EXOCYTOSIS:

REGULATION OF THE OOCYTE CORTICAL REACTION

Christiaan H. C. Zeelenberg

3075923

Bachelorscriptie Biomedische Wetenschappen

Universiteit van Utrecht

Maart 2011

Contents

Preface

An impressive broad range of cell types exhibit a number of events that show undeniable similarity. One of these events is known by several names such as secretion, degranulation, synaptic release et cetera and these are all of vital importance for cell life and tissue functioning. The common underlying principle is exocytosis, which is a cornerstone in all cell biology and has been the focus of many fields of research for a long period of time. The fundamental goal of exocytosis is the deliverance of the specific cargo, present in a membrane-surrounded vesicle, to the exterior of the cell. To this end the vesicle has to bind to its target membrane and since the cell is a fully membrane enclosed entity the plasma membrane is the primary target. This process is logically not a solitary system and is coupled to compensatory systems in intracellular trafficking, thus ensuring a steady recycling and refreshment of plasma membrane molecules and adaptation of the cell surface to certain physiological circumstances. During normal cell function there is a continuous transport of proteins, lipids and other compounds by various means of intracellular vesicular mediated membrane transport. For instance, these routes can take integral membrane proteins after their translation at the rough endoplasmatic reticulum from the location of synthesis to a functional destination. For example, starting at the endoplasmic reticulum, these proteins will first be glycosylated through the Golgi complex and later appear at the cell surface by vesicle mediated membrane transport.

Similarly, a foreign particle (for instance a virion) can be taken up from the environment by endocytosis and the endosome vesicle will be processed and matured to acidic pH so that the virion particle induces fusion with the late endosome membrane and effectively introduces its DNA or RNA into the cell's cytosol, enabling assemblage of new virions. Other endocytozed material can be routed to the lysosome for degradation into smaller products that can be used for biosynthesis of new materials or routed to the Golgi and/or the endoplasmic reticulum for specific molecular modifications. In general, the pinching off of vesicles as well as the merging fusions of vesicles to donor and target membranes have a common machinery in which calcium dependent conformational changes of membrane fusion proteins are involved. In spite of this

general principle, many cells with specific functions exhibit strongly specialized trafficking pathways, which will be dealt with in chapter two.

The cell is, as well as most organelles, a membrane enclosed entity and, in principle, these membranes form the barrier of free transport, excluding uptake diffusion of large molecules. Such larger molecules can only be taken up by vesicle transport in which the passage of a certain cargo from one location to the other involves at least one membrane fusion. This fusion of two membranes – generally a transport vesicle and a target membrane – makes the delivery of a cargo to its target possible. In this thesis, the process of exocytosis is the main topic and will be investigated with the use of studies on various expressions of the exocytotic event. The research part of the thesis is devoted to the cortical reaction of the oocyte which is an exocytotic event elicited after the fertilization by one sperm cell and is believed to prevent polyspermic fertilization. The review is constructed in such a way that first the general concept of exocytosis is dealt with in chapter one, then variations in the organization of exocytosis in neurons and secretory cells are dealt with in chapter two. Next, chapter three is devoted to the oocyte, which will deal with the physiology and maturation of the cell and give an introduction on the cortical reaction. Finally, an overview of my experimental findings on the oocyte's cortical reaction will be covered in chapter four. Major conclusions and future aspects of cell exocytosis, with special attention to the cortical reaction, will be discussed in the concluding chapter five.

CHAPTER ONE

General introduction on exocytosis

The mechanisms of exocytosis

Vesicle transport and membrane fusion

Molecular trafficking is important for a cell in order to maintain its functioning. Since the cell contains an array of membrane-enclosed organelles, certain diffusion barriers need to be overcome by large molecules and a specialized transport system is required. The distribution and transportation of proteins and lipids is executed by means of vesicle transport. This process involves the compartmentalization of the transported molecule from the donor organelle and the migration to an intracellular target, where ultimately the two membranes fuse. The targeting of these vesicles and the fusion of the membrane requires specialized machinery.

In the early 1990's a range of important proteins were purified from both yeast and mammalian brain cells (Clary *et al*. 1990). Two of these, called N-ethylmaleimide-sensitive factor (NSF) and a family of soluble NSF attachment proteins (SNAPs), were found to be essential in membrane fusion processes in the Golgi complex. Later, ranges of new proteins were purified that provide the specificity in the fusion process. Studies on neurons (which are specialized in vesicle fusion for neurotransmitter release in the synapses) led to the discovery of SNAP-receptors (SNAREs), which mediate the docking of vesicles and are essential for membrane fusion (Rothman 1994). Subsequently, a hypothesis was posed that was claimed to be common for all membrane fusion processes involving vesicle transport, which will be explained in the following sections (this excludes extracellular membrane fusion processes as well as virion membrane fusion).

Constitutive and regulated exocytosis

Besides the trafficking of proteins between intracellular compartments, also the secretion of molecules, generally termed exocytosis, is conducted by vesicle transport. Two protein secretion

pathways, namely the constitutive and the regulated pathway, have been described and Burgess and Kelly laid out an overview of the earliest designs in 1987 (Burgess & Kelly 1987). The constitutive pathway is involved in the processing of plasma membrane bound proteins, lysosomal enzymes and proteins that function outside the cell. The first steps, from the start at the rough endoplasmatic reticulum (R.E.R.) to and through the Golgi complex, are common for all these different proteins. During these steps, the most important part is to modify the proteins for their further travel through the cell. In the *trans*-Golgi network, proteins undergo several modes of processing (for example glycosylation and the unmasking of specific amino acid sequences) to direct it to its intracellular location and possibly promote secretion via the regulated pathway (Morvan & Tooze 2008). In mammals, at least, the constitutive pathway is thought to be the default pathway of protein secretion when signal sequences are absent. The regulated pathway involves the secretion in response to an extracellular stimulus and is preceded by storage in secretory granules. These granules are highly abundant near the plasma membrane of secretory cells and the amount of proteins inside these granules can rise up to a 200-fold compared to the R.E.R (Salpeter & Farquhar 1981). These properties account for the fact that these secretory cells are prepared for a quick and efficient exocytosis of their secretion load.

Various forms of exocytosis have been observed and described in many years of investigation and they all prove to be variations on the basic exocytotic process. Probably, the variation serves as an adaptation in order to comply with the function of the cell. For example, synaptic release of neurotransmitters in neurons is a well-studied regulated exocytotic process in which the first discoveries of the fusion machinery were made. The key condition for the regulatory exocytosis is the stimulation from the extracellular environment. After a secretory cell receives a signal, a rapid release of secretion products is pursued. A well-designed intracellular signalling system guides the cell during the highly organized process using a set of enzymes and through the release of second messengers. Since the clarification of the pathways involved in exocytosis, calcium was found to be an important signalling molecule and appears to play a major role in a broad range of cell types (Burgess & Kelly 1987).

The SNARE concept

The purification of proteins from the fusion particle led to the description of two classes of proteins, specific for either vesicles or target membranes (named respectively v-SNAREs and t-SNAREs). The vesicle-associated membrane protein (VAMP, also termed synaptobrevin) was classified as a v-SNARE and among the t-SNAREs syntaxin and SNAP-25 (synaptosome-associated protein of 25kDa, not to be confused with SNAPs termed earlier) were discovered (Rothman 1996). Subsequently, the "SNARE hypothesis" was postulated in which SNARE proteins form a complex that serves for both specificity of the docking of a vesicle to the appropriate target membrane as the fusion process itself. Since the formulation of this hypothesis, many isoforms and other SNARE proteins have been described and the number is still increasing at this moment. Additionally, comparative research on the amino acid sequences of SNARE proteins resulted in a novel classification of Q-SNAREs and R-SNAREs (Hong 2005). The detailed description of the SNARE mechanisms in the following sections is mainly based on experiments in neuronal cells but is increasingly proved to be generally applicable.

Preparation for the fusion event

When a vesicle is released from the donor organelle (in this case mainly from the Golgi complex), it migrates to the plasma membrane where it undergoes preparation steps before the fusion event can take place. Firstly, the vesicle needs to be docked at the plasma membrane in a process called tethering. Rab3 (member of the Rab GTPases family) acts as a tethering factor and mediates in the first contact of the two membranes (Stenmark. 2009). Fig. 1B shows this Rab3-mediated approaching of the membranes that are both loaded with the SNARE proteins contributing to the fusion process (further discussed below). The tethering is facilitated by its affinity for several effector proteins on the plasma membrane and association with the vesicle membrane lipids via its attached hydrophobic geranylgeranyl groups. As a result, the two membranes come into closer proximity to facilitate the opposing SNARE proteins to meet.

Before SNARE-SNARE interaction, the individual proteins are highly unstructured and the assembly of the SNARE complex initiates conformation changes in the SNARE motifs of the participating proteins (Fig.1) (Fasshauer. 2003).

Three proteins contribute in the SNARE complex, forming a bundle of four helices of distinct SNARE motifs, namely Qa-, Qb-, Qc- and R-motifs (Hong. 2005). All VAMP isoforms contain an R-motif and a transmembrane domain, whereas syntaxin isoforms that are located on the plasma membrane comprise a Qa-motif adjacent to the transmembrane sequence. The Qb- and Qc-motifs are found in SNAP-25 (as well as in the related SNAP-23 protein), which is linked to the plasma membrane via palmitoylation by absence of a transmembrane domain. As can be seen in *Fig. 2*, during SNARE complex assembly, the four domains interact and gradually shape into α-helices starting at their N-terminal sides (most distally from the associated membranes), forming the SNARE core complex (Fasshauer 2003, Jahn & Scheller 2006) SNAP-25 is an exception since one of its two SNARE motives interacts in a reversed order (Fig. 2). This process,

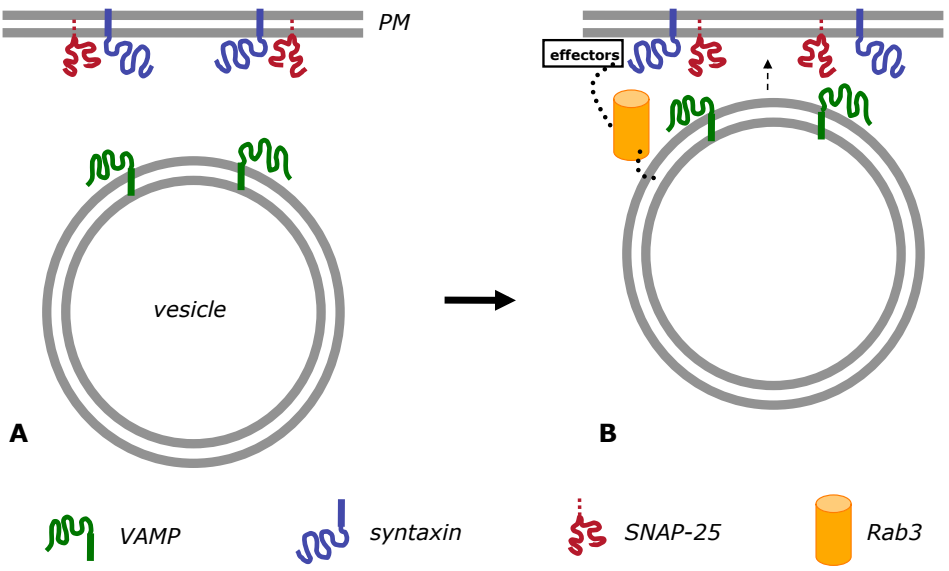

Figure 1. **Tethering of the vesicle to the plasma membrane is mediated by Rab3**. (**A**) The vesicle contains the VAMP *(green)* protein anchored in the membrane and the plasma membrane (PM) is loaded with syntaxin *(blue)* via a transmembrane domain and SNAP-25 *(red)* via palmitoylation of amino acid residues. The cytosolic chains of the SNARE proteins are highly unstructured at this point. (**B**) Rab3 *(yellow)* is associated with the vesicle by lipid interactions by geranylgeranyl groups and association with the plasma membrane is mediated by effector proteins (show in box). This facilitates the approaching of the vesicle to the plasma membrane and is called "tethering". Both interactions of Rab3 are depicted as dotted lines since the exact mechanism is not clearly understood.

which ultimately results in two coiled-coils, can be compared by the "zippering" that is observed in leucine-zipper proteins and secures the SNARE complex into a tight conformation (Matos *et al*. 2003).

When the assembly has complete, a *trans*-SNARE complex is formed, which crosses the two opposing membranes (Fig. 2B). After these preparation steps, with the secretory granules docked and ready for secretion, the cell is in a "primed" state. Recent evidence shows that the partial assembly of the SNARE complex (the C-terminal parts not yet "zipped") defines the primed state of secretory vesicles, suggesting the existence of intermediates of the SNARE complex (Walter *et al*. 2010). However, the degree of SNARE complex assembly, as well as the exact mechanism of vesicle docking, prior to the fusion event is still under debate.

Membrane fusion

When the *trans*-SNARE complex assembles into a tight formation, the mechanical force drives the membranes into closer proximity while the zippering releases a certain amount of energy. This process is suggested to overcome the first energy barriers of membrane fusion since two opposing lipid bilayers have repulsive forces (Szule & Coorssen 2003, Hong 2005, Jahn & Scheller 2006). At this fully primed state, various regulator proteins are needed to prevent spontaneous fusion, as well as to initiate the fusion event itself. Chapter two will introduce these regulation systems and the proteins involved.

As a reaction to a stimulus for secretion (mainly rise in intracellular calcium concentration) membrane fusion is initiated. The fusion event is a well-studied phenomenon and the widely accepted "hemifusion" working model consists of number of intermediates (Kozlovsky & Kozlov 2002). First, the two outer layers of the membranes make molecular contact by a slight curvature in both membranes. Next, the lipids of both outer layers start mixing and become continuous without forming an aqueous connection, an intermediate named the "hemifusion stalk" is formed (Fig. 2). Lastly, the original inner layers of the

membranes become continuous and the fusion pore is created. This last step completes the fusion process as the two membranes are fully merged and the content of the vesicle is released.

