractions. **(C):** SDS-resistant SNARE complexes emerged after capacitation when IP was performed with either anti-VAMP 3 or anti-SNAP 23 antibodies indicating the trimeric interactions between syntaxin 1B/ SNAP 23/ VAMP 3. The noted 80kDa protein complex further substantiates the observed capacitation-dependent formation of ternary *trans*-SNARE complex in Western-blot experiments. This 80kDa protein complex fully dissociated into the constituted SNARE monomer under 100ºC heat treatment in the presence of 0.1M DTT and can be detected at 36kDa when WB was performed subsequently with anti-syntaxin 1B antibody.

Discussion

Mammalian sperm must acquire the ability to fertilize the oocyte after ejaculation and this process is called ‘capacitation’ [1]. Capacitation of sperm cells involves the activation of several signalling pathways such as sperm specific adenylyl cyclase,

cAMP-dependent protein kinase A (PKA) and protein tyrosine phosphorylation [2] as well as reorganization of proteins and lipids on the plasma membrane [21]. Proteins belonging to the SNARE family have been shown to regulate sperm exocytosis and functional studies have provided evidence for a possible role in fertilization [17,18,27]. Although several studies have showed the importance of SNARE interactions in the modulation of sperm acrosome exocytosis (also known as the acrosome reaction), no information is available on the spatial-temporal rearrangements of these protein interactions during capacitation. With this respect, it is of interest to mention that the classical topologies of SNARE proteins in somatic cells on target membrane and vesicle membrane is probably more complex in sperm. This may have to do with the fact that the Golgi apparatus rolls over the growing acrosomal granule during acrosome formation in developing spermatids and exposes its *trans* Golgi side towards the acrosome granule and its *cis* side towards the plasma membrane [28]. This largely neglected Golgi reorganization phenomenon in spermatids may cause the noted aberrant topology of SNAP 23 (at the outer acrosomal membrane) and VAMP 3 (at the plasma membrane) when compared to somatic cell types.

Previously, we have demonstrated that both Q- and R-SNAREs (also known as t- and v-SNAREs) show a bicarbonate- and albumin-dependent redistribution to the apical area of the sperm head upon capacitation [20]. In the present work, we have expanded our studies to establish whether this bicarbonate- and albumin-dependent redistribution of SNARE proteins underlies the formation of *trans*-SNARE complexes resulting in stable docking and priming of the plasma membrane, and the acrosomal membrane, without the emergence of the spontaneous acrosome reaction. The stabilization of this 80kD SNARE complex probably serves to prevent AR. Indeed, only minimal amount of sperm showed spontaneous AR under control and capacitation conditions (supplementary figure 3).

Our ultrastructural data showed that capacitation induces a close apposition of the apical PM with the OAM. The two membranes could be isolated from sperm by means of nitrogen cavitation and differential centrifugation steps. These cavitate membrane fractions showed a stable bilamellar membrane structure. In control sperm, the same cell disruption technique resulted in unilamellar vesicles representing the apical plasma membrane only. These results suggest that sperm capacitation induces the interactions of the acrosome membrane with the apical sperm head PM.

Analysis of membrane specific enzyme activities in the cavitate membrane fraction confirmed this speculation as cavitated membranes from capacitated sperm contained 74 % more PNA-binding sites (indicative for the OAM [19,22]) when compared to control sperm. The efficiency of apical PM isolation from control and capacitated sperm were quantitatively and qualitatively similar and were only minimally contaminated with other sperm components. Western-blot studies showed that the acrosomal SNAP 23 was only present in the cavitated membrane fraction after

capacitation and appeared in a partially heat-resistant 80 kDa form. VAMP 3, in contrast to other isoforms of VAMP (namely VAMP1 and VAMP2; [20]), is specific for the PM and only present in monomeric form in control sperm emerged predominantly in 80 kDa band in capacitated sperm. Syntaxin 2 and 3 (unlike SNAP 23 and VAMP 3) were predominantly present in a monomeric 36 kDa form. Only a small fraction of syntaxin 2/3 was present in the high MW protein complex. However, these two syntaxin isoforms are probably are not the primary cognate Q-SNARE with SNAP 23 and VAMP 3. In stead, the presence of syntaxin 1B in the 80kDa SNARE complex formed under capacitation as well as its SDS- and thermal-stability characteristics suggest that syntaxin 1B/SNAP 23/VAMP 3 are the interacting SNARE proteins in the 80kDa *trans*-SNARE complex. The involvement of the OAM-specific SNARE protein SNAP 23 in the 80kDa protein complex of cavitate membrane fraction provides evidence that capacitation induces the docking and priming of the acrosome membrane to the PM. The exclusive presence of 80kDa SNARE complex in the capacitated cavitate membrane fraction supports the possibility of SNARE-mediated docking and priming of PM and OAM. A role of the syntaxin 1B/SNAP 23/VAMP 3 complex in the regulation of exocytosis has been reported for other cell types [25]. Moreover, the conformational switch of a specific syntaxin isoform (i.e. syntaxin 1B) is important to control the synaptic vesicle fusion [8].

SNARE interactions appear to be non-selective [29] and thus could hypothetically lead to various SNARE protein combinations. Hence, we expect more than one type of SNARE complexes in sperm cells which may explain the additional detection of syntaxin 2/3-containing SNARE protein complexes. Furthermore, different SNARE complexes in cells could play a role on the modulation of exocytosis as described in human neutrophils [30]. The various combinations of SNARE proteins may to some extent, explain the unexpected syntaxin 2 and 3-containing SNARE complexes found in both control and capacitated sperm cells. We observed that both VAMP 1 and VAMP 2 participated in protein complex formation independently from capacitation (supplementary figure 4), which suggests that syntaxin 2/3 and VAMP 1/2 could be the cognate interacting SNARE proteins and form an additional pool of SNARE complexes. We consider that these additional preformed SNARE complexes may not be directly involved in the docking and priming of the acrosome to the sperm PM, but could take part in multipoint fusions during AR to ensure the successful acrosome exocytosis upon zona binding. Hence we propose that docking, priming and fusion are distinct and segregate processes that require distinct sets of SNARE proteins [6,14].

SNAP 25 was not found in any of our sperm preparations (data not shown), which is in line with the observation that SNAP 23, an ubiquitously expressed homolog of SNAP 25, is present in non-neural tissues and is able to bind different syntaxins and VAMP isoforms [24]. In contrast to Yang et al. who showed that SNAP 23-containing SNARE complexes have an on average lower temperature resistance (71-83ºC) [29],

we found in this study stable SNAP 23-containing SNARE complexes with a higher thermal stability (up to 90 ºC). Therefore sperm capacitation, together with the different combinations of SNARE proteins, can cause variability in the stability of SNARE complexes.

Interestingly, SNAP 23 shows a three times higher raft/detergent resistant membrane (DRM)-association affinity when compared with SNAP 25 [31]. We have shown in a previous study that SNARE proteins cluster into raft membrane areas upon capacitation [20] and it is therefore possible that they gain extra protection from these large functional DRMs to withstand the changes (i.e. temperature, pH etc.) in the surrounding environment in order to assure the formation of stable SNARE complexes that can lead to a successful AR upon calcium stimulation. The idea -supported by the group of Boekhoff - is that a lipid raft associated scaffold protein is controlling the acrosome reaction in mammalian spermatozoa [32,33] and as such may serve to direct the acrosome and plasma membrane SNAREs into the right membrane topology for the formation of stable ternary complexes. In fact the same group recently reported that CaMKIIalpha interacts with multi-PDZ domain protein MUPP1 in spermatozoa to prevent spontaneous acrosomal exocytosis [34]. Future research should emphasize whether and how this interaction as well as our previously reported emergence of SNARE proteins into surface membrane rafts in capacitation sperm relate to the capacitation-dependent docking of the acrosome to the plasma membrane and that this docking does not resulting in the acrosome reaction.

In other cell types, SNARE proteins have been suggested to assemble into SNARE complexes prior to actual fusion of membranes [15,35]. The observed ternary *trans*-SNARE complex formation is in line with studies on streptolysin-O permeabilized studies on mouse sperm [33] and on see urchin sperm [36]. In both species, these SNARE interactions were functionally linked to the calcium-dependent acrosome reaction. In similarity to other cell types, other factors like Rab 3 and complexin are most likely involved in formation of the observed ternary *trans*-SNARE complexes [37-39]. The functional relevance of these SNARE interacting/associating proteins on the regulation of acrosome reaction is currently under investigation.

We here show that capacitation-dependent docking and priming of the acrosome membrane to the sperm plasma membrane is not accompanied by membrane fusions. Instead, after docking and priming, the bilamellar structure remains intact and the two interacting membranes persist together as bilamellar membrane structure as discussed above. This implies that the formation of ternary *trans*-SNARE complexes is not sufficient to induce the AR but require additional stimuli or the removal of inhibitory components to allow the fusion reaction. Thus we may conclude that the physiological acrosome reactions are not identical to the calcium ionophore-induced acrosome reaction. Calcium ionophore treatments have recently been shown to induce multiple point fusions of the PM with the OAM [40]. The main difference between capacitated

sperm and ionophore treated sperm is that acrosome fusions are not initiated by the capacitation treatment (this study) but do occur after ionophore treatment. Consequently, we propose that the working model for the acrosome reaction from Roggero et al. [38]: *(i)* the introduction of extracellular calcium between the acrosome and plasma membrane by ionophore treatment that caused formation of loose *trans*-SNARE complexes and *(ii)* an intra-acrosomal calcium efflux causing the execution of the SNARE-mediated acrosome exocytosis, should be refined as suggested in our model depicted in Fig. 7. We propose now that: (1) the acrosome is not associated with the plasma membrane in freshly ejaculated (control) sperm; (2) sperm capacitation leads to stable docking and priming of the two membranes at the apical part of the sperm head but this occurs without the execution of acrosome fusions; (3) ZP binding allows Ca^{2+} entry into the sperm [41] and the -as a consequence- elevated cytosolic calcium levels will evoke a calcium sensitive conformational change of the *trans*- to *cis*-SNARE complex. This is accompanied by the execution of the membrane fusions involved in the acrosome reaction. The involvement of SNARE proteins in these membrane fusions have been established by other groups [17,27]. Novel is the capacitation-dependent transition from step A to step B (Fig. 7), which essentially summarizes the merits of the current study.

We propose that zona binding is required for a sufficient increase in free calcium to execute the fusions of the PM with the acrosome [41] which probably involves the opening a transient receptor potential (TRP) channel allowing transiently increased intracellular calcium levels [43]. In this light, it is interesting that the entire area where the docking and priming of the PM and the acrosome takes place is the same area where the sperm cell initially will bind to the zona pellucida [1,2]. Moreover, this area coincides with the area where sperm membrane rafts have been shown to aggregate during capacitation treatments [20,44,45]. The stability and extended area of these docked and primed membranes may explain why after zona binding, the acrosome reaction is executed with its characteristic feature of multiple membrane fusions. These interactions between the two membranes involved in the acrosome reaction and the actual fusions of these membranes as result the generation of mixed vesicles (Fig. 7C1'-2') are therefore, physiologically separated processes in mammalian fertilization.

In conclusion, we have shown that SNARE proteins form stable ternary *trans*-SNARE complexes during sperm capacitation. As a result of this interaction, the acrosome becomes docked and primed to the apical sperm head plasma membrane without the occurrence of fusions between the two interacting membranes. The remarkable segregation of membrane docking/priming and fusion events as well as the extended surface area where both processes take place around the acrosome reaction makes sperm suitable as model for studying ultrastructural and biochemical aspects of exocytosis.

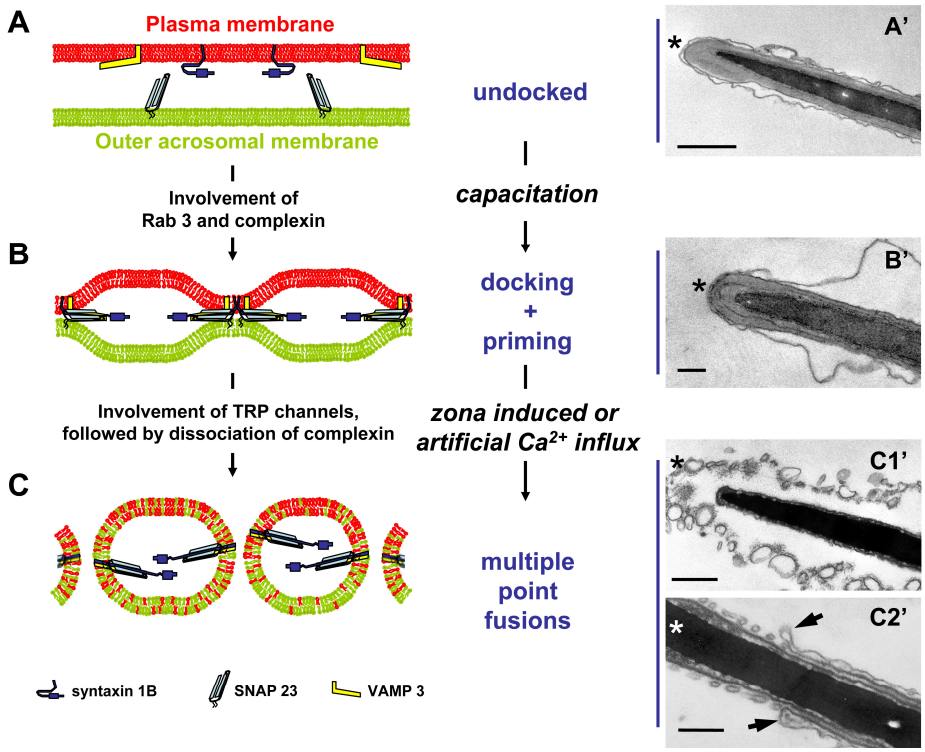

Figure 7. Hypothetical model for capacitation-induced docking and priming of the acrosome to the sperm plasma membrane. (A): In control sperm the plasma membrane (in red) and outer acrosomal membrane (in green) are not associated together. We found syntaxin 1B (purple blue) and VAMP 3 (yellow) at the plasma membrane and SNAP 23 (light blue) at the outer acrosomal membrane. The undocked plasma membrane appears as loose arrangement at the entire head area due to the osmotic effect by EM processing (A'). **(B):** *In vitro* capcitation caused the docking and priming of the acrosome to the apical sperm plasma membrane. A stable ternary- SNARE complex is formed but this did not result in exocytotic membrane fusions (B'). **(C):** Exocytotic membrane fusions are executed after sperm binding to the zona pellucida or *in vitro* by calcium ionophore treatments. Mixed vesicles of the apical plasma membrane and the outer acrosomal membrane are the result of the multipoint fusions characteristic for sperm acrosome exocytosis (C'1). However, at the equatorial sperm head area (distal from the arrows indicated in panel C'2) this fusion does not take place (C'2). This sperm surface area is specifically involved in the later occurring adhesion and fusion processes between the sperm cell and the oocyte leading to fertilization. The asterisks indicate the apical side of the sperm head. Bar represents 50 nm.

Materials and Methods

Reagents and antibodies

Chemicals were obtained from Merck (Darmstadt, Germany), unless otherwise stated. Rabbit polyclonal antibodies against syntaxin 1B, 2, 3, SNAP 23, VAMP 3 (specific for this isoform) were obtained from Synaptic Systems (Göttingen, Germany). Immunoprecipitation reagents ReliaBLOT™ and Exacta Cruz™ F were purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Biotin conjugated peanut agglutinin (Biotin-PNA) was from EY laboratory (San Mateo, CA, USA). Horseradish peroxidase (HRP) conjugated to streptavidin and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sigma (St Louis, MO, USA). EDTA free protease inhibitor was obtained from Roche (Mannheim, Germany). Boar brain tissue homogenate was prepared as previously described [20] and used as positive control.

Semen preparation and isolation of membrane vesicles

Freshly ejaculated sperms from highly fertile Sus scrofa domestica (pig) were obtained from commercial breeder (Cooperative Center for Artificial Insemination in pigs, 'Utrecht en den Hollanden', Bunnik, the Netherlands). All solutions were iso-osmotic (300±5 mOsm/ kg) at room temperature (RT) and with protease inhibitor. Sperm was subjected to two *in vitro* fertilization (IVF) conditions; *capacitation* and *control* condition as previously described [20]. In short, sperm cells were incubated in HBT (HEPES buffered Tyrode's medium: 90 mM NaCl, 21.7 mM lactate, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 1.0 mM pyruvate, 0.4 mM $MgsO_4$, 0.3 mM NaH_2PO_4, 2 mM $CaCl_2$, 100 µg/ ml kanamycine; 300 mOsm/ kg, pH 7) either supplemented with 15 mM $NaHCO_3$ in open vials for 2 hours at 38.5°C in humidified atmosphere with 5% CO_2 (capacitation) or incubated in HBT medium with the omission of bicarbonate in air-tight vials for 2 hours at 38.5 °C in a tube rack place pre-warmed water bath (control). Both conditions were prepared in the presence of the cholesterol acceptor bovine serum albumin (0.3% w/v BSA: essential fatty acid free, delipidated fraction V; Boehringer, Mannheim, Germany). Routinely, sperm were screened for cell integrity and spontaneous acrosome reactions using a flow cytometric protocol described previously [44].

For membrane vesicles isolation, a standard nitrogen cavitation protocol was followed [19]. One billion Percoll-washed sperm cells in TBSS buffer (Tris buffered sucrose solution; 5 mM Tris, 250 mM sucrose, pH 7.4) were subjected to nitrogen cavitation in a cell disruption device (Parr Instruments, Moline, IL, USA). The cavitate was slowly extruded and centrifuged at 1000g for 2 times 10 min at 4ºC. Supernatants were combined and centrifuged at 4ºC for 10 min at 6000g. Supernatant from 6000g centrifugation was carefully loaded on top of a 50µl, 80% (w/v) sucrose layer to

prevent the stickiness of membrane materials at the bottom of the tube. Membrane materials were pelleted by ultracentrifugation at 4ºC (2 times 70 min at 285000g). Membrane pellets were re-suspended in HBS (HEPES buffered saline; 5 mM HEPES, 2.7 mM KCl, 146 mM NaCl, pH 7.4). Purity of the membrane isolates were assessed by measuring the alkaline phosphatase and acrosin activities [19,46]. The remaining sperm heads (pellets that resulted from the 1000g centrifugation described above), sperm tails (pellets that resulted from the 6000g centrifugation described above) and the cavitate membrane fraction were flash-frozen in liquid nitrogen and stored at -20ºC for later use.

Transmission electron-microscopy (TEM)

Samples used for TEM purpose were recovered from Percoll-washed, cavitated or uncavitated spermatozoa. Cavitate membrane fractions (recovered from 285000g ultra-centrifugation) under two IVF treatments were pelleted. Both samples were fixed overnight at 4ºC in Karnovsky (contains 2% (v/v) paraformaldehyde and 2.5% (v/v) glutaradehyde diluted in cacodylate buffer) fixative. Pellets were washed with 0.1 M Na-cacodylate (pH 7.4) and post-fixed with 1% osmium tetraoxide in 0.1 M Na-cacodylate (pH 7.4) for 1 hour. After washing with milli-Q H_2O, pellets were incubated with 2% (w/v) uranylacetate for 1 hour. Fixed pellets were subsequently dehydrated in graded series of acetone (50-100%) and embedded in Durcupan ACM resin (Fluka, Bachs, Switzerland). Ultrathin sections of 50 nm were obtained on a Reichert UltracutS (Leica Aktiengesellschaft, Vienna, Austria) and studied using TEM (Philips CM 10, Philips, Eindhoven, the Netherlands).

Biochemical analysis

Alkaline phosphatase was measured in samples that were incubated in a buffer containing 200 mM glycine, 2 mM $MgCl_2$ and 20 mM *p*-nitrophenylphosphate with 0.05% Triton X-100 (pH 9.9) for 15 minutes, 37ºC. The reaction was stopped by adding 0.5 M NaOH and subsequently measured in a spectrophotometer (Benchmark™ micro plate reader, Bio-Rad Laboratories, Inc.) at 405 nm [46]. Acrosin activity was assayed by its esterolytic activity on N-α-benzoyl-*L*-arginine-*p*-nitroanalide hydrochloride (*L*-BAPNA, 4 mM, Sigma, St. Louis, MO) as described by Breden et al [47]. Samples were incubated in a buffer containing 55 mM Hepes, 55 mM NaCl and 0.01% (v/v) Triton X-100 (pH 8.0) for 2 hours, 22ºC. The reaction was stopped by 500 mM benzamidine. The formation product of *p*-nitroanalide was measured continuously at 410 nm during the incubation in a buffer containing 0.1 M Tris (tris[hydroxymethyl]aminomethane) and 67 mM NaCl (pH 8.0) at 25ºC.

Quantification of lectin binding

An enzyme linked lectin binding assay (ELLBA) was performed according to Flesch et al. [19] to quantify lectin binding of the samples. ELISA plates were coated with 500 ng of protein in 0.1 M Na_2CO_3 (pH 9.6) overnight at 4ºC. Plates were blocked for 10 minutes with 0.3% (v/v) Tween-20 in HBS and subsequently blocked for another hour in 0.05% (v/v) Tween-20 and 1% (v/v) casein in HBS. Biotin-PNA (0.5 µg/ ml) was used to quantify the amount of OAM material in cavitate membrane fractions. Horseradish peroxidase conjugated streptavidin (0.1 µg/ ml) was allowed to bind to the Biotin-PNA conjugates. 3,3',5,5' - tetramethylbenzidine was used as the substrate for HRP and bound enzyme activity was measured in a ELISA plate reader at 450 nm with 655 nm for reference filter [48]. Calculations were made with OD_{450} corrected for background absorption (sample without Biotin-PNA conjugate).

