# CHAPTER 6

# C2 Domains and Membrane Fusion

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## sec0005 I. OVERVIEW

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In biology, membrane fusion describes the controlled merger of two initially separated membranes. In this chapter, we discuss how cellular proteins mediate the lipid rearrangements that are necessary for fusion to occur. We focus on the $Ca^{2+}$-dependent fusion of granules and synaptic vesicles in professional secretory cells and neurons, respectively. The key molecules discussed are the SNARE-proteins and C2 domain-containing proteins of the synaptotagmin family and its relatives. Special emphasis is given to recent evidence showing that C2 domains are able to induce membrane curvature in a $Ca^{2+}$-dependent manner. We discuss how this activity in combination with the energy provided by SNARE complex assembly brings about the extremely fast and controlled fusion of granules and synaptic vesicles.

## sec0010 II. MEMBRANE FUSION

Membrane fusion or the controlled merger of two initially separated membranes is of fundamental importance in biology. This is particularly true for eukaryotic cells that contain a plethora of membrane-bound compartments. The transport of proteins, lipids, and other material between the endoplasmic

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reticulum, the Golgi apparatus, the endocytic system, and other intracellular compartments is in large part mediated by small vesicular and tubular carriers (Jahn & Scheller, 2006; Martens & McMahon, 2008; Zerial & McBride, 2001). These carriers bud off from a donor compartment and fuse with their target compartment thereby delivering membrane bound proteins, lipids, and luminal cargo. If these vesicular carriers fuse with the plasma membrane they can deliver molecules such as neurotransmitters and other signaling factors into the extracellular space. Membrane fusion can also occur between whole organelles. Thus, mitochondria undergo constant fusion and fission events (Hoppins, Lackner, & Nunnari, 2007). During cell division the Golgi apparatus fragments in order to allow it to be partitioned between the two daughter cells. After partitioning it reassembles by the controlled fusion of these membranous fragments (Shorter & Warren, 2002). Another fascinating example is the reassembly of the nuclear envelope after mitosis. During open mitosis the nuclear envelope fragments into small vesicular structures. These structures fuse after mitosis in order to reform the nuclear envelope (Guttinger, Laurell, & Kutay, 2009). However, membrane fusion processes are not limited to intracellular events as fusion can also occur between cells. For instance, myoblasts fuse to generate myotubes and trophoblasts fuse to form the syncytiotrophoblast layer (Oren-Suissa & Podbilewicz, 2007). Membrane fusion is also exploited by pathogens such as enveloped viruses that fuse with their target cells in order to gain access to the cell's intracellular space (Skehel & Wiley, 2000).

par0020 In all examples of membrane fusion events mentioned above the requirements for the actual fusion event is similar. The fusion event must be tightly controlled and it should be nonleaky, meaning that the membranes must keep their integrity and only the content of the two membrane structures should mix (Chernomordik & Kozlov, 2003; Jahn, Lang, & Sudhof, 2003; Martens & McMahon, 2008).

par0025 Most if not all membrane fusion processes are believed to proceed through the same intermediates (Chernomordik & Kozlov, 2008; Martens & McMahon, 2008). Initially the two membranes destined to fuse, but initially separated, must be brought into close contact. In most biological fusion events this will require the two membranes to be denuded of proteins in order to allow close contact to occur. Next, energy (e.g., curvature stress) must be injected into both membrane destined to fuse in order to overcome the energy barriers for the subsequent fusion intermediates. If the fusion event is initiated by highly curved membrane intermediates (e.g., buckle-like protrusions) this will facilitate both, close membrane contact and the intermediates to follow (Efrat, Chernomordik, & Kozlov, 2007; Martens, Kozlov, & McMahon, 2007). The next step entails the merger of the two contacting monolayers of each membrane into a structure referred to as hemifusion stalk. During hemifusion the two distal monolayers remain separated. The hemifusion stalk resolves by the subsequent merger of the distal monolayers allowing the formation of the fusion pore (Chernomordik & Kozlov, 2003). This is the first time the contents of the two compartments come

into contact and mix. Finally, the fusion pore dilates that results in the complete intermixing of the two membranes.

par0030 During fusion the membrane undergoes major conformational changes. However, these changes are controlled and mediated by fusion proteins. The task of the fusion proteins is to reduce the energy barriers separating the highly curved intermediates during the membrane fusion process (Chernomordik & Kozlov, 2003, 2008). Flat membranes do not like to undergo spontaneous fusion, but highly curved membranes have a high spontaneous fusion rate, likely because the energy barrier for the high curvature intermediates is lower. The more unstable the highly curved intermediate then the more likely it will want to fuse to relax to a lower energy state (McMahon, Kozlov, & Martens, 2010).

par0035 As outlined above the fundamental process of membrane fusion plays crucial roles in a wide range of biological processes. Consequently it is tightly regulated by a variety of signals that allow the fusion reaction to proceed only when needed (Martens et al., 2007). One of the most fascinating and most investigated fusion reactions is the calcium-dependent fusion of vesicles and granules in professional secretory cells such as neurons and chromaffin cells (Sorensen, 2004; Sudhof, 2004) (Fig. 1).