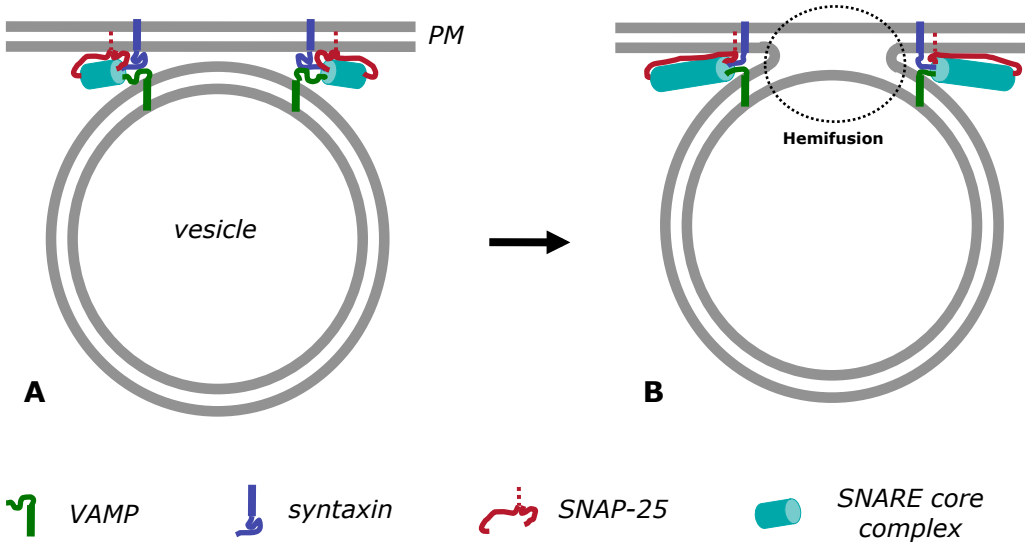

Figure 2. **SNARE complex assembly by zippering action and hemifusion state**. (**A**) The SNARE core complex *(light blue cylinder)* assembly is initiated when the SNARE motives of vesicle protein VAMP *(green)* associate with the SNARE motives of syntaxin (*blue)* and SNAP-25 (*red),* which are both attached to the plasma membrane (PM). As a results, four α-helices begin forming two coiled coils, starting most distally from the membranes. SNAP-25 contributes two helices in anti-parallel orientation (as can be seen by the binary intrusion into the complex). (**B**) The formation proceeds in a gradual manner through a "zippering" action (see text), facilitating the approaching of the membranes and releasing the energy to generate a hemifusion state, in which the two outer membrane layers become continuous. The SNARE complex (core complex plus the SNARE proteins) that is formed is called the *trans*-SNARE complex, which is arrested by SNARE regulator proteins (see text) and in anticipation of the fusion trigger. In this state, the vesicle is "primed" for fusion with the plasma membrane and releasing its content into the extracellular environment.

SNARE complex transition and protein recycling

The fusion of two membranes is highly energetically unfavourable and can therefore not occur spontaneously. Various mechanisms of membrane fusion have been studied and show the importance of protein machinery (Jahn & Sudhof 1999). SNARE protein complexes mediate this event by a structural transition in their conformation. While prior to the membrane fusion the SNARE complex is in trans orientation (Fig. 3A), post-fusion SNARE complexes show a *cis* orientation (Fig. 3B) (Jahn *et al*. 2003). In this state, all SNARE proteins are anchored in the same membrane, namely the fused vesicle and target membrane. The transition from a *trans*- to *cis*–

SNARE complex is suggested to be the mechanical force that initiates the fusion event. However, it is still under debate if and how this transition can solely be responsible for the energeticallyunfavorable event.

As explained earlier, the SNARE core complex is suggested to assemble in a gradual manner, namely from the parallel aligned N-terminals to the C-terminals of the SNARE proteins (except for one of the SNAP-25 motives, which is reversely orientated (Fig. 2 & Fig. 3). Thus, the zippering action approaches the two opposing membrane and brings them into closer proximity, until a yet undefined level. Upon receiving the signal for membrane fusion, which is mostly a rise in calcium concentration, this zippering is thought to continue beyond the SNARE core complex and subsequently creates a mechanical force that drives the fusion event (Kozlov *et al*. 2010). Two important proteins play a central role in the arresting of SNARE complex assembly and the calcium sensitivity (complexin and synaptotagmin, respectively) and are discussed in detail in chapter two.

Recent suggestions predict a situation where SNARE complex assembly is arrested by complexin in a hemifusion intermediate in which the outer membrane layers have merged (Fig. 3A) (Schaub *et al*. 2006). Subsequently, upon calcium rise, synaptotagmin is able to relieve the inhibition by complexin and initiate membrane fusion and SNARE complex transition (Fig. 3B). Moreover, synaptotagmin, as a vesicle anchored protein, has been qualified as a membrane curvature promoter, which strengthens its assumed role in the initiation of fusion (Kozlov *et al*. 2010, Martens *et al*. 2007). Chapters two and four will go into deeper detail on SNARE regulator proteins and their role in both inhibiting and promoting exocytosis.

After the fusion event is accomplished and the *cis*-SNARE complex is present, a dissociation process is required to recycle the SNARE proteins and enable subsequent membrane fusions. Paradoxically, the earlier coined essential fusion proteins NSF and SNAPs are the machinery that facilitates this disassembly of the SNARE complex and are not involved in the fusion itself (Sollner *et al*. 1993a). In general, NSF associates with α-SNAP, which in turn binds to syntaxin in the *cis*-SNARE complex, and catalyses the dissociation of the SNARE proteins by hydrolysis of ATP.

When a cell has undergone an exocytotic event, a major change has occurred in its membrane conditions as the fusion of secretory vesicles has increased the cells surface dramatically. In favour of membrane homeostasis, generation of novel vesicles and to recycle v-SNARE proteins for future exocytosis, a compensatory endocytotic pathway is needed. For instance in neuronal cells, which exhibit exocytosis in a high rate or recurrence, the importance for endocytosis for cell functioning has been shown (Augustine *et al*. 2006, Perez Bay *et al*. 2007). Mainly, the clathrin-dependent pathway is used to internalize parts of the membrane after fusion has occurred and inhibition of this pathway greatly reduces exocytotic capabilities. However, studies in chromaffin cells pose various opinions regarding the mechanism of compensatory endocytosis (Barg & Machado 2008). To date, little conclusive information exists about this process and a major niche for research is still to be occupied.

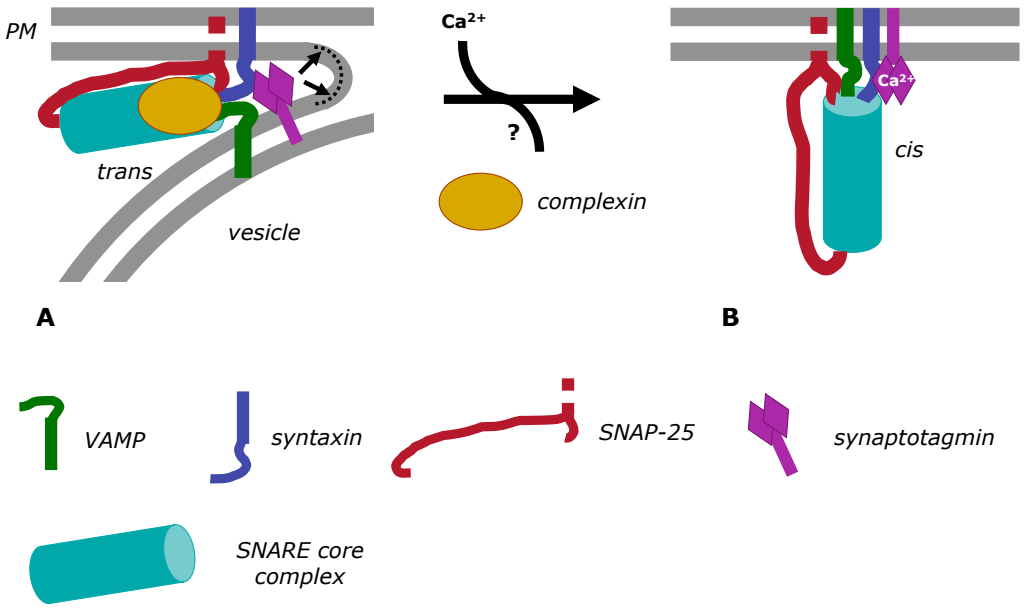

Figure 3. **SNARE complex transition and regulators**. (**A**) Prior to the fusion event, in the primed state, all three SNARE proteins have assembled into the *trans*-SNARE complex, which crosses the two membranes. To what extent the assembly of the SNARE complex proceeds until this stage remains to be clarified. The SNARE regulator protein complexin (*light brown*) is thought to arrest the complex and prevent early membrane fusion. A calcium sensitive regulator protein, synaptotagmin (*purple*), anchored in the vesicle membrane, is assumed to promote membrane curvature (shown by the arrows and dotted lines) and associates with the SNARE complex. (**B**) Upon calcium rise, the arresting action of complexin is thought to be released by intervention of synaptotagmin binding calcium. As a result, the two membranes fuse and the *cis*-SNARE complex is formed, as all SNARE proteins involved are in the same, freshly mixed, membrane. However, the possible dissociation of complexin from the SNARE complex is still under debate.

Scope of the thesis

Although many years of research on exocytosis yielded a broad range of knowledge concerning the protein machinery and its regulation, the topic is still under intense investigation. Exocytosis is an event that plays an important role in various cell types, as well as in the oocyte where it occurs in the cortical reaction (CR). Upon activation of the oocyte by a penetrating sperm cell, a massive exocytosis event occurs that served to create a modification of its surrounding protein layer, the zona pellucida (ZP). As a result, the oocyte protects itself from binding and penetration of additional sperm cells in order to successfully complete the fertilization process, the so-called "polyspermy block" (Ducibella 1996). This mechanism receives great interests from the reproductive field since polyspermic fertilization remains a returning result of *in-vitro* fertilization (IVF) treatment in animals as well as in human.

A couple of years after the first postulations of SNARE proteins as the machinery in synaptic release, evidence arose for a similar mechanism in the cortical reaction (Conner *et al*. 1997). In the following years, the role of SNARE proteins in the CR became a growing topic of investigation and many proteins were identified. However, to date, there is still little conclusive data to fully understand the mechanism and regulation of the CR. Moreover, in the case of mammals, the essence of the cortical reaction for the development of the polyspermy block is still under debate. As observed in many species, there is an additional, if not fundamental, mechanism at the level of the oocyte plasma membrane (the oolemma). To what extent the cortical reaction is either essential or facultative to the polyspermy block, or participates in a sophisticated interplay, remains to be discussed.

In this thesis, the present data concerning the biology of the cortical reaction is examined, in comparison with the knowledge of exocytotic events in other cell types. A special focus will lay on the regulatory processes and the proteins that are involved. Having presented these insights, some future prospects and directions of research are suggested to further elucidate the mechanism and regulation of the oocyte cortical reaction. Lastly, the role of the cortical reaction in the generation of a polyspermy block, essentially in mammals, will be discussed.

CHAPTER TWO

Exocytosis in various cell types

Different modes of exocytosis

Mechanisms in exocytotic events

The ultimate goal of exocytosis is the secretion of molecules from a cell to serve goals such as hormonal signalling, neurotransmitter release or immunological mediator secretion. In these different situations, several modes of exocytosis can be observed. The schematic overview in Fig. 4 illustrates two predominant modes of exocytosis. The classical mode, which in terms is simplest, exhibits the fusion of individual vesicles with the plasma membrane to release the secretory content into the extracellular environment. The other mode, which is a more extreme secretion process, is named compound exocytosis and constitutes of either a sequential (also termed cumulative) or a multivesicular manner (Lollike *et al*. 2002, Pickett & Edwardson 2006). The former is characterized by fusion of vesicles with ones that have already fused with the plasma membrane (Fig. 4). In contrast, multivesicular exocytosis exhibits mutual fusion of vesicles prior to fusion with the plasma membrane. Both manners of compound exocytosis yield effective release of molecules and can be viewed as physiological adaptation on the classical mode. Immune cells can undergo different types of exocytosis and are very suitable for research. For instance the mast cell, which is most famous for its role in allergic reactions and discussed in detail later, exhibits both types (Logan *et al*. 2003). Among cells that exhibit both sequential and multivesicular exocytosis, the majority of the secretion processes is mediated via sequential exocytotic pathway.

Another striking example is the pancreas acinar cell, which is highly polarized and secretes digestive enzymes by sequential compound exocytosis (Nemoto *et al*. 2001). Specialized vesicles names zymogen granules that reside in the periphery dock to the plasma membrane to form omega-shaped structures. They remain in this state for several minutes before secondary vesicles attach to them to continue the exocytotic process and thus ensuring the secretion at the site where the first vesicles docked to the plasma membrane. Interestingly, the hypothesized mechanism for this sequential vesicle binding is the relocation of t-SNAREs,

originally residing in the plasma membrane, onto the already fused vesicles (Guo *et al*. 1998, Nemoto *et al*. 2001). In contrast, the mechanism of mutual fusion during multivesicular compound exocytosis is less understood and could involve specific SNARE proteins present on the secretory vesicles (Takahashi & Kasai 2007).

