Fusion pore in exocytosis: more than an exit gate?

A β -cell perspective

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ABSTRACT (250 WORDS)

Exocytosis is a fundamental biological event and the process by which hormones (like insulin) are released into the blood. Considerable progress has been made in understanding the detailed steps from secretory vesicle docking to membrane hemifusion, opening of a membrane fusion pore and its regulation. This precisely orchestrated sequence of events implies a close interaction between membrane proteins and lipids in a confined space and constrained geometry to ensure physiological delivery of cargo. Here we consider some of the many (still open) questions such as the nature of the initial fusion pore, the structure of the SNARE transmembrane domains, their potential role (active or passive?), the lipid-transmembrane domain interplay and dynamics and regulation of the fusion pore. The fusion pore, via the restrictions imposed by its physical dimensions constrains hormone release. We discuss how fusion pore expansion may be affected in type-2 diabetes via changes in gene transcription and alterations in the profile of circulating lipids. Thus detailed understanding of membrane fusion and the fusion pore itself seems to be required to understand important aspects of healthy β -cell function and how it becomes disrupted under pathophysiological conditions.

1. Introduction

Pancreatic islet β -cells occupy a unique position in the physiological regulation of nutrient homeostasis [1]. Although several other organs (such as gut, brain, liver, adipocytes and muscle) are required for fine-tuning of metabolism and its adaptation to environmental cues [2-4], the β -cells of the pancreatic islets arguably represent the body's principal nutrient sensor. The β -cell exerts its function by secreting insulin, the only hormone of the body capable of lowering the plasma glucose levels. Thus, the β -cell plays both the part of the sensor and the actuator. The functional significance of the β -cell and insulin is illustrated by the devastating metabolic consequences of type 1 diabetes (T1D), an autoimmune disease associated with the destruction of the insulin-producing β -cells. However, T1D accounts for only a small fraction of diabetes (10%) and most diabetic patients instead have type 2 diabetes (T2D).

In T2D, the β -cells remain viable but they fail to dispense insulin with the right kinetics and at the right level to maintain plasma glucose levels in the normal range. The underlying disturbance remains obscure but it is now clear that the insulin secretion defect develops gradually over many years [5, 6] as a result of a conspiracy between the genetic predisposition and lifestyle factors. Because of its central role in fuel homeostasis and the evidence implicating defective insulin secretion as a cause of T2D, it is perhaps not surprising that the β -cells represent the target of many of the diabetes medicine currently in clinical use (such as sulfonylureas and GLP-1). Currently, more than 100 gene variants increasing T2D risk have been identified but – with a few exceptions – the mechanism(s) by which they act remain to be established. In some cases, the evidence points to 'unusual suspects' such as the very last steps of the insulin release process.

2. β -cell Physiology and Insulin Secretion via Exocytosis

The concept 'exocytosis' denotes the fusion of intracellular vesicles with the plasma membrane to release either soluble cargo or to insert new molecules (such as lipids or proteins) into the target membrane (Fig. 1). Physiologically, exocytosis is of very broad significance, spanning from renewal of the plasma membrane and its components to cell migration and cell division, maintenance of the membrane integrity and intercellular communication [7-9]. The term "exocytosis", was first coined in in the 1960s [10] in chromaffin cells, observed at the ultrastructural level in the 1970s there [11] as well as at synapses [12] and described molecularly (at a relatively detailed level) in the 1990s (and onwards) [13-15].

Insulin secretion involves exocytosis of large dense core vesicles (LDCVs) and show great similarity to the release of catecholamines from adrenal chromaffin cells (the archetypal neuroendocrine cells). Indeed, β -cells and insulin secretion now represent a widely used model system to study LDCV exocytosis [16]. Studies of insulin exocytosis and its regulation are of particular interest given the link to T2D and its clinical implications [1].

The process of nutrient-induced insulin secretion is well understood and will not be described in great detail here. Briefly, insulin secretion is elicited by nutrients such as glucose, lipids or certain amino acids [17]. Their metabolism, subsequent to transporter-mediated entry into the cytosol generates a number of coupling factors linking metabolism to hormone secretion. Whereas changes in the ATP/ADP ratio are the best characterized example, other are also important such as increases in cyclic AMP or specific lipids [18-20, 21]. Changes in ATP/ADP ratio lead to closure of ATP-regulated K^+ (K_{ATP}) channels [22]. K_{ATP} channel activity maintains membrane potential at the resting potential (-70mV) at low glucose concentrations not associated with stimulation of insulin secretion. Closure of these channels results in membrane depolarization and the initiation of action potential firing due to opening of voltage-dependent Ca^{2+} channels. The associated increase in cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) triggers insulin exocytosis [3] (Fig. 1).

Insulin secretion follows a characteristic biphasic time course and consists of an initial but transient 1st phase followed by a sustained/slowly developing 2nd phase. Although first described 50 years ago [23] and preserved in all species investigated (including man [24]), the cellular background of biphasic insulin secretion remains debated. It has been attributed to both sequential release of distinct pools of secretory granules and time-dependent changes in β -cell electrical activity [17, 25-27].

It is interesting to correlate the kinetics of biphasic insulin secretion with the physiological actions of insulin. Whereas insulin action on the liver is maximal after 15 min [28], a considerable further delay in insulin action on other organs is caused by the time required for transepithelial delivery of the peptide hormone to attain its site of action [29]. These considerations suggest that the biphasic release subserves a physiological function.

Impaired nutrient homeostasis is a hallmark of T2D [30]. Insulin resistance in target tissues as well as β -cell dysfunction are recognized as the major pathogenic factors. However, the view that islet failure develops mainly secondary to hormone resistance is no longer tenable on the base of functional, epidemiological and genetic data [1, 31-34].

Interestingly, T2D is associated with the loss of normal biphasic insulin secretion: 1st phase insulin secretion is nearly abolished and 2nd phase insulin secretion strongly reduced [35]. The underlying causes are not known but may be secondary to impaired metabolism [36], electrical activity or exocytosis [37-40]. T2D has also been shown to be accompanied by a loss in β -cells, especially in long-standing cases, but control and patient's post-mortem sample values largely overlap and differences are not pronounced enough to explain the progression to the disease [35, 41-43]. Thus, it seems reasonable to conclude that impaired β -cell secretory function must be taken into account in the aetiology of T2D. Of course, the two concepts are not mutually exclusive and T2D may well involve both reduced β -cell function and mass.

3. Insulin Secretion as paradigm for LDCV Exocytosis

3.1. Vesicles and pools

Exocytosis encompasses the last steps in hormone secretion (Fig. 1). It can be divided into several distinct steps [44]. The movement to and tethering of the secretory granule to the plasma membrane is referred to as 'docking' [12, 45-48]. Once the granules have docked, they undergo additional biochemical reactions to attain release competence in a process termed 'priming' [49, 50]. To allow release of the cargo, the two membranes (the plasma membrane and the secretory granule) must overcome membrane repulsion and merge [51] (Fig. 2A). Insulin containing LDCVs are formed in the trans-Golgi Network and have to undergo several maturation steps [16, 52, 53]. In this process, pro-insulin is converted into insulin [54]. The diameter of mature granules has been a matter of debate. Chemical fixation yields a diameter of around 350 nm, however, this may well be an artefact and rapid freezing techniques concluded upon a diameter of some 250 nm [55-57]. Granule maturation also involves membrane remodelling and insertion of proteins involved in the exocytotic process including certain SNARE proteins (see below) such as syntaxin 6 or VAMP4 as well as calcium-sensing synaptotagmin 4 [58-61].

The β -cell contains some 5.000 to 10.000 secretory vesicles [55, 62]. Of these, only 1% are immediately available for release ("readily releasable pool" or RRP) (see Fig. 1). The RRP granules have been proposed to underlie the first phase of biphasic insulin secretion. The remaining 99% of granules constitute a 'reserve pool' of granules [25, 26]. Reserve granules must undergo a series of priming reactions and even physical translocation within the β -cell to attain release competence. It has been proposed that this gives rise to 2nd phase insulin secretion [63]. As in other cases of LDCV

exocytosis, both the final fusion step and the transit between pools are Ca^{2+} -dependent processes, albeit with distinct affinities [64, 65].

3.2. The β -cell SNARE Machinery

Membrane fusion is driven by an evolutionary conserved set of proteins, the Soluble N-ethylmaleimide-sensitive-factor Attachment protein REceptor (SNARE) complex [13-15, 66-68]. The core of this supramolecular complex encompasses two cytosolic proteins with transmembrane domains (see Fig. 2A): VAMP/synaptobrevin on the vesicles (v- or R-SNARE, according to the central presence of an Arg in its cytosolic helix [67, 69]) syntaxin on target plasma membranes and lipid-anchored SNAP-25 (target t-SNAREs or Q-SNAREs, according to the central presence of an Gln in their cytosolic helices [67, 69]). The interaction of the cytosolic domains of these three proteins gives rise to a coiled coil [69, 70]. Zippering of the helix exerts a mechanical force and brings the vesicle and plasma membrane into close contact [15, 71]. This may overcome the forces exerted by the hydration barrier and induce membrane fusion. The complex is subject to further regulation by a number of proteins such as complexins, munc13, the SM proteins sec1/munc18 as well as chaperones that intervene at different steps [72-75].

Early work demonstrated the expression and functional role of the essential components in β -cells. These include the SNARE proteins VAMP-2, syntaxin-1 and SNAP-25 as well as chaperones [76-84]. As in neurones, syntaxin 1 and SNAP-25 bind to [85] and regulate the activity of voltage-dependent Ca^{2+} channels in β -cells [86-89], which may explain why the Ca^{2+} channels congregate at secretory granules [38]. Syntaxin also interacts with $\text{K}_v2.1$ voltage-gated K^+ channels (that are involved in action potential repolarization of the β -cell action potential) [90-92]. Similar to K_v channels, distinct SNARE protein isoforms have been shown to interact with different forms of voltage-gated Ca^{2+} channels [87, 88, 93].

Recent work led to the proposal that granules in 1st and 2nd phase insulin release may use distinct SNARE protein isoforms [94-96] (but see [97, 98]). However, it is not clear whether distinct SNARE machineries need to be implicated as events upstream of exocytosis, such as electrical activity and changes in $[\text{Ca}^{2+}]_i$, are also biphasic [27, 99] and should suffice to produce biphasic secretion patterns. The attribution of different phases of secretion to distinct sets of SNARE proteins, such as VAMP8 or syntaxin 3 and syntaxin 4 [95, 96], is also complicated by the fact that some of these are classical components of other membrane trafficking pathways. These include glucose transporter cycling [100] or fusion of endosomes, lysosomes or autophagosomes [101-105]. Interference with any of these events could indirectly affect exocytosis, as was recently

demonstrated for dynamin [106]. Indeed, a careful and quantitative analysis of distribution in the post-Golgi SNARE trafficking by immune electron microscopy, as performed for the intra-Golgi pathway [107], is still crucially lacking and light microscopy may not be sufficient in view of the highly intertwined and functionally linked post-Golgi compartments.