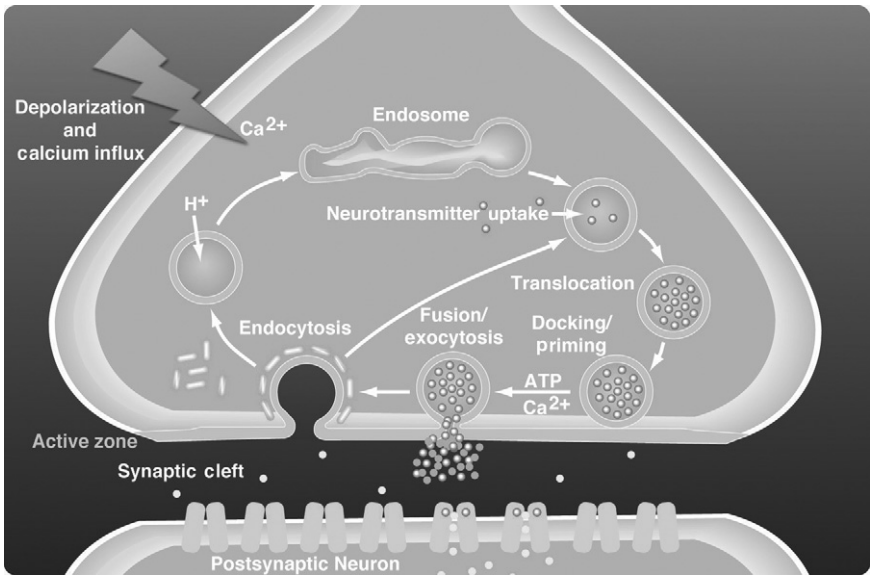


fig0005 **FIGURE 1** Synaptic vesicle exocytic/endocytic cycle. Nerves on depolarization respond to the influx of calcium by fusion of vesicles in a region called the active zone. Neurotransmitters are released into the synaptic cleft and bind to postsynaptic receptors to activate/modulate its activity. Vesicle components and membrane are recycled by endocytosis either directly to form new synaptic vesicles or via an endosome intermediate. These vesicles are then docked/primed and wait on another stimulus for SNARE-dependent fusion.

sec0015 ## A. Calcium-Dependent Membrane Fusion

par0040 Calcium or more precisely $Ca^{2+}$ ions serve as second messengers for a variety of signaling events. There is a steep gradient of the $Ca^{2+}$ concentration across the limiting membrane of the cell. Thus, the concentration of $Ca^{2+}$ in the extracellular space and some intracellular compartments such as the endoplasmic reticulum is in the mM range whereas the $Ca^{2+}$ concentration in the cytoplasm is in the sub-mM range, at least under resting conditions. The activation of certain signaling receptors and ion channels results in the further opening of channels that allow $Ca^{2+}$ to cross the plasma membrane or endoplasmic reticulum membrane. The elevated cytoplasmic $Ca^{2+}$ concentration is sensed by $Ca^{2+}$ binding proteins, which in turn trigger various downstream events. One of the most striking actions is the triggering of exocytic fusion events in neurons, chromaffin cells, and many other professional secretory cells (Fig. 1). In fact many of the hormones controlling our body functions are secreted into the extracellular space by calcium-dependent exocytosis. Furthermore, most of our neurons communicate with each other at chemical synapses. Here the signaling molecules, termed neurotransmitters are stored in small vesicles and are released by the fusion of these vesicles with the plasma membrane. Upon their release these neurotransmitters bind to receptors on the surface of the neighboring cell and thereby alter its behavior. It's fundamental importance and fascinating biophysics has made synaptic vesicle fusion one of the most heavily studied processes in biology.

sec0020 ### 1. Synaptic Vesicle Fusion

par0045 Synaptic vesicle fusion lies at the core of the communication between the vast majority of our neurons. At chemical synapses, one neuron communicates with its neighbor across a specialized site called a *synapse* (Fig. 1). Synapses are composed of pre- and postsynaptic structures where the presynaptic part is supplied by the signal-sending neuron and the postsynaptic part is provided by the signal-receiving neuron (DeFelipe, 2010). Within the presynapse the neurotransmitters are packed into small rather uniform 40–50 nm diameter vesicles termed *synaptic vesicles* (Takamori et al., 2006). Dependent on the synapse, the number of synaptic vesicles per presynaptic terminal can vary between a hundred to several thousand. If an action potential arrives at the presynapse it triggers the opening of voltage-gated $Ca^{2+}$ channels resulting in the increase of the intracellular $Ca^{2+}$ concentration. The increased $Ca^{2+}$ concentration triggers the fusion of some synaptic vesicles with the presynaptic plasma membrane that results in the release of neurotransmitters into the space between the pre- and postsynapse termed *the synaptic cleft* where they can bind to receptors on the postsynaptic plasma membrane (Neher & Sakaba, 2008; Sudhof, 2004). The probability with which an action potential can trigger the

fusion of one or more synaptic vesicle is called *release probability* and can vary from 0.1 at some central synapses to 1 at the neuromuscular junction. Changes in this release probability are one of the fundamental mechanisms by which long- and short-term memory is mediated (Citri & Malenka, 2007).