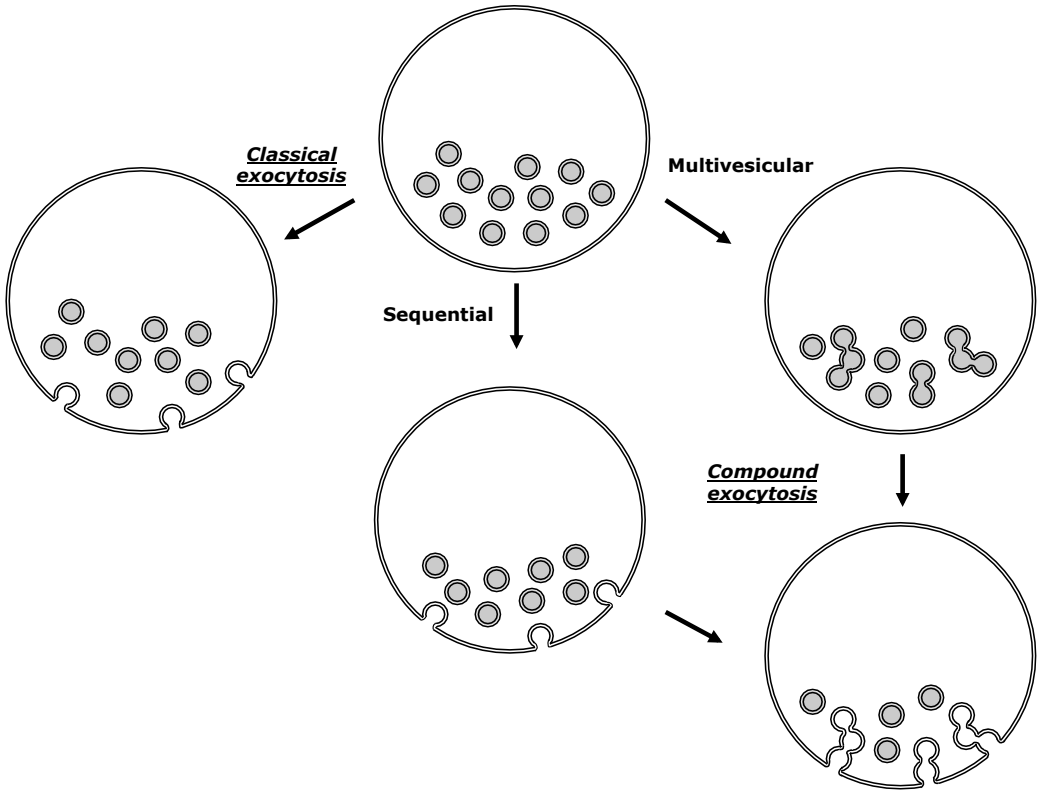

Figure 4. **Different modes of exocytosis**. The top circle in this schematic overview illustrates a cell that is loaded with secretory granules (or vesicles, grey stained circles), which are scattered throughout the cell with a predominate presence in the cell cortex. These granules are, when in proximity of the plasma membrane, able to fuse upon stimulation of the cell. The left-hand arrow points to the result of classical exocytosis, in which single granules fuse with the plasma membrane and release their content into the extracellular environment. The center arrow points to one mode of compound exocytosis (see text), namely the sequential manner. Initially, the single primary granules fuse with the plasma membrane and, subsequently, secondary granules fuse with the already-fused primary granules. The right-hand arrow points to the multivesicular manner of compound exocytosis in which, at first, granules mutually fuse and, subsequently, fuse with the plasma membrane. Both manners of compound exocytosis result in rapid and elaborate release of the secretory content after cell stimulation. Note that, suggestively, the initial situation of sequential compound exocytosis resembles the process of classical exocytosis.

Classical exocytosis

Although several variations are already described among cells that are capable of exocytosis, the classical mode is still intensively investigated. The classical mode was first examined in yeast and neuronal cells, which led to the postulations of the SNARE hypothesis (Clary *et al*. 1990, Sollner *et al*. 1993b, Rothman 1996). The essence of classical exocytosis is the fusion event of a single vesicle with the plasma membrane, usually exhibited simultaneously by a great number of other vesicles. Assumingly, with the growing amount of knowledge on exocytosis in a whole, one could believe that no distinct mode can be described as an individual mechanism. Moreover, this would only lead to a loss of scope and oppose the further insights. However, classical exocytosis is yet to be elaborately described in many cells types, such as the neurons and the oocyte, and forms a suitable starting point for research. To date, present knowledge explains that the SNARE machinery used by both classical and compound exocytosis is common, although SNARE interactions for mutual vesicle fusion remain to be characterized (Logan *et al*. 2003, Pickett & Edwardson 2006, Takahashi & Kasai 2007). In the following sections, the essential steps of priming, activation and fusion in distinct cell types are described.

Docking, priming and fusion processes in neurons

SM proteins

The first proteins that were described to regulate the synaptic release cycle were of the Sec/Munc (SM) family. Presynaptic plasma membranes contain specialized sites, named "active zones", which are electron-dense structures that consist of proteins involved in exocytosis, since the synaptic vesicle fuses exclusively at these sites (Fig. 5) (Sudhof 2004). In these active zones, mainly the proteins Munc13 and Rab3 interacting molecules (RIMs) are found, which are proved to be involved in the docking and priming of the synaptic vesicles (Sudhof 2004, Andrews-Zwilling *et al*. 2006, Rizo & Rosenmund 2008). In mice neurons, RIM1α interacts with

Munc13-1 and Rab3A on the synaptic vesicle and thus forming a scaffold for vesicle docking (Schoch *et al*. 2002). Knockout of RIM1α show a decrease in synaptic release potential, although no decrease in number of docked vesicle. This suggests that RIMs are involved in a more complicated way with the docking and priming process, especially since they also bind to synaptotagmin.

In another study in mice, Munc13-1/2 double knockouts show a deficiency in vesicle docking, although they reside in close proximity of the plasma membrane, probably by other tethering proteins (Siksou *et al*. 2009). In addition to the suggested role of RIMs, the interacting of Munc13 with syntaxin suggests a role in the functional priming of the vesicles by facilitating SNARE complex formation. Syntaxin isoforms generally consist of four domains: a transmembrane domain, a Qa-SNARE domain, H_{abc} domain and an N-terminal sequence. The H_{abc} domain forms a motif, consisting of three anti-parallel helices, that has affinity for the SNARE domain helix. In this way, two distinct conformations can be created: either "closed" with the H_{abc} domain bound to the SNARE motif, or "opened" with the two domains apart. Probably, Munc13 induces the transition of syntaxin from a closed to an opened conformation and thus facilitates SNARE complex assembly (Rizo & Rosenmund 2008). Fig. 5B illustrates this interaction with the opened conformation during the docking process.

Another important SM protein is Munc18 (neurons express isoform Munc18-1), a cytosolic protein whose absence results in reduced synaptic vesicle docking in *Caenorhabditis elegans* neurons (Weimer *et al*. 2003). In addition to Munc13, Munc18 also binds syntaxin, although it does so in a different fashion. At first, the Munc18-syntaxin interaction was observed with the closed conformation of syntaxin, rendering a stable intermediate prior to SNARE complex assembly that required the transition to the opened conformation (Dulubova *et al*. 1999). However, Munc18 was also found to interact with syntaxin-1 in an opened conformation via a highly conserved sequence in the N-terminal region, although with lower affinity than to the closed conformation (Fig. 5A & 5B) (Rickman *et al*. 2007). These finding suggests a model in which Munc18 acts as a chaperone by facilitating a stable closed conformation and preventing precipitate formation of SNARE complexes prior to delivery at the plasma membrane (Medine *et al*. 2007, Han *et al*. 2010). In this model, a role for Munc13 is reserved to induce the transition

of syntaxin to the opened conformation and assist SNARE complex assembly. To which extent these interactions occur during the docking and priming phases is not clearly known. However, data on interactions with other SNARE regulators might yield more elaborate insights on the primed vesicle state.

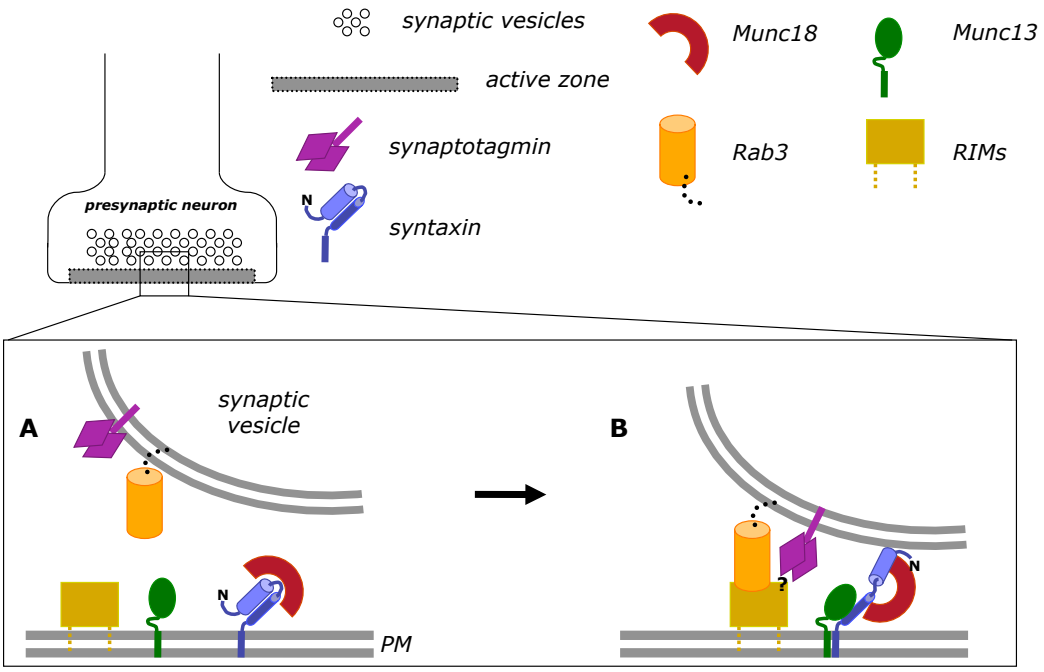

Figure 5. **SNARE regulator proteins in the active zone facilitate vesicle docking**. The presynaptic neuron has an 'active zone' that has high electron density and consists of various SNARE regulator proteins. Synaptic vesicles fuse exclusively in this area, thus these proteins must function in concert with SNAREs and other regulator proteins to form a scaffold for the docking of these vesicles. The detailed illustration (**A**) shows that Munc13 (*green*) and Rab3 interaction molecules (RIMs, *beige*) are present in the active zone on the plasma membrane (PM). Syntaxin (*blue*) is made up of a SNARE motif (*blue*) and a H_{abc} domain (*light blue*), which have affinity for each other and form a 'closed' conformation when Munc18 (*red*) is bound. Synaptotagmin (*purple*) and Rab3 (*yellow*) are attached to the synaptic vesicle by either a transmembrane domain or by geranylgeranyl groups, respectively. (**B**) When a synaptic vesicle nears the plasma membrane and starts the docking process, Munc13 is thought to interact with syntaxin to induce the switch to an 'opened' confirmation (see text). Munc18 still remains bound to syntaxin but has a lower affinity for this confirmation. The RIMs interact with Rab3 and, possibly, also with synaptotagmin (see question mark) to facilitate the docking.

Complexin-SNARE complex interactions

Another SNARE regulator is complexin, a 15–16 kDa protein that has been shown to bind to the SNARE core complex, via an α-helix domain, in between the helices of VAMP and syntaxin, thus stabilizing the whole (Chen *et al*. 2002). Early studies showed that increased amounts of complexin in nerve terminals reduced exocytosis, suggesting an inhibitory role (Ono *et al*. 1998). However, later studies demonstrated that deficiencies and prevented binding of complexin result in a significant loss of synaptic release in neurons of mice and squid, confirming its importance in exocytosis (Reim *et al*. 2001, Marz & Hanson 2002). Additionally, complexin was shown to play a positive role in synaptic release in mouse chromaffin cells and increases the number of primed vesicles by binding the SNARE complex (Cai *et al*. 2008).

Detailed functional examination showed that complexin, indeed, inhibits the membrane fusion, specifically by arresting the hemifusion state (Fig. 3A) (Schaub *et al*. 2006). Prior to the arrival of the calcium signal, which induces membrane fusion and SNARE complex transition, the SNARE complex is not fully assembled (Chen *et al*. 2001), thus providing the possibility for complexin to act as a "fusion clamp" on the primed vesicles (Melia 2007, McNew 2008). Interplay of the calcium sensitive protein synaptotagmin (further described below) can release the inhibited state and is suggested to form a switching mechanism together with complexin (Giraudo *et al*. 2006, Schaub *et al*. 2006). Moreover, complexin bound to SNARE complexes on artificial membranes was shown to be displaced by synaptotagmin in the presence of calcium (Tang *et al*. 2006). However, to what extent complexin remains bound or dissociates during SNARE complex transition and exocytotic membrane fusion is still a question to be answered.

Recently, distinct domains of complexin-1 with their binding and functional properties were described. N-terminally from the SNARE binding α-helix, an accessory helix executes the inhibitory effect by preventing further binding of VAMP to the SNARE complex and, most N-terminal, a 26 amino acid sequences facilitates a positive effect on membrane fusion and is essential for overall protein functioning (Xue *et al*. 2007). More specifically, the complexin N-terminus is able to bind the C-terminal part of the SNARE complex, which results in the release of the inhibitory accessory helix, thus facilitating the full assembly of the SNARE complex (Xue

et al. 2010). Taken together, complexin exercises a complicated regulation on the fusion mechanism that upon calcium influx (likely with the interplay of synaptotagmin) promotes membrane fusion and thus synaptic release.

Synaptotagmin and calcium signals

As stated in the previous section, synaptotagmin acts as a calcium sensitive regulator of synaptic release. It comprises a transmembrane domain, which anchors into the synaptic vesicle, two calcium sensitive domains, C_2A and C_2B. The knockout of synaptotagmin-1 in mice showed reduced calcium-triggered synchronous synaptic release, while calcium-independent release remains unaltered (Geppert *et al*. 1994). Synaptotagmin also has a strong affinity for phospholipids and the mutual interaction enhances the binding of calcium ions, confirming its regulating role in membrane fusion (Fernandez-Chacon *et al*. 2001). This phospholipid binding property has been suggested to induce membrane curvature upon calcium rises and thus facilitating bilayer fusion (Martens *et al*. 2007, Lynch *et al*. 2008). Additionally, as intracellular calcium levels rise, synaptotagmin is also able to bind the SNARE complex (Chapman *et al*. 1995, Davis *et al*. 1999, Gerona *et al*. 2000). Fig. 3A shows the suggested membrane curvature induced by synaptotagmin and the transition to Fig. 3B illustrates the calcium dependent action of synaptotagmin to interact with the SNARE complex and relieve the inhibitory effect of complexin. Besides stimulating membrane fusion most efficiently at 10 µM calcium (Lee *et al*. 2010), a recent study shows that spontaneous synaptic release, by slight increases of calcium levels, is also triggered by synaptotagmin-1 (Xu *et al*. 2009), rendering it a highly sensitive calcium sensor. Taken together, to current knowledge, synaptotagmin exerts an important regulating role in membrane fusion during synaptic release via its calcium sensitivity and interplay with complexin, probably by direct binding (Tokumaru *et al*. 2008). However, the high calcium sensitivity of synaptotagmin-1 leaves the possible involvement of multiple isoforms or other calcium-dependent proteins.