3.3. β -cell Synaptotagmins and Fusion

Several isoforms of the vesicle proteins of the synaptotagmin (Syt) family bind Ca^{2+} and phospholipids and are the main Ca^{2+} sensors for exocytosis in neurones [14]. Following the initial demonstration that these sensors are also operational in insulin exocytosis [108], almost every Ca^{2+} -sensitive synaptotagmin (i.e. Syt1, 3, 4, 5, 7 and 9 [109-111]) has been proposed to be involved in insulin release in clonal or primary β -cells using antibodies, overexpression or knockdown, although their respective subcellular localisation remains controversial [112-123]. In human β -cells the major Ca^{2+} -sensitive synaptotagmins expressed are Syt4, Syt5 and Syt7, whereas Syt2 and Syt9 are only present at minor levels [124]. Decreasing Syt4 expression levels reduces insulin secretion [121, 123], an effect which likely reflects its known TGN localisation in the trans-Golgi Network (TGN) and action in vesicle maturation rather than a role in exocytosis itself [60, 114, 125]. Whereas knock-down of Syt9 did not alter glucose homeostasis or insulin secretion in vivo [126], Syt7 knock-out mice exhibit glucose intolerance and some reduction in exocytosis [127, 128], raising the question about additional Ca^{2+} sensors. Recently Syt7 has been reported to play a novel more dominant function in the replenishment of releasable vesicle pools rather than in exocytosis per se [122]. A further complication is that Syt7 is also involved in endocytic-lysosomal traffic [129] and cholesterol delivery into the plasma membrane [130], which may impact insulin secretion via indirect effects [131]. Thus, the identity of the physiologically relevant Ca^{2+} sensor of insulin secretion remains controversial. A potential resolution to this conundrum is that either exocytosis is controlled by an array of multiple synaptotagmin isoforms and eventually at distinct steps during exocytosis [132-135] or that secretory granules are more heterogeneous than previously thought [136]. The ultrastructural co-localisation and characterisation required for such a scenario as well as the functional implication remain to be experimentally tested.

4. The Centre Stage of Exocytosis: The Fusion Pore

4.1. Hemifusion

Discrete steps during membrane fusion were initially conceptualized and demonstrated for viral fusion [137-139] and SNARE-mediated exocytosis is generally accepted to follow a similar path: from an initial membrane deformation via hemifusion (involving the apposed leaflets of the two

bilayers) stalk formation to opening of the fusion pore (see Fig. 2). At the fusion site, both lipid bilayers (that of the plasma membrane and the secretory vesicle) must transiently enter an unstable state that allows lipid mixing without disrupting membranes. The precise mechanisms of hemifusion are still debated [140]. They may involve initial deformation of vesicular and plasma membranes thus reducing intermembrane space (Fig. 2B), followed by mechanical strain on membranes exerted via full SNARE complex formation (zippering) and juxtamembrane domain mediated destabilisation of opposing membrane leaflets with acyl chain protrusion [141, 142]. The initial deformation of one or the two opposing membranes (“dimples”, see Fig. 2B) would further enhance the propensity to fuse due to increased membrane tension [143] and may involve the synaptotagmins [144, 145]. The resulting hemifusion (Fig. 2B) allows lipid mixing between the fused outer layers of the donor (vesicle) and acceptor (plasma) membrane [146]. Direct evidence for hemifusion has been provided by various experimental approaches; SNARE proteoliposomes [147-149], cell-to-cell fusion [150, 151], force measurements [152, 153], imaging in neurones and β -cells [154, 155] and evidence for its potential effect lipid structures have been provided by diffraction methods [156]. The formation of the stalk (Fig 2B and C) was predicted in protein-free systems [157, 158] and these *In silico* analyses suggest three different possible outcomes: (i) a minimal stalk with a height just greater than two bilayers; (ii) an elongated stalk; or (iii) the formation of a hemi-diaphragm [51, 157, 159]. The latter two outcomes have also been predicted in simulations including lipids and SNARE proteins [160-162]. These observations support the existence of the stalk and have greatly advanced our view of this intermediate structure although the dynamics in native protein-containing lipid membranes remain unknown.

Critically, the stalk is characterised by a high curvature (Fig. 2B). Similarly, *trans* leaflets (facing the vesicle lumen, or in case of the plasma membrane, the extracellular medium) have to bend and rearrange with large opposite curvatures to obtain a stalk [163] (see Fig. 2B and also 6). According to this model, membranes undergo a great amount of physical stress during hemifusion and its evolution that may require specific adaptation in order to preserve membrane integrity and comply with a very particular geometry. Indeed, lipid acyl chains are probably bent at the base and stretched at the neck (mid-height) of the stalk [51, 159, 164] and a relative movement of *cis* (cytosolic) versus *trans* (luminal/extracellular) leaflets has been postulated *in silico* [162]. It is often assumed that local membrane curvature could be supported by specific lipids, which may favour positive or negative curvatures according to their geometry [165, 166] (but see also [167]) and consequently influence membrane fusion [140]. The opening of the fusion pore occurs when a membrane continuum is formed consisting of the *trans* leaflets and of the *cis*-leaflets (Fig. 2C). Seen this way, fusion, opening and expansion of the fusion pore reflect the relaxation of the system and

the return to a stable state.

4.2. Fusion Pore and Full vs partial Fusion

The fusion pore (term first used by Almers and collaborators [168]) constitutes the polar continuum between vesicle lumen and cell exterior prior to further expansion. Its highly dynamic nature as well as its initial nano- or even subnanometric inner diameter excludes capture of its early form by electron microscopy [169-171]. In contrast, its kinetics can be reasonably well recorded by sophisticated electrophysiological, electrochemical and optical means [154, 172-175]. In rat β -cells, the earliest pore measured has a diameter of about 2 nm [176]. Once it has fully expanded, the fusion pore is wide enough to accommodate insulin, the diameter of which ranges between 2.6 and 5 nm in its mono- and hexameric forms, respectively [177, 178]. However, due to the lack of direct morphological and structural assays, we still do not have a precise view of its chemical composition in terms of proteins or lipids (or both) at the time of its formation and its nature is still highly debated (see below 4.4.).

The fusion pore connects electrically the granule lumen to the exterior and the ease by which electrical charges pass (conductance) is determined by the cross-sectional area of the pore, and to a lesser extent by its length. Thus, the fusion pore conductance is not linearly related to the pore diameter but depends on its square and small changes in diameter results in considerably larger changes in conductance. At its early stage the fusion pore opening is still reversible. In this unstable state, the conductance varies [179] and may flicker between an open (conducting) and closed (non-conducting) state. In some cases, the fusion pore closes permanently after a few openings and closures. This type of exocytosis has been referred to as 'kiss'-and-run' [180-183] or 'flickering' [168]. Conversely, during full fusion, the fusion pore expands completely until the entire granule membrane collapses into the plasma membrane (Fig. 3A).

According to observations in chromaffin cells, pituitary lactotrophs and astrocytes the behaviour of the fusion pore described here may be influenced by the vesicle size, which could relate to the bending energy of the membrane [82, 184-187]. It is implicit from this view that larger vesicles should be more prone to transit to full exocytosis whereas smaller vesicles may be more suited for kiss-and-run events. However, vesicle diameter also influences docking [188] and downstream mechanisms are clearly modulated by SNARE cooperativity [189] and potentially by the geometry of contact sites [190] and it is worth noting that direct comparison of fusion pores generated by the small synaptic-like microvesicles [191] or by the larger insulin granules does not lend support for such a vesicle size effect in pancreatic β -cells [192].

Given its small size, the fusion pore allows a selective release of vesicular content [193, 194]. Small molecules as ATP, GABA are able to go through the pore and once released may regulate neighbouring cells [195]. Although 'kiss-and-run' exocytosis normally represents only 10% of the fusion events measured in mouse β -cells [40], it is of physiological relevance for paracrine/autocrine regulation [192, 196]. Kiss-and-run exocytosis can also be considered as a specific type of endocytosis. In this very short amount of time, the vesicular pH is buffered from an acidic pH and small molecules can enter in the vesicle. Techniques of imaging take advantage of these two properties to study the fusion pore kinetics. Total Internal Reflection Fluorescent (TIRF) microscopy, used in combination with the pH sensitive GFP (pHluorin), allows a precise detection of the fusion pore opening since the fluorescence signal significantly increases as the vesicular pH becomes neutral [197, 198]. Two-photon confocal microscopy can be used to monitor the rate of exocytosis by using the fluorescent tracer sulforhodamine-B (SRB), which small enough to enter the granule via the fusion pore [199]. However, due to the technical challenges, the fate of these vesicles after endocytosis and their consequence on β -cell functions remain poorly understood. Recent developments in light sheet microscopy may also provide new insights especially for very rapid events [200].

Kiss-and-run exocytosis may not involve mixing of the membrane proteins of the two compartments (secretory granules and plasma membrane) whilst still allowing lipid exchange [201]. Evidence for lipid mixing can be obtained by ruthenium red staining of the membrane (Figure 3). If the granule membrane has been in direct contact with the plasma membrane, part of the granule membrane will be labelled by ruthenium red which is electron dense and thus exhibits an increased contrast by electron microscopy. When visualised by electron microscopy, the structure of the late fusion pore and attached vesicle is seen as an omega (Ω) shape (note that early fusion pores escape direct visualisation) (Fig. 1 and 3). Once the fusion pore has expanded beyond a certain diameter, its opening is irreversible, at least in the case of large vesicles as the giant granules of mast cell as well as eosinophil or neutrophil granules [179, 202, 203], and proceeds to full fusion of the vesicle with the plasma membrane and the subsequent release of the hormone cargo.

4.3. SNARE protein TMDs: Sequences and Dynamic Structures

Recent work has led to a more detailed understanding of how different domains in the SNARE proteins influence exocytosis and the opening of fusion pore. The vesicular VAMP plasma membrane syntaxin SNARE proteins contain a C-terminal transmembrane domain (TMD) preceded by a regulatory N-terminal domain, the cytosolic SNARE motif and the amphipathic juxtamembrane

domain (Fig. 4A). By contrast, the plasma membrane SNARE protein SNAP25 is attached through palmitoylated cysteines. Whereas the function of the cytosolic and juxtamembrane portions of the SNARE complex in membrane fusion is fairly well understood [69, 70, 204-208] and dynamics of their organisation during exocytosis has also been imaged in live chromaffin and β -cells [209-212], the role of the TMDs is much less clear.

During hemifusion and fusion pore opening local parameters such as protein concentration, membrane tension and lipid organisation change drastically (see 4.6. below). The analysis of how the structure and dynamics of the TMDs varies during these events requires advanced techniques and has mostly been performed in either complete absence of lipids [213], dehydrated membranes [214] or at low protein/lipid ratios [147, 215-218], conditions that do not recapitulate the SNARE protein 'crowding' during fusion [219]. Vibrational spectroscopy and its different approaches (ATR-FTIR, Attenuated Total Reflection-Fourier Transformed Infrared; PMIRRAS, Phase Modulated Infrared Reflection Absorption Spectroscopy) allow label-free simultaneous detection of the orientation and structural conformation of peptides, lipids and phospholipid head groups [220, 221]. Moreover, these approaches permit dynamic modulation of membrane pressure and peptide/lipid ratios during recording [220, 222, 223]. Aided by these methods, it has been possible to address a number of issues pertinent to membrane rearrangement or fusion in model systems and this has considerably advanced our understanding of viral membrane fusion proteins [224-227]. The effect of protein-lipid interactions can also be visualized by Brewster angle microscopy (BAM) in hydrated monolayers as models of biological membranes and provides useful information on domain organisation, membrane heights and viscosity [376].