par0050 Synaptic vesicles undergo a complex life cycle (Sudhof, 2004) (Fig. 1). Synaptic vesicles initially bud off from a donor compartment and become filled with neurotransmitters. Subsequently some of these vesicles are believed to undergo a complex series of events that are collectively called *docking* and *priming* (Verhage & Sørensen, 2008). The term docking is based on a morphological criterion and describes the phenomenon where some synaptic vesicles are deemed to be close to the presynaptic plasma membrane. The term priming is based on a functional classification and describes the ability of some synaptic vesicles to rapidly fuse after the increase in intercellular $Ca^{2+}$ concentration. It is commonly believed that during docking and priming the molecular machinery that ultimately brings about the actual fusion event is assembled. Furthermore, the primed vesicles are believed to be a subset of the docked vesicles where docked but not primed vesicles are on some intermediate stage toward the fully fusion competent state. It should be noted that most studies investigating the molecular events during docking and priming have been conducted in chromaffin cells (discussed below) and the strict classification into docked and primed vesicles in neurons should be applied with some caution (de Wit et al., 2009; Sorensen, 2004; Verhage & Sørensen, 2008). After $Ca^{2+}$-dependent fusion the membrane and proteins of the synaptic vesicles now found in the plasma membrane are recycled by *endocytosis*, after which the synaptic vesicles become re-filled with neurotransmitters and are ready for the next cycle of depolarization and release (Sudhof, 2004) (Fig. 1).

par0055 It has been observed that upon arrival of an action potential, and subsequent $Ca^{2+}$ channel opening, synaptic vesicles fuse in different phases (Barrett & Stevens, 1972; Sun et al., 2007). *Synchronous release* refers to the neurotransmitters that are released immediately after $Ca^{2+}$ channel opening. For synchronous release, the delay between the increase of the intracellular $Ca^{2+}$ concentration and synaptic vesicle fusion is surprisingly short (less than 1 ms). This short delay has prompted the belief that the molecular release machinery responsible for fusion must be largely preassembled. Synchronous release is followed by *asynchronous release*, which refers to the synaptic vesicles that fuse in a slightly delayed manner. Both synchronous and asynchronous synaptic vesicle fusion are absolutely dependent on $Ca^{2+}$ (Hagler & Goda, 2001). In addition a third form of synaptic vesicle fusion is know, the so-called *spontaneous release*. Spontaneous release is not coupled to an action potential and has long been considered as $Ca^{2+}$ independent. However, there is now compelling evidence that also this type of release is at least to a large extent triggered by $Ca^{2+}$ (Emptage, Reid, & Fine, 2001; Groffen et al., 2010; Xu, Pang, Shin, & Sudhof, 2009).

par0060 A large body of evidence suggests a common molecular mechanism underlies synchronous, asynchronous, as well as spontaneous release (Groffen et al., 2010; Schoch et al., 2001; Verhage et al., 2000). However, before we discuss the molecules that drive these fusion reactions we will briefly discuss $Ca^{2+}$-dependent fusion in non-neuronal cells.

sec0025 ## 2. Secretory Granule Fusion

par0065 Granules are relatively large secretory organelles (up to several 100 nm in diameter) found in adrenal chromaffin cells, pancreatic beta cells, mast cells, cytotoxic T cells, and many other cell types. Like the fusion of synaptic vesicles in neurons, secretory granule fusion is strictly dependent on $Ca^{2+}$. In addition, the molecular core machinery mediating the fusion of these granules is the same as the machinery mediating the fusion of synaptic vesicles, although important differences do exist (Sorensen, 2004). However, fusion of secretory granules is about 10 times slower than synaptic vesicle fusion. The most intensely studied system is the exocytosis of secretory granules in chromaffin cells. Analogous to synaptic vesicle fusion, the fusion of secretory granules in chromaffin cells occurs in different phases. Immediately after the rise of the intracellular $Ca^{2+}$ concentration fast fusion is observed. This fast phase is followed by a slow phase, which in turn is followed by a sustained phase (Sorensen, 2004). A key advantage of the study of granule fusion over the study of synaptic vesicle fusion is that the cells are more accessible. Thus, while most measurements of synaptic vesicle fusion are made indirectly by recording the postsynaptic response to the neurotransmitters that are released by these fusion events, individual exocytic events can be detected directly in chromaffin cells. One such method involves measuring plasma membrane capacitance, which allows the detection of an increase in cell surface area upon fusion of secretory granules. Another technique is amperometry that allows the detection of the signaling molecules that are released upon fusion (Haller, Heinemann, Chow, Heidelberger, & Neher, 1998). Both of these techniques are extremely sensitive and accurate and have in combination with the precise manipulation of the molecular release machinery given us deep insights into the core machinery driving $Ca^{2+}$-dependent exocytosis and membrane fusion in general.