Exocytotic events in secretion cells

The mast cell and SNARE proteins

Because the earliest, and generally the most fundamental, studies on the mechanism of exocytosis have been conducted in neurons has this cell type developed into a model cell. However, to broaden the knowledge to different cell types, it would also be interesting to investigate the procedures and specific variations in secretion cells. The mast cell, already mentioned as an immune cell involved in allergic reactions, might serve as a suitable candidate for this comparative research. Firstly, as mentioned above, the mast cell is capable of exerting compound exocytosis, which is orchestrated by the same SNAREs and regulatory proteins or alternative isoforms (Shukla *et al*. 2001). A recent study in mice on the expressed proteins VAMP-2, -3 and -8 showed that knockdown of VAMP-8 has the most reducing effect on regulated exocytosis (Puri & Roche 2008). In the same study, VAMP-8 was found to be localized to distinct secretory granules, suggesting a discrepancy between SNARE isoforms involved in different secretion pathways. Another recent study, conducted in human mast cells, showed that VAMP-7 and VAMP-8 are essential for activation-induced exocytosis while VAMP-2 and -3 were significantly present (Sander *et al*. 2008). This study also demonstrated that upon activation, these two proteins co-localize with SNAP-23 (similar to the rather neuron-specific SNAP-25 (Ravichandran *et al*. 1996)) and syntaxin-4 (syntaxin-1 appears to be neuron-specific (Shukla *et al*. 2001)) on the plasma membrane.

A possible explanation for the massive action of compound exocytosis lies in the distribution and relocation of some SNARE protein. SNAP-23 is, together with syntaxin-4, located in protrusions of rat mast cells in resting state and relocates to the cell surface upon stimulation – which is a prerequisite for exocytosis and assumingly prevents early SNARE complex formation (Guo *et al*. 1998). Moreover, SNAP-23 was found to relocate further into the cell interior upon activation (syntaxin-4 remained at the cell surface) and this suggestively promotes the mutual fusion of granules (Guo *et al*. 1998, Castle *et al*. 2002). While syntaxin-4 is

strictly a plasma membrane protein, syntaxin-3 was found together with VAMP-7 on secretory granules in RBL-2H3 cells (rat tumor mast cells) and these two proteins form binary *cis*-SNARE complexes (Puri *et al*. 2003). The same study suggested an important role for NSF in disassembling these complexes for successful exocytosis (in a compound manner) because increasing amounts of mutant NSF significantly inhibits exocytosis. Subsequently, one could hypothesize that relocated SNAP-23 (into the cell interior via the fused granule membrane) interacts with VAMP and syntaxin-3 on secondary granules and thus facilitates the compound exocytotic event.

SNARE regulator actions in mast cells

Among the two isoforms Munc18-2 and 18-3 that are expressed in RBL-2H3 cells, most abundantly present is Munc18-2 (Martin-Verdeaux *et al*. 2003, Nigam *et al*. 2005). Interestingly, the two show distinct interactions with syntaxin isoforms. Munc18-2 interacts with syntaxin-3 and strongly co-localization with secretory granules, while Munc18-3 interacts with syntaxin-4 and is generally localized at the plasma membrane (Martin-Verdeaux *et al*. 2003, Tadokoro *et al*. 2007). These findings enhance the assumption that distinct SNARE mechanisms operate in either plasma membrane-granule or granule-granule fusion to facilitate mast cell degranulation. To date, very little experimental data is present on Munc18 in mast cells and its function remains under debate.

The SNARE regulator protein complexin-2 has also been identified in mast cells although its exact role is also rather doubtful. Upon stimulation, complexin-II relocates from being dispersed in the cytoplasm to the plasma membrane and positively regulates exocytosis (Tadokoro *et al*. 2005). Interestingly, recent data shows that complexin-2 interact with syntaxin-3 (which is present on both membranes of granules and the plasma membrane) and not with syntaxin-4 (which is restricted to the plasma membrane) (Tadokoro *et al*. 2010). This interaction with syntaxin-3 was enhanced by the presence of SNAP-23 and VAMP-8. These data suggest the interaction of complexin with the SNARE complex in a similar fashion as observed in neurons,

but also point to the possible interaction on secretory granules and involvement in granule-granule fusion.

Taken together, the mast cell is observed to consist of complicated machinery for its degranulation, probably for its ability to secrete various molecules and to do so by compound exocytosis. However, the main trigger for cell activation and secretion is also the rise of calcium and synaptotagmin-2 appears to be the important calcium sensor (Melicoff *et al*. 2009). Noteworthy to point out is that instead of, like the neuron, maintaining a ready-set state for rapid exocytosis, the mast cell response to cell activation is somewhat slower and more dramatic. As a consequence, in studies on secretion cells, a rather diverse action of SNARE proteins and regulators (and cognate isoforms) should be taken into account. The experimental findings presented in chapter four will be discussed within the perspective of a more complicated situation than observed in the exocytotic model cell, the neuron.

CHAPTER THREE

The oocyte and the cortical reaction

The oocyte

As briefly mentioned in the last section of chapter one, the oocyte exerts exocytosis at an important moment during its existence. At fertilization, the oocyte needs to fuse with a single sperm cell to successfully start the development of a new organism. In order to ensure the success of this process the oocyte needs to be in a highly prepared state and be able to prevent the fusion of multiple sperm cells. To achieve this, the oocyte undergoes the cortical reaction directly after the fusion with the first sperm cell. When an oocyte is selected to mature and thus to become ovulated it undergoes a long-lasting process from an under-developed cell to a fully matured cell that is ready for fertilization and in which exocytotic vesicles become ordered at the cortex region (just under the oolemma) and are ready for fertilization-induced exocytosis. In the following section, I will give an overview of the maturation process of the oocyte and the relevant preparation as well as execution of the cortical reaction. This chapter is also the introduction to my experimental research topic, which is dealt with in chapter four.

Maturation steps

During mammalian embryonic development, a specialized group of cells called the primordial germ cells (PGC) arises. These cells are capable of forming male and female gametes. In the development of a female organism, these cells differentiate into oogonia and are grouped together in large clusters. When these cells become fully embedded in the tissue of the developing ovaries, DNA replication starts and a first meiotic cycle begins. With the onset of these developmental steps, the cell becomes an oocyte, which resides in primordial follicles. These follicles not only nurture the oocytes throughout the whole maturation stages, but also develop themselves along in time. The early oocytes pass through several sub-stages within meiosis prophase I before they are arrested at the diplotene stage. It is at this stage - due to the intrinsic quality control system – of the early oocyte maturation that a massive portion of the

oocyte pool is lost. Percentages of loss differ between species but are roughly between 60-95% with humans in the highest levels. This control system ensures the quality of the oocytes and partially contributes to the success of fertilization. The arrest during meiosis I is maintained for a long period of time, and is resumed for further oocyte maturation after the follicle has started growing for potential ovulation. Characteristic for the stage at the arrest is the organization of the genetic material. The entire replicated DNA, ready for the first meiotic division is stored in a circular orientation inside the swollen nucleus. This structure is called the germinal vesicle (GV) and is used for indication of this stage of the oocyte (van den Hurk & Zhao 2005).

At the time of the germinal vesicle stage, as major rearrangements and modifications have been made inside the nucleus, the cytoplasm has also been reorganised. During the various stages of the prophase, gene expression and protein production have fluctuated, marking the onset of cytoplasmic maturation. The development of the oocyte starts with drastic changes in metabolism and forces other cell organelles, such as mitochondria, to relocate and increase in activity. The increase of protein and lipid synthesis makes the E.R. and Golgi complex to expand tremendously and increases the intracellular vesicle transport. Among these vesicles, a specialized group of secretory granules (later named cortical granules) are formed. These vesicles, which are destined to fuse with the plasma membrane of the oocyte upon fertilization are scattered around in the ooplasm at this stage (Ferreira *et al*. 2009).

From the moment that the female becomes fertile, follicles in the ovary become periodically selected to grow further and nurture the oocyte until ovulation. Inside the follicle, the oocyte continues its maturation to ultimately form a haploid cell that is able to fuse with a sperm cell. The continuation of the maturation starts with the resumption from the meiotic arrest, which characterized by the breaking down of the GV. From then, the oocyte proceeds through the further phases of meiosis I. The replicated genetic material, which has already undergone recombination, is divided during metaphase I and the first polar body is formed. Next, further cytoplasmic maturation and genetic reorganizations are made and the oocyte prepares itself for fertilization by a sperm cell. Synthesis of proteins and lipids is low at this stage and the E.R. and Golgi complex are reduced in size. The absence of a functional nucleus could also be the reason for this reduction. The oocyte enters meiosis and the genetic material is

prepared for the final division during metaphase II. It is in this stage that the oocyte undergoes another arrest, right before the formation of the second polar body. The chromosomes are orientated in the so-called metaphase plate at the cortical area near the first polar body. One of the major cytoplasmic reorganizations is the migration of the cortical granules. From the resumption of the maturation process, these granules gradually moved to the cortical area of the oocyte and reside adjacent to the oocyte plasma membrane. This migration process is probably assisted by specific intracellular matrix (i.e. actin) and these granules later become docked to the oocyte plasma membrane to prepare for the rapid cortical reaction upon fertilization (Ferreira *et al*. 2009).

Zona binding and fertilization

The moment of fertilization, when a sperm cell encounters the oocyte, is extremely decisive and highly programmed. The target for binding of the sperm cell is the zona pellucida (ZP), which is in humans made up of four proteins (ZP1 to ZP4). It has been shown that the human sperm cell primarily binds to the ZP3 and ZP4 proteins to induce its own acrosomal reaction to ultimately enable the fusion with the oocyte plasma membrane (Gupta *et al*. 2009). During the acrosomal reaction the sperm cell releases proteolytic enzymes to digest the ZP in order to approach and bind the oolemma. Next, the sperm cell penetrates the oocyte and subsequently evokes the cortical reaction (further discussed below). This exocytotic event results in the modification of the ZP in order to prevent sperm binding and thus penetration, which is discussed in the next section. When a single sperm enters the oocyte, the two pronuclei fuse and the first somatic cell division is started. At this moment, fertilization is successful and a zygote will start to develop.

The cortical reaction

The cortical reaction is an important process that occurs after a sperm cell fuses with the oocyte and is so-called activated. The result is the release of a large amount of enzymes into the

perivitelline space in a very short matter of time. The enzymes that are secreted act on the proteins of the whole ZP surrounding the oocyte and various studies indicate that mainly the ZP2 and ZP3 proteins are modified (Ducibella 1996, Tsaadon *et al*. 2006, Gupta *et al*. 2009). As a result of these modifications, the sperm receptors in the ZP are removed and no other sperm cell is able to bind. Among other mechanisms the oocyte has, this block to polyspermy plays a major role in the success rate of fertilization and indicates the importance of research on the cortical reaction and its regulation.

As the oocyte proceeds through several phases during its maturation, properties and fertilization potential differ greatly between the GV- and M-II stages. Moreover, studies on oocytes from various species show that CR is mostly absent or abnormal after fertilization of GV-stage oocytes and high incidences of polyspermy are observed (Ducibella 1996). These facts indicate that the maturation of the oocyte is important for development of CR competence and forms a large field of research. During this maturation, there is a major rearrangement of organelles (orchestrated by the cytoskeleton) towards the centre of the oocyte and an organelle-free zone is created in the cortex. Cortical granules are an exception to this and migrate in opposite direction. Actin filaments are essential for CG migration and attach to the plasma membrane by specialized proteins. After germinal vesicle breakdown, cortical granules start the migration along these filaments and this is dependent on association with Rho proteins until they reach the cortex (Sun & Schatten 2006). When CGs arrive at the plasma membrane, they dock independent of actin and by association with other specialized proteins.

Studies in sea urchins and mice show that the docking of the cortical granules to the oolemma is, like in many other exocytotic events, performed by proteins of the SNARE family, as is the subsequent exocytosis (Ikebuchi *et al*. 1998, Sun & Schatten 2006). There is a wide range of SNARE proteins which all have their specific role in the so-called tethering of the vesicles to the plasma membrane and the fusion of the membrane. Since these SNARE proteins are part of the SNARE hypothesis, which prevails for many exocytotic events (e.g. synaptic release), a fascinating observation is that they are differentially distributed as maturation progresses and even after fertilization has occurred (Conner *et al*. 1997). In this way, by studying

the localization of proteins at different moments in the oocyte's lifetime, a more detailed understanding can be created of how the cortical reaction is executed.

However, while a number of observations of SNARE proteins in sea urchins and mice have been made, there is still a major blank concerning the role of SNARE proteins in mammalian oocytes. Consequentially, there is a niche of experimental knowledge to support the importance of SNARE proteins in the cortical reaction and thus the construction of a polyspermy blocking mechanism. This was the motivation for conducting the experiments, in which the distribution of SNARE regulators was examined, that are discussed in chapter four.

The biology of the cortical reaction

The role of calcium

When the sperm cell and the oocyte have fused and the oocyte is activated, a wide range of processes is set in action to start the development of the zygote. Besides the suggested importance of the cortical reaction in the polyspermy block, most important of this activation is the resumption of meiosis, which is characterized by the formation of the second polar body and marks the onset of development. More than two decades ago, early studies on fertilization demonstrated significant intracellular increases in calcium concentrations, which were soon thought to be essential for the activation of the oocyte (Kline & Kline 1992). Since then, the knowledge of the role of calcium in oocyte activation has been expanding in high pass.

In mouse oocytes, a rise of calcium was observed approximately ten minutes after fertilization, which is followed by series of short peaks in concentration with intervals of five to ten minutes (Fig. 6) (Kline & Kline 1994). Various methods of inducing these transient of calcium were discovered, such as injection of Ca^{2+}, electropermeabilization and treatment with calcium ionophore, all leading to egg activation (Kline 1996, Wang *et al*. 1999). However, both artificial and sperm-induced activation yielded different results in calcium transients depending on

maturation phase of the oocyte (Kline 1996, Cheung *et al*. 2000). Assumingly, oocytes develop competence to generate calcium transients as maturation progresses, resulting in increasing success rates of development. This is largely due to the cytoplasmic maturation and will be explained in the following sections.