Immuno- blotting

Protein concentration in boar sperm and boar brain samples was determined and standardized for all experiments according to the Lowry method [49]. For both sperm and control brain samples, equal amount of total protein extract was re-suspended with appropriate amount of lithium dodecyl sulfate (LDS) loading buffer (Invitrogen, Carlsbad, CA, USA). Samples were separated into (1) non-reducing condition: in the absence of reducing agent and without boiling treatment; (2) reducing conditions: in the presence of 0.1 M dithiothreitol (DTT) and heated for either 5 minutes at 90ºC or 10 minutes at 100ºC prior to western blotting. Proteins were separated in a 4% stacking and 12% running SDS (sodium dodecyl sulfate)-PAGE gel and wet-blotted onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). After blocking for 1 hour with ReliaBLOT® at RT, blots were incubated with primary antibodies diluted in ReliaBLOT® for overnight at 4ºC. After washing the blots in TBS (Tris buffered solution) with 0.2% v/v Tween-20 (TBST), secondary antibodies were subsequently added for 60 minutes. After rinse with TBST, protein staining was visualized by using chemiluminescence (ECL-detection kit; Supersignal West Pico, Pierce, Rockford IL, USA).

Co-immunoprecipitation (IP)

Cavitate membrane fraction from capacitated and control sperm cells were subjected to strong solubilisation: samples were first lysed with 1% (w/v) SDS solution in HBS for 30 minutes, then lysed with 5% (v/v) Nonidet-P40 (NP-40) in HBS for another 30 minutes. Solubilised membrane suspensions were subsequently centrifuged (10000g, 20 minutes). Supernatants were collected and measured for protein concentration prior to immunoprecipitation. For IP procedures, lysed membrane supernatants were first subjected to pre-clearing matrix (Santa Cruz Biotechnology) for 30 minutes at 4ºC. To form the antibody-IP matrix complex, 100 µl of 25% (v/v) IP matrix suspension

(Santa Cruz Biotechnology) was pre-incubated with 10 µg of antibody on an end-to-end rotor overnight at 4ºC. Antibody-coated beads were rinsed with phosphate buffered saline (PBS, pH 7.4) to remove unbound antibody, pre-cleared membrane suspensions were then added to the antibody-IP matrix mixture and rotated overnight at 4ºC. As for negative control, an equivalent amount of protein from purified rabbit IgG was incubated with primary antibodies. After IP, the complex was pelleted by centrifugation (200g, 5 minutes); the supernatants were used to check the efficiency of the IP reaction. The pellet was subsequently rinsed with PBS. Appropriate amounts of freshly prepared LDS loading buffer was added after the last wash. To improve the detection of immunoprecipitated proteins assayed via Western blotting, IP/Western Blot reagents (ReliaBLOT®) were used to reduce the recognition of the IgG heavy/light chain at 55 and 25kDa, respectively.

Statistical analysis

Statistical analyses were carried out in SPSS 12.0. Statistical significance for 2-tailed tests is set at $p \leq 0.05$. For two-group comparison, non-parametric test for two-independent-samples (Mann-Whitney *U*-test) was used.

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Competing interests. The authors declare no competing financial interests.

Authors contributions. PST has designed and carried out most experiments and was as primary writer involved in the preparation of this manuscript. NGG has made the ultrathin sections of sperm and helped with the TEM analysis. TH was involved in daily supervision of the project and corrected the manuscript, BMG acted as senior investigator in the design of experiments and in the preparation of the manuscript and was involved in obtaining the University grant for this part of the PhD project of PST.

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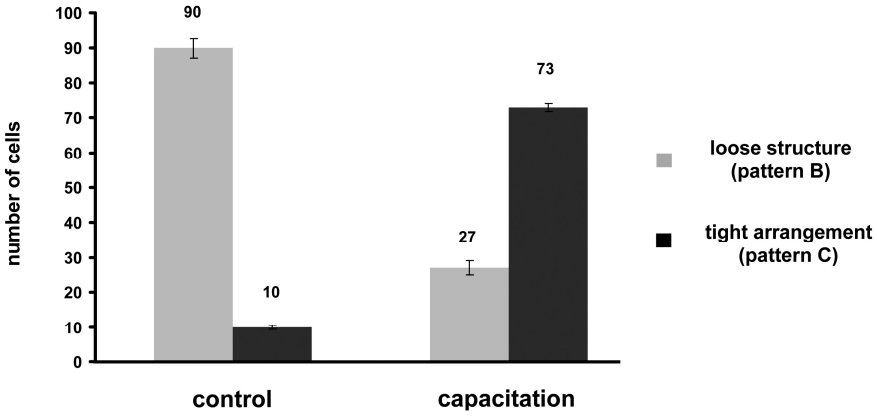
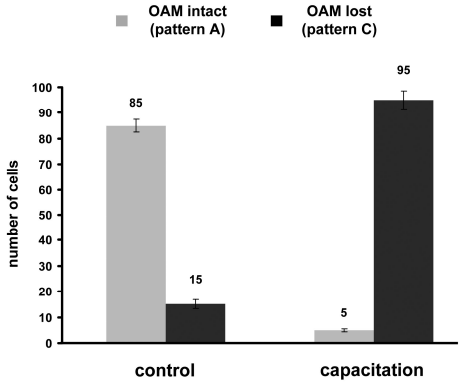


Section number		1	2	3	SD	# sperm
Control	loose structure	27	32	31	2.7	90
	tight arrangement	4	3	3	0.6	10
	total count	31	35	34		100
Capacitation	loose structure	9	7	11	2	27
	tight arrangement	25	23	25	1.2	73
	total count	34	30	36		100

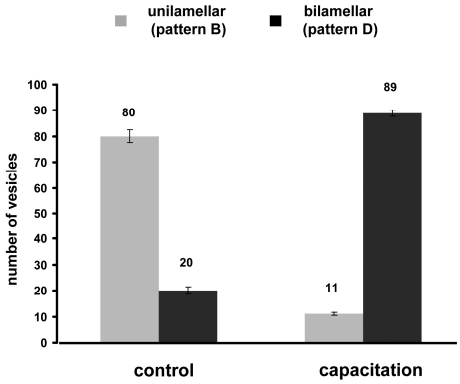
Supplementary Figure 1: Quantification of the capacitation-dependent interaction of PM and OAM at the apical head of boar sperm. Morphology of the plasma membrane and acrosome at the apical head of boar sperms is quantified via the inspection of ultrathin plastic sections using transmission electron microscopy (TEM). Patterns are classified into two categories (1) pattern B (the same morphology as in figure 1B) with the loose structure of the plasma membrane at the apical area of the sperm head; (2) pattern C (identical morphology as showing in figure 1C) with the tight and parallel arrangement of the plasma membrane and the acrosome at the apical area of the sperm head. One hundred sperm cells from at least 3 different sections are examined.

S 2-1



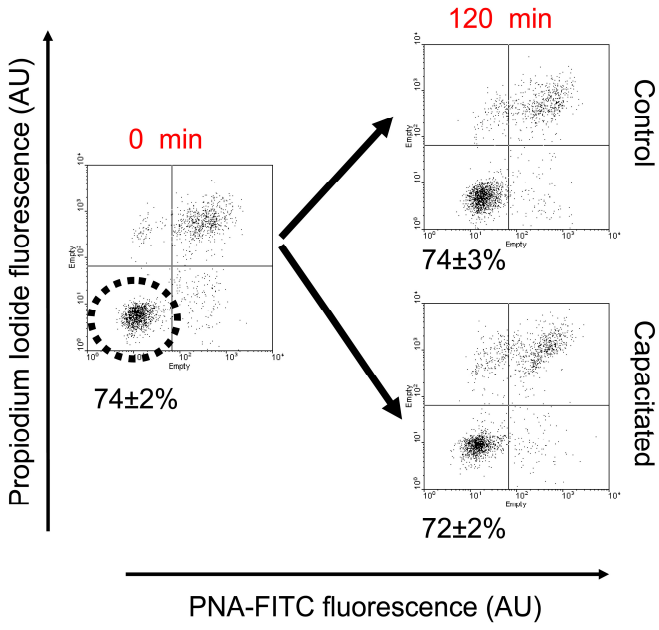
Section number		1	2	3	SD	# sperm
Control	OAM intact	28	31	26	2.5	85
	OAM lost	4	7	4	1.7	15
	total count	32	38	30		100
Capacitation	OAM intact	2	2	1	0.6	5
	OAM lost	32	35	28	3.5	95
	total count	31	35	34		100

S 2-2

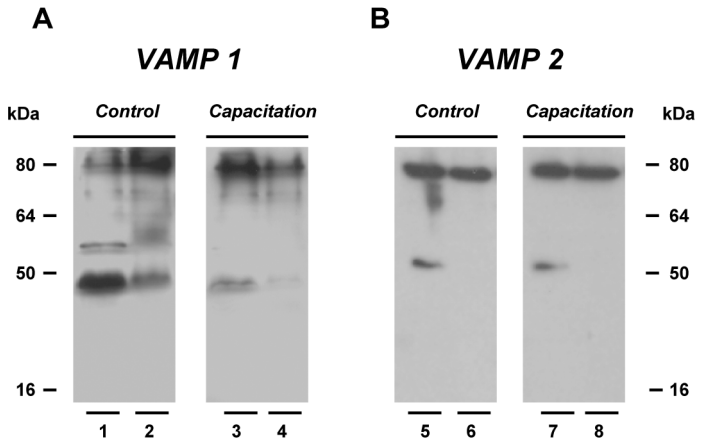


Section number		1	2	3	SD	# vesicles
Control	unilamellar	29	24	27	2.5	80
	bilamellar	7	8	5	1.5	20
	total count	36	32	32		100
Capacitation	unilamellar	4	4	3	0.6	11
	bilamellar	31	29	29	1.2	89
	total count	35	33	34		100

Supplementary Figure 2: Quantification analysis on the nitrogen cavitated boar sperm sample. In combination of nitrogen cavitation and differential centrifugation, remaining sperm head (without apical membrane), tail and membrane vesicles from the apical area of the sperm head are separated into different fractions. **(2-1)** Remaining sperm heads are sub-classified into (1) pattern A (the same morphology as in figure 2A) with outer acrosome membrane (OAM) remains intact around the entire sperm head; (2) pattern C (identical morphology as showing in figure 2C) with OAM is lost at the apical sperm head while OAM remains intact at the equatorial area of the sperm head. One hundred sperm cells from at least 3 different sections are quantified manually; cells or sections that do not show clear morphology are excluded from this analysis. **(2-2)** Quantification analyses on the morphology of the cavitate membrane fractions. Membrane structures are classified into (1) pattern B (the same morphology as in figure 2B where vesicles recovered from the same sperm cells of those showing in figure 2A): unilamellar vesicles; (2) pattern D (the same morphology as in figure 2D, vesicles recovered from the same sperm cells of those showing in figure 2C) the biilamellar vesicles. Quantification of the vesicles is proceeded via manually inspection on the ultrathin sections using TEM. Percentage of the membrane vesicles is quantified from at least 3 sections.



Supplementary Figure 3: Control and *in vitro* capacitation treatments did not result in spontaneous acrosome reactions. Percoll washed sperm suspensions were diluted in (A-C) control or capacitation media and (D-E in capcitation media and stained for cell integrity (propidium iodide on the Y-axis) and for spontaneous acrosome reaction (PNA-FITC X axis) as described before [20,44]. The percentage of acrosome intact and life sperm are indicated ± SD (n=4).



Supplementary Figure 4: Capacitation-independent involvement of VAMP 1 and VAMP 2 in the SNARE protein complex. Apical membranes of control (lane 1-2, 5-6) and *in vitro* capacitated (lane 3-4, 7-8) sperms were isolated via nitrogen cavitation in combination with differential ultracentrifugation and used for the detection of other two VAMP isoforms. All samples were loaded under either non-reducing condition (lane 2, 4, 6, 8) or treated with reducing agent (0.1M DTT) and heated at 90ºC for 5 minutes (lane 1, 3, 5, 7). Both VAMP 1 **(A)** and VAMP 2 **(B)** appeared in a 80 kDa SDS-resistant protein complex under non-reducing condition irrespectively to the capacitation treatment (lane 2, 4, 6, 8). These protein complexes partially dissociated into an iontermediate 45-55kDa VAMP1/2-containing protein bands under 90ºC heat-treated condition (lane 1, 3, 5, 7). 10 µg of total protein extract was used for all samples.

Chapter 5

SNARE protein and complexin interaction dynamics during the acrosome reaction

In preparation

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Abstract

Acrosomal exocytosis is an intracellular multipoint fusion reaction of the sperm plasma membrane with the outer acrosomal membrane. This specific exocytotic event enables the penetration of the sperm through the zona pellucida of the oocyte. Here we demonstrate that calcium ionophore-induced acrosome exocytosis results in the formation of unilamellar membrane vesicles containing a mix of components originating from the two fused membranes. These so called mixed vesicles carried a 80kDa *cis*-SNARE complexes of syntaxin 2, SNAP 23 and VAMP 3, and 118kDa protein complexes that beyond these three SNARE proteins contained additionally the SNARE complex interacting protein complexin 2. The formation of this 118 kDa complex was exclusively detected in the lipid-ordered membrane area of capacitated and ionophore-treated sperm but not in control ionophore-treated sperm. Therefore it appears that the previously reported raft and capacitation dependent docking of the acrosome with the sperm's surface allows the recruitment of special SNARE complex stabilizing proteins in these lipid-ordered microdomains. The dissociation of complexin from the SNARE complex during the ionophore-induced intracellular calcium level elevation allows the accomplishment of the acrosome reaction. The specific docking of the acrosome with the sperm surface probably also allows the recruitment of a number of secondary zona binding proteins at the zona interacting interface of the sperm immediately after the zona induced AR. Candidates for this scenario were identified using a proteomic strategy and this approach also led to the identification of additional SNARE interacting proteins. Taken together this indicates that the execution of the acrosome reaction is more complicated than the solely dissociation of complexin from the 118 kDa complex.

Keywords: sperm, acrosome reaction, SNARE, complexin

Introduction

Capacitation and acrosomal exocytosis (also known as the acrosome reaction, AR) are processes required for sperm to fertilize the oocyte. A number of changes occur at the sperm surface during sperm capacitation and cause an increase in plasma membrane fluidity and thereby allow protein and lipid rearrangements and the clustering of lipid-ordered microdomains (better known as membrane rafts) at the apical area of the sperm head surface [1-5]. These capacitation-induced sperm surface changes are effectuated *in vitro* by two capacitation factors, namely fatty acid free bovine serum albumin (BSA) and bicarbonate. Both factors activate protein kinase A and tyrosine kinase signalling pathways which are required for the specific depletion of cholesterol from the sperm surface [6-8].

Under physiological conditions, capacitated sperm remains intact for an extended period and will not undergo a spontaneous acrosome reaction unless an additional acrosome reaction inducer is added [9]. Commonly calcium ionophores, lysophosphatidylcholine and progesterone are used to induce the acrosome reaction [10-12]. Before the execution of the acrosome reaction, SNARE (soluble *N*-ethylmaleimide-sensitive factor [NSF] attachment protein receptor) proteins from the acrosome vesicle and the plasma membrane are docked into a *trans*-SNARE protein complex that serves to position the two membranes for eventual fusion [13]. During the acrosome reaction elevated calcium levels induce a conformational shift of the *trans*-SNARE protein complex into a *cis*-SNARE protein complex and thus the merging of the two docked membranes by executing the membrane fusion [14;15]. In our lab we have established that SNARE proteins migrate into the area where membrane rafts accumulate during sperm capacitation [16]. Recent findings by the group of Ackermann et al. support this finding as they reported that a multi-PDZ domain (a common structural protein subdomain found in many types of signaling proteins in variety cell types) protein MUPP1 is crucial for the relocalization of required proteins (such as SNAREs) into detergent resistant membranes (DRMs) and that this relates to the regulation of the acrosome reaction [17]. Hence, this protein may play a role in recruiting SNARE proteins into the area where we have detected membrane raft clustering.

Moreover, during *in vitro* capacitation the plasma membrane and the outer acrosomal membrane are stably docked and primed [9]. Despite this tight interaction the two membranes do not fuse and could even be isolated as bilamellar structures after cell subfractionation [9]. The absence of acrosome fusion with the plasma membrane strongly suggests that the *trans*-SNARE complex is stabilized by a SNARE interacting protein. Complexin is a protein known to have dual functions (preventing and promoting membrane fusion) including stabilizes *trans*-SNARE complexes and thus allows stable docking of vesicles to their target membrane [18-20] and this

protein has been identified and described to regulate the acrosome reaction [21]. Complexin can dissociate from the *trans*-SNARE complex in a Ca^{2+}-dependent matter and thereby allow synaptotagmin interaction with the *trans*-SNARE complex [22]. In turn synaptotagmin allows the calcium-dependent configuration shift of the SNARE complex enabling acrosomal exocytosis [23]. Interestingly, the above mentioned multi-PDZ domain protein MUPP1 has recently been shown to interact with CaMKII [24]. This interaction prevents spontaneous acrosomal exocytosis and moreover its release by Ca^{2+} calmodulin from the PDZ scaffolding protein is required to facilitate the acrosome reaction by precisely adjusting an increase in Ca^{2+} to synchronized fusion pore formation [25].

In the present study we have determined whether or not the primed *trans*-SNARE complexes - that we detected in membrane rafts that were aggregated in capacitated sperm - are stabilized by complexin. To this end, we have incubated sperm with Ca^{2+} and calcium-ionophore to evoke the required calcium levels for the acrosome reaction. We investigated the *in vitro* capacitated sperm and control treated sperm (i.e. the condition where SNARE proteins of the two membranes do not yet interact with each other) to detect the differences in their responses to the calcium ionophore as well as the involvement of complexin in these responses. The mixed vesicles that are formed by both the calcium/calcium ionophore treatments were also analysed. Furthermore, the possible presence of complexins in the mixed vesicles as well as in isolated DRMs of control and capacitated sperm was .investigated. The role of sperm membrane rafts in the (de)stabilization of *trans*-SNARE complexes formed after capacitation -by recruiting complexin 2 to the complex- and the proteomic detection of relevant SNARE associating proteins are discussed.

Results

Ultrastructure of calcium ionophore dependent formation of mixed vesicles

Incubating sperm cells with 1 mM Ca^{2+} and Ca^{2+} ionophore A21387 induced acrosome vesiculation (Fig. 1 A and B) both in control (Fig. 1A) and in capacitated sperm (Fig. 1B) albeit that the efficiency in capacitated sperm is much higher (5% in control versus 95% in capacitated sperm see also [26]). The resulting unilamellar vesicles contain both plasma membrane and outer acrosomal membrane material and hence are called mixed vesicles (MV). The emerged MVs could be isolated after differential centrifugation from other sperm structures and their ultrastructural appearance was visualized using transmission electron microscopy (TEM) on ultrathin sections (Fig. 1C and D). Membrane vesicles obtained under both conditions have a unilamellar structure. However, in the absence of bicarbonate, membrane vesicles were less homogeneous in size and shape (Fig. 1C) when compared with vesicles

formed in the presence of bicarbonate (Fig. 1D). Besides the observed differences in size and shape, it appears that more acrosome material is present on the MV of capacitated sperm than on those of control sperm (compare Fig. 1D with 1C; additional material layer is indicated with arrow heads).

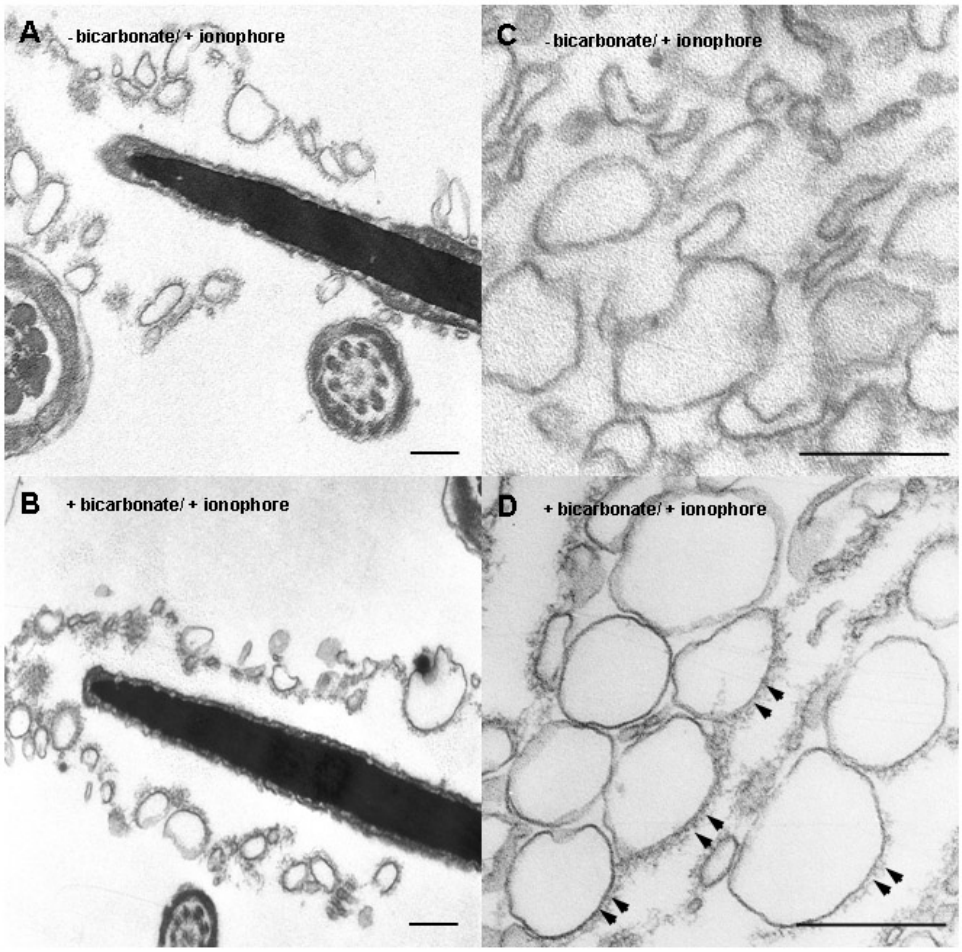

Figure 1. Ultrastructural morphology of Percoll-washed spermatozoa using transmission electron microscopy (TEM). (A): Mixed vesicles (MV) are present around the sperm head after acrosome vesiculation was induced by calcium ionophore in the absence of bicarbonate. **(B)** MVs were observed around the sperm head when capacitated sperm (in the presence of bicarbonate) cells were treated with additional calcium ionophore. **(C)** MVs from control sperm appeared as unilamellar membrane vesicles but varied in size and shape **(D)** Unilamellar vesicles from capacitated sperm cells were isolated via ultracentrifugation. They appeared as homogeneous vesicle population with two morphological distinct membrane regions (area with or without arrow heads) suggesting the co-purification of additional proteins (e.g. proteins involve in the secondary sperm-zona binding). Bar represents 200 nm.