sec0030 # III. THE MOLECULAR MACHINERY MEDIATING CALCIUM-DEPENDENT MEMBRANE FUSION

A wealth of studies over the last decades has unearthed a plethora of molecules that are required for the precisely controlled and fascinating events outlined above. In fact we now have an accurate picture of the molecular composition of a complete synaptic vesicle and although it does not excluded that new molecules playing key role during $Ca^{2+}$ dependent will be discovered it is likely

that the major players are known (Rizo & Rosenmund, 2008; Sudhof, 2004; Takamori et al., 2006). The task now is therefore to assign precise functions and mechanisms to these molecules. In our subsequent discussion we will limit ourselves to the so-called SNARE complex, the synaptotagmins and related C2 domain containing proteins.

sec0035 ## A. The SNARE Complex

par0075 The SNARE complex is at the core of all $Ca^{2+}$-dependent exocytic fusion events including synaptic vesicle fusion and secretory granule fusion (Borisovska et al., 2005; Jahn & Scheller, 2006; Schiavo et al., 1992; Schoch et al., 2001; Südhof & Rothman, 2009; Washbourne et al., 2002) (Fig. 2). The

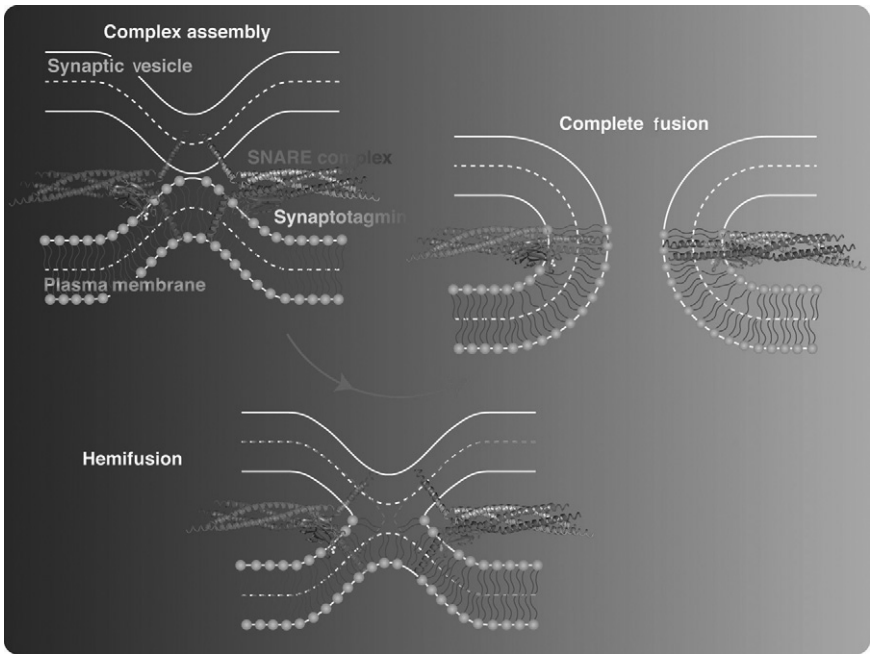


fig0010 **FIGURE 2** SNARE and synaptotagmin-dependent membrane fusion. Zippering of SNARE proteins pulls the synaptic vesicle membrane into close apposition with the plasma membrane. The C2 domains of synaptotagmin/DOC proteins bind to the SNARE complex, and so are positioned at the fusion site, where they can dip into the membrane and promote membrane curvature. This will help reduce the energy barrier that is needed to get to the hemifusion intermediate. The synaptotagmin will still prefer to promote positive membrane curvature and so will help to drive the fusion reaction to complete fusion, where the membranes can relax on collapse of the vesicle.

SNARE complex is an extremely stable four-helical bundle and the fully formed complex likely represents the postfusion state of these proteins. The individual helices are provided by separate SNARE proteins (Stein, Weber, Wahl, & Jahn, 2009; Sutton, Fasshauer, Jahn, & Brunger, 1998). In neurons and chromaffin cells these SNARE proteins are syntaxin1, SNAP25, and synaptobrevin. Each of these SNAREs contain an approximately 60 amino acid long SNARE domain that is largely disordered in isolation (Fasshauer, Otto, Eliason, Jahn, & Brünger, 1997; Stein et al., 2009; Sutton et al., 1998). Syntaxin1 and synaptobrevin are transmembrane domain proteins localized to the plasma membrane and vesicular membrane, respectively (Fig. 2). Syntaxin1 and synaptobrevin each provide one helix to the four-helical SNARE complex. In contrast to syntaxin1 and synaptobrevin, SNAP25 is localized to the plasma membrane via a series of palmitoylated cysteines and provides two helices to the complex (Fasshauer et al., 1997; Stein et al., 2009; Sutton et al., 1998). The discovery that two transmembrane proteins localized to the two membranes destined to fuse have the ability to participate in an extremely stable complex, immediately suggested a mechanism by which these molecules could mediate membrane fusion (Sollner et al., 1993; Weber et al., 1998) (Fig. 2). The SNARE domains of synatxin1 and synaptobrevin are located to the N-terminus of their transmembrane domains and only a few amino acids separate the SNARE domains from the membrane spanning regions (Stein et al., 2009; Sutton et al., 1998). Structural evidence suggests that upon SNARE complex formation the SNARE helices extend into the transmembrane domain transducing at least some force, generated by the SNARE complex formation, into the membranes (Stein et al., 2009). In fact it is now widely accepted that SNARE complex formation is the driving force bring the two membranes destined to fuse into close contact. Furthermore, tetanus and botulinum toxins have been found to specifically cleave SNARE proteins and thereby inhibit synaptic vesicle fusion (Davletov, Bajohrs, & Binz, 2005; Schiavo et al., 1992; Sutton et al., 1998). SNARE proteins are not limited to the synapses. Most, but not all, intracellular membrane fusion events are SNARE-dependent and it is widely assumed that the $Ca^{2+}$-dependent fusion of synaptic vesicles and granules is a special adaptation of a general mechanism (Jahn & Scheller, 2006; Martens & McMahon, 2008; McMahon et al., 1993).