While the direct effects of calcium on activation processes were gradually elucidated, new hypotheses were posed to explain the calcium oscillations (also termed calcium spikes). Inositol 1,4,5 trisphosphate (IP_3) was found to be the second messenger that induces the release of calcium from the endoplasmatic reticulum (E.R.) stores and injection of IP_3 into the oocyte leads to activation (Miyazaki *et al*. 1993, Kline & Kline 1994). IP_3 receptors (IP_3R) are located on the E.R. and the sensitivity of oocytes for IP_3 augments as maturation progresses. This can be explained by the increase of IP_3R type 1 that is associated with oocyte maturation (Xu *et al*. 2003).

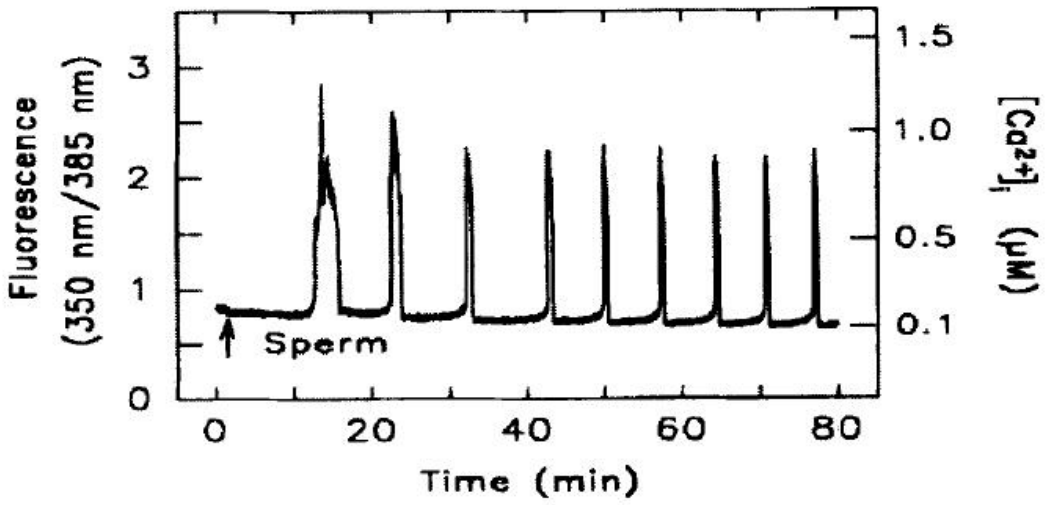

Figure 6. **Calcium oscillations observed in the mouse oocyte after fertilization**. Approximately ten minutes after fertilization with sperm (indicated with the arrow), the intracellular calcium levels increases to about a tenfold of the concentration at resting state. The first transient lasts for a couple of minutes and returns to normal levels again. Next, an oscillatory return of peaks in calcium concentration continues with intervals of five to ten minutes. Image from J.T. Kline & D. Kline, *Biology of Reproduction* **50**, 1994.

The sperm factor

As observed in many cell types, IP_3 is produced by cleavage of the membrane bound phosphatidylinositol 4,5 bisphosphate (PIP_2), which is catalysed by a phospholipase. Investigations showed that injections of mammalian intracellular sperm extracts into the oocyte were able to induce activation similar to normal fertilization and eventually a soluble and sperm-specific isoform, phospholipase C-ζ (PLC-ζ, zeta), was identified (Saunders *et al*. 2002). Injections of PLC-ζ cRNA and both purified and recombinant PLC-ζ protein triggered calcium oscillations and oocyte activation in a non species-specific manner (Swann *et al*. 2006). In spite of minor sequence differences in the PLC-ζ gene between primates and mice, crosses-species treatment of oocytes with PLC-ζ cRNA was able to induce oocyte activation and development until blastocyst stage in both species (Cox *et al*. 2002). Altogether, PLC-ζ is, at least among mammals, a highly conserved protein that is able to trigger the calcium oscillations and subsequent activation of the oocyte. Endogenous PLC of the oocyte might alternatively function as a trigger, although the definition of the exact mechanism is still under debate.

Calcium oscillations and targeting

As reviewed by Stricker in 1999, calcium release and concentration patterns upon fertilization vary among spies but calcium remains to be the common signal in oocyte activation (Stricker 1999). However, certain fish and amphibian species exhibit a single calcium transient, while in all mammals subsequent oscillations followed an intense primary rise. During normal fertilization in all species, the calcium release is initiated at the sperm entry point and propagates in a wave-like pattern across the whole oocyte (Whitaker 2006). Interestingly, artificially induced calcium transients by injection of calcium, as well as sperm-induced in calcium-free media, show a similar pattern as observed in normal fertilization experiments. This indicates that oocytes initiate these waves from internal calcium stores and propagate by intrinsic systems, which include the positive feedback of calcium on the IP_3R and the calcium sensitive activity of PLC-ζ (Whitaker 2006, Swann & Yu 2008).

With the knowledge that calcium is an essential player in oocyte activation, it must have various cellular targets that involve the diverse activation processes. Some of the proteins that are known to be targeted by calcium are protein kinase C (PKC) and calmodulin kinase II (CaMKII) (Whitaker 2006). The latter is specifically known to act on cyclins and other related proteins that control the cell cycle and are responsible for resumption of the meiosis. Assumingly, various proteins that are involved in the onset of the cortical reaction are also likely to be targeted, which will be discussed further in this thesis. However, there are still plenty of questions to be answered about the downstream targets of the calcium waves at oocyte activation.

Cytoplasmic maturation

It is commonly known that maturation of the oocyte increases the success of fertilization and ability to undergo activation. Since the activation is initiated by calcium oscillations, one can assume that these are best generated when the oocyte possesses the correct cytoplasmic properties. One of the major maturation-associated intracellular changes is the reorganization of the cell organelles, especially the endoplasmatic reticulum. Observations in many different species show that while maturation progresses the E.R. undergoes morphological changes to form clusters that localize in the cortical and subcortical area (Stricker 2006). Together with reduced calcium waves upon fertilization of immature oocytes, this suggests an important role for the development and redistribution of intracellular calcium stores in activation competence (Stricker 2006). In addition to the formation of E.R. clusters, a maturation-associated increase in IP_3R is observed in mouse oocytes (Xu *et al*. 2003). These findings attribute to the fact that the development of the ability of oocytes to generate calcium oscillations depends heavily on cytoplasmic maturation.

The cytoskeleton has a pivotal role in oocyte maturation and the reorganization of cell organelles. For instance, microtubules are responsible for the segregation of the chromosomes and formation of the distinct nuclear patterns (Sun & Schatten 2006, Ferreira *et al*. 2009).

Experiments in mouse oocytes show that upon germinal vesicle breakdown (GVBD), the formation of cortical E.R. clusters is orchestrated by microtubules and microfilaments (FitzHarris *et al*. 2007). Additionally, cortical granules are also translocated by mediation of the filamentous structures, mainly by actin filaments (Wessel *et al*. 2002, Sun & Schatten 2006). The migration of these granules and the subsequent processes of docking and exocytosis are of great importance for the cortical reaction and are discussed in the following sections.

Cortical reaction upon activation

As described above, calcium can clearly be held responsible for the activation of the oocyte in general. However, the direct causal relation between calcium and specific activation events (i.e. cortical reaction) necessitate further experimental evidence. The question arises: is the cortical reaction directly triggered by calcium, or do calcium oscillations commence another signalling route coupled to the cortical reaction?

In a study performed by Ducibella *et al.* in 2002, onset of various activation events in mouse oocytes was investigated by differentiation of the amount of calcium pulses. The principle findings show that each event depends on a different number of calcium oscillations (Ducibella *et al*. 2002). With these results, a chronological table of oocyte activation can be created since the calcium oscillations steadily accumulate over time. In this timetable, the exocytosis of CGs is the first activation event that comes into view. A single calcium pulse was enough for secretion of 20% of the initially present cortical granules and after four pulses (with eight minutes interval) half of them were secreted.

A subsequent study treated mouse oocytes with 3, 10 or 20 calcium pulses with two minutes intervals to examine activation events, independent of endogenous calcium release as observed in normal fertilization. All three treatments show a similar release of CGs within one hour after stimulation (Ozil *et al*. 2005). Moreover, even the shortest stimulation showed the quickest onset of the CR. Thus, one can conclude that the cortical reaction is not only directly coupled to the rise of intracellular calcium, but also highly sensitive to an initial calcium

transient. Suggestively, a calcium sensitive molecular system probably triggers the exocytosis of CGs. This will be discussed in the next chapter, which focuses on the proteins involved in exocytosis.

Alternative signalling routes

Studies on pancreatic acinar cells give an interesting insight into intracellular signalling to evoke exocytosis. As reviewed by Peterson and Tepikin, calcium oscillations can be elicited in these cells by binding of two different molecules to their specific plasma membrane receptors (Petersen & Tepikin 2008). Both trigger the activation of distinct pathways to ultimately release calcium from the E.R. stores. Besides production of IP_3 by PLC action that is involved in one pathway, another signalling route generates the second messengers nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPR). Both these molecules act on the ryanodine receptor (RyR) on the E.R., which is also a calcium channel, similar to the IP_3R.

Interestingly, while the generation of calcium waves in the oocyte has largely been assigned to the IP_3 pathway, the importance of NAADP in the initiating of calcium waves has been described in experiments on starfish (Moccia *et al*. 2006). However, the same experiments showed that blockage of the RyR had no effect on polyspermy rates. This indicates that the role of NAADP in fertilization success in played in a more complicated system and involves a delicate interplay with the IP_3R route. Additionally, this challenges the role for the recently discovered sperm-derived activator molecule PLC-ζ. Although a role for another calcium release route in oocyte activation in mammals has not been elucidated yet, the importance of calcium to activate the oocyte, especially triggering the cortical reaction, remains the same.

Maturation and cortical granules

During oocyte development, the reorganization of the cytoplasm is of vital importance and involves the migration of cortical granules. In several species, the structural distribution of CGs

has been investigated at maturation stages. Studies in mouse (Ducibella *et al*. 1988), porcine (Wang *et al*. 1997), goat (Velilla *et al*. 2004) and horse (Carneiro *et al*. 2002) oocytes show similar structural results. At GV-stage, cortical granules generally appear to be randomly distributed in the cytoplasm with a mild accumulation at the cortex. While at M-I stage a monolayer is already created adjacent to the plasma membrane, especially in porcine oocytes, at M-II the distinct monolayer of CGs is completed. These structural differences have created a criterion for maturation progress and this method of observation could be used to assess and optimize maturation protocols for IVF.

With these results, the development of competence to successfully undergo cortical reaction, which increases from the moment of germinal vesicle breakdown, cannot be directly linked to CGs migration. The presence of CGs in the cortical area in early maturation stages suggests that probably the development of the signalling pathways is the restricting factor (Ducibella 1996). As explained above, the formation of E.R. clusters in the cortical area could play an important role in this competence development.

Additionally, while the migration of CGs is mediated by microfilaments, the last steps of anchoring to the plasma membrane are independent of the cytoskeleton (Sun & Schatten 2006). This process of CGs docking is executed by specialized proteins and essential for the construction of a highly regulated and calcium sensitive exocytotic mechanism. The next chapter will focus on SNAREs and their related proteins involved in the regulation of this docking process as well the fusion event itself.

Cortical reaction and polyspermy block

As ready mentioned earlier in this chapter, the secretion that is yielded by the cortical reaction is thought to have a major effect on the zona pellucida (ZP), termed "zona hardening". A recent study by Papi *et. al* investigated the differences in morphology and physical properties of the ZP in immature, matured and fertilized bovine oocytes. They found that maturation resulted in the loss of elasticity while fertilized oocytes recovered this elasticity, though being more compact

and rigid (Papi *et al*. 2010). With these properties the ZP is more resistant to digestion, for instance by a secondary sperm cell. This zona hardening is achieved through proteolytic modification by enzymes that are released into the perivitelline space. For a review on the composition of ZPs in mammals and modifications of ZP proteins, see (Gadella 2010).

It has been widely established that the cortical reaction induces a functional alteration of the ZP (thus participating in the block to polyspermic fertilization), although still many uncertainties remain. For instance in mouse oocytes, N-acetylglucosaminidase was found to be essential for ZP protein modification and localized in cortical granules (Miller *et al*. 1993), the exact content that is secreted during the cortical reaction has not yet been described. Additionally, there is increasing evidence that indicates the importance of pre-fertilization hardening of the ZP in potentiating the polyspermic block (Coy *et al*. 2008a, Coy *et al*. 2008b, Canovas *et al*. 2009). These early modifications normally take place in the oviduct, where *in vivo* the oocyte resides prior to fertilization, and are bypassed by *in vitro* fertilization (IVF). Also, while in the polyspermy block in mammals the emphasis is placed on the zona pellucida, a role for the oolemma properties and modifications (as mainly observed in marine animals) has not extensively been considered. Chapter five will continue in the discussion of this topic.

CHAPTER FOUR

SNAREs and regulators in the oocyte

EXPERIMENTAL REPORT

Experimental Report

This chapter presents the experimental part of this thesis with the background presented in the previous chapters, which have served as in-depth introduction on the topics of exocytosis in general, the role of SNARE proteins and the properties of the oocyte, research on the cortical reaction and its importance.

Redistribution and role of SNARE regulator proteins in preparation of the cortical reaction in maturing porcine oocytes: an immunostaining study.

Introduction

The cortical reaction in the oocyte is part of the many processes that is set in action upon fertilization, termed oocyte activation. For successful fertilization, the oocyte must be penetrated by a single sperm cell after which the two pronuclei fuse to ultimately form a diploid zygote. This condition is secured by means of the so-called "polyspermy block" and the cortical reaction (CR) is assumed to play an important role in mammals (Sun 2003). Cortical granules are a special type of secretory vesicles that migrate from a dispersed distribution to a cortical localization during oocyte maturation (Ducibella *et al*. 1990, Tsai *et al*. 2010). This migration is mediated by cytoskeleton structures and is part of a larger redistribution of the cytoplasm (Wessel *et al*. 2002, Sun & Schatten 2006, Ferreira *et al*. 2009). As a result, the cortical granules arrive at sites adjacent to the oocyte plasma membrane (oolemma) where they are primed to rapidly fuse and release their content when the oocyte is activated. This activation is normally induced by fertilization (thus penetration) of sperm cell, which is thought to introduce phospholipase C-ζ(zeta) (PLC-ζ) (Saunders *et al*. 2002, Swann *et al*. 2006). However, the

injection of ectopic PLC-ζ (Cox *et al*. 2002) or administration of calcium ionophore (Wang *et al*. 1999) can mimic this activation. Generally, the activation results in a rise of intracellular calcium and subsequent oscillatory transients of increased concentration (Swann & Yu 2008). In response to these calcium oscillations, cortical granules fuse with the oolemma and release their enzymatic content, modifying the surrounding zona pellucida (ZP) to prevent binding of additional sperm cells (Ducibella 1996, Sun 2003).