Single membrane spanning TMDs, as present in most but not all VAMP and syntaxin isoforms, are classically described as hydrophobic anchors adopting α -helical structures. The amino acid sequence of the VAMP TMD is rather short, consists of only 19-21 residues [228] and contains an unusually high percentage of rigid C_β -branched amino acids (mainly hydrophobic Ile and Val as well as the small, slightly polar Thr, see Fig. 4B). These amino acids tend to destabilise α -helices and favour β -sheet conformation [229]. Accordingly protein structure predictions, such as Chou-Fassman, GOR or position-specific scoring matrices [230-232], indicate a large preference for β -sheets over α -helices for VAMP and syntaxin transmembrane domains.

It appears indeed that the TMDs of VAMP1 and VAMP2 contain a significant amount of β -sheets and that these not only occur in micelles or organic solvents but also in synthetic bilayers and using the full-length protein [233-235, 236]. Interestingly, they shift from a predominantly α -helical

to a β -sheet configuration solely depending on lipid/protein ratios, and this transition is fully reversible [234-236]. This structural flexibility is very unusual and may be highly relevant to exocytosis given that the local TMD concentration increases during membrane fusion [189, 219, 237, 238]. The syntaxin TMD (see Fig. 4C) also contains equally a high percentage of C_β -branched amino acids but the few structural studies undertaken to date suggest a far lesser degree of structural diversity and alterations than for the VAMP TMD [236, 239].

Why have these structural diversity and dynamics not been observed previously? It may be relevant that in most instances lipid membranes were either completely absent [213], dehydrated and thus rigid [214] or present only at a low peptide/lipid ratio [147, 240], conditions that all favour the presence of α -helices. Finally, computational constraints do not permit the *in silico* study of such transitions.

Many transmembrane domains are not straight rods but more often kinked or tilted and this deviation from the canonical α -helical structure may be functionally significant [241]. *In silico* simulations [242] provide evidence for a highly tilted TMD for VAMP2 as already observed by structural approaches [214, 236, 240]. In addition these simulations revealed the presence of a kink at G₁₀₀ [242]. This residue is probably situated close to the centre of the bilayer as the preceding amphipathic juxtamembrane domain (JMD) is already partially embedded in the membrane [242, 243]. Simulations indicate that the helix was partially unwound around G₁₀₀, suggesting some flexibility, and this unwinding was also observed *in vitro*, even when using highly dehydrated membrane [214]. Indeed, when the TMD is mutated to substitute this glycine with a rigid C_β -branched valine, a large diminution of its structural dynamics is observed [234]. The G₁₀₀ residue of VAMP2 is highly conserved among all species examined [234] and similarly situated glycine residues are present in VAMP1, 3 and 5 and may be replaced by other small residues (A, C) in VAMP4 and 8 (see Fig. 4 B). The presence of a small amino acid at these positions is indeed typical for SNARE proteins and forms a statistical hallmark for SNARE TMDs together with the high prevalence of C_β -branched amino acids (I, V and L). The latter characteristics are also shared with viral fusion proteins [229]. A functional importance of this signature is suggested by the positive correlation in artificial or in biological systems between membrane fusion and structural mobility, β -sheet content and central helix breakers such as glycine [234, 244-246].

Membrane density, packaging and thickness and the relative amount of lipids species such as cholesterol or phosphatidylserine exposed to the cytosolic face (which influence the membrane's capacity to bend) increase considerably from the ER to the plasma membrane [247-249].

Interestingly, this correlates with variations in the SNARE TMD sequences. Accordingly, the small central and C_β-branched amino acids are absent in the TMDs of the endoplasmic reticulum/Golgi R-SNARE sec22. Thus the potential role of these TMD characteristics may be constrained by specific membrane architecture.

VAMPs may form dimers at defined contact sites [250, 251]. Initially debated on grounds of affinity [252], high local concentrations are clearly present and further increased during fusion [219, 237, 253] and may even be more pronounced in large dense core vesicles as compared to synaptic vesicles [189]. Dimers have been well documented for VAMP2 by biochemical assays and as well as *in silico* [218, 254-258]. These interactions may be modulated by lipids competing as solvents or via repulsive head group charges [259]. Whereas some evidence indicates a role of TMD homodimerisation in fusion events depending on sec22 [260], a VAMP isoform implicated in the early secretory pathway (see above), the physiological relevance remains unknown at the level of exocytosis.

4.4. Are SNARE transmembrane domains lining the fusion pore?

The nature of the fusion pore remains hotly debated and it is still unclear whether the pore is lined by proteins (such as SNARE TMDs), lipids or both. The assumption of a “protein only” nature of the initial fusion pore was intuitively deduced because the fusion pore undergoes rapid conductance changes (openings, closures, flickers) resembling those seen for ion channels [170, 171, 179, 261, 262]. However, these characteristics can also be obtained using protein-free lipid membranes [263] or been modelled as lipid pore [264]. It should be noted, however, that fusion pores in protein-free systems were of larger conductance than seen in cellular systems (i.e. during synaptic-like vesicle exocytosis [265]). However, these pores are still within the range of those observed for insulin granules and there is no strong evidence in β -cells that vesicle size (LDCV vs SLMV) would influence fusion pore stability [192]. The absence of lipid flow upon initial pore opening using fusogenic hemagglutinin and phospholipid probes [266] is often taken as a further argument in favour of a protein pore. This led to the proposal of the dispersing subunit model [267]: specific fusion proteins form a proteinaceous bridge spanning both membranes and lipid influx ensues late upon subunit dissociation and pore expansion. However, lipids and corresponding lipid dyes may be contained by high protein concentrations [268] and fluxes difficult to observe. Using the amphiphilic dye FMI-43, entry of the probe into the granule membrane is observed prior to the uptake of an aqueous fluorophore into the granule lumen during insulin exocytosis [199]. This suggests that the early fusion pore is indeed of lipidic nature. The extent of lipid flow required to

form an initial lipid fusion pore obviously depends on the geometry and size of the stalk, which has only been simulated *in silico* with different models [162, 269, 270].

The fusion pore allows exit of polar or charged substances (H^+ , ATP, insulin upon equilibration of vesicular and extracellular pH, e.g.) and therefore needs a corresponding surface, which is provided either by polar phospholipid head groups or suitable amino acid residues. With regard to the possible proteinaceous nature of the fusion pore, it is worth noting that TMDs of VAMP2/syntaxin 1 appear to lack polar residues except for the most C-terminal portion (see Fig. 4B). The situation is not different in other isoforms which contain, at most, one or maximally two polar residues (in general serines or threonines) more centrally located within the TMD. In fact, serine may behave more like apolar amino acids when in a transmembrane helix as its side chain protons can bind back to the carbonyl oxygen of a residue preceding it by one helical turn they [271]. Although multimeric aggregates of synthetic amphipathic 21-mer peptides (containing solely Leu and Ser) have been shown to mimic ion channels, at least a total of 18 to 24 serines were required to form the pore, thus largely exceeding the number present in VAMP or syntaxin TMDs [272]. From a protein chemistry point of view, a plausible conformation consists of the polar C-termini sticking out towards the pore lumen. Such an arrangement had been proposed as scaffold model [273] and has some *in silico* [274] and experimental support. Addition of residues at this site delays the opening of the fusion pore and reduces burst amplitude which reflects flux through the pore. This may point to a reduced mobility of the TMD [275] but may also occur if the flux of cargo through the fusion pore was impeded by steric hindrance. However, at least in insulin exocytosis, the attachment of a glycine linker and a large protein (phluorin) to the C-terminus of VAMP2 did not alter fusion pore kinetics for a small molecule (ATP) when comparing endogenous VAMP2 with phluorin-tagged wild-type VAMP2 [234]. This argues against a lining of the fusion pore by the C-terminus of VAMP2. Finally, it is worth noting that two copies of SNARE proteins suffice for synaptic exocytosis [276] and in certain intracellular fusion steps only one protein SNARE TMD is available [277], which is clearly insufficient to produce a hydrophilic central pore.

A number of studies have been performed using truncated peptides, point- or “en-bloc” mutations or addition of lipid anchors (see Fig. 5). As a general caveat, almost all of these studies lack dynamic structural studies, an important point also in view of the general difficulty to model complex lipid membranes and to predict structural effects of mutations *in silico* [278, 279]. Therefore, crucial information is often lacking for precise interpretation. Mutations in the TMDs of either syntaxin 1 or VAMP2 in PC12 or chromaffin cells affects the kinetics of the fusion pore as deduced from amperometric analyses [280-283] (Fig. 5). These data are consistent with a largely (but not

exclusively) proteinaceous fusion pore but it remains unclear how the corresponding hydrophobic residues (L/Leu, V/Val, I/Ile, C/Cys) could form a central water-filled pore in the native peptides [284, 285]. Similarly covalent labelling of several central residues in the VAMP2 TMD by polar tracers was interpreted as their exposure to the pore lumen [286] (see Fig. 5). However, as VAMP was only present in two copies, it remains difficult to arrange any polar lining of the pore. Note that another set of proteins had been proposed as pore former, the F_0 subunit of the v-ATPase [287], however, its pore size is far too large for an initial fusion pore [288] and functional data suggest a control of vesicle filling [289]. In conclusion, it cannot be completely excluded that the fusion pore is in part proteinaceous but compelling evidence for such an arrangement is still missing.

4.5. SNARE TMDs: a role in membrane fusion beyond anchoring?

Membrane fusion and fusion pore opening and expansion have a number of important constraints in terms of time, geometry, pressure and tension [143] as well as packaging of hydrophobic and polar compounds in a highly crowded environment [253]. This implies close interaction between lipids and proteins and raises the first obvious question: are SNARE protein TMDs required for fusion beyond function as membrane anchor?

Previous studies in yeast suggest that at least one of the TMDs can be replaced by long-chain lipids or unrelated protein sequences in intracellular membrane trafficking events [290-292]. Similar observations were reported for exocytosis [293] but data are confounded by unspecific effects of a protein linker [246, 280] and the use of supraphysiological Ca^{2+} . It is now believed that membrane fusion proceeds in a sequential fashion; initiated as hemifusion with lipid mixing and followed by fusion pore opening with content release [157]. From these considerations it follows that lipid mixing cannot be equated to fusion pore opening and that both events have to be independently determined.

The concept that the VAMP2 TMD influences exocytosis beyond a role as membrane anchor is based in part on the fusogenicity of VAMP TMD-mimicking peptides [229, 233, 244, 245]. Although membrane lipid mixing persisted in reconstituted and cellular systems after replacement of the TMD with lipids or unrelated transmembrane protein sequences, both the expansion and lifetime of the fusion pore were affected [149, 150, 294, 295]. Mutational analysis provides further evidence for a role of TMDs (see Fig. 5). Point mutations along the VAMP2 or syntaxin 1 TMD interfere with the emptying of the vesicles of their cargo [234, 246, 281-283, 296].

Fusion pore kinetics is also influenced by proteins interacting with SNAREs or associated components (such as CSP or Munc-18) and 'tuneable' by phosphorylation [187, 297-301] but it remains unclear whether these effects reflect indirect effects (long range actions such as differences in SNARE assembly pathways and complex cooperativity) or more direct post-fusion modulation of the pore.

4.6. Lipids and the fusion pore

Lipids are amphiphilic molecules with polar heads, facing the aqueous lumen, and acyl chains of high hydrophobicity, which results in strong interactions between chains. This amphiphilic nature of lipids drives the structure of the membrane bilayer. The hydrophobic acyl tails compose the core, which amounts to about one half of the membrane thickness. The hydrated membrane interfaces account of the other half and its polar headgroups face either the cytosol, the lumen of an organelle, or the extracellular medium [302]. Membranes are composed of a mixture of different types of lipids [303, 304] and the inner and out leaflets vary in their lipid composition [305-307].