par0080 Syntaxin1 and SNAP25 are also called target-SNAREs (tSNAREs) and have been reported to form a tSNARE *acceptor complex* in the plasma membrane for the SNARE motif of synaptobrevin. There is good evidence suggesting a model where synaptobrevin initially makes contact with the preformed tSNARE complex with its N-terminus (Sorensen et al., 2006; Walter, Wiederhold, Bruns, Fasshauer, & Sørensen, 2010). Thus, the regions furthest from the transmembrane domains make contact and as the proteins zipper together the transmembrane domains are pulled closer together. This model is also referred to as N- to C-terminal

zippering. It is an appealing model because it explains how the two membranes are brought into proximity and further suggests a mechanism by which the full zippering could be stalled at certain stages. It now appears that accessory molecules regulate the formation of the SNARE complex at various stages (Jahn & Scheller, 2006; Rizo & Rosenmund, 2008; Südhof & Rothman, 2009).

par0085 Two lines of evidence point to the SNARE complex as the major driving force during membrane fusion. First, disruption of SNARE function *in vivo* severely inhibits $Ca^{2+}$-dependent exocytosis. The targeted deletion of individual SNAREs almost completely abolished synaptic vesicle and secretory granule fusion (Borisovska et al., 2005; Schiavo et al., 1992; Schoch et al., 2001; Washbourne et al., 2002). Furthermore, mutations of critical amino acids within the SNARE complex reduce fusion (Kesavan, Borisovska, & Bruns, 2007; Sorensen et al., 2006; Walter et al., 2010). Second, membrane fusion mediated by SNAREs has been successfully reconstituted *in vitro*. Starting with a seminal study by Rothman and colleagues (Weber et al., 1998) membrane fusion mediated by the neuronal SNAREs has been reconstituted and the role of SNAREs has been analyzed (Chen et al., 2006; Domanska, Kiessling, Stein, Fasshauer, & Tamm, 2009; Fix et al., 2004; Kiessling, Domanska, & Tamm, 2010; Liu, Tucker, Bhalla, Chapman, & Weisshaar, 2005; Montecucco, Schiavo, & Pantano, 2005; Pobbati, Stein, & Fasshauer, 2006; van den Bogaart et al., 2010). The results from these *in vitro* systems have led to various and often conflicting interpretations. In some cases the fusion rates observed were extremely low or dependent on nonphysiological conditions. It was therefore suggested that the SNAREs are not the minimal machinery for membrane fusion. In other studies it was observed that SNAREs alone suffice to mediate fast fusion and it was thus concluded that they are the minimal machinery mediating membrane fusion *in vivo* (Chen et al., 2006). In addition, the number of SNARE complexes required for an individual fusion events was found to range from 15 to just a few (Domanska et al., 2009; Montecucco et al., 2005). It has recently been suggested that as few as one SNARE complex is sufficient for membrane fusion *in vitro* prompting the question why each synaptic vesicle carries about 70 synaptobrevin molecules (Takamori et al., 2006; van den Bogaart et al., 2010). Titration experiments of wild type and mutant SNAP25 expression in SNAP25 knockout cells have now proposed that as at least three SNARE complexes are required for fast fusion but less than three SNARE complexes may be sufficient to drive slow fusion (Mohrmann, de Wit, Verhage, Neher, & Sørensen, 2010).

par0090 In all *in vitro* reconstitutions described above one crucial feature of synaptic vesicle and secretory granule fusion was missing, that is the strict $Ca^{2+}$ dependence of the fusion reaction. It became thus clear that although the SNAREs are at the core of the fusion event they are neither $Ca^{2+}$ sensitive nor sufficient for $Ca^{2+}$-dependent fusion (Chen, Tang, Sudhof, & Rizo, 2005).