The docking and fusion of vesicles in exocytotic events in various cell types, especially in neurons, has been extensively studied and the importance of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and associated proteins is generally known (Jahn & Scheller 2006). However, scarce data has surfaced over the years that describe the role of SNARE proteins in the cortical reaction. Reports are available that describe the presence of SNARE in sea urchin (Conner *et al*. 1997) and in mouse oocytes SNAP-25 was found to be essential in the cortical reaction (Ikebuchi *et al*. 1998).

Recently, in a study performed by Tsai *et. al,* the distribution of SNAREs and regulator proteins in porcine oocytes has been examined to investigate the relocation during oocyte maturation (Tsai *et al*. 2010). This research group found that during maturation from the germinal vesicle (GV) stage to the metaphase-II (M-II) stage, the SNARE proteins VAMP-1 and SNAP-23 relocate to the cortical area and ultimately, together with syntaxin-2, colocalize with the cortical granules. However, little is known about the localization of SNARE regulator proteins in the preparation of the CR. In this present study, we investigate the (re)distribution of the SNARE regulators complexin, Munc18 and synaptotagmin by immunostaining and confocal imaging techniques.

Materials and Methods

Reagents and antibodies

All used chemicals, except when stated, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Affinity purified rabbit polyclonal antibodies (dilution stated in brackets) against synaptotagmin-1 and -2 (1:500, specific for both isoforms), Munc18-2 (1:250), complexin-3 (1:100) and complexin-4 (1:100) were purchased from Synaptic System (Göttingen, Germany).

Oocyte obtainment, culturing and in vitro *maturation (IVM)*

Ovaries were obtained from adult fertile sows (*Sus scrofa*) from a slaughterhouse in Groenlo, the Netherlands. Cumulus-oocyte-complexes (COCs) were collected by aspiration of follicles with 3-6 mm diameter and selected by criteria as reported by Schoevers *et. al* (Schoevers *et al*. 2007). This procedure yielded approximately 100 oocytes per session.. COC selection was done in HEPES buffered M199 (Gibco Laboratories Inc., Grand Island, NY, USA) and subsequently washed three times in pre-equilibrated M199 supplemented with 2.2mg/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). Following *in vitro* maturation (IVM) was conducted as previously described (Schoevers *et al*. 2007). For obtaining germinal vesicle (GV) stage oocytes (20-30 oocytes per staining group, approximately 100 in total), COCs were repeatedly resuspended and washed in 80 mM PIPES, 5 mM EGTA, 2 mM MgCl2, pH 6.8, supplemented with 0.3% (w/v) PVP (PEM-PVP) to denude oocytes prior to immunolabeling. Oocytes that could not be identified with the presence of germinal vesicle structure were excluded from the study. For oocyte maturation until metaphase-II (M-II), COCs were cultured at 38 °C with 5% CO_2 for 22 hours in OMM provided with 0.05 IU/ml recombinant human FSH (rhFSH, obtained from Organon, Oss, the Netherlands). Following the 22 hours IVM, COCs were similarly cultured for another 22 hours in OMM, free of rhFSH. After complete IVM (total 44 hours) COCs were denuded, similarly as GV stage oocytes, and selected for presence of metaphase plate and first polar body (20-30 oocytes per staining group, approximately 100 in total).

Immunofluorescent straining for SNARE regulator proteins

Immunolabeling was performed as previously described (Holzenspies *et al*. 2009). Primary and secondary antibodies were diluted in blocking buffer. To prevent formation of aggregates, antibodies were centrifuged at 45,000 g for 1 hour before use. Incubation with primary antibodies (for negative control group, purified rabbit IgG (BD Bioscience) was used, diluted 1:100) was done overnight at 4 °C. Subsequently, oocytes were washed three times with PBSS (0.1 M PBS, pH 7.4 containing 0.1 % Saponin) before incubating for 1 hour with Alexa-488 conjugated with goat-anti-rabbit IgG (Molecular Probes, Eugene, OR) at room temperature. After three washes with PBSS, DNA was labelled with 1:100 ToPro-3 iodide (Molecular Probes, Leiden, the Netherlands) for 20 minutes in PBSS and washed similarly three times. Next, oocytes were transferred into 0.12 mm, 8 well Secure-Seal Spacer (Molecular Probes, Leiden, the Netherlands) on a cover slip, protected with 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

Procedures for microscopy and image processing were followed as previously described (Tsai *et al*. 2010). 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.

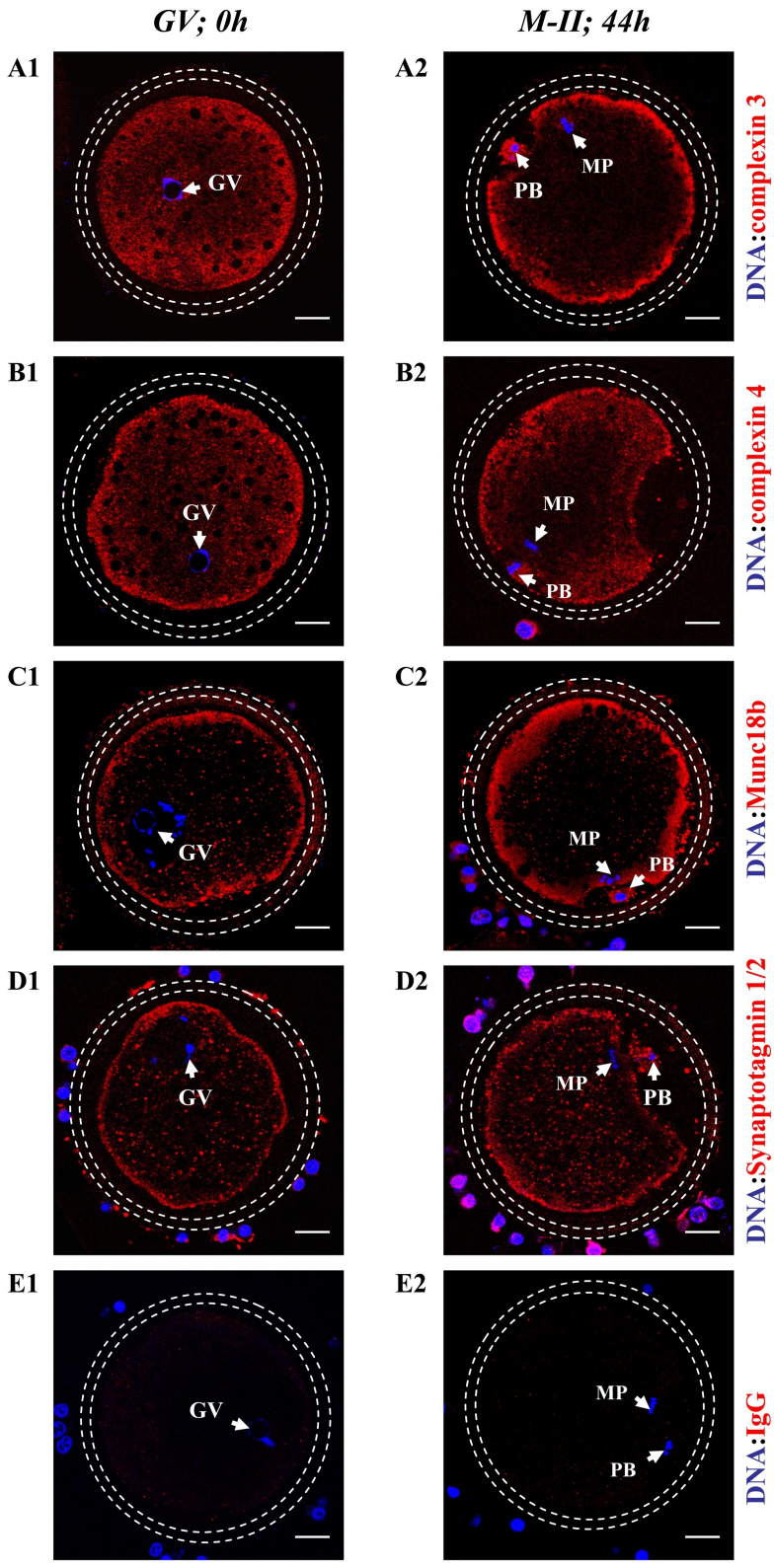

Figure 7. **SNARE regulator proteins (re)distribute during oocyte maturation in preparation for the cortical reaction.** Staining of SNARE regulator proteins (*red*) in germinal vesicle (GV) stage or metaphase-II (M-II) stage show selective redistribution during oocyte maturation. DNA staining (*blue*) shows either GV structure or metaphase plate and first polar body. Complexin-3 shows a dispersed distribution throughout the ooplasm in germinal vesicle (GV) stage (**A1**) but migrates into the cortical area when the oocyte matures to metaphase-II (M-II) stage (**A2**) where it can bind to the SNARE complex at the docked cortical granules (CGs). Complexin-4 shows rather similar distribution in both stages (**B1**, **B2**). At GV stage, Munc18-2 is generally dispersed through the ooplasm with remarkable cortical abundance (**C1**), although this varied among the oocytes in the sample. In M-II stage, Munc18-2 showed a slightly stronger cortical localization (**C2**), where it could associate with CGs, but this cannot be ascertained. Synaptotagmin showed in both GV (**D1**) and M-II (**D2**) stage a rather dispersed distribution in the ooplasm. Although a slight predominant localization in the cortical area could be observed, this distribution was not common among the sample. Additionally, the signal of the staining in the entire sample was relatively low, rendering a doubtful presence for these two isoforms.

Results and Discussion

To investigate the localization of SNARE regulator proteins in both GV and M-II stage oocytes, we fixed oocytes and subsequently stained them with fluorescent antibodies. Using confocal microscopy we examined the differences in distribution patterns of complexin, Munc18 and synaptotagmin and found that there is a remarkable relocalization of some of these proteins during oocyte maturation. Representative images of the staining patterns are shown in Fig. 7.

Complexin

The distribution of complexin was examined using antibodies against two isoforms, namely complexin-3 and -4, which do not cross-interact. In GV oocytes stained for complexin-3 we found a regular distribution across the ooplasm. Although some oocytes in the imaged sample showed a weaker signal, we determined that complexin-3 is located throughout the ooplasm during the GV stage (Fig. 7A1). Oocytes in M-II stage showed a significant different staining pattern for complexin-3. The signal was observed as a relatively sharp line at the cortical area of the oocyte, creating a demarcation of the cell (Fig. 7A2). These observations suggest the migration of complexin 3 to a position adjacent to the oolemma during maturation. In this position complexin-3 could interact with the SNARE complex to regulate the fusion event, probably by preventing precipitate fusion of the cortical granules with the oolemma. Whether complexin-3 is truly interacts with the SNARE complex and cortical granules could be examined by co-localization studies.

Staining for complexin-4 showed a different pattern of distribution. In both GV and M-II stage oocytes, the staining is observed throughout the whole ooplasm. Additionally, within the sample, the staining showed slightly differences in patterns and Fig. 7B1 and 7B2 illustrate the intermediate distribution. We conclude that there is no distinct difference in distribution of complexin-4 between the two stages of the oocytes and the role of this isoform is likely to be inferior to the assumed role of complexin-3.

Munc18

The localization of Munc18 was examined by the use of an antibody specific against the isoform Munc18-2. In the GV stage oocytes we found a somewhat scattered distribution of Munc18-2 with a slight predominant staining in the cortical area and Fig. 7C1 shows a representative image of the observation. The staining pattern in the M-II oocytes sample resembled the observation of GV oocytes, although the cortical localization was stronger. In Fig. 7C2 a more abundant signal is illustrated in the cortical area, although this cannot suggest a distinct redistribution during oocyte maturation. Moreover, we cannot strictly couple these observations with a functional explanation for this isoform. Suggestively, as a soluble protein, Munc18-2 could interact with both the plasma membrane and the cortical granules. Co-localization studies with specific SNARE proteins could give more insight in the role of Munc18.

Synaptotagmin

For the staining of synaptotagmin we used an antibody that was specific for two isoforms, synaptotagmin-1 and -2. The distribution of synaptotagmin showed no significant difference between GV of M-II stage oocytes. The staining pattern in both samples was distributed throughout the entire oocyte and no distinct localization could be observed (Fig. 7D1 & 7D2). Additionally, the signal of the staining in both samples was considerably weaker than observed in the other staining experiments, although this was corrected by image processing. Therefore, we are not able to determine the presence of these two synaptotagmin isoforms since the staining could be caused by aspecific binding of the antibody. This is not fully surprising given the fact these isoforms are mainly observed in neurons throughout literature. However, similar immunostaining studies on other isoforms, such as synaptotagmin-7 and -8, might yield interesting results to suggest a role for this protein.

CHAPTER FIVE

Conclusions and future prospects

The role of SNARE proteins in exocytotic events has been well established over the many years of research that have passed since their first identification. Initially, as their name (SNAP-receptors) suggests, these proteins were identified as receptors for a protein that was found to be of high importance for membrane fusion processes and received most attention. However, as their functions gradually became clearer and their abundance and variety of isoforms grew, they claimed the focus of research on exocytotic events. Present data shows the presence of SNARE proteins in many cell types, especially in cells capable of secretion namely in the regulated exocytotic pathway. The formation of SNARE complexes is explanatory for the mechanistic part of membrane fusion. From the moment that a secretory vesicle docks at the plasma membrane, the complementary SNARE proteins interact to construct a situation that is primed to respond to a trigger that initiates the fusion of the opposing membranes. This event of membrane fusion is accompanied, and assumingly facilitated, by a structural transition of the SNARE complex. Prior to the fusion, the SNARE complex is in a *trans* orientation and after fusion it has transited to a *cis* complex, which is inserted in the newly merged membrane. Since the fusion of membranes is known to be energetically unfavourable, this transition must generate enough energy of compromise this. The zippering model with a hemifusion intermediate, as discussed in chapter one, provides an explanation for how the fusion process is orchestrated. However, this model is still hypothetical and can therefore not be considerate as a common property.