Fatty acid length and saturation modify the properties of the lipids. The nature of the acyl chains affects not only the molecular interactions and packaging density, but also impacts on the molecular shape of the lipids. This shape, defined by a packing parameter (P) [308], can vary from cylindrical ($P=1$) to conical ($P>1$ or $P<1$). Lipids with $P\neq 1$ induce a spontaneous curvature to the bilayer provided their distribution is anisotropic, i.e. differs between the two leaflets. In cells, the curvature of a membrane reflects to the sum of spontaneous curvatures of each lipid [308]. However, both the strength of this correlation and its relevance in biological membranes remain debated [167]. The capacity of membranes to adapt to mechanical stress depends on their lipid contents [166]. These mechanical properties of the membrane are likely to be highly relevant to exocytosis [309] as it will affect the curvature of the stalk structure [310].

Cholesterol is one of the essential lipids of membrane bilayers and in membrane fusion [311]. While its basic properties were described early on [312], our understanding of exocytosis and technological advances continue to reveal the importance of its role during exocytosis and membrane fusion [311, 313]. Cholesterol is most abundant in secretory vesicles, synaptic vesicles and the plasma membrane where it is present in similar molar amounts as phospholipids [253, 314]. Cholesterol plays an important role in trafficking all along the secretory pathway [315, 316] as well as secretory vesicle budding and shaping [317, 318]. Thus, data obtained by chronic cholesterol addition or by its depletion using methyl- β -cyclodextrin in cellular systems should be interpreted

with caution. More direct evidence pointing to a role of cholesterol in membrane fusion and fusion pore opening comes from acute manipulation by microinjection of cholesterol [319, 320]. These experiments also suggest that cholesterol located in the cytoplasmic leaflet is the most relevant pool in membrane fusion and pore opening.

The geometry of cholesterol favours membrane bending but other properties of the molecule are also important as phospholipids of the same shape cannot restore the kinetics of fusion after cholesterol depletion [321]. Due to its polycyclic amphipathic structure, cholesterol modifies the biophysical properties of the surrounding phospholipids. Cholesterol contains a rigid ring structures that straightens the neighbouring lipid acyl chains and increases the viscosity of the membrane by reducing the mobility and the structural dynamics of the neighbouring phospholipids [322-325]. These properties increase the thickness of the lipid bilayer [326] and promotes the formation of phase separations in a mixture of saturated and un saturated phospholipids [311, 327, 328]. The junction of the two phases promotes membrane curvature [329]. These biophysical properties are hypothesized to support also the budding and the creation of new vesicles [311, 330, 331].

Cholesterol can form dimers with other lipids and also interact with protein juxta- and transmembrane domains [332, 333]. Cholesterol Recognition/interaction Aminoacid Consensus sequence (CRAC sequence: L/V-(X₁₋₅)-Y-(X₁₋₅)-R/K) [333, 334] as well as its reverse sequence (CARC) [335] and the short amino acid sequence R-W-L may provide interaction sites and play an essential role in partitioning cholesterol and other lipids in conjunction with specific transmembrane domain. A potential effect of cholesterol on TMDs has also been deduced from its ability to stimulate not only of hemifusion but also of the fusion pore opening and expansion [313, 336]. The SNARE protein VAMP2 contains these two overlapping putative CRAC and CARC motifs [337] in the juxtamembrane and N-terminal transmembrane domain and VAMP2 has been identified as cholesterol binding protein in a mass spectrometric survey [338] but functional significance in intact cells (if any) has not been studied.

A number of effects have been demonstrated for cholesterol on SNARE proteins and exocytosis in both reconstituted fusion assays and intact cells. Cholesterol promotes the interaction between SNAP25 and syntaxin 1 prior to SNARE complex formation [339]. Topologically, the structure of cholesterol (small hydroxyl head, large hydrophobic portion) imposes an intrinsic negative curvature to the surrounding bilayer [340]. It is therefore tempting to hypothesise that cholesterol is involved in the generation of lipidic fusion intermediates (lipid stalk, see previous section), where membranes are highly curved [159, 163, 310, 341]. Observations on purified cortical

vesicles identified two main functions of cholesterol: membrane organizer and fusion trigger [342]. The former function would promote the recruitment of component essential for Ca^{2+} sensing whereas the latter is mainly supported by its negative curvature and its high concentration [321]. More recently, progressive addition of cholesterol to protein-free giant unilamellar liposomes has shown that cholesterol stabilised and decelerated the opening of the fusion pore [343], placing cholesterol at the centre stage as a critical regulator of the core fusion machinery.

Lipid subdomains driven by either lipid-lipid or lipid-protein interactions ensure membrane heterogeneity, which is quite different from the original fluid mosaic model where membrane proteins diffuse freely [344]. This heterogeneity in protein and lipid partition provides the membrane with mechanical properties such as lateral tensions. The latter (also termed membrane tension) corresponds to the force needed to deform the membrane. In cells, this tension is influenced by several parameters such as the osmotic force between cytosol and extra cellular medium, the submembrane actin/myosin cortex and its links to vesicles [345, 346] as well as forces resulting from cell-to-cell or cell-to-surface adhesion [143]. Biophysical and *in-silico* approaches indicate an important contribution of membrane tension to fusion [347-349] but it is not clear whether there is an optimal membrane tension and (if so) how cells measure it. During vesicle fusion, the surface tension contributes to the expansion of the fusion pore that precedes the complete merging of the vesicle into the plasma membrane [143]. Although it was assumed that this occurs spontaneously, recent work in neurones indicates it requires ATP hydrolysis and actin polymerisation [350]. Therefore, any mechanism that influences membrane tension may affect both the stability and kinetics of the fusion pore.

4.7. Lipids and proteins in fusion and fusion pore dynamics

The initial events in hemifusion are thought to be initiated by close membrane apposition and mechanical forces exerted by zippering of the cytosolic SNARE complex [351]. Ensuing movement of the TMD may pull phospholipids out of the membrane plane and thus create a focus for hemifusion of the apposed membranes layers [70, 71, 204, 213]. This capacity would account for the fusogenicity of TMDs and derived model peptides in proteoliposomes [233, 244]. However, an increased local concentration of VAMP2 TMDs does not disturb polar phospholipid headgroups [236] and “canonical” SNARE TMD sequences are not an obligatory requirement for hemifusion [147, 149, 295]. Interestingly, the arrest of exocytosis when using short rigid acyl chains instead of TMDs [294], situated probably at the level of hemifusion [149], can be overcome by the addition of inverse conical ($P < 1$) lysophosphatidylcholine [294]. This suggests that membrane lipid rearrangements may

not proceed correctly in the absence of specific TMDs and thereby impedes fusion pore opening. Interestingly the increase in local SNARE TMD concentration at the fusion site changes the tilt of the TMD [235, 236]. An increase in its perpendicular insertion is further promoted by cholesterol in model membranes [218]. Such a change in insertion angle may provide not only adaptation to different membrane thickness but also increase the curvature in conjunction with a direct action of cholesterol on membrane bending (see Fig 2 and 6).

The relative mobility between lipids and TMDs plays a major role. Functional assays and *in silico* predictions of TMD mobility provided important insight after replacing “en-bloc” native VAMP2 TMD (including the G₁₀₀ glycine kink [242]) or by either multiple C_β-branched isoleucines (Poly-Ile) or by multiple leucines (Poly-Leu). Flexibility of TMDs in membranes was clearly linked to fusion pore opening and expansion: whereas increased TMD mobility favours fusion pore expansion, a decrease in flexibility reduces the number of fusion events, and slows pore opening and expansion [246]. In fact, just replacing two small residues that may provide TMD mobility around the G₁₀₀ glycine kink [242] had similar effects on fusion pore opening and stability [234]. These mutations impede structural mobility of the TMD, interfere with the α -helix to β -sheet transitions, and irreversibly increase membrane viscosity upon an increase in local TMD concentration. The importance of lateral movements of the TMD is also compatible with observations on VAMP2 TMD mutations introducing additional charged residues at the extreme C-terminus and thus impeding its movement [275].

These observations on lipid-protein interactions also raise the question as to whether SNARE TMDs exert an active, eventually catalytic role [246]? Initial models of hemifusion stalk formation contained (highly unlikely) void interstices between the membrane leaflets, a conformation that could only be overcome by the assumption of locally highly disordered acyl chains [159]. Interestingly the fusogenicity of VAMP2-derived TMD model peptides [233, 352] correlates with increased acyl chain disorder [353, 354]. More importantly, an increased local concentration of VAMP2 TMDs, as expected to occur during hemifusion and pore opening, promotes disordered arrangement of the acyl chains without disturbing headgroups [236]. This argues that the physicochemical nature of the VAMP TMD actively influences the lipid phase. Collectively, these observations provide evidence that the TMD exerts a critical and catalytic role in membrane fusion by altering membrane viscosity and peptide-lipid interactions that influence the establishment and expansion of the fusion pore [234]. The TMD dynamics may be arranged around G₁₀₀, located in the centre of the bilayer, which could also permit relative movement between the two leaflets during fusion.

5. SNAREs, Lipids and Fusion Pore in Diabetes

5.1. Lipids, Ion Channels and Second Messengers

There is a strong correlation between obesity and T2D risk [355, 356] and elevated plasma levels of free fatty acids [357]. Early work incubating cell lines with different fatty acids revealed an inhibitory effect on exocytosis [312, 358]. Similarly, chronic incubation of human and mice islets with palmitate inhibited glucose-induced insulin secretion (GIIS) by dispersion of the Ca^{2+} channel aggregates close to release-competent secretory granules [38, 359, 360].

The dyslipidaemia associated with T2D can be also accompanied with a low level of HDL-cholesterol [361]. These observations prompted studies in which islets or clonal β -cells were cultured in the presence of extreme levels of cholesterol (addition or depletion). Accumulation of cholesterol was found to inhibit GIIS either by affecting glucose metabolism [362], trafficking [363] or actin organisation [364]. Rescue by cholesterol depletion using methyl- β -cyclodextrin (M β CD) restored insulin secretion [362]. However, when M β CD was used on its own, it also impaired insulin secretion by modifying SNAP25 localization [131]. A genetic model of cholesterol distribution offers another way to address its role. The efflux of cellular cholesterol and phospholipids involves ABCA1. ABCA1-KO mice accumulate cholesterol and exhibit impaired GIIS [365] that correlated with reduced depolarization-evoked Ca^{2+} entry and exocytosis [366]. On the other hand, decreasing endogenous cholesterol by squalene epoxidase inhibitors, which exert a milder effect than M β CD, affects exocytosis independent of Ca^{2+} entry [367]. Recently, accumulation of cholesterol modulated also the proportion of kiss-and-run and full fusion exocytosis (more kiss-and-run) [368]. These studies emphasize the central role of cholesterol in membrane dynamics, organisation, and the critical cholesterol set point that support membrane function. However, a caveat of the experimental protocols used to date is that they produce increases in membrane cholesterol that are far larger than those encountered *in vivo* (even in the diseased state).