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### B. Synaptotagmins and Doc2 Proteins

#### 1. Synaptotagmins

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The strict $Ca^{2+}$ dependence of synaptic vesicle and secretory granule fusion prompted the search for $Ca^{2+}$ sensors that transmit the $Ca^{2+}$ signal into the actual fusion event. The first such sensor identified was synaptotagmin-1 (Geppert et al., 1994; Matthew, Tsavaler, & Reichardt, 1981; Perin, Fried, Mignery, Jahn, & Sudhof, 1990; Perin et al., 1991). Synaptotagmin-1 is localized to synaptic vesicles and secretory granules by an N-terminal transmembrane domain. Its main functional modules are two C-terminal C2 domains (Chapman, 2002; Fernandez et al., 2001; Sutton, Davletov, Berghuis, Sudhof, & Sprang, 1995). C2 domains are composed of two beta sheets with each sheet composed of four beta strands. The beta strands are connected by variable loops (Rizo & Sudhof, 1998). The C2 domain fold is conserved from yeast to humans. In synaptotagmins, the N-terminal C2 domain is called C2A and the C-terminal C2 domain is called C2B domain. In synaptotagmin-1 and several other synaptotagmins, the C2A domain is able to bind three $Ca^{2+}$ ions whereas the C2B domain binds two $Ca^{2+}$ ions (Chapman, 2002). $Ca^{2+}$ binding is mediated by one pocket in each domain located between the loops connecting the beta strands. Acidic residues make these pockets highly negatively charged, allowing the pocket to coordinate $Ca^{2+}$ ions (Cheng et al., 2004; Fernandez et al., 2001; Sutton et al., 1995). $Ca^{2+}$ binding reverses the net negative charge to a net positive charge enabling the C2 domains to bind membranes. In solution, the $Ca^{2+}$ affinity of synaptotagmins is very low, in fact too low to be able to serve as a $Ca^{2+}$ sensor *in vivo*. However, in the presence of negatively charged membranes its $Ca^{2+}$ affinity increases dramatically to the low mM range. The C2 domains of synaptotagmin-1 are thus $Ca^{2+}$-dependent membrane binding modules (Zhang, Rizo, & Sudhof, 1998). Furthermore as the C2 domains insert into one monolayer of the membrane in a shallow manner they induce membrane curvature, triggering the membrane to bend toward them (Herrick, Sterbling, Rasch, Hinderliter, & Cafiso, 2006; Hui, Bai, & Chapman, 2006; Hui, Johnson, Yao, Dunning, & Chapman, 2009; Martens et al., 2007; McMahon et al., 2010). They thereby act analogously to amphipathic helices found in other membrane trafficking proteins such as epsins, arfs, some BAR domain proteins, or HIV Nef (Beck et al., 2008; Ford et al., 2002; Gerlach et al., 2010; Lee et al., 2005; Peter et al., 2004). In addition to $Ca^{2+}$-dependent membrane binding, the C2 domains of synaptotagmin-1 can bind to the SNAREs (Chapman, 2002; Martens & McMahon, 2008). Some of the SNARE interactions with the synaptotagmins appear to be $Ca^{2+}$ dependent (Lai, Huang, Herrick, Epp, & Cafiso, 2011). It is generally observed that the interaction with SNAREs is relatively weak (Choi et al., 2010; Lai et al., 2011; Tang et al., 2006; Vrljic et al., 2010). The C2B domain binds to the tSNARE complex in a $Ca^{2+}$-independent manner (Rickman et al., 2006), an activity that has been

implicated in secretory granule docking (de Wit et al., 2009). It can also bind to the fully assembled SNARE complex but here the interaction appears to be enhanced by $Ca^{2+}$ (Choi et al., 2010; Lai et al., 2011; Tang et al., 2006; Vrljic et al., 2010). The C2A domain has been reported to bind to SNAP-25 within the SNARE complex. This interaction is of low affinity and is dependent on $Ca^{2+}$ (Lynch et al., 2007).

par0100 In synaptotagmin-1 knockout neurons the synchronous phase of neurotransmitter release is completely lost (Geppert et al., 1994). In some neurons synaptotagmin-2 and synaptotagmin-9 can take over the function of synaptotagmin-1 (Xu, Mashimo, & Sudhof, 2007). When they are deleted in the respective cells the synchronous phase is lost. In these knockout cells asynchronous release is still present and spontaneous release is increased (Fernandez-Chacon et al., 2001; Geppert et al., 1994; Xu et al., 2009). In chromaffin cells deletion of synaptotagmin-1 results in the loss of the fast phase of release whereas the slow and sustained phases are still present (Schonn, Maximov, Lao, Südhof, & Sørensen, 2008; Voets et al., 2001).