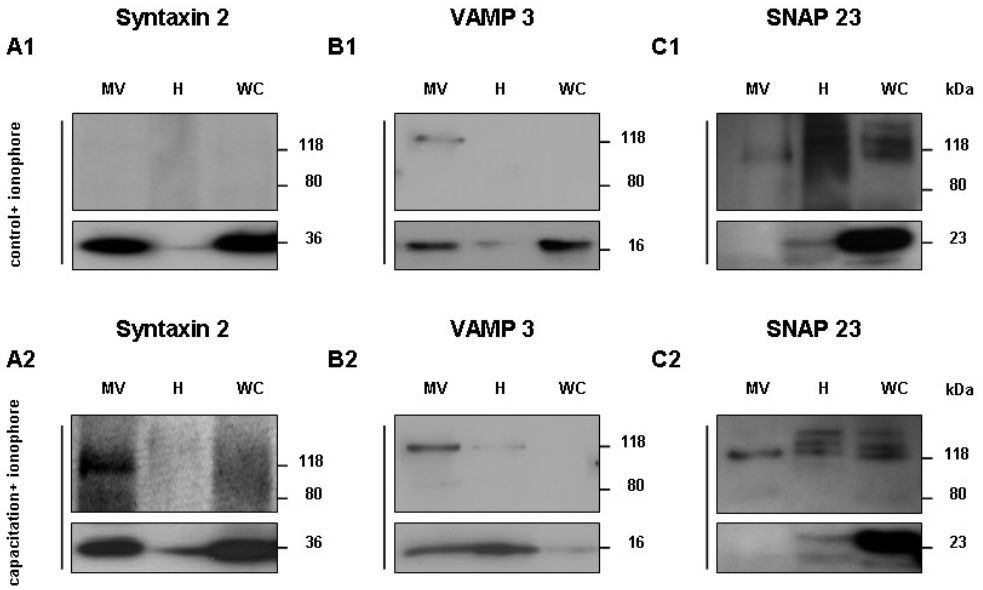

Figure 2. Mixed vesicles formed in the presence of bicarbonate contain both monomeric SNARE proteins and SNARE containing protein complexes. A1-2: syntaxin 2; **B1-2:** VAMP 3; **C1-2:** SNAP 23. When MVs were formed in the presence of bicarbonate (as isolated from the capacitated sperm that was subsequently treated with additional calcium ionophore), a considerable amount of SNARE proteins was present in the protein complexes at 118kDa **(A2-C2)**. No stable protein complexes were observed in mixed vesicles from control sperm when acrosome vesiculation was induced in the absence of bicarbonate **(A1, C1)**. However, with the exception of VAMP 3, trace amount of VAMP 3 are observed at 118kDa position. Thirty five µg of total crude protein was used per loading lane. WC: whole sperm cell lysate; H: sperm head fraction; MV: mixed vesicles.

Subcellular partitioning of SNARE proteins in mixed vesicles and the remaining sperm head

During the differential centrifugation of ionophore treated sperm we were able to separate the remaining sperm fraction and the MV fraction. We characterized the SNARE proteins in both fractions and in the parent whole cell homogenate. In control cells monomeric forms of the plasma membrane specific syntaxin 2 and VAMP 3 were observed with only very weak signs of SNARE protein complexes (Fig. 2A1, B1, lower panels). A weak signal for the acrosome specific SNAP 23 was observed at a higher molecular weight (MW) (Fig. 2C1, upper panel). The majority of SNAP 23 was recovered in the remaining sperm fraction (Fig. 2C1, lower panel) and therefore it is possible that SNAP 23 is complexed to protein(s) at the inner acrosomal membrane and that some of that membrane fraction is co-isolated with the MV fraction. In contrast, MV isolated from capacitation treated sperm showed a high amount of

SNARE complexes of both 80 kDa but also of a higher MW of 118 kDa suggesting that an additional protein is interacting with the SNARE complex (Fig. A2- C2). It is of considerable interest that these higher molecular weight forms were exclusively recovered in MV and were absent in the remaining sperm fraction. The exception is SNAP 23, this could be attributed to its presence in the inner acrosomal membrane which may be partly co-purified in the MV fraction.

We have characterized the SNARE proteins and SNARE containing protein complexes in the DRM fraction (Figure 3). In addition to Figure 2 and to what we have determined previously for the monomeric form of SNAREs [27] we here show that the SNARE complexes found in the MV fractions (Fig. 2) are recovered in the DRM fraction. Again, besides a 80kDa *trans*-SNARE complex, a 118 kDa SNARE containing protein complex was found to which another protein must be associated beyond syntaxin 2, VAMP 3 and SNAP 23 (Fig. 3).

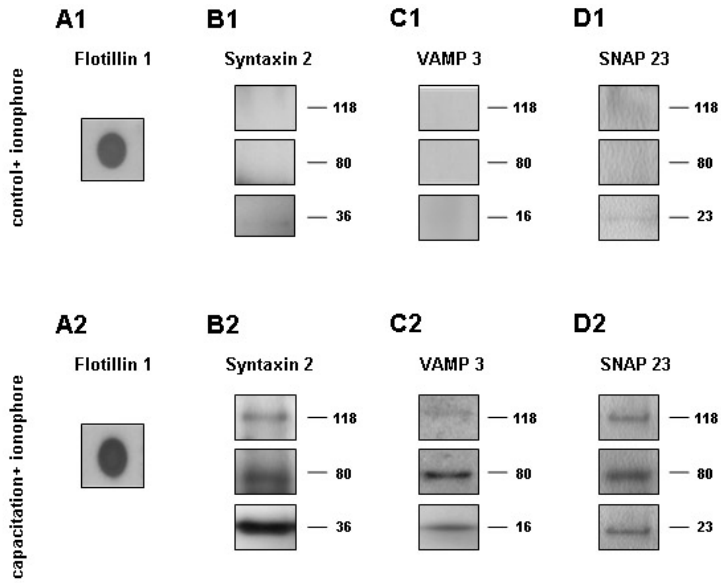

Figure 3. Bicarbonate-dependent migration of SNARE proteins and the formation of DRM-specific SNARE containing protein complexes. (A): Identification of the membrane raft fraction using the raft specific marker protein Flotillin-1. Dot blot figures show only the 5th fraction out of in total 13 fractions of sucrose gradient as Flotillin 1 was found to be enriched in this fraction irrespective of the acrosome reaction was induced in the absence **(A1)** or the presence **(A2)** of bicarbonate. **(B1-D1)** Neither the migration of monomeric SNARE proteins nor the formation of SNARE complexes were identified in the control sperm even in the presence of additional calcium ionophore. **(B2-D2)** Raft-specific SNARE containing protein complexes were present at 80 and 118kDa when capacitated sperm were treated with additional calcium ionophore indicating that additional protein becomes associated with *trans*-SNARE complex. The presence of monomeric SNARE proteins in the DRM fraction further confirms the bicarbonate- and BSA-dependent migration of SNARE proteins upon capacitation. Pane B: syntaxin 2; pane C: VAMP 3; pane D: SNAP 23.

Capacitation causes complexin 2 interactions with the SNARE complex.
When membrane preparations from control and capacitated sperm were compared, we noted that capacitation (either in absence or presence of Ca^{2+} ionophore A21387) induced a *trans* trimeric SNARE complex of 80 kDa (Fig. 4B, D, upper panels). Beyond this, a 118 kDa SNARE containing complex was observed suggesting the presence of another SNARE interacting protein (Fig. 4B, D, upper panels). One putative SNARE complex interacting protein is complexin [28;29] and we tested whether or not this protein was present in membrane associated 118 kDa complex. In control sperm this 118 kDa form was not identified in any of the membrane preparations (plasma membrane cavitate, MV fraction or remaining sperm fraction, Fig. 4C, upper panel).

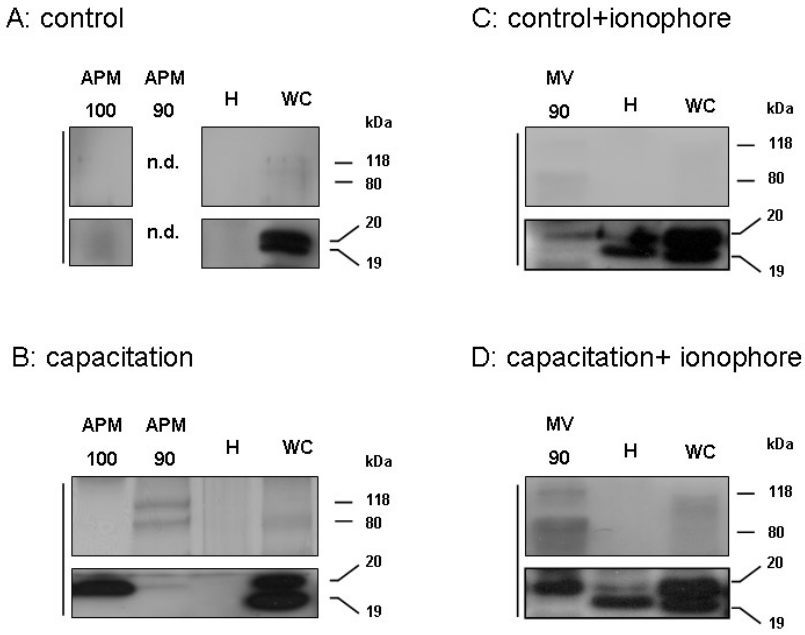

Figure 4. Complexin associated with *trans*-SNARE complex during capacitation and was released by calcium ionophore upon acrosome reaction. When an antibody against complexin SNARE binding domain was used, only the monomer complexins (19kDa for complexin I and 20kDa for complexin II) can be observed in control, non-activated whole sperm lysate (WC). Despite the absence **(A)** or the presence of calcium ionophore **(C)** no complexed complexins were observed in head (H) or mixed vesicle (MV) fractions. **(B)** High molecular weight complexins emerged in the apical plasma membrane (APM) fraction upon capacitation (APM 90) and were released from these protein complexes when the APM preparation was heated to 100 ºC (APM 100). **(D)** After the acrosome reaction, the associated complexin II was released from the SNARE protein complexes before the true boiling at 100 ºC and detected as a monomer (20kDa) indicating that the presence of additional calcium ionophore *in vitro* (and zona pellucida-induced calcium elevation *in vivo*) during the acrosome reaction is crucial for the release of complexin from the SNARE complexes. n.d.: not determine.

This indicates that in control sperm complexin is predominantly present in its soluble form (two forms were detected one of 19 and one of 20 kDa, Fig. 4A, C, lower panel) irrespective of ionophore treatment. When sperm were subjected to capacitation, this resulted not only in the docking of the acrosome (and thus the formation of a *trans* trimeric SNARE complex of 80 kDa) but also in the formation of 80 and 118 kDa forms of membrane associated complexin (only observed for the 20 kDa isoform, Fig. 4B, D).

For sperm cells that were capacitated in the absence of ionophore we noted that the complexed complexin was insensitive to treatment at 90 ºC but dissociated completely from the complex when the samples were heated at 100 ºC (Fig. 4B). This is in contrast to ionophore treated samples where the majority of complexin was recovered in the monomeric form at 90 ºC (Fig. 4D). Probably this monomeric form represents a soluble form of complexin that is purified with the MV fraction as it is present in the encapsulated cytosol within the MV. We anticipate that the higher Ca^{2+} levels that are established by the use of Ca^{2+} ionophore cause the dissociation of complexin from the SNARE complex. Interestingly, the 19 kDa form was not observed in the MV or APM membrane preparation but was recovered almost exclusively in the remaining sperm material. Both isoforms were detected in whole cell lysates in approximately equal amounts (Fig. 4).

We also observed the 118 and 80 kDa forms of complexin in the DRM fraction (fraction 5) of capacitated sperm and found the monomeric 20 kDa complexin in the detergent soluble fractions (data not shown). These complexes were only observed for capacitated sperm that have been incubated in presence of ionophore (Fig. 5).

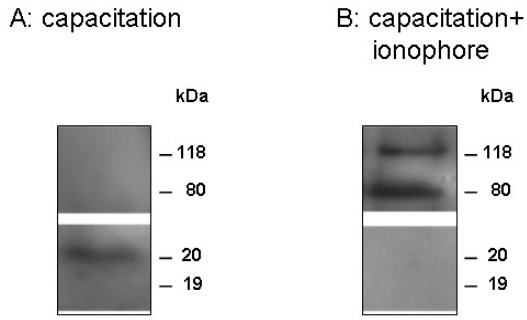

Figure 5. Detergent-based isolation of membrane raft altered the stability of complexin to the SNARE complex. (A) The majority of the complexin was present as the monomer in the soluble fraction (13th fraction, data not shown). A small fraction of complexin II migrated into DRM fraction (the 5th fraction as presented in the figure) during sperm capacitation whereas no complexin was detected at any of the three molecular weights (monomer, 80kDa, 118kDa). **(B)** Similar to that of capacitated sperm, the majority of the complexin in acrosome reacted sperm was present as the monomer in the soluble fraction (13th fraction, data not shown). However, in contrast to Fig. 4 where the 80kDa and 118kDa complexed complexin was observed in capacitated sperm, the G1 condition (capacitated and acrosome reacted) is the only condition that caused the complexed complexin suggesting an artifact that altered the stability of complexin to the SNARE complex when using Triton X-100.

This result is in contrast to what was found for the membrane fractions in the absence of the cold Triton X-100 extraction method. We therefore believe that the altered stability of these complexes is an artifact from the cold Triton X-100 extraction treatment. Nevertheless the presence of these complexes support the findings of Figure 3 and indicate that the 118 kDa syntaxin 2/VAMP 3/SNAP 23 containing protein complexes are indeed interacting with complexin. The absence of such complexes in treatments without Ca^{2+} ionophore may indicate that the tighter association of complexin (Fig. 4) under those artificial conditions causes the complexin antibody binding epitope to become cryptic for the antibody.

Proteomic identification of proteins in the apical plasma membrane (APM)
With advanced proteomics we have determined the composition of APM and identified VAMP 3, syntaxin binding protein 2 and Rab-2A with high probability scores and more than one peptide match to the proteins identified (Table 1, highlighted in bold). Lower probability scores were found for syntaxin 12. The APM fractions were fairly pure and a number of proteins routinely found to be specific of apical plasma membrane origin were also detected in these vesicles (Table 1). Of specific interest is the detection of a part of the PDZ domain-containing protein (C16orf65, highlighted in red, Table 1). This supports the recent observation [30] that this protein indeed recruits SNAREs into the DRM fraction during capacitation.

Capacitation dependent SNARE complex formation recruits specific acrosome proteins to the mixed vesicles.
When proteins from MV fractions were determined by advanced proteomics technology, we detected major plasma membrane specific proteins but also membrane proteins unique to the acrosomal membrane (Table 2a) which was expected as the two interacting membranes merge their membrane proteins into the formed MVs. However, a number of proteins with glycoconjugate binding specificities were also recovered from the MVs isolated from capacitated sperm (Table 2b). Most of them have been previously reported as acrosome specific proteins and some of them can be assumed to be of acrosomal origin as they were not recovered from the APM vesicles and are known to be secretory proteins (as highlighted in grey, Table 2b). Since these proteins were not observed in MVs from control sperm, we speculate that the additional material observed in Fig. 1 contains these additional secretory proteins. It is noteworthy that a number of the additional proteins characterized have a function in secondary sperm-zona binding (interaction and digestion of the zona peullucida of an acrosome reacted sperm using intra-acrosomal proteins; for instance acrosin and its interacting proteins and arylsulfatase A [31;32]) (Table 2b).

Table 1 LC-MALDI MS-based protein identifications of apical plasma membrane vesicles of capacitated porcine spermatozoa

Presented are a subset of those proteins identified that correspond to previously identified sperm proteins relevant to sperm-oocyte interactions. SNARE-related protein IDs are highlighted in bold. A protein with a putative function in the recruitment of SNARE proteins into membrane rafts [54;55] is shown in red. Number signs indicate proteins which are routinely found in apical plasma membrane cavitates of porcine sperm (See Van Gestel et al., 2007). Asterisks indicate proteins that are recovered as plasma membrane proteins in MV preparations. Protein ID was considered to be conclusive when two or more peptides were identified (e<0.005) (all proteins above the line).

Protein Name	Peptide Count	Accession Number	Sequence 1	Expect Value 1	Sequence 2	Expect Value 2	Database
Zonadhesin#	17	ZAN_PIG	FVELQTAFGLR	2,8E-07	QEGVSCLSK	8,1E-06	Swiss Prot v.57.10
Epididymal sperm-binding protein 1#*	9	ESPB1_PIG	NCIVEGSFFGK	3,2E-06	TNSLSPWCATR	1,6E-06	Swiss Prot v.57.10
Carbohydrate-binding protein AWN#*	6	AWN_PIG	SSSNIATIK	5,7E-07	QTIIATEK	3,0E-06	Swiss Prot v.57.10
Carbohydrate-binding protein AQN-3#*	5	AQN3_PIG	GSDDCGGFLK	3,9E-07	NYSGWISYYK	2,6E-06	Swiss Prot v.57.10
ADAM3b#	4	A5HJZ3_PIG	GLLCVSAQLR	1,8E-04	NFDTQYTYYK	1,3E-06	Trembl v.40.10
Fertilin beta#	4	Q866A8_PIG	CHPNDLR	9,4E-03	TDESGACGLTA	1,2E-09	Trembl v.40.10
PDZ domain-containing protein C16orf65	4	CP065_BOVIN	DINCDVMIHR	2,4E-06	APSPYWTMVK	4,0E-03	Swiss Prot v.57.10
Acrosomal protein SP-10#*	3	ASPX_HUMAN	NQSFCNKI	5,2E-04	KIFEGGK	2,6E-02	Swiss Prot v.57.10
ADAM3a#	3	A5A4F6_PIG	QCAELFGK	1,1E-03	SEVVPFK	1,6E-02	Trembl v.40.10
CD44	3	CD44_BOVIN	YAGVFHVEK	1,2E-04	TEAADLCK	1,5E-04	Swiss Prot v.57.10
Carbohydrate-binding protein AQN-1#	2	AQN1_PIG	ISTYEGPK	1,3E-04	EYVEVQDGLP	8,0E-03	Swiss Prot v.57.10
Ras-related protein Rab-2A	2	RAB2A_HUMAN	GAAGALLVYDI	1,5E-04	MITIDGK	2,2E-02	Swiss Prot v.57.10
Sperm adhesion molecule 1 (SPAM1)#*	2	Q8MI02_PIG	ESTALFPSIYLN	8,5E-04	TFMQETLK	6,6E-03	Trembl v.40.10
Syntaxin-binding protein 2	2	Q2NL10_BOVIN	DLSHILK	2,7E-03	ADTPSLGEGP	1,6E-03	Swiss Prot v.57.10
VAMP 3	2	VAMP3_HUMAN	ADALQAGASQF	4,3E-17	LQQTQNQVD	9,7E-03	Swiss Prot v.57.10
Syntaxin 12	1	STX12_HUMAN	ISQATAQIK	3,9E-02			Swiss Prot v.57.10

Table 2 LC-MALDI MS-based protein identifications of mixed vesicles derived from acrosome reacted porcine sperm
(a) Presented is a subset of those proteins identified, that correspond to sperm proteins previously determined to be relevant to sperm-oocyte interactions. Asterisks indicate acrosomal membranes proteins, others are plasma membrane specific. **(b)** Other protein IDs that could have a putative role in the secondary sperm-zona binding. Proteins that are not detected in the apical plasma membrane and are exclusively present in the MV are highlighted in grey. Most of these proteins have been previously reported to be acrosomal membrane specific.