Ca^{2+} entry via voltage-gated Ca^{2+} -channels triggers exocytosis [3, 369] There is evidence that GIIS requires the close proximity of the Ca^{2+} channel clusters with the secretory granules. As described in the preceding section, dyslipidaemia reduces GIIS by the disassembly of these Ca^{2+} -channel/granule aggregates [359]. Very recent evidence suggests that the Ca^{2+} -channels are recruited to docked granules with pre-formed SNARE complexes and that this process is perturbed in β -cells from T2D donors [38]. Similar aggregates have also been described for $\text{K}_v2.1$ voltage-gated K^+ channels and overexpression of these channels rescues reduced insulin exocytosis from T2D β -cells

[92]. Thus, clustering of ion channels in the plasma membrane appears essential for proper exocytosis in the healthy β -cell.

Fusion pore kinetics is regulated by Ca^{2+} [132-135] and cAMP [192, 194, 370]. Both signalling pathways are activated by glucose metabolism [17, 18, 371] and affected by long-term exposure to high glucose [372-375], a situation that emulates the increase in plasma glucose seen in T2D. Glucose metabolism also regulates sumoylation, which may control levels and function of exocytotic proteins [376-379]. As sumoylation is regulated by the redox potential and given that T2D is associated with disturbed glucose metabolism, this provides a potential link between diabetes and impaired insulin exocytosis [380].

5.2. SNARE proteins, GWAS and Islets

The limited access to human islets in the past, especially to those from diabetic patients, has prompted the development of cellular models related to T2D. Chronic exposure to elevated glucose levels *in vitro* (glucotoxicity) induces a number of biochemical alterations resulting in loss of glucose stimulated insulin secretion [381-383]. Using this model within an acceptable glucose concentration range (5-20 mM), a decreased expression of VAMP2 and (to a lesser degree) syntaxin 1 along with an increased expression of SNAP-25 were observed in INS-1 cells and in human islets that correlated with reduced Ca^{2+} -induced exocytosis in permeabilised cells [372]. VAMP2 downregulation by elevated glucose was also reported for mouse islets [384], rat islets from Wistar or non-obese diabetic Goto-Kakizaki (GK) rats [385, 386] and MKR mouse, a diabetes model induced by insulin resistance [387]. Interestingly expression of one SNAP25 isoform, SNAP25b, was required for physiological $[\text{Ca}^{2+}]_i$ oscillations in mouse β -cells, which may point to a role in transport of components required for physiological islet cell coupling and signalling [388]. In humans, considerable downregulation of all three SNARE proteins in diabetic islets was apparent in one study [389], whereas other studies reported only a very modest reduction in syntaxin 1 without alterations in VAMP2 [123] or only in the TGN synaptotagmin Syt4 [390]. As access to diabetic human islets is still limited and these data were based on a small number of patients, it remains to be conclusively established that the expression of SNARE proteins is reduced in T2D.

T2D is a complex disease involving the interplay of both genetic and environmental factors (including lifestyle, diet and age). The advent of Genome Wide Association Studies (GWASs) proposed an unbiased genetic approach based on the whole genome sequence [391, 392]. It is based on the genotyping of Linkage Disequilibrium (LD) blocks (parts of the genome that are always

inherited together) and captures all common single nucleotide polymorphisms (SNP) in different loci across the whole genome. Whereas this approach is extremely powerful for SNP mapping, linking a given SNP with the causal transcript in the same LD block remains challenging [393]. Each locus encompasses coding and non-coding regions and each non-coding variant can influence the expression of the neighbouring genes [394-396].

More than 100 loci show statistically significant association with T2D susceptibility [397], with most of these proposed to elicit their affect via altered β -cell function [398-400]. However, the majority of the genetic variants associated with T2D risks are non-coding and common. When taken individually, they have a modest impact on T2D risk [397, 401]. Whereas it seems established that T2D and exocytosis can be associated [389, 402, 403], the molecular mechanism through which they influence hormone release and specially the fusion pore remains poorly described. The non-coding variant CDKAL1 (rs7756992) is associated with a 50% increase in T2D risk [400] and increased expression of the transcription factor Sox4 [395]. Increase in Sox4 expression correlated with a reduced insulin secretion in mouse and human β -cells, an effect that was attributed to a dramatic 5-fold increase (from 10% to 50%) in kiss-and-run exocytosis at the expense of full fusion [40]. Electron microscopy performed on the islets incubated with Ruthenium red also confirmed the increase in kiss-and-run events: membrane labelling was observed in some vesicles that retained a central insulin core. The occurrence of such granules suggests that the fusion pore was open long enough for ruthenium to label the granular membrane but not expanding sufficiently to allow the exit of insulin (Figure 3 A, B arrowheads).

The effect of Sox4 overexpression on exocytosis is mediated by an increase in STXBP6/amisyn expression, which stabilizes the fusion pore and prevents its expansion. The precise mechanism by which amisyn acts on the fusion pore has not been clarified yet. However, some hypotheses can be drawn among which, due to its structure with R-SNARE-like coiled-coil domain, amisyn could compete in the formation of the SNARE complex and prevent the zippering of the SNARE complex [196]. This may in itself restrict fusion pore expansion but it will also result in a lower TMD concentration at the fusion sites that in turn (as discussed above) affects membrane viscosity consequential effect on fusion pore establishment and expansion.

6. Conclusions

Although our knowledge about membrane fusion has made very significant progress during the last 20 years, our understanding of the complex interaction between different lipid species and

transmembrane domains remains rudimentary. A number of questions are still open and it remains to define which mechanisms are universal throughout the secretory pathway and which represent 'variations on the theme' to allow adaptation to specific biochemical, geometric and time constraints. Another layer of complexity is added by the intertwined traffic and fusion/fission events occurring in the post-Golgi compartments and here we lack a high-quality view of the distribution gradient of proteins, not to speak about lipid species. It has also become evident recently that the fusion pore opening requires a complex environment, it is highly regulated and can lead to very diverse outcomes as well as differential release of compounds. It is tempting to describe the fusion pore as the Cerberus of exocytosis, guarding the exit of the cargo (including insulin) with his three heads: membrane viscosity, structural dynamics and lateral tension. Subtle mechanical alterations at this crucial event may have great impact on the amounts of cargo released and may (as we have discussed) contribute to the secretory deficiency in T2D.

REFERENCES

- [1] F.M. Ashcroft, P. Rorsman, Diabetes mellitus and the beta cell: the last ten years, *Cell*, 148 (2012) 1160-1171.
- [2] B. Thorens, Glucose sensing and the pathogenesis of obesity and type 2 diabetes, *Int. J. Obesity* (2005), 32 Suppl 6 (2008) S62-71.
- [3] P. Rorsman, M. Braun, Regulation of insulin secretion in human pancreatic islets, *Annu. Rev. Physiol.*, 75 (2013) 155-179.
- [4] M. Soty, A. Gautier-Stein, F. Rajas, G. Mithieux, Gut-Brain Glucose Signaling in Energy Homeostasis, *Cell Metab.*, 25 (2017) 1231-1242.
- [5] R.A. DeFronzo, E. Ferrannini, L. Groop, R.R. Henry, W.H. Herman, J.J. Holst, F.B. Hu, C.R. Kahn, I. Raz, G.I. Shulman, D.C. Simonson, M.A. Testa, R. Weiss, Type 2 diabetes mellitus, *Nature Rev. Disease primers*, 1 (2015) 15019.
- [6] R.A. DeFronzo, Pathogenesis of type 2 diabetes mellitus, *International Textbook of Diabetes Mellitus*, John Wiley & Sons, Ltd 2015, pp. 371-400.
- [7] A.M. Moe, A.E. Golding, W.M. Bement, Cell healing: Calcium, repair and regeneration, *Sem. Cell Dev. Biol.*, 45 (2015) 18-23.
- [8] B. Wu, W. Guo, The Exocyst at a Glance, *J. Cell Sci.*, 128 (2015) 2957-2964.
- [9] B. Hausott, L. Klimaschewski, Membrane turnover and receptor trafficking in regenerating axons, *Eur. J. Neurosci.*, 43 (2016) 309-317.
- [10] W.W. Douglas, Stimulus-secretion coupling: the concept and clues from chromaffin and other cells, *Br. J. Pharmacol.*, 34 (1968) 451-474.
- [11] O. Grynspan-Winograd, Morphological aspects of exocytosis in the adrenal medulla, *Philos. Trans. R. Soc. Lond., B, Biol. Sci.*, 261 (1971) 291-292.
- [12] J.E. Heuser, T.S. Reese, M.J. Dennis, Y. Jan, L. Jan, L. Evans, Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release, *J. Cell Biol.*, 81 (1979) 275-300.
- [13] J.E. Rothman, The principle of membrane fusion in the cell (Nobel lecture), *Angew. Chem. Int. Ed.*, 53 (2014) 12676-12694.
- [14] T.C. Sudhof, The molecular machinery of neurotransmitter release (Nobel lecture), *Angew. Chem. Int. Ed.*, 53 (2014) 12696-12717.

723 [15] R. Jahn, D. Fasshauer, Molecular machines governing exocytosis of synaptic vesicles, *Nature*,
724 490 (2012) 201-207.

725 [16] P.V. Roder, X. Wong, W. Hong, W. Han, Molecular regulation of insulin granule biogenesis and
726 exocytosis, *Biochem. J.*, 473 (2016) 2737-2756.

727 [17] P.E. MacDonald, J.W. Joseph, P. Rorsman, Glucose-sensing mechanisms in pancreatic beta-cells,
728 *Philos. Trans. R. Soc. Lond., B, Biol. Sci.*, 360 (2005) 2211-2225.

729 [18] M.A. Charles, R. Fanska, F.G. Schmid, P.H. Forsham, G.M. Grodsky, Adenosine 3',5'-
730 monophosphate in pancreatic islets: glucose-induced insulin release, *Science*, 179 (1973) 569-
731 571.

732 [19] F. Schwede, O.G. Chepurny, M. Kaufholz, D. Bertinetti, C.A. Leech, O. Cabrera, Y. Zhu, F. Mei, X.
733 Cheng, J.E. Manning Fox, P.E. MacDonald, H.-G. Genieser, F.W. Herberg, et al., Rp-cAMPS
734 prodrugs reveal the cAMP dependence of first-phase glucose-stimulated insulin secretion, *Mol.*
735 *Endocrinol.*, 29 (2015) 988-1005.

736 [20] L.E. Fridlyand, L.H. Philipson, Coupling of metabolic, second messenger pathways and insulin
737 granule dynamics in pancreatic beta-cells: a computational analysis, *Progr. Biophys. Mol. Biol.*,
738 107 (2011) 293-303.

739 [21] M. Prentki, Franz M. Matschinsky, S.R.M. Madiraju, Metabolic signaling in fuel-induced insulin
740 secretion, *Cell Metab.*, 18 (2013) 162-185.

741 [22] J.S. McTaggart, R.H. Clark, F.M. Ashcroft, SYMPOSIUM REVIEW: The role of the K_{ATP} channel in
742 glucose homeostasis in health and disease: more than meets the islet, *J. Physiol.*, 588 (2010)
743 3201-3209.

744 [23] D.L. Curry, L.L. Bennett, G.M. Grodsky, Dynamics of insulin secretion by the perfused rat
745 pancreas, *Endocrinology*, 83 (1968) 572-584.

746 [24] R. Nesher, E. Cerasi, Modeling phasic insulin release: immediate and time-dependent effects of
747 glucose, *Diabetes*, 51 Suppl 1 (2002) S53-59.

748 [25] G. Grodsky, H. Landahl, D. Curry, L. Bennett, A two-compartmental model for insulin secretion,
749 *Adv. Metab. Disord.*, 1 (1970) Suppl 1:45-50.