par0105 In addition it was observed that deletion or expression of mutant synaptotagmin-1 has a direct affect on the kinetics of release. Effects on the kinetics of release have so far only been observed for certain SNARE mutations and the synaptotagmins (Kesavan et al., 2007; Sorensen, 2004; Sorensen et al., 2006). These results suggest that synaptotagmins-1, -2, and -9 are integral parts of the release machinery and several models have been proposed for the final events leading from the $Ca^{2+}$ signal to membrane fusion. Thus, there is now good evidence that the SNARE complex is at least partially assembled before the $Ca^{2+}$ trigger for release arrives (Sorensen et al., 2006; Walter et al., 2010). Due to its $Ca^{2+}$-independent interaction via the C2B domain, synaptotagmin-1 may already be bound to this partially assembled SNARE complex (Rickman et al., 2006). In fact it has been proposed that the $Ca^{2+}$-independent interaction of synaptotagmin-1 with the tSNARE complex composed of syntaxin-1 and SNAP-25 may be required for the docking of secretory granules in chromaffin cells (de Wit et al., 2009). Whether synaptotagmin-1 has a similar function in neurons is less clear (Burgalossi et al., 2010). It is thus conceivable that during docking synaptotagmin-1 binds to the tSNARE complex and that during priming synaptobrevin joins this complex to from a partially zippered SNARE complex. This partially assembled SNARE complex may be prevented from complete zippering by accessory molecules such as the complexins (Giraudo et al., 2009; Tang et al., 2006). The preassembly of the fusion apparatus offers a good explanation for the short time delay between the $Ca^{2+}$ trigger and the fusion event. It should be stressed however that most of the studies examining the molecular events during docking and priming have been conducted in chromaffin cells. It is therefore possible that the molecular events in neurons are different. In fact it has recently been suggested that synaptotagmin-1 is not

required for the priming of synaptic vesicles (Burgalossi et al., 2010). Upon $Ca^{2+}$ channel opening triggered by an action potential, the local $Ca^{2+}$ concentration rises to the low mM range (Neher & Sakaba, 2008). At this $Ca^{2+}$ concentration the C2 domains of synaptotagmin-1 are able to bind membranes, which is likely the final trigger for fusion. As the C2 domains insert into the membrane they will locally change the curvature of the bilayer inducing the bending of the membrane toward them (Herrick et al., 2006; Hui et al., 2006, 2009; Martens et al., 2007; McMahon et al., 2010) (Fig. 2). Since the plasma membrane contains more negative charge than the vesicular membrane the C2 domains are likely to bind to the plasma membrane. It is however not excluded that one of the C2 domain, possibly the C2A domain, binds the vesicular membrane (Herrick et al., 2009). As synaptotagmin-1 is associated with the SNARE complex and assuming that several SNARE complexes are located in a ring-like manner close to the future fusion site, the C2 domain are sufficiently concentrated to induce a buckle-like protrusion of the plasma membrane toward the vesicular membrane (Martens et al., 2007). Due to the interaction with the SNAREs the C2 domains are confined to the circumference of the buckle and thus the end caps are free from C2 domains (Choi et al., 2010; Lai et al., 2011; Tang et al., 2006; Vrljic et al., 2010) (Fig. 2). As the membrane in this end cap is highly curved in the absence of insertions the lipids are under curvature stress in this area. This curvature stress reduces the energy barriers for the later stages of the fusion process (Hui et al., 2009; Martens et al., 2007; McMahon et al., 2010). Concomitant with membrane binding the C2 domains may change their mode of binding to the SNARE complex and thereby induce the complete zippering of the complex (McMahon et al., 2010). The C2 domains of synaptotagmin-1 may also displace a hypothetical fusion clamp, such as complexin (Giraudo, Eng, Melia, & Rothman, 2006; Tang et al., 2006). The complete zippering of the SNARE complexes will further bring the membrane into close contact (Stein et al., 2009). As the helical SNARE complex is likely to extend into the membrane via syntaxin1 and synaptobrevin the complete zippering will act synergistically with the curvature induced by synaptatogmin-1 (McMahon et al., 2010; Stein et al., 2009) (Fig. 2). This model offers an explanation for the $Ca^{2+}$-dependent induction of membrane fusion by synaptotagmin-1 and is supported by *in vitro* and *in vivo* evidence. However, the lack of structural data on key intermediates as well as structural information about the fusion apparatus prior to fusion precludes a complete understanding of the events during fusion.

par0110 As mentioned above synaptotagmin-2 and synaptotagmin-9 act analogously to synaptoatgmin-1 during synchronous release. Given the central role these synaptotagmins play during synchronous release it was assumed that other synaptotagmins or related molecules trigger asynchronous release. An interesting candidate is synaptotagmin-7, which is a major $Ca^{2+}$ sensor for granule fusion in chromaffin cells (Schonn et al., 2008). However, in neurons it does not

seem to play a role during synaptic vesicle fusion (Maximov et al., 2008). Until now the $Ca^{2+}$ sensor for asynchronous synaptic vesicle fusion has remained elusive. In contrast significant progress has been made understanding spontaneous release, the third form of release at the synapse.