Table 2a

From mixed vesicle samples

Protein Name	Peptide Count	Accession Number	Sequence 1	Expect Value 1	Sequence 2	Expect Value 2	Database
Epididymal sperm-binding protein 1	9	ESPB1_PIG	AVYDGQWK	3,4E-05	YCLIEDYPR	5,0E-05	Swiss Prot v.57.10
Zona pellucida-binding protein 1*	5	ZPBP1_PIG	FFNQQVEVLGR	5,4E-07	IVGSPNFPVK	6,5E-05	Swiss Prot v.57.10
Carbohydrate-binding protein AWN	5	AWN_PIG	IFNSDGPQK	8,1E-06	QTIIATEK	3,4E-05	Swiss Prot v.57.10
Carbohydrate-binding protein AQN-3	4	AQN3_PIG	GSDDCGGFLK	2,1E-07	NYSGWISYYK	2,6E-07	Swiss Prot v.57.10
Acrosomal protein SP-10	2	ASPX_HUMAN	NQSFCNKI	2,6E-03	KIFEGGK	4,3E-03	Swiss Prot v.57.10
Sperm adhesion molecule 1 (SPAM1)	2	Q8MI02_PIG	ESTALFPSIYLN	2,2E-04	QSIELVQQK	2,4E-02	Trembl v.40.10
Sperm inner acrosomal membrane protein IAM38*	2	Q2PMM0_BOVIN	FFNQQVEVLGR	1,2E-06	VYVMLHQK	5,1E-03	Swiss Prot v.57.10

Table 2b

Other interesting proteins in MV

Protein Name	Peptide Count	Accession Number	Sequence 1	Expect Value 1	Sequence 2	Expect Value 2	Database
Fibronectin*	15	FINC_HUMAN	SSPVVIDAST	6,9E-08	ESKPLTAQQ	5,2E-08	Swiss Prot v.57.10
Cumulus cell-specific fibronectin 1*	13	B8Y9T0_BOVIN	SYTITGLQPG	4,7E-10	VPGTSASATL	8,1E-09	Trembl v.40.10
Acrosin*	10	ACRO_PIG	YVSGLEINDI	1,2E-10	APQTCWVTG	2,0E-09	Swiss Prot v.57.10
Acrosin-binding protein*	10	ACRBP_PIG	AWQYLEDET	8,5E-12	LEQCHSETN	9,6E-09	Swiss Prot v.57.10
Alpha-1-acid glycoprotein*	5	A1AG_BOVIN	WFYIGSAFR	1,5E-05	EFLDVIK	1,7E-04	Swiss Prot v.57.10
Alpha-2-HS-glycoprotein*	5	FETUA_BOVIN	QQTQHAVE	9,4E-11	QDGQFSVLF	6,1E-08	Swiss Prot v.57.10
Sperm-associated acrosin inhibitor*	5	IACS_PIG	KEPDCDVY	4,4E-05	SHLFFCTR	7,7E-04	Swiss Prot v.57.10
Testis cDNA clone: QtsA-14886, similar to human acrosin binding protein	5	Q4R933_MACFA	TMSQLSSTL	2,6E-05	IYYENILLGVP	2,1E-08	Trembl v.40.10
Alpha-1B-glycoprotein*	4	A1BG_BOVIN	ALWTGALTP	6,1E-05	FPLGPVTSTT	1,1E-04	Swiss Prot v.57.10
Arylsulfatase A*	2	Q8WNR3_PIG	TLFFYPAYPD	4,6E-04	GYLTGMAGK	8,8E-04	Trembl v.40.10
Pancreatic secretory granule membrane major glycoprotein*	2	Q29209_PIG	YCTDPTTAIV	3,3E-07	LESTPQCNL	7,7E-04	Trembl v.40.10
Spermatid-specific heat shock protein 70	3	Q9R2A1_MOUSE	TTPSYVAFT	2,0E-06	NQVAMNPQN	6,5E-10	Trembl v.40.10
Acrosomal vesicle protein 1*	2	Q32KR2_BOVIN	NQSFCNKI	1,1E-03	NQSFCNK	8,1E-03	Trembl v.40.10
Synaptotagmin-4	**1**	**SYT4_HUMAN**	**AFVVNIKEAR**	**8,8E-02**			Swiss Prot v.57.10

The possibility that capacitation-dependent SNARE complex formation at the lipid-ordered membrane recruits specific acrosomal membrane proteins to the area where acrosome fusion is executed should be considered. This capacitation-dependent recruitment may serve to have the acrosome reacted sperm with the secondary zona binding and digesting proteins at the forefront enabling the immediate exposure of such proteins once the acrosome fusion is initiated by the zona pellucida. In the MVs, we found evidence for presence of synaptotagmin 4 but this was not unequivocal. Synaptotagmin 4 is considered to be important for secretory membrane fusions as this protein belongs to a Ca^{2+} sensor protein family normally present on a secretory vesicle (the equivalent in sperm is the acrosome vesicle) that can interact with syntaxin and complexin and is known to be involved in the *trans* to *cis* configuration of SNARE complexes and thus execute the fusions involved in the acrosome reaction [33]. Hence this is worthy of further investigation.

Discussion

In this study, we extended our biochemical approach to elucidate the involvement of SNARE protein interactions in the acrosome reaction. In previous studies we found the capacitation-dependent formation of a *trans*-SNARE complex that serves to dock the apical sperm plasma membrane with the outer acrosomal membrane. Such docking results in the bilamellar membrane structure [9], a specific phenomenon for the lipid-ordered sperm surface area [34;35]. In the whole sperm homogenate and membrane isolates, the interactions of syntaxin 1b/VAMP 3/SNAP 23 were established. However, these interactions did not result in membrane fusions and no signs of the acrosome reaction were detected.

In the current study, we have evoked the acrosome reaction in control and capacitated sperm by using calcium ionophore. This resulted in the emergence of mixed unilamellar vesicles containing material from both membranes (plasma membrane and outer acrosomal membrane) that fused at multiple sites. Both control and capacitated sperm showed signs of acrosome vesiculation, albeit that the efficiency for the induction of the acrosome reaction was profoundly higher after capacitation. In line with the formation of SNARE complexes in capacitated sperm we found a 80 kDa ternary SNARE complex that contained syntaxin 1b/2/VAMP 3/SNAP 23 (which corresponds to observations in *chapter 4*, [9]). However, additionally a 118 kDa complex was identified that also contained these three interacting SNARE proteins indicating that another protein is interacting with the *trans*-SNARE complex. The complexes were specifically recovered in the MVs and were absent in the remaining sperm material. In control sperm, the two SNARE complexes were almost absent. Interestingly, the complexes were recovered in the DRM of capacitated and

ionophore treated sperm which supports the notion that SNARE complexes are established in the area where raft aggregation is taking place [36].

Previous literature suggests that complexin is involved in interactions with SNARE proteins [37-41]. We indeed established that the 118 kDa complexes from the capacitated sperm contain complexin (one of the two isoforms present in sperm is specifically interacting in the MVs). In control sperm, complexin was only present in a soluble monomeric form and was not associated to a protein complex (data not shown). Interestingly, the stability of the 118 kDa complex was Ca^{2+} sensitive. In ionophore treated sperm the majority of complexin did not interact with the SNARE complex whereas in the bilamellar structures isolated from capacitated sperm in absence of Ca^{2+} ionophore complexin was completely complexed to the 118 and 80 kDa protein multimers. Heating of this preparation to 100ºC resulted in a complete dissociation of the complexin. We therefore believe that Ca^{2+} influx into the sperm cell (normally evoked after sperm-zona binding) results in the dissociation of complexin. In line with this we speculate that complexin binding to the raft specific *trans* ternary SNARE complex prevents preliminary acrosome fusion events. The altered interaction of complexin with this complex by elevated Ca^{2+} may be of relevance for the initiation of the AR after zona binding.

From the proteomic analysis of the mixed vesicles we elucidated that a number of proteins from the outer acrosomal membrane (and luminal matrix) are only observed in the MVs from the capacitated sperm. These proteins have affinity for glycosylated proteins such as the zona pellucida proteins and thus may serve to establish the firm secondary sperm-zona binding during the acrosome reaction. The fact that such proteins were not detected in MVs from control sperm provides an indication that the sperm surface rearrangement and the concomitant reordering of the interacting outer acrosomal membrane are functionally relevant for the secondary sperm-zona binding. The proteomic data also delivered a number of proteins that are known to be involved in the regulation of SNARE mediated exocytosis. Indeed, Rab 2a, syntaxin binding protein 2 and VAMP 3 are present in boar sperm and in addition there is some evidence that syntaxin 12 and synaptotagmin 4 are also present although this is not unequivocal. The role of Rab 2a, synaptotagmin 4 (a Ca^{2+} sensor known to be important for the execution of secretory vesicle fusion) and syntaxin binding protein 2 (Munc 18b) is a matter for future research. It indicates that the execution of the AR is complicated and that a Ca^{2+} sensing protein (like synaptotagmin 4) is competing with complexin for the binding with the SNARE complex and altered competing affinities may well be relevant for preventing or stimulating the AR. Interestingly, introduction of a membrane soluble form of Rab3a induced spontaneous AR [42].

In summary, we have detected the capacitation-dependent formation of complexin stabilized SNARE protein complexes in the lipid-ordered membrane area where the sperm surface and acrosome are docked. When the acrosome reaction was

induced by a Ca^{2+}-ionophore, complexin dissociated from the complex and the acrosome reaction was accomplished. In control sperm, these protein complexes were not discovered. We postulate that the specific docking of the acrosome with the sperm surface is required to recruit certain secondary zona binding proteins at the surface as soon as the AR is initiated by the ZP. We identified a number of secondary zona binding proteins in MV derived from capacitated sperm that in contrast were not observed in MV from control sperm. The additional SNARE interacting proteins identified in the MV of capacitated sperm indicate that the execution of the acrosome reaction is more complicated because it depends on more events than solely the dissociation of complexin from the SNARE complex.

Materials and Methods

Reagents and antibodies

All chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated. Rabbit polyclonal antibodies against syntaxin 2, SNAP 23, VAMP 3 (specific for cellubrevin, no cross reactivity to VAMP 1or 2) and complexin I/II (recognized both isoforms; immunogen is located inside the mapped binding domain of complexin 2 to the SNARE complex, aa 45-81) were obtained from Synaptic Systems (Göttingen, Germany). Mouse IgG1 monoclonal antibody against raft specific protein flotillin 1 was obtained from BD Biosciences (San Jose, CA, USA). Ca^{2+} ionophore (A23187) was from Sigma.

Sperm preparation

Freshly ejaculated sperm cells from highly fertile boars (*Sus scrofa domestica*) were obtained from a commercial breeder (Cooperative Center for Artificial Insemination in Pigs, 'Utrecht en den Hollanden', Bunnik, the Netherlands). Sperm cells were washed through a discontinuous (70% v/v and 35% v/v) Percoll gradient (GE Helathcare, Diegem, Belgium) as previously described [43]. All solutions were iso-osmotic (300±5 mOsm/kg) at room temperature (RT) and with protease inhibitor (EDTA free, Roche, Mannheim, Germany).

Two billion matured sperm cells per condition were used for the following *in vitro* fertilization (IVF) compatible protocols: (1) G1: capacitated and acrosome reacted: sperm cells were incubated in HEPES- buffered Tyrode's medium (HBT: 90 mM NaCl, 21.7 mM lactate, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 1.0 mM pyruvate, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 2 mM $CaCl_2$, 100 µg/ml penicillin-streptomycin sulphate) with addition 15 mM $NaHCO_3$ in a open-capped tube at 38.5 ºC in humidified atmosphere with 5% CO_2 for 2 hours to induce capacitation. Acrosomal exocytosis of capacitated sperm cells was subsequently induced by the addition of 5 µM of calcium

ionophore A21387 and incubation under same condition for extra 1 hour; (2) G3: non-capacitated but acrosome reacted: sperm cells were incubated under the same IVF condition as G1, only without $NaHCO_3$ in the capacitation incubation medium. All incubation media were prepared in the presence of the cholesterol acceptor bovine albumin (0.3% w/v BSA).

Transmission Electron Microscopy (TEM)

Sperm cells as well as mixed vesicles from both treatments were pelleted and fixed overnight at 4 ºC in Karnovsky (2 % (v/v) paraformaldehyde with 2.5 % (v/v) glutaraldehyde, diluted in cacodylate buffer) fixative. Pellets were washed with 0.1 M Na-cacodylate (pH 7.4) and post-fixed with 1% osmium tetraoxide in 0.1 M Na-cacodylate (pH 7.4) for 1 hour. After washing with milliQ H_2O, pellets were incubated with 2 % (w/v) uranylacetate for 1 hour. Fixed pellets were subsequently dehydrated in graded series of acetone (50%-100%) and embedded in Durcupan ACM resin (Fluka, Bachs, Switzerland). Ultrathin sections of 50 nm were obtained on a Reichert UltracutS (Leica Aktiengesellschaft, Vienna, Austria) and studied using TEM (Philips CM10, Philips, Eindhoven, the Netherlands).

Detergent- Resistant Membrane (DRM) Isolation

DRMs were isolated according to van Gestel et al. [44]. In short, sperm cells were washed in HBS, lysed in 2-(N-morpholino)ethanesulfonic acid (Mes) buffer (25 mM Mes, 150 mM NaCl, 1 mM EGTA), 1 % (v/v) Triton-X 100; pH 6.5) and kept on ice for 30 min. The suspension was subsequently mixed with an equal volume of 80 % (w/v) buffered sucrose solution. The mixture was overlaid with 8 ml 30 % (w/v) sucrose followed by 4 ml 5 % (w/v) sucrose in Mes buffer and centrifuged for 18 hour at 200000 g at 4 ºC. The DRMs appeared as an opalescent band in the low-density fraction of the gradient. Starting from the top of the gradient 1 ml/ fraction was collected (in total 13 fractions).

Membrane vesicle isolation

Membrane vesicles from capacitated and control sperm cells were obtained via nitrogen cavitation as previously described [45]. For mixed vesicle isolation, after the required incubation period, shed membrane vesicles from conditions G1 and G3 were obtained via differential centrifugations at 4 ºC. Sperm cells from both conditions were centrifuged at 1000 g, 10 min. Pellets were subjected to strong solubilization as described previously [46]. Supernatants were collected and were centrifuged at 6000 g, 10 min. Supernatants from 6000 g centrifugation were carefully loaded on top of the 100 µl, 80% sucrose layer in SW 60 (Beckman, Palo Alto, CA, USA) tubes followed by ultracentrifugation at 285000 g for 70min. A whitish band can subsequently be observed above the sucrose layer. Supernatants were removed carefully until 1ml was

left, remaining material was resuspended with HBS (5 mM HEPES, 2.7 mM KCl, 146 mM NCl, pH 7.4) and subjected to a second ultracentrifugation (285000 g, 70 min). Pellets from mixed vesicles were resuspended in ice-cold HBS, flash-frozen in liquid nitrogen and stored at -20 ºC for later use. Purity of these membrane isolates were checked as previously described [47-50].

Immuno-blotting

Protein concentrations from positive control (pig brain homogenate) and all samples were standardized according to the Lowry method [51]. For mixed vesicle experiments, an equal amount of total protein extract was resuspended with an appropriate amount of lithium dodecyl sulfate loading buffer (Invitrogen, Carlsbad, CA) in the presence of 0.1M dithiothreitol (DTT) and heated for 5 min at 90 ºC or 10min at 100 ºC prior to immuno-blotting. For detergent resistant membrane experiments, an equal volume (15µl) of different fractions was used. Proteins were separated in a 4 % stacking and 10 % running SDS-PAGE gel and wet-blotted onto nitrocellulose membranes (Protran BA 85, Whatman, Dassel, Germany). After blocking for 1 hour with ReliaBLOT® Block (Bethyl Lab., Inc., Montgomery, TX, USA) at RT, blots were incubated with primary antibodies diluted in ReliaBLOT® for overnight at 4 ºC. After washing the blots in TBS (5 mM Tris, 250 mM sucrose, pH 7.4) with 0.2 % v/v Tween-20 (TBST), secondary antibodies were subsequently added for 30 min. After rinsing with TBST, protein was visualized by using chemiluminescence (ECL-detection kit; Supersignal West Pico, Pierce, Rockford IL, USA).

Dot-blotting

Dot blotting was performed by using the Easy-Titer™ ELIFA dot blot system (Pierce, Rockford IL, USA). Nitrocellulose membranes were rinsed in Milli Q water and equal volumes of DRM fractions were subsequently pipetted into separate wells. Membranes and adhering proteins were dried using a vacuum system (flow rate 100 µl/ 1.5min/well). Immuno-blotting procedures were followed as described below. Quantitative analysis of dot blot labeling was performed by scanning the blots with a GS-700 densitometer (Bio-Rad Laboratories, Hercules, CA, USA) using Quantity One acquisition software (version 4.3, Biorad). Densitometric quantitation was carried out with the use of Gel-Pro Analyzer software (version 3.0, MediaCybernetics, Silver Spring, MD, USA). Statistical analyses were performed with the use of SPSS 12.0 statistical software (SPSS Inc., Chicago, IL, USA) and/or SigmaPlot 9.0 (Systat Software Inc. Richmond, CA, USA).

LC-MALDI MS-based protein identifications of membrane vesicles

Two types of membrane vesicles were used for the proteomic analyses; (1) apical plasma membrane vesicles from capacitated sperm were prepared as described

previously [52], (2) mixed vesicles from capacitated and acrosome reacted sperm were prepared as mentioned above (in the "*membrane vesicle isolation*" section). Suspensions containing membrane vesicles were pelleted at 118,000 g for 45 min at 4°C in a TLA-110 rotor, Optima-Max Ultracentrifuge (Beckman Coulter, Palo Alto, CA, USA). The pellets were solubilized in 100 µl triethylammonium bicarbonate (TEAB) lysis buffer (20 mM TEAB) containing 20 mM DTT and 1% (w/v) SDS at RT for 10 min, and then heated to 95°C for 10 min. The samples were left at RT for 10 min before samples were subjected to protein precipitation using a 2D Clean-up kit (GE Healthcare, Buckinghamshire, UK). The pellets were resuspended in 20 mM TEAB and left overnight at 4°C. The protein content was then determined using a bicinchoninic acid (BCA) protein assay kit (Sigma, St. Louis, MO, USA). Samples were then reduced, denatured and alkylated using an Applied Biosystems iTRAQ (isobaric tag for relative and absolute quantitation) labelling kit with standard protocol (Foster City, USA). The proteins were subjected to digestion with trypsin (ratio of 1 µg trypsin: 50 µg of sample), and incubated at 37°C for 12-16 h. The samples were then dried and resuspended in water with 0.1 % (v/v) trifluoroacetic acid (TFA).

Digested peptides were separated on a nano-LC (liquid chromatography) system (UltiMate 3000, Dionex, Sunnyvale, USA) using a two-dimensional salt plug method as previously described [53]. Mass spectrometry was performed using an Applied Biosystems (Warrington, UK) 4800 MALDI (Matrix-assisted laser desorption/ionization) TOF/TOF (time-of-flight) mass spectrometer as previously described [53]. The MS/MS data was used to search the Swiss-Prot database (Version 57.10; mammalian taxonomy) in the first instance but also the Trembl database (Version 40.10; mammalian taxonomy) using the multiple alignment system for protein sequences (MASCOT) database search engine v2.1.04 (Matrix Science Ltd, London, UK), embedded into GPS Explorer software v3.6 Build 327 (Applied Biosystems) (default GPS parameters, 1 missed cleavage allowed, fixed modification of MMTS(C), variable modifications of oxidation (M), pyro-glu (N-term E) and pyro-glu (N-term Q), 150 ppm mass tolerance in MS and 0.3 Da mass tolerance for MS/MS which are recommended published tolerances for LC-MALDI [53]. A minimum of two peptides with MASCOT e-values less than 0.05 was considered to be conclusive for protein identification. Where more than one protein was identified, the protein with the highest MOWSE (molecular weight search) score in MASCOT is reported.

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Chapter 6

Preparation of the cortical reaction: maturation-dependent migration of SNARE proteins, clathrin and complexin to the oocyte's surface blocks membrane traffic until fertilization

Submitted

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Summary sentence: SNARE protein-mediated docking of cortical granules to the oolemma is temporarily arrested by SNARE-complexin interaction; this subsequently arrests clathrin-dependent endocytosis. Fertilization fusion-induced calcium oscillations will lead to the resumption of both processes.

Abstract

The cortical reaction is a calcium-dependent exocytotic process in which the content of secretory granules is released into the peri-vitellin space immediately after fertilization and serves to prevent polyspermic-fertilization. In this study we investigated the involvement and the organization of Soluble N-ethylmaleimide-sensitive [NSF] factor Attachment Protein Receptor (SNARE) proteins in the docking and fusion of the cortical granule membrane with the oolemma. During meiotic maturation secretory vesicles (labeled with a granule specific binding lectin, PNA) migrated toward the oocyte's surface. This surface orientated redistribution behavior was also observed for the oocyte-specific SNARE proteins SNAP 23 and VAMP 1 that co-localized with the PNA-labeled structures in the cortex area just under the oolemma and to the exclusive localization area of complexin (a *trans*-SNARE complex stabilizing protein). The coming together of these proteins serves to prevent the spontaneous secretion of the docked cortical granules and to prepare the oocytes surface for the cortical reaction which should immediately be compensated, probably by a clathrin-mediated endocytosis. *In vitro* fertilization resulted in the secretion of the cortical granule content and the concomitant release of complexin and clathrin into the oocyte's cytosol and this is considered to stimulate the observed endocytosis of SNARE containing membrane vesicles.

Keywords: SNARE, complexin, clathrin, oocyte, cortical reaction

Introduction

Cortical granules (CGs) are a specialized group of secretory vesicles that are randomly dispersed throughout the cytoplasm of immature oocytes and migrate towards the cortical cytoplasm during meiotic maturation [1]. This relocalization is mediated by specific association with components of the cytoskeleton, e.g. microfilaments [2]. Once the granules are positioned at the cortical area of the oocyte, they dissociate from the cytoskeleton structures and remain in close proximity to the egg plasma membrane (oolemma) [3;4]. The redistribution of secretory granules is a preparative step for the cortical reaction (CR), a calcium-dependent exocytotic process. The trigger for the CR is the fusion of the sperm plasma membrane with the oolemma. This fertilization fusion activates a series of intracellular signaling pathways [3]. In mammals, the sperm cell introduces phospholipase C zeta (a sperm specific isoform) into the oocyte cytoplasm (ooplasm), which serves to produce inositol 1,4,5-triphosphate (IP_3) by breaking down of phosphatidylinostol 4,5 bisphosphate (*PtdIns(4,5)P*$_2$; PIP_2) [5;6]. On its turn the produced IP_3 binds to receptors on the endoplasmic reticulum (ER) of the oocyte causing an oscillatory release of Ca^{2+} into the ooplasm. The resulting Ca^{2+}-oscillations in the ooplasm induce the fusion of cortical granules with the oolemma over the entire oocyte's surface [7;8]. These calcium oscillations can *in vitro* be mimicked by addition of calcium ionophore (A23187), by stimulation with electrical pulses, or by a more physiological approach namely the incubation of matured meiosis-II (M-II) oocytes with sperm cells [9]. During the CR, the enzyme content of the cortical granules is secreted into the peri-vitelline space and these released enzymes subsequently either modify the zona pellucida (ZP) proteins resulting in a structural change of the ZP (known as "zona hardening") and/or form a specific coating on the oolemma [10;11]. Both changes are known to inhibit polyspermic fertilization which is incompatible with normal development of the zygote. A further understanding of cellular dynamics of proteins that involved in the CR is considered important to reveal the mechanisms that contribute to the regulation of CR and the subsequent events that are relevant for the normal development of zygote.