750 [26] P. Rorsman, E. Renstrom, Insulin granule dynamics in pancreatic beta cells, *Diabetologia*, 46
751 (2003) 1029-1045.

752 [27] J.C. Henquin, Regulation of insulin secretion: a matter of phase control and amplitude
753 modulation, *Diabetologia*, 52 (2009) 739-751.

754 [28] C. Dalla Man, R.A. Rizza, C. Cobelli, Meal simulation model of the glucose-insulin system, IEEE
755 Trans. Biomed. Eng., 54 (2007) 1740-1749.

756 [29] R.S. Sherwin, K.J. Kramer, J.D. Tobin, P.A. Insel, J.E. Liljenquist, M. Berman, R. Andres, A model of
757 the kinetics of insulin in man, J. Clin. Invest., 53 (1974) 1481-1492.

758 [30] C. Bommer, E. Heesemann, V. Sagalova, J. Manne-Goehler, R. Atun, T. Bärnighausen, S. Vollmer,
759 The global economic burden of diabetes in adults aged 20–79 years: a cost-of-illness study, The
760 Lancet Diabetes & Endocrinology, 5 (2017) 423-430.

761 [31] C. Weyer, C. Bogardus, D.M. Mott, R.E. Pratley, The natural history of insulin secretory
762 dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus, J. Clin.
763 Invest., 104 (1999) 787-794.

764 [32] V. Lyssenko, P. Almgren, D. Anevski, R. Perfekt, K. Lahti, M. Nissen, B. Isomaa, B. Forsen, N.
765 Homstrom, C. Saloranta, M.R. Taskinen, L. Groop, T. Tuomi, Predictors of and longitudinal
766 changes in insulin sensitivity and secretion preceding onset of type 2 diabetes, Diabetes, 54
767 (2005) 166-174.

768 [33] S.E. Kahn, M.E. Cooper, S. Del Prato, Pathophysiology and treatment of type 2 diabetes:
769 perspectives on the past, present, and future, Lancet, 383 (2014) 1068-1083.

770 [34] D. Yabe, Y. Seino, M. Fukushima, S. Seino, beta cell dysfunction versus insulin resistance in the
771 pathogenesis of type 2 diabetes in East Asians, Curr. Diab. Rep., 15 (2015) 602.

772 [35] L. Marselli, M. Suleiman, M. Masini, D. Campani, M. Bugliani, F. Syed, L. Martino, D. Focosi, F.
773 Scatena, F. Olimpico, F. Filipponi, P. Masiello, U. Boggi, et al., Are we overestimating the loss of
774 beta cells in type 2 diabetes?, Diabetologia, 57 (2014) 362-365.

775 [36] M.F. Brereton, M. Rohm, K. Shimomura, C. Holland, S. Tornovsky-Babeay, D. Dadon, M. Iberl,
776 M.V. Chibalina, S. Lee, B. Glaser, Y. Dor, P. Rorsman, A. Clark, et al., Hyperglycaemia induces
777 metabolic dysfunction and glycogen accumulation in pancreatic β -cells, Nature
778 Communications, 7 (2016) 13496.

779 [37] A.H. Rosengren, M. Braun, T. Mahdi, S.A. Andersson, M.E. Travers, M. Shigeto, E. Zhang, P.
780 Almgren, C. Ladenvall, A.S. Axelsson, A. Edlund, M.G. Pedersen, A. Jonsson, et al., Reduced
781 insulin exocytosis in human pancreatic beta-cells with gene variants linked to type 2 diabetes,
782 Diabetes, 61 (2012) 1726-1733.

783 [38] N.R. Gandasi, P. Yin, M. Riz, M.V. Chibalina, G. Cortese, P.E. Lund, V. Matveev, P. Rorsman, A.
784 Sherman, M.G. Pedersen, S. Barg, Ca^{2+} channel clustering with insulin-containing granules is
785 disturbed in type 2 diabetes, J. Clin. Invest., (2017).

786 [39] G. Cortese, N.R. Gandasi, S. Barg, M.G. Pedersen, Statistical frailty modeling for quantitative
787 analysis of exocytotic events recorded by live cell imaging: Rapid release of insulin-containing
788 granules is impaired in human diabetic beta-cells, *PLoS One*, 11 (2016) e0167282.

789 [40] S.C. Collins, H.W. Do, B. Hastoy, A. Hugill, J. Adam, M.V. Chibalina, J. Galvanovskis, M. Godazgar,
790 S. Lee, M. Goldsworthy, A. Salehi, A.I. Tarasov, A.H. Rosengren, et al., Increased expression of
791 the diabetes gene SOX4 reduces insulin secretion by impaired fusion pore expansion, *Diabetes*,
792 65 (2016) 1952-1961.

793 [41] A.E. Butler, J. Janson, S. Bonner-Weir, R. Ritzel, R.A. Rizza, P.C. Butler, Beta-cell deficit and
794 increased beta-cell apoptosis in humans with type 2 diabetes, *Diabetes*, 52 (2003) 102-110.

795 [42] J. Rahier, Y. Guiot, R.M. Goebbels, C. Sempoux, J.C. Henquin, Pancreatic beta-cell mass in
796 European subjects with type 2 diabetes, *Diabetes Obes. Metab.*, 10 Suppl 4 (2008) 32-42.

797 [43] P. Marchetti, E. Ferrannini, β -Cell mass and function in human type 2 diabetes, *International*
798 *Textbook of Diabetes Mellitus*, John Wiley & Sons, Ltd 2015, pp. 354-370.

799 [44] J.C. Hou, L. Min, J.E. Pessin, Chapter 16: Insulin granule biogenesis, trafficking and exocytosis,
800 *Vit. Horm.*, Academic Press 2009, pp. 473-506.

801 [45] T. Sollner, M.K. Bennett, S.W. Whiteheart, R.H. Scheller, J.E. Rothman, A protein assembly-
802 disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle
803 docking, activation, and fusion, *Cell*, 75 (1993) 409-418.

804 [46] M. Verhage, J.B. Sorensen, Vesicle docking in regulated exocytosis, *Traffic*, 9 (2008) 1414-1424.

805 [47] J. Lang, Molecular mechanisms and regulation of insulin exocytosis as a paradigm of endocrine
806 secretion, *Eur. J. Biochem.*, 259 (1999) 3-17.

807 [48] S. Barg, A. Lindqvist, S. Obermuller, Granule docking and cargo release in pancreatic beta-cells,
808 *Biochem. Soc. Trans.*, 36 (2008) 294-299.

809 [49] L. Eliasson, E. Renstrom, W.G. Ding, P. Proks, P. Rorsman, Rapid ATP-dependent priming of
810 secretory granules precedes Ca^{2+} -induced exocytosis in mouse pancreatic B-cells, *J. Physiol.*, 503
811 (Pt 2) (1997) 399-412.

812 [50] T.F. Martin, Role of $\text{PI}_{4,5}\text{P}_2$ in vesicle exocytosis and membrane fusion, *Sub-cell. Biochem.*, 59
813 (2012) 111-130.

814 [51] P.I. Kuzmin, J. Zimmerberg, Y.A. Chizmadzhev, F.S. Cohen, A quantitative model for membrane
815 fusion based on low-energy intermediates, *Proc. Nat'l Acad. of Sci. USA*, 98 (2001) 7235-7240.

816 [52] M. Molinete, J.C. Irminger, S.A. Tooze, P.A. Halban, Trafficking/sorting and granule biogenesis in
817 the beta-cell, *Semin. Cell Dev. Biol.*, 11 (2000) 243-251.

818 [53] J.C. Hou, L. Min, J.E. Pessin, Insulin granule biogenesis, trafficking and exocytosis, *Vitam. Horm.*,
819 80 (2009) 473-506.

820 [54] L. Orci, M. Ravazzola, M.J. Storch, R.G. Anderson, J.D. Vassalli, A. Perrelet, Proteolytic
821 maturation of insulin is a post-Golgi event which occurs in acidifying clathrin-coated secretory
822 vesicles, *Cell*, 49 (1987) 865-868.

823 [55] E. Fava, J. Dehghany, J. Ouwendijk, A. Muller, A. Niederlein, P. Verkade, M. Meyer-Hermann, M.
824 Solimena, Novel standards in the measurement of rat insulin granules combining electron
825 microscopy, high-content image analysis and in silico modelling, *Diabetologia*, 55 (2012) 1013-
826 1023.

827 [56] A.B. Noske, B.J. Marsh, Mapping the β -Cell in 3D at the Nanoscale Using Novel Cellular Electron
828 Tomography and Computational Approaches, in: B. Booß-Bavnbek, B. Klösigen, J. Larsen, F.
829 Pociot, E. Renström (Eds.) *BetaSys: Systems biology of regulated exocytosis in pancreatic β -cells*,
830 Springer New York, New York, NY, 2011, pp. 147-183.

831 [57] A.B. Noske, A.J. Costin, G.P. Morgan, B.J. Marsh, Expedited approaches to whole cell electron
832 tomography and organelle mark-up in situ in high-pressure frozen pancreatic islets, *J.*
833 *Struct.Biol.*, 161 (2008) 298-313.

834 [58] F. Wendler, L. Page, S. Urbe, S.A. Tooze, Homotypic fusion of immature secretory granules
835 during maturation requires syntaxin 6, *Mol. Biol. Cell*, 12 (2001) 1699-1709.

836 [59] M. Steegmaier, J. Klumperman, D.L. Foletti, J.S. Yoo, R.H. Scheller, Vesicle-associated membrane
837 protein 4 is implicated in trans-Golgi network vesicle trafficking, *Mol. Biol. Cell*, 10 (1999) 1957-
838 1972.

839 [60] M. Ahras, G.P. Otto, S.A. Tooze, Synaptotagmin IV is necessary for the maturation of secretory
840 granules in PC12 cells, *J. Cell Biol.*, 173 (2006) 241-251.

841 [61] W. Du, M. Zhou, W. Zhao, D. Cheng, L. Wang, J. Lu, E. Song, W. Feng, Y. Xue, P. Xu, T. Xu, HID-1 is
842 required for homotypic fusion of immature secretory granules during maturation, *eLife*, 5
843 (2016).

844 [62] A. Shomorony, C.R. Pfeifer, M.A. Aronova, G. Zhang, T. Cai, H. Xu, A.L. Notkins, R.D. Leapman,
845 Combining quantitative 2D and 3D image analysis in the serial block face SEM: application to
846 secretory organelles of pancreatic islet cells, *J. Microsc.*, 259 (2015) 155-164.

847 [63] J. Dehghany, P. Hoboth, A. Ivanova, H. Mziaut, A. Muller, Y. Kalaidzidis, M. Solimena, M. Meyer-
848 Hermann, A spatial model of insulin-granule dynamics in pancreatic beta-cells, *Traffic*, 16 (2015)
849 797-813.

850 [64] E. Neher, R.S. Zucker, Multiple calcium-dependent processes related to secretion in bovine
851 chromaffin cells, *Neuron*, 10 (1993) 21-30.

852 [65] J. Gromada, M. Hoy, E. Renstrom, K. Bokvist, L. Eliasson, S. Gopel, P. Rorsman, CaM kinase II-
853 dependent mobilization of secretory granules underlies acetylcholine-induced stimulation of
854 exocytosis in mouse pancreatic B-cells, *J. Physiol.*, 518 (Pt 3) (1999) 745-759.