The first studies on SNARE proteins were conducted in neuron and constructed a strong hypothesis on which all subsequent research was based. Although, to date, the most elaborated descriptions of the mechanism in literature are based on observations in neurons, the role of SNARE proteins in, among others, secretion cells is gradually being established. Recent studies on mast cells show the presence of isoforms of VAMP, syntaxin and SNAP-25. However, the isoforms studied in these cells, which could be of essence among many other secretion cells, differ from the ones observed in neuron. Thus, one might suggest that secretion cells utilize a distinct subset of SNARE proteins to orchestrate their membrane fusion events. This is a

compelling assumption, especially because this could explain the different manifestations of exocytotic events of these cells, compared to the somewhat straightforward neuronal secretion.

While the function of SNAREs has become remarkably clear to date, the properties of SNARE regulator proteins is still to be fully unveiled. Although countless studies have proved these proteins to be of essential function, the debate of their precise functions is ongoing. Moreover, with experimental data increasing, the hypotheses start to diversify. The family of Sec/Munc proteins are assumed to be involved in vesicle docking and stimulation of SNARE complex assembly. Nonetheless, the unclearness about the exact interactions with other proteins and their cellular localization as a cytoplasmic protein obstruct the confirmation of these assumptions. Complexin has been suggested as a stabilizer of the SNARE complex and thereby inhibiting precipitate membrane fusion. In contrast, other studies have shown that it could also stimulate membrane and the fact whether and when it dissociates from the SNARE complex is still under debate. Additionally, complexin acts in tight interplay with synaptotagmin, which has been posed as the calcium sensor in exocytosis. However, a great number of isoforms of synaptotagmin has been identified, with differences in calcium sensitivity. Altogether, this renders a complicated situation in which SNARE regulators undoubtedly have essential functions, leaving a niche for future research.

In short, the data discussed in this thesis suggest a diversification in exocytosis both from a physical point of view (classical versus compound exocytosis, discussed in chapter one) as the diversity of SNARE protein isoforms. Still, there is a gap of knowledge about which isoforms of SNARE proteins function in which cells to create these physiological differences. Future research should focus on the investigation of the expression patterns and localization of these proteins in individual cell types, mainly in cells other than neurons, and accurately examine their function. More important, the knowledge about SNARE regulator protein might require even greater attention, since they are the key players in successful exocytosis.

SNARE proteins in oocytes

Given the fact that SNARE protein are broadly identified as the mediating proteins in exocytosis, it is not surprising that they are increasingly observed in oocytes. The cortical reaction is undoubtedly a secretion event, comparable to others described in chapters one and two. The most interesting facet of studies on SNARE proteins on the cortical reaction has to do with the preparation of the event. With the suggested role of zona hardening (as a result of cortical granule release) in the block to polyspermy, the cortical reaction needs to be precisely executed upon fertilization. In this perspective, the physiological state of the oocyte, that is the distribution of SNARE proteins and the docking of cortical granules, is crucial. However, to date, scarce data is available on SNAREs in the oocyte, especially during the maturation to a fully potential gamete.

At present, studies on SNAREs in oocytes are increasingly being conducted. Tsai *et. al* has conducted interesting experiments on the migration of cortical granules and SNARE proteins in porcine oocytes (Tsai *et al*. 2010). This marked a new direction in the understanding of the exocytotic aspect of the cortical reaction by focussing on differences between underdeveloped (GV stage) and fully developed gametes (M-II stage). Continuing in this line of research, I have conducted some experiments on the (re)distribution of SNARE regulator proteins in porcine oocytes as described in chapter four. The results of these experiments indicate an important role for complexin-3, probably associating with cortical granules and the SNARE complex since it migrates from a cytoplasmic distribution to a cortical localization. However, examination of complexin-4 yielded no remarkable redistribution, suggesting that this isoform is of less importance to the cortical reaction. Munc18-2 also showed a cortical localization in M-II stage, although this was not a dramatic redistribution as observed on complexin-3. Together with synaptotagmin, these two proteins require further investigation, especially in the examination of various isoforms.

Taken together, SNARE proteins carry out an important function in the cortical reaction. However, to better understand the construct of this exocytotic event, in special with regard to

the polyspermy block, SNARE regulator proteins should form a pivotal part of in research. A remarkable niche exists in the presence and localization, and thus in the precise functioning, of these proteins, which so tightly regulate this important exocytotic event.

Polyspermy block and the role of the cortical reaction

Lastly, one could discuss of how much interest the study on the cortical reaction is for the block to polyspermy. In artificial reproductive techniques, it is widely observed that polyspermic fertilizations are a common phenomenon. Initial research on mechanisms to prevent polyspermic fertilizations was conducted on marine animals and resulted in the suggestion of a membrane-based mechanism. Interestingly, in mammals, the zona pellucida and its hardening has received the greatest attention in research. By doing so, the possible importance of the oolema seems to be bypassed. Additionally, and even most interestingly, is that the question arises whether the zona hardening can be held responsible for complete preventing of polyspermic fertilization. One might suggest that the zona hardening, and thus the cortical reaction, functions as a facultative mechanism, prior to a most essential polyspermy block at the level of the oolema. Moreover, the exact mechanism of zona hardening, the composition of the zona itself and the precise contents of cortical granules remain to be indisputably unclear. Conclusively, this discussing is remarkably interesting and deserves respectable attention in the reproductive field. Nonetheless, the cortical reaction remains an interesting mystery to be solved.

Acknowledgements

For accomplishing this thesis, now it has found its final form, I would like to express my gratitude to some people in particular. Bart Gadella, my supervisor, for giving me the opportunity to spend the past months on the Biochemistry Department and gaining all the knowledge in both the reproductive and biochemical field to write this thesis, and more. Jason Tsai, my practical and daily supervisor, for introducing me into the wonderful experimental side of scientific research, assisting me on the experiments, teaching me the plentiful techniques and the interesting discussions. Lastly, I want to thank everybody at the Biochemistry Department for all the birthday cakes, candy in the tin and their kindness.

References

Andrews-Zwilling YS, Kawabe H, Reim K, Varoqueaux F & Brose N 2006 Binding to Rab3A-interacting molecule RIM regulates the presynaptic recruitment of Munc13-1 and ubMunc13-2. *The Journal of biological chemistry* **281** 19720-19731.

Augustine GJ, Morgan JR, Villalba-Galea CA, Jin S, Prasad K & Lafer EM 2006 Clathrin and synaptic vesicle endocytosis: studies at the squid giant synapse. *Biochemical Society transactions* **34** 68-72.

Barg S & Machado JD 2008 Compensatory endocytosis in chromaffin cells. *Acta physiologica (Oxford, England)* **192** 195-201.

Burgess TL & Kelly RB 1987 Constitutive and regulated secretion of proteins. *Annual Review of Cell Biology* **3** 243-293.

Cai H, Reim K, Varoqueaux F, Tapechum S, Hill K, Sorensen JB, Brose N & Chow RH 2008 Complexin II plays a positive role in Ca2+-triggered exocytosis by facilitating vesicle priming. *Proceedings of the National Academy of Sciences of the United States of America* **105** 19538-19543.

Canovas S, Romar R, Grullon LA, Aviles M & Coy P 2009 Pre-fertilization zona pellucida hardening by different cross-linkers affects IVF in pigs and cattle and improves embryo production in pigs. *Reproduction (Cambridge, England)* **137** 803-812.

Carneiro GF, Liu IK, Hyde D, Anderson GB, Lorenzo PL & Ball BA 2002 Quantification and distribution of equine oocyte cortical granules during meiotic maturation and after activation. *Molecular reproduction and development* **63** 451-458.

Castle JD, Guo Z & Liu L 2002 Function of the t-SNARE SNAP-23 and secretory carrier membrane proteins (SCAMPs) in exocytosis in mast cells. *Molecular immunology* **38** 1337-1340.

Chapman ER, Hanson PI, An S & Jahn R 1995 Ca2+ regulates the interaction between synaptotagmin and syntaxin 1. *The Journal of biological chemistry* **270** 23667-23671.

Chen X, Tomchick DR, Kovrigin E, Arac D, Machius M, Sudhof TC & Rizo J 2002 Three-dimensional structure of the complexin/SNARE complex. *Neuron* **33** 397-409.

Chen YA, Scales SJ & Scheller RH 2001 Sequential SNARE assembly underlies priming and triggering of exocytosis. *Neuron* **30** 161-170.

Cheung A, Swann K & Carroll J 2000 The ability to generate normal Ca(2+) transients in response to spermatozoa develops during the final stages of oocyte growth and maturation. *Human reproduction (Oxford, England)* **15** 1389-1395.

Clary DO, Griff IC & Rothman JE 1990 SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell* **61** 709-721.

Conner S, Leaf D & Wessel G 1997 Members of the SNARE hypothesis are associated with cortical granule exocytosis in the sea urchin egg. *Molecular reproduction and development* **48** 106-118.

Cox LJ, Larman MG, Saunders CM, Hashimoto K, Swann K & Lai FA 2002 Sperm phospholipase Cζ from humans and cynomolgus monkeys triggers Ca2+ oscillations, activation and development of mouse oocytes. *Reproduction (Cambridge, England)* **124** 611-623.

Coy P, Canovas S, Mondejar I, Saavedra MD, Romar R, Grullon L, Matas C & Aviles M 2008a Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proceedings of the National Academy of Sciences of the United States of America* **105** 15809-15814.

Coy P, Grullon L, Canovas S, Romar R, Matas C & Aviles M 2008b Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction (Cambridge, England)* **135** 19-27.

Davis AF, Bai J, Fasshauer D, Wolowick MJ, Lewis JL & Chapman ER 1999 Kinetics of synaptotagmin responses to Ca2+ and assembly with the core SNARE complex onto membranes. *Neuron* **24** 363-376.

Ducibella T 1996 The cortical reaction and development of activation competence in mammalian oocytes. *Human reproduction update* **2** 29-42.

Ducibella T, Anderson E, Albertini DF, Aalberg J & Rangarajan S 1988 Quantitative studies of changes in cortical granule number and distribution in the mouse oocyte during meiotic maturation. *Developmental biology* **130** 184-197.

Ducibella T, Duffy P, Reindollar R & Su B 1990 Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin-stimulated meiotic maturation and aging in vivo. *Biology of reproduction* **43** 870-876.

Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, Fissore R, Madoux S & Ozil JP 2002 Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. *Developmental biology* **250** 280-291.

Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Sudhof TC & Rizo J 1999 A conformational switch in syntaxin during exocytosis: role of munc18. *The EMBO journal* **18** 4372-4382.

Fasshauer D 2003 Structural insights into the SNARE mechanism. *Biochimica et biophysica acta* **1641** 87-97.

Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C & Sudhof TC 2001 Synaptotagmin I functions as a calcium regulator of release probability. *Nature* **410** 41-49.

Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA & Navarro PA 2009 Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* **71** 836-848.

FitzHarris G, Marangos P & Carroll J 2007 Changes in endoplasmic reticulum structure during mouse oocyte maturation are controlled by the cytoskeleton and cytoplasmic dynein. *Developmental biology* **305** 133-144.

Gadella BM 2010 Interaction of sperm with the zona pellucida during fertilization. *Reproduction (ahead of publication)*.

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF & Sudhof TC 1994 Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. *Cell* **79** 717-727.

Gerona RR, Larsen EC, Kowalchyk JA & Martin TF 2000 The C terminus of SNAP25 is essential for Ca(2+)-dependent binding of synaptotagmin to SNARE complexes. *The Journal of biological chemistry* **275** 6328-6336.

Giraudo CG, Eng WS, Melia TJ & Rothman JE 2006 A clamping mechanism involved in SNARE-dependent exocytosis. *Science (New York, N.Y.)* **313** 676-680.

Guo Z, Turner C & Castle D 1998 Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell* **94** 537-548.

Gupta SK, Bansal P, Ganguly A, Bhandari B & Chakrabarti K 2009 Human zona pellucida glycoproteins: functional relevance during fertilization. *Journal of reproductive immunology* **83** 50-55.

Han GA, Malintan NT, Collins BM, Meunier FA & Sugita S 2010 Munc18-1 as a key regulator of neurosecretion. *Journal of neurochemistry* **115** 1-10.

Holzenspies JJ, Stoorvogel W, Colenbrander B, Roelen BA, Gutknecht DR & van Haeften T 2009 CDC2/SPDY transiently associates with endoplasmic reticulum exit sites during oocyte maturation. *BMC developmental biology* **9** 8.

Hong W 2005 SNAREs and traffic. *Biochimica et biophysica acta* **1744** 120-144.

Ikebuchi Y, Masumoto N, Matsuoka T, Yokoi T, Tahara M, Tasaka K, Miyake A & Murata Y 1998 SNAP-25 is essential for cortical granule exocytosis in mouse eggs. *The American Journal of Physiology* **274** C1496-500.

Jahn R, Lang T & Sudhof TC 2003 Membrane fusion. *Cell* **112** 519-533.

Jahn R & Scheller RH 2006 SNAREs--engines for membrane fusion. *Nature reviews.Molecular cell biology* **7** 631-643.

Jahn R & Sudhof TC 1999 Membrane fusion and exocytosis. *Annual Review of Biochemistry* **68** 863-911.

Kline D & Kline JT 1992 Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Developmental biology* **149** 80-89.

Kline D 1996 Activation of the mouse egg. *Theriogenology* **45** 81-90.

Kline JT & Kline D 1994 Regulation of intracellular calcium in the mouse egg: evidence for inositol trisphosphate-induced calcium release, but not calcium-induced calcium release. *Biology of reproduction* **50** 193-203.

Kozlov MM, McMahon HT & Chernomordik LV 2010 Protein-driven membrane stresses in fusion and fission. *Trends in biochemical sciences*.

Kozlovsky Y & Kozlov MM 2002 Stalk model of membrane fusion: solution of energy crisis. *Biophysical journal* **82** 882-895.