Several studies have shown that exocytosis in gametes is controlled by interactions between specific sets of proteins which belong to the SNARE (Soluble N-ethylmaleimide-sensitive factor [NSF] Attachment Protein Receptor) protein family [12-14]. Functional studies in mouse oocytes have provided solid evidence that SNAP 25 is involved in the CR [13]. However, scarce information is available on the (re)localization of SNARE proteins during specific stages of oocyte development, maturation and fertilization. In the present study, we report on the relocalization of secretory granules and the spatial-temporal reorganization of proteins that are involved in the regulation of CR pre- and post-fertilization. Furthermore, complexin

has been shown to associate with SNARE complexes in neurons and thus not only stabilizes the formed SNARE complexes but also prevents premature fusion of interacting membranes [15]. Here we provide data that support SNARE interactions contribute to the docking process of CGs to the oolemma and that the subsequent SNARE-complexin interactions are responsible for the arresting of CGs at the oolemma. Once this secretion is temporary inhibited at the oocyte's surface prior to fertilization, this also will arrest endocytosis, therefore, we investigated the cellular organization of proteins involved in clathrin-dependent (clathrin) and clathrin-independent (flotillin and caveolin) endocytosis during oocyte maturation. The possible functional implications of cortical granule docking for the cortical reaction and for its role in the prevention of polyspermic fertilization are discussed. The involvement of clathrin and complexin in the re-establishment of membrane trafficking are also investigated and discussed.

Materials and Methods

Reagents and antibodies

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. Affinity purified mouse monoclonal antibody against syntaxin 1, rabbit polyclonal antibodies against syntaxin 2, SNAP 23, VAMP 1, 2 and 3 (specific for each isoform, no cross reactivity with the other two) and complexin 1/2 (binds to both isoforms) were purchased from Synaptic System (Göttingen, Germany). Rabbit polyclonal antibody against SNAP 25 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse IgG1 monoclonal antibodies against raft marker protein flotillin 1 and against a marker protein for caveolae, caveolin 1 were obtained from BD Biosciences (San Jose, CA, USA). Mouse IgG antibody against clathrin heavy chain (X22) was a kind gift from J.J. Hölzenspies (Utrecht University, the Netherlands).

In vitro maturation (IVM) and in vitro fertilization (IVF)

Sow (*Sus scrofa*) ovaries were obtained from adult fertile sows at a special slaughterhouse (Groenlo, the Netherlands). Cumulus-oocyte complexes (COCs) were collected by aspiration of 3-6 mm follicles and subsequently selected using well-established morphological criteria [16]. *In vitro* maturation (IVM) was performed as previously described [16]. COCs were collected in HEPES buffered M199 (Gibco Laboratories Inc., Grand Island, NY, USA) and washed in pre-equilibrated M199 supplemented with 2.2 mg/ml $NaHCO_3$, 0.1 % (w/v) polyvinylpyrrolidone (PVP), 100 µM cysteamine, 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulphate (oocyte maturation medium; OMM). Selected COCs were cultured at 38 ºC with 5%

CO_2 for 22 h in OMM supplemented with 0.05 IU/ml recombinant human FSH (rhFSH, a kind gift from Organon, Oss, the Netherlands). After 22 h IVM, COCs were transferred into OMM without rhFSH for another 22 h to reach meiosis II stage. The cortical reaction was induced by *in vitro* fertilization (IVF) of co-incubating M-II oocytes with freshly ejaculated porcine sperm cells (from fertile boars, Cooperative Boar Artificial Insemination Center, Bunnik, the Netherlands). Based on overall morphology and DNA staining pattern -assessed as described before [17] by confocal laser scanning microscopy (Leica TCS SP2, (Leica Microsystems GmbH, Wetzlar, Germany). Oocytes could be divided into three categories: (1) germinal vesicle (0h, GV), as indicated by the presence of the germinal vesicle structure, (2) meiosis-II (M-II, 44h after IVM), as indicated by the presence of the metaphase plate and the first polar body, (3) fertilized (52h, IVF), as indicated by the presence of two pronuclei. Oocytes that could not be categorized according to criteria described above were excluded from this study.

Immunoblotting

Protein concentrations from all samples were standardized according to the Lowry method [18]. For both sperm and control brain samples, 15 µg of total protein extract was mixed with an appropriate amount of lithium dodecyl dulfate (LDS) loading buffer (Invitrogen, Carlsbad, CA, USA) in the presence of 0.1M dithiothreitol (DTT). Cumulus-free oocytes were obtained as previously described [17] and 100 oocytes were used per sample. All samples were heated for 10 min at 100 ºC prior to immunoblotting. Proteins were separated in a 4% stacking and 12% running SDS-PAGE gel and wet-blotted onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK). After blocking for 1 h with ReliaBLOT® (Bethyl Laboratories, Inc., Montgomery, TX, USA) at room temperature (RT) blots were incubated with primary antibodies diluted in ReliaBLOT® overnight at 4 ºC. After washing the blots in TBS (Tris-buffered solution; 50 mM Tris, 250 mM sucrose, pH 7.4) with 0.2 % v/v Tween-20 (TBST), secondary antibodies were added for 30 min. After rinsing with TBST, protein was visualized using chemiluminescence (ECL-detection kit; Supersignal West Pico, Pierce, Rockford IL, USA).

Labeling of cortical granules (CGs)

Labeling of cortical granules was performed as previously described [1;19] with minor modifications. For efficient lectin binding, partially denuded oocytes were used. To remove surrounding cumulus cells, oocytes of different maturation stages were gently pipetted in HEPES buffered M199; partially denuded oocytes were separately collected and washed two times in HEPES buffered M199. After washing, oocytes were fixed in freshly prepared fixative (4 % (v/v) para-formaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS) for 1 h at RT. After fixation, oocytes

were washed twice in PBS-PVP (0.1 M PBS, pH 7.4 containing 0.3 % (w/v) PVP) and permeabilized in PBSS (0.1 M PBS, pH 7.4 containing 0.1 % Saponin) for 10 min. Aspecific binding sites were blocked using blocking buffer glycine (PBSS with 1% (w/v) BSA, 2% (v/v) normal goat serum (Vector Lab, Burlingame, CA) supplemented with 100 mM glycine) for 2 h at RT. After rinsing in PBS, oocytes were incubated in fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC; EY laboratory, San Mateo, CA) diluted in PBSSc (PBSS containing 0.1g/l $CaCl_2$) for 1h, RT in dark. As negative control, PNA-FITC was pre-incubated in PBSSc containing 100 mM lactose for 30 min at RT before use.

Immunofluorescent staining for SNARE proteins, complexin and clathrin

Immunolabeling was performed as previously described [17]. Both primary and secondary antibodies were diluted in blocking buffer and centrifuged at 100,000 g for 1 h before use to prevent aggregation of the antibody. Oocytes were incubated with primary antibodies overnight at 4 ºC. As negative controls, purified mouse or rabbit IgG (BD Biosciences) matching the host species of primary antibodies were used and the dilutions of negative controls were identical to the dilution of the primary antibodies used in the same experiment. Oocytes were rinsed three times in PBS after primary antibodies incubation. SNARE-labeled oocytes were subsequently incubated with Alexa-568 conjugated goat anti mouse IgG or goat anti rabbit IgG (Molecular Probes, Eugene, OR) for 1 h at RT. DNA was labeled with 10 µM ToPro-3 iodide (Molecular Probes, Leiden, the Netherlands) in PBSS for 20 min. After three rinses in PBSS, oocytes were mounted in a 0.12 mm, 8 well Secure-Seal Spacer (Molecular Probes, Leiden, the Netherlands) on a cover slip, covered in a drop of Vectashield (Vector Lab, Burlingame, CA) and sealed with a microscope slide (Superfrost Plus; Menzel, Braunschweig, Germany).

Confocal laser scanning microscopy and image acquisition

Images were obtained through a 40x oil immersion objective using a Leica TCS SP2 confocal system (Leica Microsystems GmbH, Wetzlar, Germany), equipped with 488, 568, and 633 nm lasers. Dual and triple channel images were obtained by sequential scanning. ImageJ (NIH; http://rsb.info.nih.gov/ij/) software was used for analysis of images. Laser power and acquisition settings were adjusted to produce submaximal pixel values in the oocyte and settings used to image control IgG stainings were matched to the highest settings used to image primary antibody staining in the same experiment. Images presented in this study were selected based on the presence of representative DNA structures (germinal vesicle for GV stage and metaphase plate/first polar body for M-II stage) at the equatorial region of the oocyte. Background subtraction and contrast/ brightness enhancement (up to ~20% enhancement using

the maximum slider in Image J) were performed identically for all images in the same experiment.

Results

Distribution of SNARE proteins in porcine oocytes

To observe if and how SNARE proteins are organized just prior to the cortical reaction, we used meiosis-II arrested oocytes (the final maturation stage of mammalian oocytes leaving them ready to become fertilized by sperm). With indirect immunofluorescence and immunoblotting techniques, we demonstrated that the complementary SNARE proteins syntaxin 2, SNAP 23, VAMP 1 and VAMP 2 were present in porcine M-II oocytes, whereas syntaxin 1, SNAP 25 and VAMP 3 were absent (Fig. 1A). Therefore, all SNARE members that are required to form a SNARE complex which enables intracellular membrane fusion were present in porcine oocytes.

In M-II oocytes, SNARE proteins were predominately observed in the cortical region of the oocyte and showed a punctuate staining pattern which was characteristic for SNARE proteins (Fig. 1A). The fact that SNARE staining patterns coincided with that of the PNA-FITC (Fig. 1B) indicated an association of SNARE proteins with the cortical granules. To our surprise, immunoblotting studies showed that porcine oocytes did not contain SNAP 25 and only the low molecular weight form of VAMP 1 (similar to porcine sperm, Fig. 1A immunoblotting), which was in contrast to our observations in control brain tissues and the report on mouse oocytes [13]. Moreover, porcine oocytes did not contain VAMP 3 and had only a lower molecular weight form of SNAP 23 (Fig. 1A, immunoblotting).

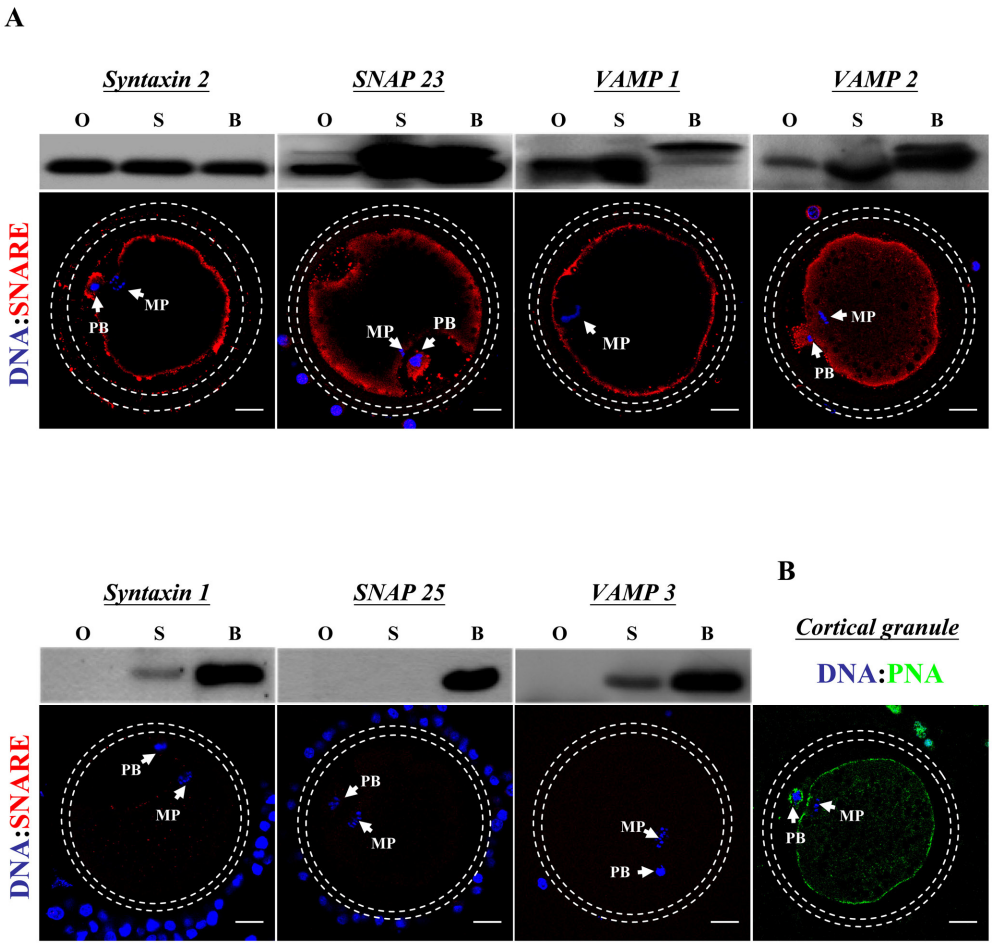

Figure 1 Cortex orientation of SNARE proteins in M-II porcine oocytes
(A) Immunofluorescent labeling of Q-SNARE proteins (red) syntaxin 2 and SNAP 23, R-SNARE proteins (red) VAMP 1 and VAMP 2 in M-II porcine oocytes (O) and the absence of labeling for syntaxin 1, SNAP 25 and VAMP 3. Western blot detection of SNARE proteins present in the M-II oocytes show comparable molecular weights as in control brain tissues (B) and of boar sperm cells (S). **(B)** PNA-FITC staining (green) indicates a defined cortex arrangement of cortical granules adjacent to oolemma at M-II arrested oocyte. PB: first polar body. MP: metaphase plate. The dashed circles indicate the area of zona pellucida. Chromatin was stained with Topro-3 (blue). Bars represent 20µm.

Peripheral migration of secretory granules and VAMP during oocyte maturation indicate SNARE-mediated docking of cortical granules to the oolemma

Since we found that SNARE proteins are enriched in the cortex of M-II arrested oocytes, we further analyzed whether this localization pattern was caused by the

redistribution of SNARE proteins during oocyte maturation. To this end, immature GV stage oocytes were harvested from follicles and were stained for the SNARE proteins that have been detected in figure 1. Simultaneously, cortical granules were identified by secretory granule specific binding lectin PNA-FITC labeling. During maturation, cortical granules moved to a sharply defined region under the oolemma as shown by changes in PNA labeling (Fig. 2A1-2). A similar relocalization was observed for VAMP 1 labeled structures (Fig. 2B1-2). SNAP 23 labeled structures, however, showed a similar peripheral redistribution but were not present under the oolemma (Fig. 2C1-2). In contrast to the maturation-dependent redistribution of secretory granules and the above mentioned SNARE proteins (VAMP 1 and SNAP 23), we observed syntaxin 2 labeled structures did not show the relocation upon meiotic maturation and were observed to associate with the oolemma under all conditions tested (Fig. 2D1-2).

The noted concentration of VAMP 1 and PNA positive structures just under the surface of the oolemma in M-II stage oocytes suggested that the cortical granules become docked at the oolemma by the use of SNARE proteins. SNARE-mediated membrane priming and docking requires additional components such as complexin to stabilize the formed SNARE complex [15]. We subsequently analyzed maturing oocytes for the presence of complexin. Our results showed that the presence of complexin was confined to a sharply defined region underneath the oolemma in M-II oocytes (Fig. 2E2). However, in contrast to the redistribution of PNA and VAMP 1 positive structures, no redistribution of complexin was observed since it was already present near the oolemma in the GV stage oocytes (Fig. 2E1). This suggests that docking of secretory granules to the oolemma is mediated by SNARE proteins and the subsequent involvement of complexin present in close proximity to the oolemma could contribute to the stabilization of this interaction. To sustain this possibility, we performed a series of co-localization studies in both immature (GV) and matured (M-II) unfertilized oocytes. We found in the majority of the immature oocytes, only the partial co-localization of PNA containing cortical granules with syntaxin 2 and VAMP 1 underneath the oolemma (Fig. 3 arrows). However, in the matured M-II oocytes, the co-localization between PNA positive structures (cortical granules) and SNARE proteins was substantially increased at the oolemma over the entire surface area (Fig. 4 indicated with arrows and enlarged panels) and this area exclusively matches the specific site where complexin was detected.

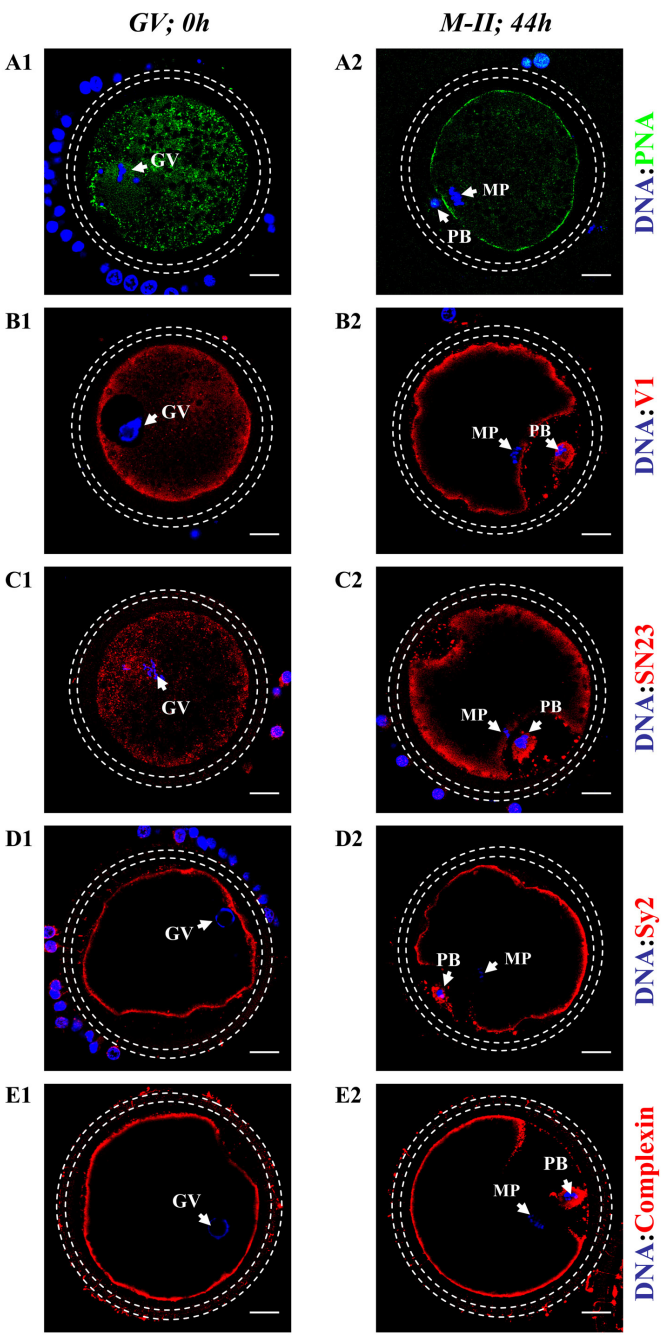

Figure 2 Secretory granules and SNARE proteins migrate to the oocyte surface upon meiotic maturation.
PNA-FITC was used to label secretory granules (green) and to demonstrate the migration of these granules upon oocyte maturation. Antibodies against different SNARE proteins and SNARE regulator (complexin) were used to indicate the localization of these proteins (red) at different maturation stages. DNA of the maturating oocytes was labeled with ToPro-3 (blue). Oocytes of two maturation stages (0h: GV and 44h IVM: M-II) were used. PNA positive secretory granules and SNARE positive membrane vesicles showed dispersed punctuate pattern throughout the entire ooplasm at GV stage **(A1-C1)**, syntaxin 2 was observed already in association with oolemma in GV oocyte **(D2)**. Both PNA positive secretory granules and SNARE positive membrane showed either pronounced oocyte surface-enriched (or oolemma-associated) staining pattern **(A2-B2, D2)** or was concentrate to the cortex of the oocyte in M-II arrested oocytes **(C2)**. Complexin in both GV and M-II stages appeared sharply plasma membrane associated pattern **(E1-2)**. GV: germinal vesicle; PB: first polar body; MP: metaphase plate. V1: VAMP 1; SN23: SNAP 23; Sy2: syntaxin 2. Dashed circles indicate the relative position for zona pellucida. Representative images of three independent experiments (n=20 per group per experiment) are presented. Bars represent 20µm.