855 [66] V. Malhotra, L. Orci, B.S. Glick, M.R. Block, J.E. Rothman, Role of an N-ethylmaleimide-sensitive
856 transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack,
857 *Cell*, 54 (1988) 221-227.

858 [67] T. Sollner, S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, J.E.
859 Rothman, SNAP receptors implicated in vesicle targeting and fusion, *Nature*, 362 (1993) 318-
860 324.

861 [68] W. Wickner, R. Schekman, Membrane fusion, *Nat. Struct. Mol. Biol.*, 15 (2008) 658-664.

862 [69] D. Fasshauer, R.B. Sutton, A.T. Brunger, R. Jahn, Conserved structural features of the synaptic
863 fusion complex: SNARE proteins reclassified as Q- and R-SNAREs, *Proc. Nat'l Acad. Sci. U S A*, 95
864 (1998) 15781-15786.

865 [70] M.A. Poirier, W. Xiao, J.C. Macosko, C. Chan, Y.K. Shin, M.K. Bennett, The synaptic SNARE
866 complex is a parallel four-stranded helical bundle, *Nat.Struct. Biol.*, 5 (1998) 765-769.

867 [71] X. Lou, Y.-K. Shin, SNARE zippering, *Biosci. Rep.*, 36 (2016).

868 [72] J. Rizo, J. Xu, The Synaptic Vesicle Release Machinery, *Annu. Rev. Biophys.*, 44 (2015) 339-367.

869 [73] T. Trimbuch, C. Rosenmund, Should I stop or should I go? The role of complexin in
870 neurotransmitter release, *Nat. Rev. Neurosci.*, 17 (2016) 118-125.

871 [74] M. Camacho, J. Basu, T. Trimbuch, S. Chang, C. Pulido-Lozano, S.-S. Chang, I. Duluvova, M. Abo-
872 Rady, J. Rizo, C. Rosenmund, Heterodimerization of Munc13 C2A domain with RIM regulates
873 synaptic vesicle docking and priming, *Nat. Comm.*, 8 (2017) 15293.

874 [75] G.J. Evans, A. Morgan, R.D. Burgoyne, Tying everything together: the multiple roles of cysteine
875 string protein (CSP) in regulated exocytosis, *Traffic*, 4 (2003) 653-659.

876 [76] R. Regazzi, C.B. Wollheim, J. Lang, J.M. Theler, O. Rossetto, C. Montecucco, K. Sadoul, U. Weller,
877 M. Palmer, B. Thorens, VAMP-2 and cellubrevin are expressed in pancreatic beta-cells and are
878 essential for Ca^{2+} -but not for $\text{GT}\gamma\text{S}$ -induced insulin secretion, *EMBO J.*, 14 (1995) 2723-2730.

879 [77] K. Sadoul, J. Lang, C. Montecucco, U. Weller, R. Regazzi, S. Catsicas, C.B. Wollheim, P.A. Halban,
880 SNAP-25 is expressed in islets of Langerhans and is involved in insulin release, *J. Cell Biol.*, 128
881 (1995) 1019-1028.

882 [78] C.E. Kiraly-Borri, A. Morgan, R.D. Burgoyne, U. Weller, C.B. Wollheim, J. Lang, Soluble N-
883 ethylmaleimide-sensitive-factor attachment protein and N-ethylmaleimide-insensitive factors
884 are required for Ca^{2+} -stimulated exocytosis of insulin, *Biochem. J.*, 314 (1996) 199-203.

885 [79] J. Lang, H. Zhang, V.V. Vaidyanathan, K. Sadoul, H. Niemann, C.B. Wollheim, Transient
886 expression of botulinum neurotoxin C1 light chain differentially inhibits calcium and glucose
887 induced insulin secretion in clonal beta-cells, *FEBS Lett.*, 419 (1997) 13-17.

888 [80] G. Jacobsson, A.J. Bean, R.H. Scheller, L. Juntti-Berggren, J.T. Deeney, P.O. Berggren, B. Meister,
889 Identification of synaptic proteins and their isoform mRNAs in compartments of pancreatic
890 endocrine cells, *Proc. Nat'l Acad. Sci. U S A*, 91 (1994) 12487-12491.

891 [81] M.B. Wheeler, L. Sheu, M. Ghai, A. Bouquillon, G. Grondin, U. Weller, A.R. Beaudoin, M.K.
892 Bennett, W.S. Trimble, H.Y. Gaisano, Characterization of SNARE protein expression in beta cell
893 lines and pancreatic islets, *Endocrinology*, 137 (1996) 1340-1348.

894 [82] W. Zhang, A. Efanov, S.N. Yang, G. Fried, S. Kolare, H. Brown, S. Zaitsev, P.O. Berggren, B.
895 Meister, Munc-18 associates with syntaxin and serves as a negative regulator of exocytosis in
896 the pancreatic beta-cell, *J. Biol. Chem.*, 275 (2000) 41521-41527.

897 [83] H. Zhang, W.L. Kelley, L.H. Chamberlain, R.D. Burgoyne, J. Lang, Mutational analysis of cysteine-
898 string protein function in insulin exocytosis, *J. Cell Sci.*, 112 (1999) 1345-1351.

899 [84] F. Boal, M. Laguerre, A. Milochau, J. Lang, P.A. Scotti, A charged prominence in the linker
900 domain of the cysteine-string protein Csp α mediates its regulated interaction with the
901 calcium sensor synaptotagmin 9 during exocytosis, *FASEB J.*, 25 (2011) 132-143.

902 [85] X. Jing, D.-Q. Li, C.S. Olofsson, A. Salehi, V.V. Surve, J. Caballero, R. Ivarsson, I. Lundquist, A.
903 Pereverzev, T. Schneider, P. Rorsman, E. Renström, CaV2.3 calcium channels control second-
904 phase insulin release, *J. Clin. Invest.*, 115 (2005) 146-154.

905 [86] J. Ji, S.N. Yang, X. Huang, X. Li, L. Sheu, N. Diamant, P.O. Berggren, H.Y. Gaisano, Modulation of
906 L-type Ca^{2+} channels by distinct domains within SNAP-25, *Diabetes*, 51 (2002) 1425-1436.

907 [87] S.N. Yang, O. Larsson, R. Branstrom, A.M. Bertorello, B. Leibiger, I.B. Leibiger, T. Moede, M.
908 Kohler, B. Meister, P.O. Berggren, Syntaxin 1 interacts with the L_D subtype of voltage-gated Ca²⁺
909 channels in pancreatic beta cells, *Proc. Nat'l Acad. Sci. U S A*, 96 (1999) 10164-10169.

910 [88] O. Wiser, M. Trus, A. Hernandez, E. Renstrom, S. Barg, P. Rorsman, D. Atlas, The voltage
911 sensitive L_c-type Ca²⁺ channel is functionally coupled to the exocytotic machinery, *Proc. Nat'l*
912 *Acad. Sci. U S A*, 96 (1999) 248-253.

913 [89] W.A. Catterall, Signaling complexes of voltage-gated sodium and calcium channels,
914 *Neuroscience Letters*, 486 (2010) 107-116.

915 [90] Y.M. Leung, Y. Kang, X. Gao, F. Xia, H. Xie, L. Sheu, S. Tsuk, I. Lotan, R.G. Tsushima, H.Y. Gaisano,
916 Syntaxin 1A binds to the cytoplasmic C terminus of K_v2.1 to regulate channel gating and
917 trafficking, *J. Biol. Chem.*, 278 (2003) 17532-17538.

918 [91] Y.M. Leung, E.P. Kwan, B. Ng, Y. Kang, H.Y. Gaisano, SNAREing voltage-gated K⁺ and ATP-
919 sensitive K⁺ channels: tuning beta-cell excitability with syntaxin-1A and other exocytotic
920 proteins, *Endocr. Rev.*, 28 (2007) 653-663.

921 [92] J. Fu, X. Dai, G. Plummer, K. Suzuki, A. Bautista, J.M. Githaka, L. Senior, M. Jensen, D. Greitzer-
922 Antes, J.E. Manning Fox, H.Y. Gaisano, C.B. Newgard, N. Touret, et al., Kv2.1 Clustering
923 Contributes to Insulin Exocytosis and Rescues Human beta-Cell Dysfunction, *Diabetes*, (2017).

924 [93] L. Xie, S. Dolai, Y. Kang, T. Liang, H. Xie, T. Qin, L. Yang, L. Chen, H.Y. Gaisano, Syntaxin-3 binds
925 and regulates both r- and l-type calcium channels in insulin-secreting INS-1 832/13 cells, *PLoS*
926 *One*, 11 (2016) e0147862.

927 [94] H.Y. Gaisano, Here come the newcomer granules, better late than never, *Trends Endocrinol.*
928 *Metab.*, 25 (2014) 381-388.

929 [95] D. Zhu, Y. Zhang, P.P. Lam, S. Dolai, Y. Liu, E.P. Cai, D. Choi, S.A. Schroer, Y. Kang, E.M. Allister, T.
930 Qin, M.B. Wheeler, C.C. Wang, et al., Dual role of VAMP8 in regulating insulin exocytosis and
931 islet beta cell growth, *Cell Metab.*, 16 (2012) 238-249.

932 [96] L. Xie, D. Zhu, S. Dolai, T. Liang, T. Qin, Y. Kang, H. Xie, Y.C. Huang, H.Y. Gaisano, Syntaxin-4
933 mediates exocytosis of pre-docked and newcomer insulin granules underlying biphasic glucose-
934 stimulated insulin secretion in human pancreatic beta cells, *Diabetologia*, 58 (2015) 1250-1259.

935 [97] S. Nagamatsu, H. Sawa, Y. Nakamichi, Y. Kondo, S. Matsushima, T. Watanabe, Non-functional
936 role of syntaxin 2 in insulin exocytosis by pancreatic beta cells, *Cell. Biochem. Funct.*, 15 (1997)
937 237-242.

938 [98] M. Ohara-Imaizumi, T. Fujiwara, Y. Nakamichi, T. Okamura, Y. Akimoto, J. Kawai, S. Matsushima,
939 H. Kawakami, T. Watanabe, K. Akagawa, S. Nagamatsu, Imaging analysis reveals mechanistic
940 differences between first- and second-phase insulin exocytosis, *J. Cell Biol.*, 177 (2007) 695-705.

941 [99] M. Raoux, E. Bertin, A. Pirog, R. Perrier, D. Bosco, B. Catargi, D. Cattaert, S. Renaud, J. Lang,
942 Biphasic electric coupling of islets determines secretion profile, *Diabetologia*, 59 (2016) P 419.

943 [100] H. Zong, C.-C. Wang, B. Vaitheesvaran, I.J. Kurland, W. Hong, J.E. Pessin, Enhanced energy
944 expenditure, glucose utilization, and insulin sensitivity in *vamp8* null mice, *Diabetes*, 60 (2011)
945 30-38.

946 [101] S. Nagamatsu, Y. Nakamichi, T. Watanabe, S. Matsushima, S. Yamaguchi, J. Ni, E. Itagaki, H.
947 Ishida, Localization of cellubrevin-related peptide, endobrevin, in the early endosome in
948 pancreatic beta cells and its physiological function in exo-endocytosis of secretory granules, *J.*
949 *Cell Sci.*, 114 (2001) 219-227.

950 [102] Y. Brunner, Y. Couté, M. Iezzi, M. Foti, M. Fukuda, D.F. Hochstrasser, C.B. Wollheim, J.-C.
951 Sanchez, Proteomics analysis of insulin secretory granules, *Mol. Cell. Prot.*, 6 (2007) 1007-1017.