sec0050
## 2. Double C2 Domain Proteins (DOC2)

par0115 Spontaneous release has long been regarded as some oddity, which may be the result of an accidental trigger of the release machinery. In synaptotagmin-1 knockout neurons it was observed that the frequency of spontaneous release was increased (Littleton, Stern, Perin, & Bellen, 1994; Maximov & Sudhof, 2005; Pang, Sun, Rizo, Maximov, & Sudhof, 2006; Xu et al., 2009). In recent years it has emerged that spontaneous release is at least to a large extent $Ca^{2+}$ dependent (Emptage et al., 2001; Llano et al., 2000) (Groffen et al., 2010). Recently, the DOC2A and DOC2B proteins have been identified as $Ca^{2+}$ sensors that are responsible for a large fraction of the $Ca^{2+}$-dependent release events (Groffen et al., 2010). DOC2 comprises a small protein family of three proteins in mammals (Martens, 2010; Martens & McMahon, 2008). Like the synaptotagmins, the DOC2 proteins contain two C-terminal C2 domains called C2A and C2B domains. Unlike the synaptotagmins the DOC2 proteins contain no transmembrane domain at their N-terminus. The C2 domains of DOC2A and DOC2B are able to bind $Ca^{2+}$ (Groffen, Friedrich, Brian, Ashery, & Verhage, 2006; Groffen et al., 2004, 2010). However, their $Ca^{2+}$ affinity is in the high nM range and thus significantly higher than for the C2 domains of synaptotagmin-1 (Groffen et al., 2004, 2006, 2010). The expression of DOC2A and DOC2B is restricted to neuronal cells and other professional secreting cells. Within the brain DOC2A and DOC2B are expressed in an overlapping manner (Verhage et al., 1997). In cells in which the two proteins are coexpressed, they appear to function in a redundant manner (Groffen et al., 2010). Biochemically the DOC2 proteins show striking analogies to synaptoatgmin-1 and the other $Ca^{2+}$-dependent synaptotagmins. Thus, both the C2A and C2B domain of DOC2A and DOC2B are able to bind to negatively charged membranes (Groffen et al., 2010). Interestingly, the C2B domain of DOC2B shows a strong phosphatidylinositol bisphosphate dependence for $Ca^{2+}$-dependent membrane binding (Groffen et al., 2010). By analogy to synaptotagmin-1, a fragment containing both, the C2A and C2B domains of DOC2B (C2AB) is a potent inducer of membrane curvature (Groffen et al., 2010). Furthermore, the C2AB domain of DOC2B strongly promotes SNARE-dependent membrane fusion in a reconstituted system. Both membrane curvature induction and fusion promotion are $Ca^{2+}$ dependent (Groffen et al., 2010). In addition the C2 domains of DOC2B have been shown to bind to the neuronal SNARE complex. When expressed in DOC2B knockout hippocampal neurons, DOC2B that harbors mutations in the C2 domains, that interfere with $Ca^{2+}$-dependent membrane

binding and SNARE complex binding, are unable to restore wild-type frequency of spontaneous release. Furthermore, a mutant DOC2B that is able to bind membranes at lower $Ca^{2+}$ concentrations compared to the wild-type protein supports a higher frequency of spontaneous release compared to the wild type protein under condition where $Ca^{2+}$ is limiting (Groffen et al., 2010). These results strongly suggest that DOC2A and DOC2B function as $Ca^{2+}$ sensors for spontaneous neurotransmitter release and furthermore that they act in the same mechanistic manner as synaptoatgmin-1 during synchronous release. The finding that DOC2B affects the kinetics of individual fusion events in chromaffin cells further supports the hypothesis that it is an integral part of the fusion machinery (Friedrich et al., 2008).

par0120 Synaptotagmin-1 and DOC2B proteins compete with each other for binding to the neuronal SNARE complex *in vitro* (Groffen et al., 2010). Therefore, a complex picture is emerging where different $Ca^{2+}$ sensors that act with the same general mechanism but differ in their kinetics, $Ca^{2+}$ affinity and precise regulation, coexist at the synapse. In an extreme view, many different $Ca^{2+}$ sensors coexist that function in an overlapping manner. The reason no sensor for asynchronous release has been identified so far may be due to several such sensors functioning in a redundant manner. Among the calcium sensors, synaptotagmin-1 may be uniquely positioned to rapidly respond to the rise in $Ca^{2+}$ on depolarization (de Wit et al., 2009), whereas DOC2A/B with its high $Ca^{2+}$ affinity may be ideally positioned to respond to very low fluctuations in calcium concentrations (Groffen et al., 2010).

## sec0055 IV. CONCLUSION

While the SNARE proteins and synaptotagmins are both implicated in the kinetics of membrane fusion and thus in the fusion event itself there are many other accessory proteins that likely regulate the placement of the fusion site, the assembly of fusion complexes and other proteins may also participate directly in the fusion event. Thus, proteins showing phenotypes in docking or priming of vesicle fusion may not be excluded them from playing a role in later stages. Indeed, synaptotagmin-1 has now been proposed to play a role in docking, fusion, and in the post fusion opening of the fusion pore (collapse of the vesicle). Finally while synaptic vesicle fusion is calcium-dependent many other fusion event are constitutive and do not involve a calcium trigger, but yet are still likely to require membrane identity selection and membrane priming by accessory proteins of the SNARE complex in an analogous manner to C2 domains.

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