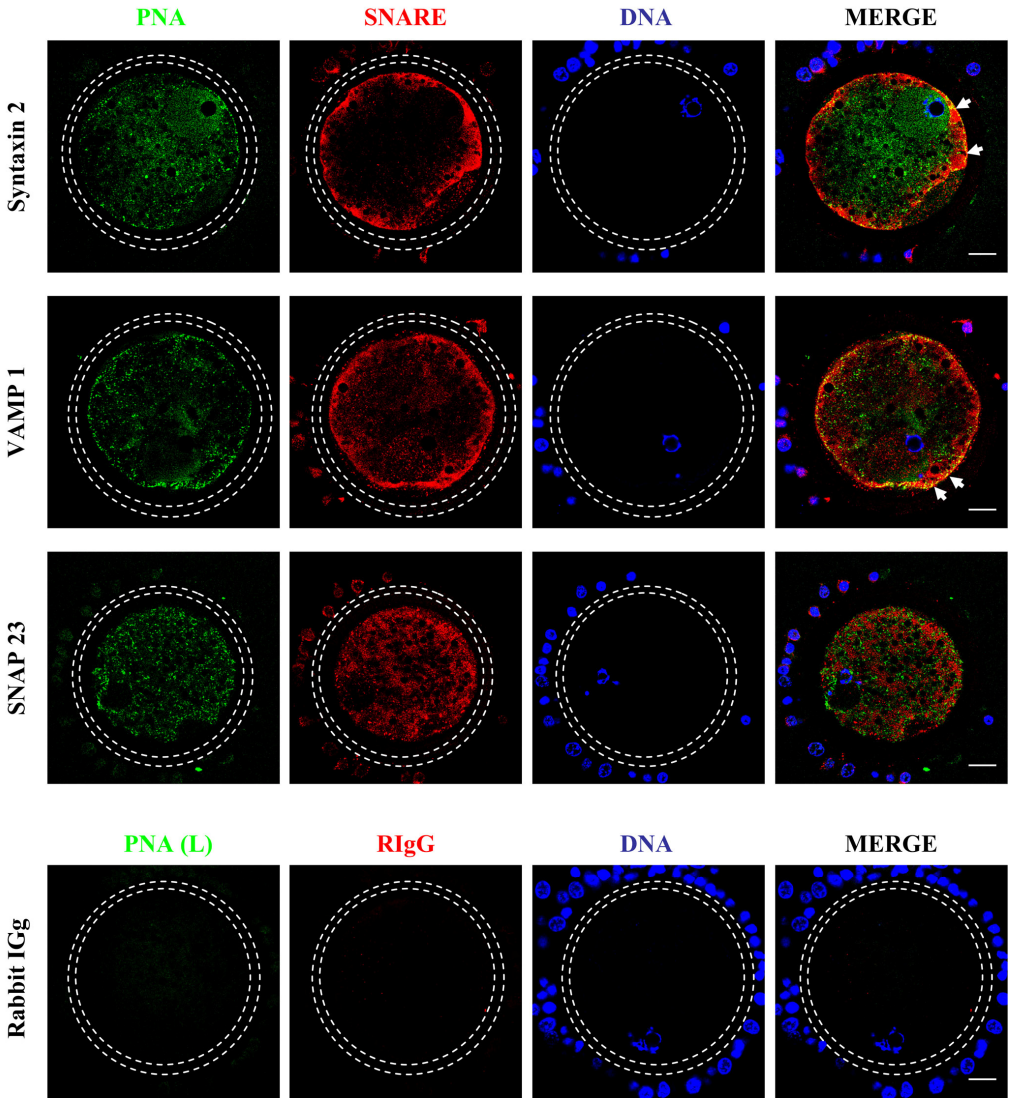

Figure 3 Minimal co-localization of cortical granules and SNARE proteins in GV oocyte.
Oocytes of germinal vesicle stage were used to demonstrate the distribution of cortical granules (labeled with PNA-FITC; red) and SNARE proteins (indirect immunolabeling; green). Chromatin was stained with ToPro-3 (blue). The majority of the secretory granules did not associate with SNARE proteins in GV arrested oocytes, minimal of co-localization between cortical granules and SNARE proteins can be observed just underneath the oolemma. Rabbit IgG was used as negative control for SNARE proteins and showed the absence of signal. PNA (L): PNA binding sites were inhibited with 100mM lactose and used as negative control for PNA labeling. Dashed circles indicate the relative position for zona pellucida. Representative images of three independent experiments (n=20 per group per experiment) are presented. Bars represent 20μm.

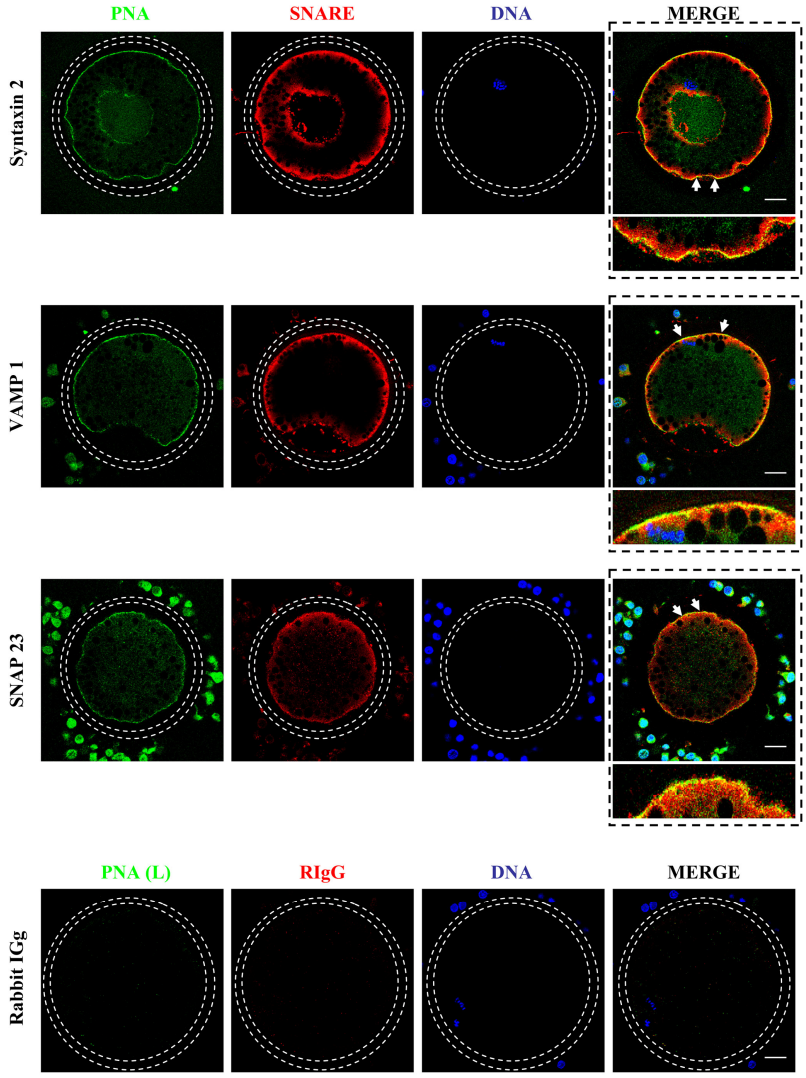

Figure 4 Cortical granules are docked to the oolemma and co-localized with SNARE proteins in M-II arrested oocytes.
Oocytes of meiosis-II stage were used to demonstrate the co-localization of cortical granules (labeled with PNA-FITC; red) and SNARE proteins (indirect immunolabeling; green). Chromatin was stained with ToPro-3 (blue). Cortical granules are positioned in the close proximity to the oolemma and showed co-localization (indicated with arrows) with SNARE proteins over the entire oocyte surface. Areas indicated with arrows are showed in the enlarged panels for the indication of co-localization. Rabbit IgG was used as negative control for SNARE proteins and showed the absence of signal. PNA (L): PNA binding sites were inhibited with 100mM lactose and used as negative control for PNA labeling. Dashed circles indicate the relative position for zona pellucida. Representative images of three independent experiments (n=20 per group per experiment) are presented. Bars represent 20µm.

Functional implications of cortical granule docking to the oolemma for endocytosis in maturing oocytes

Cortical reaction is a calcium-dependent exocytotic process and in most of the cell types, exocytosis and endocytosis are indirectly coupled processes. Therefore, we extended our investigations with the immunolocalization of endocytotic proteins during oocyte maturation. Immunolocalization of clathrin (for clathrin-dependent endocytosis) showed that clathrin was not only present at the oolemma but also in intracellular vesicular structures in GV oocytes (Fig 5A1). Clathrin migrated to the same sharply defined area just under the oolemma during oocyte maturation (Fig. 5A2). This indicated that clathrin was targeted to the oolemma (and/or oolemma docked structures) in M-II oocytes and was not sequestered anymore by endocytosis or released deeper in the oocyte. Another protein-mediated endocytotic pathway is initiated by the formation of membrane caveolae; structures enriched with flotillin and caveolin. Both membrane raft marker proteins were present in oocytes but did not show obvious redistribution upon oocyte maturation (Fig. 5B-C).

Cortical reaction leads to clathrin and complexin dissociation from the oolemma

The fusion of a sperm cell with the oocyte leads to the initiation of the CR. During the CR, cortical granules fuse with the oolemma and because of the resulting enlargement of the oocyte surface, a compensatory reduction of this surface must be accomplished by the immediate induction of endocytosis, which enables the re-establishment of proteins involved in membrane transport. For this reason, we have analyzed the fate of SNARE proteins, complexin and clathrin in fertilized oocytes until 8 hrs after *in vitro* fertilization. At this stage, fertilized oocytes contain both a male and female pronucleus (Fig. 6A). As expected, the intracellular PNA staining decreased below the detection level when compared to M-II oocytes as the cortical granules fused with the oolemma and the granule contents were released and diluted (Fig. 6A).

In the fertilized oocytes, the CR coincided with the release of complexin from the oocyte's surface into the cytosol (Fig. 6B; and partly associated to lipid droplets), most likely due to the dissociation of complexin from the SNARE protein complex. When SNARE distributions of fertilized and unfertilized mature M-II oocytes were compared, VAMP 1 was present at intracellular structures (Fig 6C) indicated that VAMP 1 was endocytosed after fertilization. The noted release of clathrin into the cytosol may be of importance for re-establishing endocytosis at the oolemma after the CR (Fig 6D).

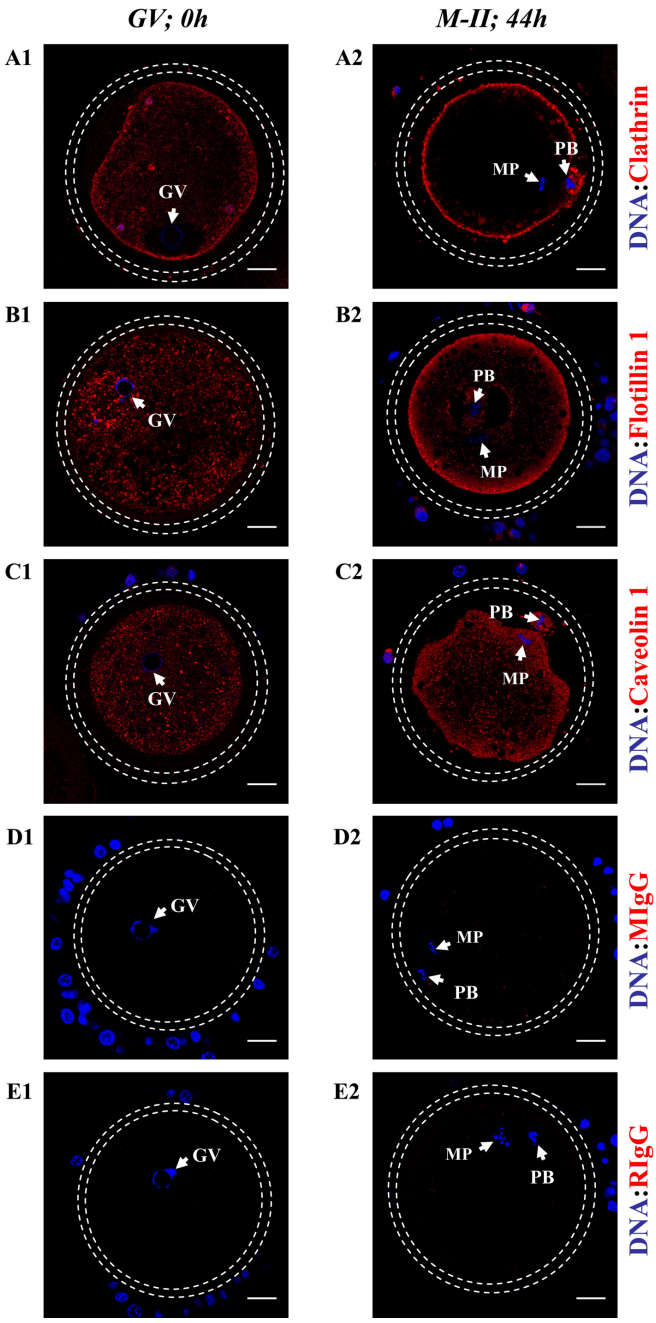

Figure 5 Clathrin-dependent endocytosis is arrested in meiosis-II oocyte.
Indirect immunolabeling of endocytosis related proteins (red) and chromatin (blue) in two maturation stages (GV and M-II) oocytes. **(A)** Clathrin migrated from cytoplasm (A1) to the surface of the oocyte where it becomes arrested adjacent to the oolemma at M-II stage (A2). **(B1-2)** Flotillin-1 concentrates to the cortex region of the oocyte, but does not associate to or reside underneath the oolemma. **(C1-2)** Caveolin 1 does not show meiotic maturation-dependent redistribution. **(D1-2; E1-2)** Purified mouse IgG (MIgG) or rabbit IgG (RIgG) were used as negative controls and showed the absence of signal. GV: germinal vesicle; PB: first polar body; MP: metaphase plate. Dashed circles indicate the relative position for zona pellucida. Representative images of three independent experiments (n=20 per group per experiment) are presented. Bars represent 20μm.

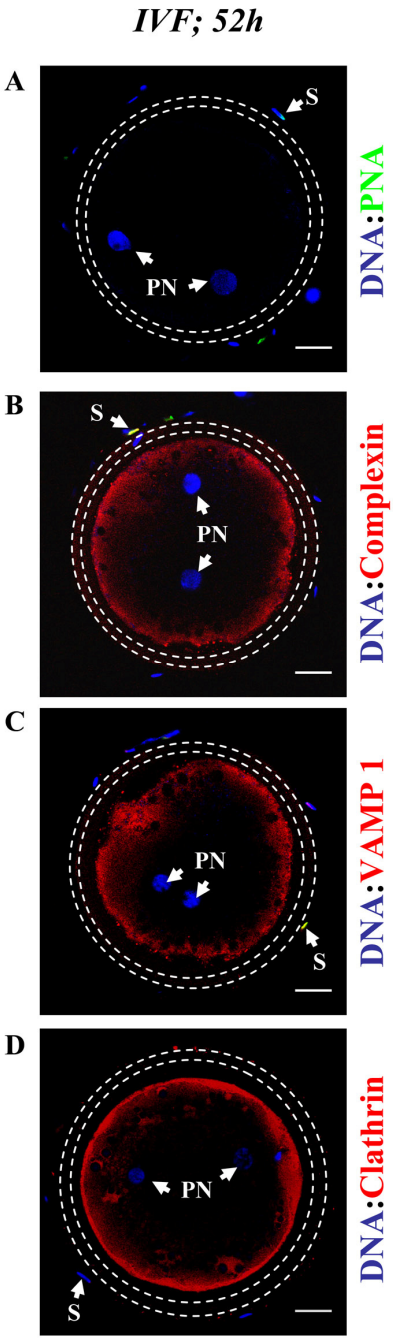

Figure 6 Cortical reaction releases enzymatic content and initiates the endocytosis-driven recycling of VAMP 1 and the disassociation of complexin into cytosol.
Indirect immunolabeling of endocytosis related proteins (red), chromatin (blue) and cortical granules (green, stained with PNA-FITC) in oocytes eight hours after *in vitro* fertilization. **(A)** The cortical reaction has taken place (absence of PNA labeling). **(B)** Complexins are released from the SNARE complex after sperm-induced fertilization and therefore lost their oolemma specific orientation. A portion of complexin appears to be liberated in the cytosol while another part the labeling is detected around lipid droplets. **(C)** VAMP 1 is endocytosed into a deeper area of the oocyte as the defined oolemma specific pattern is lost and VAMP1 is detected at intracellular structures inside the ooplasm. **(D)** Clathrin is released from the oocytes surface after fertiization and diffuses into the oocyte's cytosol when compared to the oolemma-specific pattern in M-II oocytes. IVF: *in vitro* fertilization; PN: pronuclei. S: sperm cell (indication of IVF). Dashed circles indicate the relative position for zona pellucida. Representative images of three independent experiments (n=20 per group per experiment) were presented. Bars represent 20µm.

Discussion

Precise regulation on the cortical reaction is crucial since premature or spontaneous cortical reaction blocks the entry of the sperm cell and belated cortical reaction leads to polyspermic fertilization, both results in the failure of fertilization. The cortical reaction is executed immediately after the incidence of sperm fusing with the oolemma [3] and involves fusion of a multitude of cortical granules with the oolemma. Most intracellular membrane fusion events are driven by a conformational change of ternary *trans*-SNARE complexes on the two interacting membranes into a *cis*-SNARE complex merging the two fusing membranes (for review see [20]). Therefore, in this study we have investigated matured oocytes that are ready to be fertilized and detected the presence and

the spatial organization of SNARE proteins in relation to the secretary granules.

The maturation-dependent migration of secretory granules to the cortical area of the oocyte serves to prepare the oocyte surface for the cortical reaction. After their arrival at the oolemma in M-II oocyte, these cortical granules await for the proper signal (i.e. Ca^{2+}-oscillations in the ooplasm induced by sperm specific phospholipase C zeta [5;21]) without the occurrence of spontaneous exocytosis. It is known that SNARE and their associate proteins (e.g. complexin, synaptotagmin) regulate acrosome reaction in the sperm cell [12;22]. Thus the interactions between SNARE proteins and their regulators ad well as their importance for the regulation of the cortical reaction was studied. SNAP 25 has been shown to be required for cortical granule exocytosis that the sperm-induced CG exocytosis is significantly inhibited in botulinum neurotoxin A (BoNT/A) treated mouse oocytes [13]. However, the actual mechanism behind the regulation of cortical reaction is essentially unknown. The absence of SNAP 25 in the porcine oocyte (this study) indicates that gametes from different mammals employ distinct subsets of SNARE proteins for this highly specialized exocytosis process. Further support for the idea that SNARE proteins are involved in the CR is the detection of SNARE complex composing proteins (syntaxin 2, VAMP 1 and SNAP 23) at the restricted cortical region upon meiotic maturation and the significantly increase in co-localization of SNARE proteins with the cortical granules at the oocyte's surface. The consistent presence of complexin at the oolemma further also sustains the possibility that SNARE protein complexes are stabilized by complexin and that this interaction could prevents premature or spontaneous cortical reaction.

Complexin has been shown to associate with ternary *trans*-SNARE complex and can subsequently inhibit the fusion reaction of the interacting membranes [15;23]. We found that complexin is released to the cytosol with a concomitant occurrence of VAMP1 endocytosis that was detected in intracellular membrane structures. This indicates that SNARE-complexin interaction contributes to the temporary arresting of the docked cortical granules at the oolemma and thus further prevents the spontaneous fusion of granule membrane with the oolemma (this has been reported for other cell types [15]). The fertilization-dependent increase in cytosolic Ca^{2+}-levels probably releases complexin from the SNARE complex and thereby allows the onset of the SNARE-mediated cortical reaction. Most likely, the dissociation of complexin is mediated by Ca^{2+} mobilization from the oocyte's ER by soluble signaling factors originating from the fertilizing sperm [21]. Consequently, during the CR, Ca^{2+}-dependent dissociation of complexin from the *trans*-SNARE complex triggers the onset of massive exocytosis of granules. The CR-induced release of complexin into cytosol can lead to interactions of complexin with SNARE proteins at intracellular membranes.

Exocytosis and endocytosis are indirectly coupled processes that occur simultaneously in cells in order to maintain a similar size in cell surface area and cell volume. Therefore, if exocytosis is inhibited by complexin this also will inhibit endocytosis in M-II oocytes. Of interest is that clathrin concentrates in the same area where the cortical granules and the oolemma are docking and stabilized with complexin. Probably this serves to allow a rapid onset of endocytosis at the oocyte surface to compensate for the surface enlargement which explains the accumulation of surface-associated clathrin in matured unfertilized (M-II) oocyte. Taken together, the oocyte undergoes preparative changes during meiotic maturation which prepare its surface for the cortical reaction and the subsequent compensatory endocytosis that required for the re-establishment of normal cellular processes. The less apparent migration of caveolin and flotillin during meiotic maturation may indicate that caveolin- and flotillin-mediated endocytosis are not directly involved in the compensation of the enlarged oolemma surface that results from the CR but instead, these proteins are more relevant to the later development of the embryo [24].

The absence of PNA binding site in fertilized oocytes was due to the release of granule contents during the exocytosis of cortical granules, intriguingly, the released PNA was also not detected in the peri-vitelline space after CR and might be explained by (i) a dilution and diffusion of PNA binding sites through the zona pellucida, (ii) a post-CR modification of the lectin binding site that reduces or eliminates the affinity for PNA.

In conclusion, we showed that secretory granules required for the cortical reaction become associated with a SNARE complex containing syntaxin 2, VAMP 1 and SNAP 23 in M-II arrested oocytes. We hypothesize that the docked CGs are stabilized and temporary arrested by complexin in order to prevent pre-fertilization exocytosis (which would cause a premature fertilization block and results in the failure of fertilization). This then also indirectly shuts down membrane recycling by arresting clathrin-mediated endocytosis at the oocyte surface. Once these processes are resumed, namely immediately following fertilization, the induced CR allows (1) secretion of PNA binding sites, (2) compensatory recycling of VAMP 1 containing membranes vesicles, (3) dissociation of clathrin and complexin from the oocyte's surface into the cytosol and the subsequent re-association of clathrin and complexin with intracellular membranes. Both VAMP 1 recycling as well as clathrin and complexin redistribution are considered relevant for renewed membrane trafficking in the fertilized oocyte. This is the first report showing the dynamics of SNARE proteins and complexin to form an arrested docking stage of cortical granules at the surface of the maturing oocyte. Furthermore, it shows the fertilization-dependent removal of complexin and clathrin in order to execute an immediate cortical reaction to achieve a polyspermic fertilization block.