Lee HK, Yang Y, Su Z, Hyeon C, Lee TS, Lee HW, Kweon DH, Shin YK & Yoon TY 2010 Dynamic Ca2+-dependent stimulation of vesicle fusion by membrane-anchored synaptotagmin 1. *Science (New York, N.Y.)* **328** 760-763.

Logan MR, Odemuyiwa SO & Moqbel R 2003 Understanding exocytosis in immune and inflammatory cells: the molecular basis of mediator secretion. *The Journal of allergy and clinical immunology* **111** 923-32; quiz 933.

Lollike K, Lindau M, Calafat J & Borregaard N 2002 Compound exocytosis of granules in human neutrophils. *Journal of leukocyte biology* **71** 973-980.

Lynch KL, Gerona RR, Kielar DM, Martens S, McMahon HT & Martin TF 2008 Synaptotagmin-1 utilizes membrane bending and SNARE binding to drive fusion pore expansion. *Molecular biology of the cell* **19** 5093-5103.

Martens S, Kozlov MM & McMahon HT 2007 How synaptotagmin promotes membrane fusion. *Science (New York, N.Y.)* **316** 1205-1208.

Martin-Verdeaux S, Pombo I, Iannascoli B, Roa M, Varin-Blank N, Rivera J & Blank U 2003 Evidence of a role for Munc18-2 and microtubules in mast cell granule exocytosis. *Journal of cell science* **116** 325-334.

Marz KE & Hanson PI 2002 Sealed with a twist: complexin and the synaptic SNARE complex. *Trends in neurosciences* **25** 381-383.

Matos MF, Mukherjee K, Chen X, Rizo J & Sudhof TC 2003 Evidence for SNARE zippering during Ca2+-triggered exocytosis in PC12 cells. *Neuropharmacology* **45** 777-786.

McNew JA 2008 Regulation of SNARE-mediated membrane fusion during exocytosis. *Chemical reviews* **108** 1669-1686.

Medine CN, Rickman C, Chamberlain LH & Duncan RR 2007 Munc18-1 prevents the formation of ectopic SNARE complexes in living cells. *Journal of cell science* **120** 4407-4415.

Melia TJ,Jr 2007 Putting the clamps on membrane fusion: how complexin sets the stage for calcium-mediated exocytosis. *FEBS letters* **581** 2131-2139.

Melicoff E, Sansores-Garcia L, Gomez A, Moreira DC, Datta P, Thakur P, Petrova Y, Siddiqi T, Murthy JN, Dickey BF, Heidelberger R & Adachi R 2009 Synaptotagmin-2 controls regulated exocytosis but not other secretory responses of mast cells. *The Journal of biological chemistry* **284** 19445-19451.

Miller DJ, Gong X, Decker G & Shur BD 1993 Egg cortical granule N-acetylglucosaminidase is required for the mouse zona block to polyspermy. *The Journal of cell biology* **123** 1431-1440.

Miyazaki S, Shirakawa H, Nakada K & Honda Y 1993 Essential role of the inositol 1,4,5-trisphosphate receptor/Ca2+ release channel in Ca2+ waves and Ca2+ oscillations at fertilization of mammalian eggs. *Developmental biology* **158** 62-78.

Moccia F, Nusco GA, Lim D, Kyozuka K & Santella L 2006 NAADP and InsP3 play distinct roles at fertilization in starfish oocytes. *Developmental biology* **294** 24-38.

Morvan J &Tooze SA 2008 Discovery and progress in our understanding of the regulated secretory pathway in neuroendocrine cells. *Histochemistry and cell biology* **129** 243-252.

Nemoto T, Kimura R, Ito K, Tachikawa A, Miyashita Y, Iino M & Kasai H 2001 Sequential-replenishment mechanism of exocytosis in pancreatic acini. *Nature cell biology* **3** 253-258.

Nigam R, Sepulveda J, Tuvim M, Petrova Y, Adachi R, Dickey BF & Agrawal A 2005 Expression and transcriptional regulation of Munc18 isoforms in mast cells. *Biochimica et biophysica acta* **1728** 77-83.

Ono S, Baux G, Sekiguchi M, Fossier P, Morel NF, Nihonmatsu I, Hirata K, Awaji T, Takahashi S & Takahashi M 1998 Regulatory roles of complexins in neurotransmitter release from mature presynaptic nerve terminals. *The European journal of neuroscience* **10** 2143-2152.

Ozil JP, Markoulaki S, Toth S, Matson S, Banrezes B, Knott JG, Schultz RM, Huneau D & Ducibella T 2005 Egg activation events are regulated by the duration of a sustained [Ca2+]cyt signal in the mouse. *Developmental biology* **282** 39-54.

Papi M, Brunelli R, Sylla L, Parasassi T, Monaci M, Maulucci G, Missori M, Arcovito G, Ursini F & De Spirito M 2010 Mechanical properties of zona pellucida hardening. *European biophysics journal : EBJ* **39** 987-992.

Perez Bay AE, Ibanez LI & Marengo FD 2007 Rapid recovery of releasable vesicles and formation of nonreleasable endosomes follow intense exocytosis in chromaffin cells. *American journal of physiology.Cell physiology* **293** C1509-22.

Petersen OH & Tepikin AV 2008 Polarized calcium signaling in exocrine gland cells. *Annual Review of Physiology* **70** 273-299.

Pickett JA & Edwardson JM 2006 Compound exocytosis: mechanisms and functional significance. *Traffic (Copenhagen, Denmark)* **7** 109-116.

Puri N, Kruhlak MJ, Whiteheart SW & Roche PA 2003 Mast cell degranulation requires N-ethylmaleimide-sensitive factor-mediated SNARE disassembly. *Journal of immunology (Baltimore, Md.: 1950)* **171** 5345-5352.

Puri N & Roche PA 2008 Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. *Proceedings of the National Academy of Sciences of the United States of America* **105** 2580-2585.

Ravichandran V, Chawla A & Roche PA 1996 Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. *The Journal of biological chemistry* **271** 13300-13303.

Reim K, Mansour M, Varoqueaux F, McMahon HT, Sudhof TC, Brose N & Rosenmund C 2001 Complexins regulate a late step in Ca2+-dependent neurotransmitter release. *Cell* **104** 71-81.

Rickman C, Medine CN, Bergmann A & Duncan RR 2007 Functionally and spatially distinct modes of munc18-syntaxin 1 interaction. *The Journal of biological chemistry* **282** 12097-12103.

Rizo J & Rosenmund C 2008 Synaptic vesicle fusion. *Nature structural & molecular biology* **15** 665-674.

Rothman JE 1994 Mechanisms of intracellular protein transport. *Nature* **372** 55-63.

Rothman JE 1996 The protein machinery of vesicle budding and fusion. *Protein science : a publication of the Protein Society* **5** 185-194.

Salpeter MM &Farquhar MG 1981 High resolution analysis of the secretory pathway in mammotrophs of the rat anterior pituitary. *The Journal of cell biology* **91** 240-246.

Sander LE, Frank SP, Bolat S, Blank U, Galli T, Bigalke H, Bischoff SC & Lorentz A 2008 Vesicle associated membrane protein (VAMP)-7 and VAMP-8, but not VAMP-2 or VAMP-3, are required for activation-induced degranulation of mature human mast cells. *European journal of immunology* **38** 855-863.

Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K & Lai FA 2002 PLC ζ: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development (Cambridge, England)* **129** 3533-3544.

Schaub JR, Lu X, Doneske B, Shin YK & McNew JA 2006 Hemifusion arrest by complexin is relieved by Ca2+-synaptotagmin I. *Nature structural & molecular biology* **13** 748-750.

Schoch S, Castillo PE, Jo T, Mukherjee K, Geppert M, Wang Y, Schmitz F, Malenka RC & Sudhof TC 2002 RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature* **415** 321-326.

Schoevers EJ, Colenbrander B & Roelen BA 2007 Developmental stage of the oocyte during antral follicle growth and cumulus investment determines in vitro embryo development of sow oocytes. *Theriogenology* **67** 1108-1122.

Shukla A, Berglund L, Nielsen LP, Nielsen S, Hoffmann HJ & Dahl R 2001 Regulated exocytosis in immune function: are SNARE-proteins involved? *Respiratory medicine* **95** 773-780.

Siksou L, Varoqueaux F, Pascual O, Triller A, Brose N & Marty S 2009 A common molecular basis for membrane docking and functional priming of synaptic vesicles. *The European journal of neuroscience* **30** 49-56.

Sollner T, Bennett MK, Whiteheart SW, Scheller RH & Rothman JE 1993a A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75** 409-418.

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P & Rothman JE 1993b SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362** 318-324.

Stenmark H 2009 Rab GTPases as coordinators of vesicle traffic. *Nature reviews.Molecular cell biology* **10** 513-525.

Stricker SA 1999 Comparative biology of calcium signaling during fertilization and egg activation in animals. *Developmental biology* **211** 157-176.

Stricker SA 2006 Structural reorganizations of the endoplasmic reticulum during egg maturation and fertilization. *Seminars in cell & developmental biology* **17** 303-313.

Sudhof TC 2004 The synaptic vesicle cycle. *Annual Review of Neuroscience* **27** 509-547.

Sun QY 2003 Cellular and molecular mechanisms leading to cortical reaction and polyspermy block in mammalian eggs. *Microscopy research and technique* **61** 342-348.

Sun QY & Schatten H 2006 Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction (Cambridge, England)* **131** 193-205.

Swann K, Saunders CM, Rogers NT & Lai FA 2006 PLCζ(zeta): a sperm protein that triggers Ca2+ oscillations and egg activation in mammals. *Seminars in cell & developmental biology* **17** 264-273.

Swann K & Yu Y 2008 The dynamics of calcium oscillations that activate mammalian eggs. *The International journal of developmental biology* **52** 585-594.

Szule JA & Coorssen JR 2003 Revisiting the role of SNAREs in exocytosis and membrane fusion. *Biochimica et biophysica acta* **1641** 121-135.

Tadokoro S, Kurimoto T, Nakanishi M & Hirashima N 2007 Munc18-2 regulates exocytotic membrane fusion positively interacting with syntaxin-3 in RBL-2H3 cells. *Molecular immunology* **44** 3427-3433.

Tadokoro S, Nakanishi M & Hirashima N 2005 Complexin II facilitates exocytotic release in mast cells by enhancing Ca2+ sensitivity of the fusion process. *Journal of cell science* **118** 2239-2246.

Tadokoro S, Nakanishi M & Hirashima N 2010 Complexin II regulates degranulation in RBL-2H3 cells by interacting with SNARE complex containing syntaxin-3. *Cellular immunology* **261** 51-56.

Takahashi N & Kasai H 2007 Exocytic process analyzed with two-photon excitation imaging in endocrine pancreas. *Endocrine journal* **54** 337-346.

Tang J, Maximov A, Shin OH, Dai H, Rizo J & Sudhof TC 2006 A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. *Cell* **126** 1175-1187.

Tokumaru H, Shimizu-Okabe C & Abe T 2008 Direct interaction of SNARE complex binding protein synaphin/complexin with calcium sensor synaptotagmin 1. *Brain cell biology* **36** 173-189.

Tsaadon A, Eliyahu E, Shtraizent N & Shalgi R 2006 When a sperm meets an egg: block to polyspermy. *Molecular and cellular endocrinology* **252** 107-114.

Tsai PS, van Haeften T & Gadella BM 2010 Preparation of the Cortical Reaction: Maturation-Dependent Migration of SNARE Proteins, Clathrin, and Complexin to the Porcine Oocyte's Surface Blocks Membrane Traffic until Fertilization. *Biology of reproduction*.

van den Hurk R & Zhao J 2005 Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology* **63** 1717-1751.

Velilla E, Izquierdo D, Rodriguez-Gonzalez E, Lopez-Bejar M, Vidal F & Paramio MT 2004 Distribution of prepubertal and adult goat oocyte cortical granules during meiotic maturation and fertilisation: ultrastructural and cytochemical study. *Molecular reproduction and development* **68** 507-514.

Walter AM, Wiederhold K, Bruns D, Fasshauer D & Sorensen JB 2010 Synaptobrevin N-terminally bound to syntaxin-SNAP-25 defines the primed vesicle state in regulated exocytosis. *The Journal of cell biology* **188** 401-413.

Wang WH, Machaty Z, Ruddock N, Abeydeera LR, Boquest AC, Prather RS & Day BN 1999 Activation of porcine oocytes with calcium ionophore: effects of extracellular calcium. *Molecular reproduction and development* **53** 99-107.

Wang WH, Sun QY, Hosoe M, Shioya Y & Day BN 1997 Quantified analysis of cortical granule distribution and exocytosis of porcine oocytes during meiotic maturation and activation. *Biology of reproduction* **56** 1376-1382.

Weimer RM, Richmond JE, Davis WS, Hadwiger G, Nonet ML & Jorgensen EM 2003 Defects in synaptic vesicle docking in unc-18 mutants. *Nature neuroscience* **6** 1023-1030.

Wessel GM, Conner SD & Berg L 2002 Cortical granule translocation is microfilament mediated and linked to meiotic maturation in the sea urchin oocyte. *Development (Cambridge, England)* **129** 4315-4325.

Whitaker M 2006 Calcium at fertilization and in early development. *Physiological Reviews* **86** 25-88.

Xu J, Pang ZP, Shin OH & Sudhof TC 2009 Synaptotagmin-1 functions as a Ca2+ sensor for spontaneous release. *Nature neuroscience* **12** 759-766.

Xu Z, Williams CJ, Kopf GS & Schultz RM 2003 Maturation-associated increase in IP3 receptor type 1: role in conferring increased IP3 sensitivity and Ca2+ oscillatory behavior in mouse eggs. *Developmental biology* **254** 163-171.

Xue M, Craig TK, Xu J, Chao HT, Rizo J & Rosenmund C 2010 Binding of the complexin N terminus to the SNARE complex potentiates synaptic-vesicle fusogenicity. *Nature structural & molecular biology* **17** 568-575.

Xue M, Reim K, Chen X, Chao HT, Deng H, Rizo J, Brose N & Rosenmund C 2007 Distinct domains of complexin I differentially regulate neurotransmitter release. *Nature structural & molecular biology* **14** 949-958.