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Chapter 7

Summarizing Discussion

Fertilization is an event that involves highly dynamic intra- and intercellular processes which enable the merge of two gametes with the aim to ultimately form a new organism. Preparative changes are required in both gametes prior to fertilization since freshly ejaculated sperm cells and germinal vesicle (GV) stage arrested oocytes are not capable to form a zygote. Only functionally matured sperm can fertilize the oocyte and specific modifications have to be accomplished at the surface of the sperm head. Like sperm, the oocyte has to be prepared for gamete fusion and to this end resumes meiosis prior to ovulation. Immediately after sperm binding to the oocyte plasma membrane (oolemma), the oocyte initiates a restrictive process - the cortical reaction (CR) - to prevent polyspermic fertilization which is incompatible with subsequent normal development of the zygote. Aberration of any of these processes will lead to the failure of fertilization; therefore, the aim of this thesis is to contribute to the knowledge of specific intracellular processes (namely the acrosome reaction in the sperm cell and the CR in the oocyte) that are crucial in gametes for successful fertilization.

Constant remodelling of the sperm surface during epididymal maturation and migration in the male and female genital tract allow sperm cells to become functional mature. This reorganization of proteins and lipids in the sperm plasma membrane (PM) results in the assemblage of functional membrane protein complexes and the aggregation of membrane microdomains (membrane rafts); the subsequent activation of signalling pathways are also critical for the sperm cell function. Therefore, the current understanding of molecular kinetics of proteins at the surface of porcine sperm before and after fertilization is reviewed in *chapter 1*. Membrane fusion is a crucial event in many intracellular processes including in the acrosome reaction (AR) and the CR and is governed by the interactions of specific sets of proteins called “SNAREs” (soluble N-ethylmaleimide-sensitive factor [NSF] attachment protein receptors). In this thesis, the role of SNARE proteins in pre- and post-mammalian fertilization processes is investigated with special emphases on the SNARE-mediated exocytosis in gametes. To this end, the general concept of SNARE interactions that facilitate membrane fusion and the important SNARE interacting and regulating molecules are reviewed in *chapter 2*. This chapter also does focus on the knowledge on the SNARE proteins-regulated AR in sperm cells and the putative function of SNARE proteins in the CR of the oocyte. The porcine species has served as animal model because in contrast to other mammalian species, relatively large quantities of mature gametes can be obtained from the boar and the sow; the porcine species is therefore an excellent animal allowing a biochemical and cell biological experimental approach to study gamete exocytosis. The events at the sperm cell surface that are relevant for the AR are studied (*chapter 3-5*). In addition, oocyte maturation and post-fertilization events which are relevant for the CR and the block for polyspermic fertilization of the oocyte are investigated in *chapter 6*.

Dynamics of membrane microdomains and SNARE proteins during sperm capacitation

Before the sperm cell meets the oocyte, it must undergo extensive and continuous reorganization; this process (a collective term called capacitation) enables morphologically mature sperm cells to acquire the ability to fertilize the oocyte [1]. After sperm cells are capacitated, they can undergo a sperm specific type of exocytosis (the acrosome reaction). This calcium-dependent multipoint fusion of the PM and the outer acrosome membrane (OAM) at an extended area of the sperm head surface occurs once in the life span of a sperm cell and the subsequent release of acrosomal enzymes is essential for sperm penetration of the oocyte's zona pellucida (ZP). Upon capacitation, the raft marker proteins flotillin and caveolin migrate to the apical ridge of the sperm head [2], the specific site where the AR is initiated after the sperm binding to the ZP. The distribution of SNARE proteins that are required for the AR was investigated (*chapter 3*) and uncovered that upon capacitation, SNARE proteins (syntaxin and VAMP) also redistribute to the apical area of the sperm head. Thus the SNARE proteins migrate to the same defined sperm head area as where the raft marker proteins move to [2]. Furthermore, the bicarbonate- and albumin-dependent redistribution of these SNARE proteins into detergent resistant membrane (DRM; considered to reflect pre-existing 'membrane rafts' in live cells) fractions during capacitation suggests a functional link of membrane rafts in the regulation of SNARE interactions in the sperm cell.

Two possible mechanisms may explain the migration pattern of SNARE proteins into membrane rafts that is observed during sperm capacitation:
(1) *SNARE-RAFT interactions:* one of the causes of membrane raft reorganization is the loss of sterols [3] and the loss of cholesterol from the sperm surface upon capacitation has been shown to occur exclusively in the non-raft area [4], thus the non-affected cholesterol- and sphingolipid-content at the raft area of capacitating sperm cells could serve as the driving force for the recruitment of SNARE proteins into membrane rafts. This cholesterol-dependent lateral segregation is likely due to the fact that the rigid sterol ring favors interaction with straighter, stiffer hydrocarbon chains of saturated lipids [5]. Most of the SNARE proteins have multiple palmitoylated cysteine residues (e.g. SNAP 23 with 6 palmitoylation sites and VAMP 3 with 2 of these sites). These covalently linked saturated acyl chains may give SNARE proteins a stronger lipid anchor property and as a result, such anchored proteins favorably concentrate in lipid-ordered membrane microdomains that are composed of cholesterol, glycolshpingolipid and saturated glycerophospholipids [6].
(2) *SNARE-SNARE interactions:* syntaxins either have one (syntaxin 2) or none (syntaxin 3) palmitoylation sites -unlike SNAP or VAMP (see (1)) and are, therefore, less favorable to be targeted to the membrane raft. However, by interacting with the

more heavily palmitoylated SNARE proteins, syntaxin may be recruited into membrane raft upon capacitation. This can be tested by incubating cells with botulinum neurotoxin E that has been shown to disrupt SNAP 25/syntaxin 1A interactions (it cleaves the C-terminus of SNAP 25 which interacts with syntaxin) in bovine chromaffin cells and thus compromised the co-redistribution of these proteins [7].

Membrane rafts have been shown to function as signaling platforms for intracellular processes (for review see [8]). Upon capacitation, the emergence of SNARE proteins in the membrane rafts may safeguard SNARE interactions for subsequent biological events. The consequences of SNARE migration to the apical area of the sperm head as well as their redistribution into membrane rafts upon capacitation were further investigated in *chapter 4-5*.

Capacitation induced SNARE interactions seal the apical sperm head surface to the acrosome membrane

Capacitation significantly alters the ultrastructure of the apical PM and the OAM resulting in the close apposition of these two membranes (*chapter 4*). Ultrastructural studies and biochemical investigations revealed that the acrosomal membrane material together with the PM were co-purified as bilamellar vesicles from capacitated sperm cells, confirming the tight and stable interaction of the PM and the OAM induced by sperm capacitation. Further immunochemical studies revealed not only the pairwise SNARE interactions upon capacitation but also the capacitation-dependent formation of *trans*-SNARE complexes. Moreover, these SNARE interactions were observed exclusively in the membrane samples that solely contained the apical sperm head membranes. The reorganization of SNARE proteins as well as the tight interactions between stable SNARE complexes at the apical sperm head lead to the priming and docking of the PM with the OAM and are important for the AR. The area where these proteins and membrane rafts concentrate is exactly matching the ZP binding site and the initiation site for AR. The stable primed and docked membranes without the occurrence of spontaneous fusions is crucial for the sperm cells to prevent a premature AR, but to be prepared to undergo AR upon proper stimulation (e.g. ZP *in vivo* or calcium-ionophore *in vitro*).

Preventing premature acrosome reaction: functional relevance of membrane rafts for SNARE interactions

A further investigation on the intriguing finding namely that sperm capacitation did lead to the priming and docking of PM and OAM but that this interaction did not result in fusion and subsequent release of the acrosomal content (*chapter 4*) by studying whether complexin plays a role in arresting the docking of the PM to the OAM (*chapter 5*). Complexin has been shown to associate with the SNARE complex and by this interaction prevents the formation of a *cis*-SNARE complex thus appears to inhibit the fusion of the interacting membranes [9]. Indeed, the study *(chapter 5)* supported the fact that following the capacitation-dependent formation of *trans*-SNARE complexes, complexin 2 becomes associated with the *trans*-SNARE complex and then not only stabilizes the formed *trans*-SNARE complex but in this way also prevents the spontaneous fusions of the PM and the OAM. In addition, complexin is released from the SNARE complexes during the AR in a Ca^{2+}-dependent manner, likely via a calcium-dependent conformational change of SNARE complexes. Moreover, the complexin-SNARE complexes which were specific for capacitated sperm and appeared to be raft-specific as complexin is present only in the floating DRM fractions representing the membrane rafts is shown.
Proteomic identifications of purified mixed vesicles (MV), formed after the fusions of the PM and the OAM during the AR, gave additional protein IDs which have a putative function in acrosome exocytosis. The most important identified protein is synaptotagmin 4 that could operate as the calcium sensor in porcine sperm and might be important for the subsequent release of complexin from the SNARE complexes upon elevation of cytosolic calcium. Another protein of interest is the syntaxin binding protein 2 (Munc 18b) which might be involved in docking and/or fusion of the OAM with the PM and in the release of complexin. Acrosomal exocytosis is physiologically imposed by sperm-ZP binding and results in the *trans* to *cis* conformational change of the SNARE complex upon AR (a Ca^{2+}-dependent process) and can also be induced by calcium ionophore treatments. Remarkably, control sperm (i.e. where the OAM is not docked to the PM) also showed acrosome exocytosis after calcium-ionophore treatment thus illustrating that the capacitation-induced membrane raft-dependent PM and OAM docking is not a prerequisite for calcium-mediated acrosome exocytosis. The explanation of the capacitation-dependent membranes docking without subsequent acrosome exocytosis is that the sperm has prepared itself for an instant response to the physiological induction of the AR (e.g. by the ZP binding). The fact that complexin 2 containing SNARE complexes are only present in capacitated sperm and are exclusively recovered from the DRM (representing the raft specific membrane fractions) indicate that the zona binding region of the sperm surface [2;10] - where membrane rafts and SNARE proteins aggregations have been identified (*chapter 3*) -

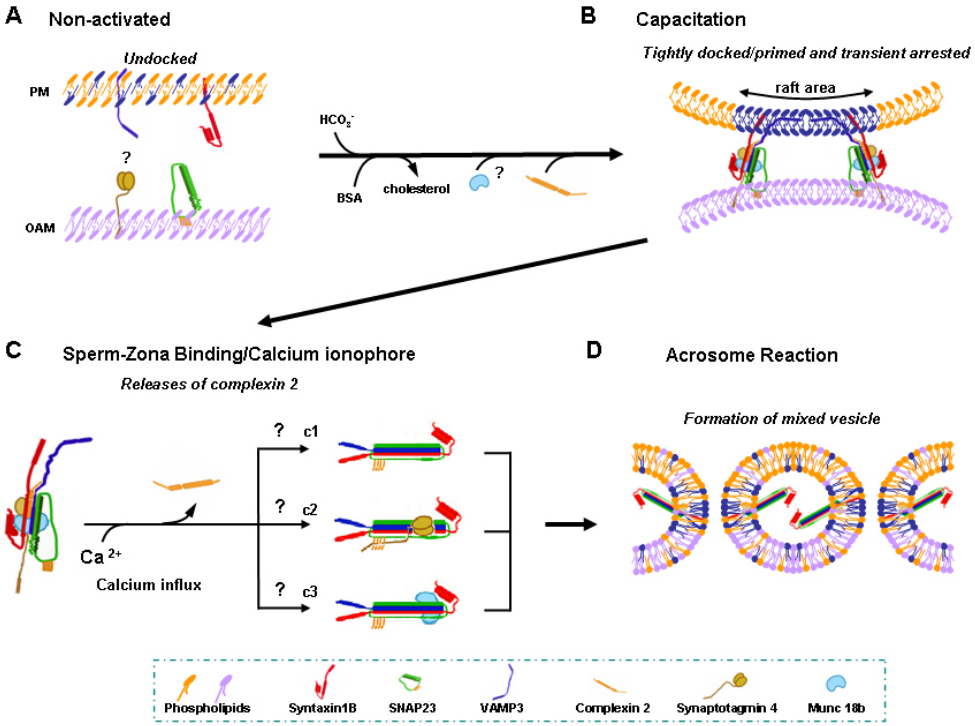
is involved in the stabilization process of the docked membranes. The temporary silencing of the AR by complexin prevents spontaneous fusion of the PM and the OAM. Premature AR would lead to the release of acrosomal contents before sperm reach the zona pellucida and as a consequence, the failure of fertilization.

During the fusion of the PM and the OAM, unilamellar membrane vesicles are generated that contain both membranes (henceforth called mixed vesicles; MV). The protein composition of these vesicles generated from capacitated sperm cells differs from that generated from non-activated control sperm cells. Capacitation-dependent formation of *trans*-SNARE complexes at the apical ridge coincide with the lateral recruitment of proteins at the luminal site of the OAM.

The identification of these proteins could provide a list of candidates which have a putative role in ZP binding and ZP-mediated AR. The protein recruitment in the generated MV may serve ZP binding immediately after the AR (by secondary ZP proteins from the acrosome). A summary of possible SNARE interactions and their relationship to the AR in the sperm cells is provided in figure 1.

SNARE interactions in the oocyte are crucial to prevent polyspermic fertilization

After fertilization which is mediated by a single fusion between a sperm cell and the oolemma, the oocyte must prevent itself from being fertilized by additional sperm. To this end, oocyte executes a sophisticated exocytotic phenomenon known as the cortical reaction (CR), immediately after the fusion of the first sperm with the oolemma. During oocyte maturation, secretory granules migrate towards the surface (cortex) of the oocyte. The rationale for this migration is to prepare for the calcium-dependent CR [11]. The granules remain closely adjacent to the oolemma in meiosis II (M-II) oocytes and await the proper signal (the raise of cytosolic Ca^{2+} resulting from soluble signaling factors in the oocyte's cytosol originating from the sperm) to undergo the cortical exocytosis. The successful fertilizing sperm cell thus induces the release of cortical granules (CGs) into the peri-vitelline space. The released cortical granule content modifies the ZP proteins and results in zona hardening which is required for the blockage of the zona penetration by subsequent sperm cells and/or forms a specific coating on the oolemma and thereby prevents the fusion of additional sperm cell with the oolemma [12]. Although little is known about proteins involved in the CR, it is likely that a highly regulated mechanism is required to coordinate the translocation of CGs towards and association with the oolemma during oocyte maturation. Like in other intracellular fusion events, SNARE proteins might be crucial for the exocytotic membrane fusion process during the CR.



Model 1 SNARE-mediated membrane interactions in the sperm cell
(A) Plasma membrane (PM) and outer acrosome membrane (OAM) of fresh non-activated sperm do not associate with each other. Uncomplexed SNARE proteins locate on opposite sides of the membranes (syntaxin 1B and VAMP 3 at the PM and SNAP 23 at the OAM); transmembrane protein synaptotagmin 4 may reside on the OAM and could facilitate the priming and docking of the OAM to the PM in the next stage. **(B)** Upon capacitation, due to bicarbonate-initiated remodeling of the sperm surface and the removal of the cholesterol from the non-raft area, sphingolipid and cholesterol-enriched microdomains (membrane rafts, dark blue) are able to aggregate into a large platform and can actively or passively recruit SNARE proteins/protein complexes into raft areas. Syntaxin binding protein Munc 18b associates with syntaxins and can directly or indirectly regulate SNARE interactions. After a SNARE core complex is formed, complexin is able to stabilize *trans*-SNARE complex and as a consequence, leads to the tight interaction of *trans*-SNARE complexes resulting in the close apposition of the PM and the OAM. The interaction between complexin and the SNARE complex further prevents the spontaneous fusion of the PM and the OAM. **(C)** Sperm-ZP binding (*in vivo*) or calcium ionophore (*in vitro*) induces the influx of extracellular calcium that leads to the release of complexin. The subsequent *trans* to *cis* conformational change of the SNARE complex is required for the fusion of the PM with the OAM. The resulting *cis*-SNARE complex may contain solely the SNARE complex (c1) or also include the calcium sensor synaptotagmin (c2) or alternatively the syntaxin binding protein, Munc 18 (c3). **(D)** The *trans* to *cis* conformational change of SNARE complexes allows the fusion of the PM and the OAM and as a result, mixed vesicles that contain both membranes and *cis*-SNARE complexes are generated.

The importance of proper timing of the CR is to avoid a premature CR (which would prevent the entry of the sperm and thus fertilization) or a belated CR (which is involved in the stabilization process of the docked membranes. The temporary silencing of the AR by complexin prevents spontaneous fusion of the PM and the OAM. Premature AR would lead to the release of acrosomal contents before sperm reach the zona pellucida and as a consequence, the failure of fertilization.

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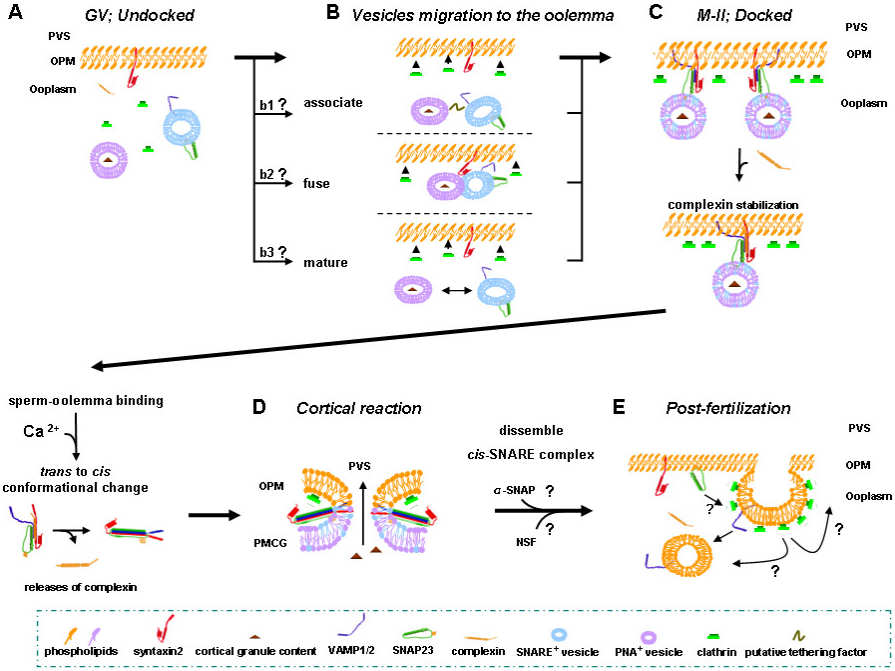
The importance of proper timing of the CR is to avoid a premature CR (which would prevent the entry of the sperm and thus fertilization) or a belated CR (which would result in polyspermic fertilization). However, scarce information is available on the spatial-temporal (re)localization of SNARE proteins during the specific stages of oocyte development, maturation and fertilization [13]. To this end, the role of SNARE proteins in the regulation of the CR is investigated (*chapter 6*). How SNARE proteins are organized during the specific stages of oocyte maturation and post-fertilization in the porcine species is elucidated for the first time and supporting data that SNARE interactions contribute to the docking process of CGs to the oolemma and that the subsequent SNARE-complexin interactions are crucial to arrest the CGs at the oolemma is provided. In contrast to the constant oolemma distribution of syntaxin 2, the redistribution of other SNARE protein (SNAP 23 and VAMP 1) positive vesicles from the cytosol to the cortex area of the oocyte matches the relocation pattern of peanut agglutinin (PNA; a secretory vesicle specific binding lectin) positive CGs. Moreover, in M-II oocytes the co-localization of PNA positive structures (CGs) and SNARE proteins just underneath the oolemma over the entire surface area is detected. This massive increase in co-localization of PNA positive and SNARE positive vesicles can be explained by the fact that the oocyte must relocate the CGs in close proximity to the oolemma during meiotic maturation for a fast and complete onset of the CR at the entire oocyte surface upon elevation of the cytosolic Ca^{2+} level. An ultrastructural approach (e.g. transmission electron microscopy; TEM) in combination with immuno (gold) labeling could provide additional information on the topological relationship between these granules/vesicles and the oolemma before, during and after the CR. This co-localization of PNA positive granules and SNARE positive vesicles at the restricted surface area in M-II oocytes supports the involvement of SNARE proteins in the docking of CGs to the oolemma for a subsequent CR.

However, co-localization of PNA and SNARE proteins was only observed at a defined cortex area underneath the oolemma in M-II oocytes but not in the cytosol of GV or M-II oocytes. This suggests the existence of two distinct vesicle populations (one that is SNARE positive and the other is PNA positive). The co-localization of SNARE proteins and PNA positive vesicles at the restricted oolemma area of M-II oocytes indicates that the vesicle content of both vesicles is merged which may be due to fusion of both vesicles. In this thesis, the evidence for this possibility was not provided which remains to be studied in further detail using e.g. an ultrastructural approach or adapting a fluorescent labeling technique in which the M-II maturation dependent emergence of fluorescence resonance energy transfer (FRET) couples could support the possibility of vesicle fusion (in this case, microinjection with the complementary RNA encoding specific tagged-SNARE proteins or CG content into the oocyte will be necessary [14]). If so, PNA/SNARE positive vesicles formed at a later stage of oocyte maturation (M-II) could dock to the oolemma by virtue of SNARE-

SNARE interactions of the two opposing membranes. Interestingly, like the primed and docked membranes in sperm cells, CGs are stably adhered to the oolemma without the emergence of exocytosis. Further investigation revealed that complexin may also play a role in the regulation of the SNARE-mediated CR. Complexin was already concentrated at the cortical area of the oocyte in GV arrested oocytes. The pronounced localization of complexin in M-II oocytes at that restricted surface area of the oocyte where both CGs and SNARE proteins are located at the M-II stage suggests that complexin can stabilize the *trans*-SNARE complexes. Fertilization induced calcium mobilization into the oocytes cytosol causes the removal of complexin from the *trans*-SNARE complex and thus triggers the onset of the CR.

VAMP 1 redistributed back into intracellular membrane structures which indicates the post-fertilization initiation of membrane traffic and recycling. To investigate whether the observed recycling of SNARE proteins was mediated by endocytosis, the distribution of clathrin (for clathrin-dependent endocytosis) during meiotic maturation and post-fertilization in porcine oocytes was investigated. The endocytosis mechanism becomes temporarily arrested in the area adjacent to the oolemma, likely due to the tight coupling of the arrested exocytosis with endocytosis. This was accompanied with a sharply-defined PM association of clathrin prior to fertilization (M-II) whereas clathrin dissociates again into the oocyte's cytosol after fertilization. After the fertilization fusion by a sperm cell, the elevated cytosolic Ca^{2+} initiates the CR. Probably this massive exocytotic event causes an increase of the oocyte's surface area and thus an immediate compensatory reduction of this surface area should be accomplished by a concomitant induction of endocytosis. The onset of endocytosis not only enables the recycling of the proteins or components required for other processes during zygote development but also re-establishes normal intra-oocyte membrane trafficking.

Other putative proteins involved in endocytosis are the raft marker proteins flotillin and caveolin that may be involved in the formation of caveolae - an alternative endocytotic pathway to the clathrin-mediated endocytosis [15]. The presence of both membrane raft marker proteins flotillin 1 and caveolin 1 in the porcine oocyte was detected but unlike the pronounced redistribution of SNARE proteins and unlike the clathrin and complexin dissociation, both raft marker proteins do not show obvious redistribution during oocyte maturation and fertilization. This suggests that raft-mediated endocytosis (formation of caveolae) is not directly involved in the regulation of the CR in porcine oocytes. For a hypothetic model proposed for SNARE-interactions in the oocyte and the consequent exocytosis that subsequently drives the clathrin-dependent endocytosis, see figure 2.



Model 2 SNARE-mediated membrane interactions in the oocyte
(A) The majority of the secretory vesicles are randomly distributed throughout the ooplasm in the germinal vesicle (GV) stage oocyte. Oocytes of this stage may contain two vesicle populations (PNA positive [in purple] and SNARE positive [in blue]). These vesicles can be detected indirectly by labeling with granule specific PNA-FITC or antibodies against SNARE proteins. Syntaxin 2 is located in the oolemma [OPM]; VAMP1/2 and SNAP 23 are located in the cytoplasm and may associate with SNARE positive vesicles. The cytosolic SNARE complex stabilizing protein complexin is found underneath the oolemma while clathrin can be detected both at the proximity of the oolemma and in the ooplasm. No SNARE-related proteins are considered to be present in the perivitelline space (PVS) at this stage. **(B)** During meiotic maturation, secretory granules migrate to the oocyte surface; three hypothetic action modes are proposed. Two distinct vesicles may associate with each other via a cytosolic tethering factor (b1); two vesicles fuse into a larger vesicle at the cytosolic site adjacent to the oolemma thus show the co-localization of CG substances and SNARE proteins at the later maturation stage (M-II) (b2); vesicles that contain different components (CG substances or SNARE proteins) mature into the other population (b3) **(C)** In the M-II oocyte, secretory vesicles are docked underneath the oolemma via SNARE interactions. *Trans*-SNARE complexes are stabilized by complexin and therefore adhere the cortical granules to the oolemma. At this stage, clathrins are also associating at the same surface area adjacent to the oolemma, which therefore is ready for the fast induction of exocytosis-driven clathrin-dependent endocytosis. Sperm binding to the oolemma mobilizes the cytosolic calcium of the oocyte and causes the release of complexin. **(D)** The release of complexin results in the *trans* to *cis* conformational change of the SNARE protein complex and further promotes the fusion of oolemma (OPM) with the PM of the CGs (PMCG). Cortical granule content is subsequently released into the perivitelline space (PVS). **(E)** While SNARE proteins can be reused later on during the development of the zygote, dissembly of *cis*-SNARE complexes by NSF and alpha SNAP can takes place after the CR. The CR results in not only the release of granule content, but also the dissociation of complexin into the cytosol and the recycling of VAMP 1 containing vesicles (probably via clathrin-dependent endocytosis). Clathrin may also reassociate with membrane vesicles or the oolemma at the cytosolic side and thus may induce additional vesicular membrane transport.

Terra incognita: SNARE-mediated membrane fusion in gametes

Beyond the experimental data presented in this thesis (*chapters 3-6*) the molecular basis of SNARE interactions as well as the SNARE-mediated membrane fusion machinery during fertilization should be approached by a combination of genetic tools and intervention strategies. Prevention of gene expression by a knock out (KO) approach could be a research strategy. However, traditional KO approach can lead to the abnormality of the gametes (e.g. spermatogenesis is disturbed in syntaxin 2 KO mice which resulted in a complete lack of acrosome formation [16]). Another limitation of the traditional KO technique is that developmental compensations may occur. Nevertheless, with an inducible KO strategy, a gene targeting technology allows the timing of expression of a gene to be regulated [17], so that the specific protein can be studied after acrosome formation in order to demonstrate its effect on the acrosome secretion. Recently, by this approach, genes encoding for specific SNARE proteins and/or SNARE regulators were knocked down which demonstrated the importance of the calcium sensor synaptotagmin 1 in the docking of secretory vesicles to their target membrane (in chromaffin cells) [18]. Thus, inducible KO systems specific for testis or ovary could be used to allow normal development of both gametes in the genetic modified animals and allow the production of morphologically normal gametes with the loss of function on the protein of interest at the later stage by the time of induction. Beyond genetic silencing, the inducible KO system also will provide more insights in the functional domains of proteins by introducing site specific mutations in the area of genes coding for such domains. Besides these genetic approaches, intervention strategies using botulinum neurotoxins or specific blocking peptides have been shown to be the useful tools for studying SNARE interactions upon exocytosis [13;19]. However, these approaches may have induced artifacts as they were carried out under non-physiological sperm incubation conditions with the addition of calcium ionophore and/or use of streptolysin-O permeabilized sperm. These treatments may well bypass the physiological capacitation process as they cause the formation of *cis*-SNARE complexes and thus execute the fusion of interacting membranes (not observed in capacitating sperm) and concomitant the loss of cell integrity with possible artificial protein interactions. Therefore, it is important to apply these intervention strategies in non-permeabilized cells that represent more physiological conditions. Examples of such approaches are co-incubation of sperm cells with neurotoxins, blocking peptides or antibodies against specific SNARE proteins prior to the induction of capacitation or AR or by applying microinjection techniques to introduce specific blocking peptides or neurotoxins into the oocyte at specific stages of oocyte maturation prior to the induction of CR.

Combining genetic tools (inducible KO), intervention strategies (neurotoxin or blocking peptides) and microinjection of *in vitro* transcribed complementary RNA into the oocytes, future research could focus on

(1) The dynamics and interactions of SNARE regulators in the fertilization processes. The observed involvement of complexin in the stabilization of interacting membranes (PM and OAM, *chapter 4-5*; CGs and oolemma, *chapter 6*) shows the importance of complexin in the temporary silencing of the AR of the sperm cells and CR of the oocyte. With proteomic approaches, additional information on protein candidates (e.g. syntaxin binding protein 2 (Munc 18 b) and calcium sensor synaptotagmin 4) that may be involved in the regulation of the AR are elucidated. Whether and if so, where and when these proteins are playing a part in the regulation of the AR and CR remains to be investigated.

Combining the FRET technique and microinjection of (1) *in vitro* transcribed complementary RNA encoding a specific fluorescence tagged-SNARE protein or SNARE regulator, (2) specific neurotoxin or blocking peptides into the oocyte can provide functional evidence for SNARE-SNARE and SNARE-regulator interactions on the docking of CGs to the oolemma. However, then an additional quantity of proteins is introduced into the gamete and the original balance of protein interactions can be disturbed.

(2) The involvement of membrane rafts in the regulation of SNARE interactions. Exocytoses in gametes are calcium-regulated and SNARE-mediated processes; the involvement of membrane rafts indicates their functional relevance in the regulation of these processes. Moreover, a recent study showed that CaMKII-alpha (Ca^{2+}-calmodulin kinase II subunit alpha) interacts with MUPP1 that is located in the same membrane microdomains as caveolin-1 (a raft marker protein) and maintains a low cytosolic calcium level in the sperm cell and thus prevents the spontaneous AR [20]. Therefore, SNARE-raft-calcium interactions are important for the regulation of the AR. However, it is challenging to investigate membrane rafts in gametes (especially in the sperm cells) in situ; harsh disruption of membrane rafts by the most commonly used cholesterol depletor methyl-beta-cyclodextrin (MBCD) causes considerable cell dysfunction and cell death due to the disruption of membrane integrity [4], thus new candidates are required and novel approaches are needed in order to address the functional relevance of membrane rafts in fertilization. Future research to resolve this issue could focus on:

(i) Inhibition or disruption of the formation of membrane rafts. Membrane rafts are tightly packed liquid-ordered microdomains; interference on lipid ordering disrupts the structure of membrane rafts without affecting total cholesterol content or membrane integrity [21]. This can be done by lidocaine, a local anesthetic that diffuses into the lipid bilayer and increases the space between lipids [21]. Furthermore, such disruption could be reversed by adding bovine

albumin (BSA) that restores the ordering of membrane microdomains and this may be considered a suitable approach for sperm research.

(ii) Prevention of membrane rafts aggregation upon cell activation. Raft aggregation can be induced by lipid- or protein-mediated activation events [8;22]. These clustered membrane microdomains can further be stabilized by cytoskeletal elements (e.g. actin [8]). Dissociation of the cytoskeleton network (e.g. by using cytochalasins to inhibit actin assembly [23]) could destabilize large functional membrane domains and advance the understanding of the role of membrane rafts in cellular events that are relevant for specific reproductive process.

In conclusion, the investigations presented in this thesis elucidated the importance and the involvement of SNARE proteins, SNARE regulators and membrane rafts in the regulation of gamete exocytosis. However, the knowledge which is described in this thesis is only the tip of an iceberg. Future research on SNARE-SNARE interactions, SNARE-SNARE regulator interplays and SNARE-lipid communications will give additional insights into SNARE-mediated exocytosis in gametes and can shed further light on SNARE-mediated membrane interactions during fertilization which are relevant to understand the molecular basis of gamete interactions.

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Appendix

Dutch summary (Nederlandse samenvatting)

Acknowledgement

Curriculum Vitae

List of publications

Nederlandse samenvatting

Bij de fertilisatie smelten twee gameten samen met als doel om uiteindelijk een nieuw organisme te vormen. Voorafgaand aan de dit proces worden de oppervlaktes van beide gameten zodanig gemodificeerd dat fertilisatie succesvol kan verlopen.

Capacitatie is het proces waarbij de *spermacel* het vermogen verkrijgt om de eicel te bevruchten. Bij capacitatie wordt de spermacel niet alleen hypermotiel maar ondergaat het spermaoppervlak ook opmerkelijke verandering in de organisatie van lipiden en eiwitten, met name in het apicale gedeelte van de spermakop. Deze oppervlakte veranderingen zijn tevens van belang ter voorbereiding van de acrosoomreactie. Bij deze sperma-specifieke exocytose komen de acrosomale enzymen vrij die essentieel zijn voor de uiteindelijke penetratie van de extracellulaire matrix van de eicel (de zona pellucida) door de spermacel.

De *eicel* ondergaat veranderingen gedurende de meiotische rijping ter voorbereiding op de latere fertilisatie. Bij dit proces worden intracellulaire secretie granula verplaatst in de richting van het oppervlak van de eicel. Deze corticale granula zijn na de fertilisatie betrokken bij een eicel-specifieke exocytose: de corticale reactie. De vrijgekomen inhoud van de corticale granula draagt er zorg voor dat het eiceloppervlak en de structuur van de zona pellucida zodanig veranderen dat een tweede spermacel niet meer de eicel kan binnen dringen (voorkómen van polyspermie).

Wanneer één van deze twee gameet-specifieke exocytose processen – de acrosoom c.q. de corticale reactie - niet goed verloopt zal tevens de fertilisatie verstoord zijn. Bij beide exocytose processen heeft een groep gespecialiseerde eiwitten genaamd SNARE eiwitten (soluble N-ethylmaleimide-sensitive factor [NSF] attachment protein receptors) een essentiële rol. In dit proefschrift is deze rol van SNARE eiwitten bestudeerd. Daarbij is specifiek aandacht besteed aan de dynamische veranderingen van SNARE eiwitten gedurende de capacitatie van de spermacel enerzijds en gedurende de meiotische rijping en in aansluiting op de fertilisatie van de eicel anderzijds.

In ***hoofdstuk 1***, wordt een overzicht gegeven van de huidige kennis omtrent de moleculaire kinetiek van eiwitten aan het oppervlak van varkensspermacellen voorafgaand aan de fertilisatie. Membraanfusies zijn belangrijke gebeurtenissen waarbij intracellulaire processen regulerend zijn voor de acrosoomreactie in de spermacel. Centraal hierin is de organisatie van SNARE eiwitten.

In ***hoofdstuk 2***, wordt beschreven hoe SNARE eiwitten membraanfusie kunnen faciliteren en welke eiwitten interacteren en deze fusie reguleren.

In de literatuur zijn aanwijzingen te vinden dat membraan microdomeinen (beter bekend als lipid rafts) betrokken zijn bij de SNARE eiwit-gemedieerde exocytose.

Deze mogelijkheid is nader onderzocht in ***hoofdstuk 3***, waarbij capaciterend varkenssperma als model werd gebruikt. Immers het was uit eerder onderzoek reeds bekend dat de capacitatie van sperma een herverdeling en aggregatie van lipid rafts in de spermakop te weeg brengt. Deze herverdeling blijkt van belang te zijn voor de zona bindende eigenschappen van varkenssperma. SNARE eiwitten bleken in het aggregerende lipid raft gedeelte van het spermaoppervlak te verschijnen. Dit is opmerkelijk omdat SNARE eiwitten zowel op de acrosoom als op de plasmamembraan van de spermacel een vergelijkbare redistributie lieten zien naar het zona-bindende domein (apicale rand) van de spermakop. Dit suggereert dat niet alleen de lipid raft aggregatie in de sperma kop nodig is voor de zona binding maar ook voor de daar direct op volgende en daardoor geïnduceerde acrosoom reactie. De waarneming van eenzelfde herverdeling van SNARE eiwitten op de acrosomale en op de plasmamembraan gedurende de capacitatie gaf een eerste aanwijzing dat deze membranen contact hebben met elkaar en zijn een aanwijzing voor de mogelijkheid dat de acrosoomreactie via SNARE eiwit docking zou plaatsvinden.

In ***hoofdstuk 4*** blijkt dat, na capacitatie, deze twee membranen op het apicale rand gedeelte van de spermakop sterk met elkaar interacteren, iets dat niet het geval was bij onbehandelde spermacellen..De twee interacterende membranen konden gemakkelijk als bilamellaire structuren (twee membranen die met elkaar een blijvende interactie aangaan) geïsoleerd worden. Dit in tegenstelling tot de onbehandelde spermacellen waaruit monolamellaire structuren met alleen plasmamembraan materiaal konden worden geïsoleerd. Zowel in gecapaciteerd sperma als in de daaruit geïsoleerde bilamellaire membraanstructuren konden stabiele trans-SNARE eiwit complexen worden aangetoond met behulp van Western blotting. Opmerkelijk is dat deze sterke interactie tussen de plasmamembraan en acrosoommembraan niet leidde tot de fusies karakteristiek voor de acrosoomreactie.

De onverwachte ontdekking van een uitstel van de acrosoomreactie vormde de aanleiding voor de studie in ***hoofdstuk 5*** waarin eiwitten die de acrosoomfusies tijdelijk kunnen tegenhouden zijn getest. Het blijkt dat het *trans*-SNARE eiwit complex dat in hoofdstuk 4 beschreven is een interactie aangaat met het eiwit complexine dat daardoor het complex stabiliseert en de acrosoomreactie tegengaat. De formatie van het *trans*-SNARE/complexine eiwit complex is uitsluitend waargenomen in de lipiden-geordende membraandomeinen maar niet in de andere membraandomeinen. In ongecapaciteerd sperma was dit complex niet waarneembaar. Wanneer de acrosoomreactie geïnduceerd is door een Ca^{2+}-ionofoor behandeling blijkt het complexine los te komen van het SNARE complex en kunnen monolamellaire vesicles worden geïsoleerd die het gevolg zijn van de karakteristieke multipunt- fusies tussen de plasmamembraan en de acrosoommembraan. Het lijkt er dus op dat bij gecapaciteerd sperma bij de apicale rand een aggregatie van lipid rafts plaats vindt waar zowel stabiele docking van de acrosoom met het oppervlak optreedt alsmede de

organisatie benodigd voor de zona binding. In de literatuur staan duidelijke aanwijzingen dat deze zona binding calcium kanalen openzet in de sperma plasmamembraan. Het is waarschijnlijk dat daardoor het complexine van het trans-SNARE eiwit complex los komt zodat calcium-afhankelijke configuratieverandering naar een cis-SNARE complex kan plaatsvinden leidende tot de acrosoomreactie.

In ***hoofdstuk 6*** is de SNARE eiwit afhankelijkheid van de corticale reactie van eicellen bestudeerd. Gedurende de eicelmaturatie bleken SNARE-eiwit positieve secretoire granula in de richting van het oppervlak (cortex regio) van de eicel te migreren. Deze zogenaamde corticale granula bleven gedurende de eindfase van de maturatie dicht bij de oolemma (plasmamembraan van de eicel) gelokaliseerd zonder dat spontane corticale fusies optraden. Interessant is dat alle SNARE eiwitten die een trans SNARE-eiwit complex kunnen vormen co-localiseren in hetzelfde nauwe corticale en oppervlakte gebied onder en bij de oolemma. De hypothese dat deze SNARE eiwitten een trans-SNARE eiwit complex vormen en daarmee de corticale granula docken aan de oolemma is verder onderzocht. Een duidelijke ondersteuning van deze mogelijkheid is de co-localisatie van de SNARE eiwitten en het complexine. Tevens bleek ook het clathrine zich uitsluitend in dezelfde regio vlak onder de oolemma te lokaliseren in eicellen in het meiose II stadium (eindstadium van eicelmaturatie). Direct na de fertilisatie en de daarbij geïnduceerde corticale reactie blijken - naast de secretie van de inhoud van deze vesicles naar de perivitelline ruimte- het clathrine en complexine los te laten van het oppervlak en te diffunderen via het cytosol naar intracellulaire membraanstructuren. Een verklaring hiervoor is dat de spermacel na de fertilisatie een verhoging van het Ca^{2+} gehalte in de cytosol van de eicel veroorzaakt waardoor het complexine loslaat. Hierdoor kan de corticale fusiereactie plaatsvinden (waarbij het trans SNARE eiwitcomplex een cis configuratie aangaat). Of en hoe het clathrine voor de fertilisatie aan het trans SNARE eiwitcomplex bindt en vervolgens na fertilisatie daarvan loslaat is niet opgehelderd. Het is echter mogelijk dat deze interactie voor de fertilisatie er zorg voor draagt dat naast de blokkering van corticale exocytose er ook geen endocytose optreedt. Dat zou een verklaring kunnen geven voor het ontbreken van vesicle gemedieerde membraan recycling aan het oolemma van de meiose II gematureerde eicel. Deze membraan recycling zou in een keer opgeheven zijn wanneer beide eiwitten zoals waargenomen los zouden laten van het SNARE complex na de fertilisatie. Zoals bekend is het exacte tijdstip waarop de corticale reactie plaats vindt overigens van belang bij het voorkómen van polyspermie bij fertilisatie van de eicel en juist dat laatste vormt bij varkens een probleem bij in vitro fertilisatie.

Conclusies

De SNARE-eiwitten en eiwitten die daaraan assembleren reguleren twee belangrijke exocytose processen bij de fertilisatie van (zoogdier)gameten. De

reorganisatie van deze eiwitten gedurende de spermacapacitatie en de eicelmaturatie dienen om beide gameetoppervlakken te prepareren voor een succesvolle fertilisatie. Verder onderzoek naar de interacties van SNARE eiwitten en hun regulerende factoren (zoals het complexine, synaptotagmine) en de betrokkenheid van lipide geordende membraan-microdomeinen zal in de toekomst bijdragen aan een beter begrip van deze processen. Omdat succesvolle fertilisatie onder andere door deze twee exocytose processen wordt bewerkstelligd is deze kennis van belang zijn voor eventuele behandeling van subfertiliteit of voor het ontwikkelen van een nieuwe generatie anticonceptiva.

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Finally, I've reached this part of the path. Over the 7 years in this lovely country, many people have helped me, and I know if I try to name each and everyone of you, I will miss someone, therefore

I THANK EVERYONE IN CASE I FORGOT ANYONE!

YOU'VE MADE ME STRONGER,
SCIENTIFICALLY and MENTALLY

YOU'VE MADE MY LIFE COLORFUL,
AT WORK and OFF WORK

Nice to have you all part of my life

Curriculum Vitae

- ***Born:*** Pei-Shiue Tsai (a.k.a. Jason) was born on the 4th, March, 1976 Taipei, Taiwan.
- ***Bachelor degree:*** 1995 – 2000. National Chung-Hsing University Taichung, Taiwan; there he also obtained his DVM.
- ***Military service:*** 2000 – 2002. Taiwan
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- In 2005 he became a ***PhD student***, and from 2006, he started the "SNARE project" under the supervision of Prof. dr. B. Colenbrander and Dr. B.M. Gadella, which formed the basis of this thesis.
- ***Post-doctoral:*** 2010 – 2011, under the supervision of Dr. B.M. Gadella. Department of Farm Animal Health, Utrecht University, the Netherlands.

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2. **Tsai PS**, Garcia-Gil N, Colenbrander B, Van Haeften T, Gadella BM. How pig sperm prepares to fertilize: acrosome docking and priming to the plasma membrane. (Submitted)

3. **Tsai PS**, Brewis I, Gadella BM. SNARE protein and complexin interaction dynamics during the acrosome reaction.

4. **Tsai PS**, van Haeften T, Gadella BM. Preparation of the cortical reaction: maturation-dependent migration of SNARE proteins, clathrin and complexin to the oocyte's surface blocks membrane traffic until fertilization. (Submitted).

You've learned me when to look closer,
when to step back

To my supervisors