952 [103] E. Itakura, C. Kishi-Itakura, N. Mizushima, The hairpin-type tail-anchored snare syntaxin 17
953 targets to autophagosomes for fusion with endosomes/lysosomes, *Cell*, 151 (2012) 1256-1269.

954 [104] M.R. Marshall, V. Pattu, M. Halimani, M. Maier-Peuschel, M.L. Muller, U. Becherer, W. Hong,
955 M. Hoth, T. Tschernig, Y.T. Bryceson, J. Rettig, VAMP8-dependent fusion of recycling endosomes
956 with the plasma membrane facilitates T lymphocyte cytotoxicity, *J. Cell Biol.*, 210 (2015) 135-
957 151.

958 [105] T. Kimura, J. Jia, A. Claude-Taupin, S. Kumar, S.W. Choi, Y. Gu, M. Mudd, N. Dupont, S. Jiang, R.
959 Peters, F. Farzam, A. Jain, K.A. Lidke, et al., Cellular and molecular mechanism for secretory
960 autophagy, *Autophagy*, 13 (2017) 1084-1085.

961 [106] F. Fan, C. Ji, Y. Wu, S.M. Ferguson, N. Tamarina, L.H. Philipson, X. Lou, Dynamin 2 regulates
962 biphasic insulin secretion and plasma glucose homeostasis, *J. Clin. Invest.*, 125 (2015) 4026-
963 4041.

964 [107] A. Volchuk, M. Ravazzola, A. Perrelet, W.S. Eng, M. Di Liberto, O. Varlamov, M. Fukasawa, T.
965 Engel, T.H. Sollner, J.E. Rothman, L. Orci, Countercurrent distribution of two distinct SNARE
966 complexes mediating transport within the Golgi stack, *Mol. Biol. Cell*, 15 (2004) 1506-1518.

967 [108] J. Lang, M. Fukuda, H. Zhang, K. Mikoshiba, C.B. Wollheim, The first C2 domain of
968 synaptotagmin is required for exocytosis of insulin from pancreatic beta-cells: action of
969 synaptotagmin at low micromolar calcium, *EMBO J.*, 16 (1997) 5837-5846.

970 [109] C. Li, B. Ullrich, J.Z. Zhang, R.G. Anderson, N. Brose, T.C. Sudhof, Ca^{2+} -dependent and -
971 independent activities of neural and non-neural synaptotagmins, *Nature*, 375 (1995) 594-599.

972 [110] M. Fukuda, T. Kojima, K. Mikoshiba, Phospholipid composition dependence of Ca^{2+} -dependent
973 phospholipid binding to the C2A domain of synaptotagmin IV, *J. Biol. Chem.*, 271 (1996) 8430-
974 8434.

975 [111] O.H. Shin, A. Maximov, B.K. Lim, J. Rizo, T.C. Sudhof, Unexpected Ca^{2+} -binding properties of
976 synaptotagmin 9, *Proc. Nat'l Acad. Sci. U S A*, 101 (2004) 2554-2559.

977 [112] M. Mizuta, T. Kurose, T. Miki, Y. Shoji-Kasai, M. Takahashi, S. Seino, S. Matsukura, Localization
978 and functional role of synaptotagmin III in insulin secretory vesicles in pancreatic beta-cells,
979 *Diabetes*, 46 (1997) 2002-2006.

980 [113] H. Brown, B. Meister, J. Deeney, B.E. Corkey, S.N. Yang, O. Larsson, C.J. Rhodes, S. Seino, P.O.
981 Berggren, G. Fried, Synaptotagmin III isoform is compartmentalized in pancreatic beta-cells and
982 has a functional role in exocytosis, *Diabetes*, 49 (2000) 383-391.

983 [114] A. Gut, C.E. Kiraly, M. Fukuda, K. Mikoshiba, C.B. Wollheim, J. Lang, Expression and localisation
984 of synaptotagmin isoforms in endocrine beta-cells: their function in insulin exocytosis, *J. Cell*
985 *Sci.*, 114 (2001) 1709-1716.

986 [115] M. Iezzi, G. Kouri, M. Fukuda, C.B. Wollheim, Synaptotagmin V and IX isoforms control Ca^{2+} -
987 dependent insulin exocytosis, *J. Cell Sci.*, 117 (2004) 3119-3127.

988 [116] M. Iezzi, L. Eliasson, M. Fukuda, C.B. Wollheim, Adenovirus-mediated silencing of
989 synaptotagmin 9 inhibits Ca^{2+} -dependent insulin secretion in islets, *FEBS Lett.*, 579 (2005) 5241-
990 5246.

991 [117] X. Xiong, K.M. Zhou, Z.X. Wu, T. Xu, Silence of synaptotagmin I in INS-1 cells inhibits fast
992 exocytosis and fast endocytosis, *Biochem. Biophys. Res. Comm.*, 347 (2006) 76-82.

993 [118] F. Grise, N. Taib, C. Monterrat, V. Lagree, J. Lang, Distinct roles of the C2A and the C2B domain
994 of the vesicular Ca^{2+} sensor synaptotagmin 9 in endocrine beta-cells, *Biochem. J.*, 403 (2007)
995 483-492.

996 [119] C. Monterrat, F. Grise, M.N. Benassy, A. Hemar, J. Lang, The calcium-sensing protein
997 synaptotagmin 7 is expressed on different endosomal compartments in endocrine,
998 neuroendocrine cells or neurons but not on large dense core vesicles, *Histochem. Cell Biol.*, 127
999 (2007) 625-632.

1000 [120] B.R. Gauthier, D.L. Duhamel, M. Iezzi, S. Theander, F. Saltel, M. Fukuda, B. Wehrle-Haller, C.B.
1001 Wollheim, Synaptotagmin VII splice variants alpha, beta, and delta are expressed in pancreatic
1002 beta-cells and regulate insulin exocytosis, *FASEB J.*, 22 (2008) 194-206.

1003 [121] D. Martin, F. Allagnat, G. Chaffard, D. Caille, M. Fukuda, R. Regazzi, A. Abderrahmani, G.
1004 Waeber, P. Meda, P. Maechler, J.-A. Haefliger, Functional significance of repressor element 1
1005 silencing transcription factor (REST) target genes in pancreatic beta cells, *Diabetologia*, 51
1006 (2008) 1429-1439.

1007 [122] S. Dolai, L. Xie, D. Zhu, T. Liang, T. Qin, H. Xie, Y. Kang, E.R. Chapman, H.Y. Gaisano,
1008 Synaptotagmin-7 Functions to Replenish Insulin Granules for Exocytosis in Human Islet beta-
1009 Cells, *Diabetes*, 65 (2016) 1962-1976.

1010 [123] S.A. Andersson, A.H. Olsson, J.L. Esguerra, E. Heimann, C. Ladenvall, A. Edlund, A. Salehi, J.
1011 Taneera, E. Degerman, L. Groop, C. Ling, L. Eliasson, Reduced insulin secretion correlates with
1012 decreased expression of exocytotic genes in pancreatic islets from patients with type 2
1013 diabetes, *Mol. Cell. Endocrinol.*, 364 (2012) 36-45.

1014 [124] D.M. Blodgett, A. Nowosielska, S. Afik, S. Pechhold, A.J. Cura, N.J. Kennedy, S. Kim, A.
1015 Kucukural, R.J. Davis, S.C. Kent, D.L. Greiner, M.G. Garber, D.M. Harlan, et al., Novel
1016 Observations from next-generation rna sequencing of highly purified human adult and fetal islet
1017 cell subsets, *Diabetes*, 64 (2015) 3172-3181.

1018 [125] M. Fukuda, A. Yamamoto, Effect of forskolin on synaptotagmin iv protein trafficking in PC12
1019 cells, *J. Biochem.*, 136 (2004) 245-253.

1020 [126] N. Gustavsson, X. Wang, Y. Wang, T. Seah, J. Xu, G.K. Radda, T.C. Sudhof, W. Han, Neuronal
1021 calcium sensor synaptotagmin-9 is not involved in the regulation of glucose homeostasis or
1022 insulin secretion, *PLoS One*, 5 (2010) e15414.

1023 [127] N. Gustavsson, Y. Lao, A. Maximov, J.C. Chuang, E. Kostromina, J.J. Repa, C. Li, G.K. Radda, T.C.
1024 Sudhof, W. Han, Impaired insulin secretion and glucose intolerance in synaptotagmin-7 null
1025 mutant mice, *Proc. Nat'l Acad. Sci. U S A*, 105 (2008) 3992-3997.

1026 [128] B. Wu, S. Wei, N. Petersen, Y. Ali, X. Wang, T. Bacaj, P. Rorsman, W. Hong, T.C. Sudhof, W. Han,
1027 Synaptotagmin-7 phosphorylation mediates GLP-1-dependent potentiation of insulin secretion
1028 from beta-cells, *Proc. Nat'l Acad. Sci. U S A*, 112 (2015) 9996-10001.

1029 [129] N.W. Andrews, S. Chakrabarti, There's more to life than neurotransmission: the regulation of
1030 exocytosis by synaptotagmin VII, *Trends Cell Biol.*, 15 (2005) 626-631.

1031 [130] B.-B. Chu, Y.-C. Liao, W. Qi, C. Xie, X. Du, J. Wang, H. Yang, H.-H. Miao, B.-L. Li, B.-L. Song,
 1032 Cholesterol transport through lysosome-peroxisome membrane contacts, *Cell*, 161 (2015) 291-
 1033 306.

1034 [131] J. Vikman, J. Jimenez-Feltstrom, P. Nyman, J. Thelin, L. Eliasson, Insulin secretion is highly
 1035 sensitive to desorption of plasma membrane cholesterol, *FASEB J.*, 23 (2009) 58-67.

1036 [132] R. Fernandez-Chacon, G. Alvarez de Toledo, Cytosolic calcium facilitates release of secretory
 1037 products after exocytotic vesicle fusion, *FEBS Lett.*, 363 (1995) 221-225.

1038 [133] J. Hartmann, M. Lindau, A novel Ca^{2+} -dependent step in exocytosis subsequent to vesicle fusion,
 1039 *FEBS Lett.*, 363 (1995) 217-220.

1040 [134] S. Scepek, J.R. Coorssen, M. Lindau, Fusion pore expansion in horse eosinophils is modulated
 1041 by Ca^{2+} and protein kinase C via distinct mechanisms, *EMBO J.*, 17 (1998) 4340-4345.

1042 [135] T. Haller, P. Dietl, K. Pfaller, M. Frick, N. Mair, M. Paulmichl, M.W. Hess, J. Furst, K. Maly, Fusion
 1043 pore expansion is a slow, discontinuous, and Ca^{2+} -dependent process regulating secretion from
 1044 alveolar type II cells, *J. Cell Biol.*, 155 (2001) 279-289.

1045 [136] Z. Zhang, Y. Wu, Z. Wang, F.M. Dunning, J. Rehfuss, D. Ramanan, E.R. Chapman, M.B. Jackson,
 1046 Release mode of large and small dense-core vesicles specified by different synaptotagmin
 1047 isoforms in PC12 cells, *Mol. Biol. Cell*, 22 (2011) 2324-2336.

1048 [137] M.M. Kozlov, V.V. Chernyi, V.S. Sokolov, A. Ermakov Iu, V.S. Markin, Theory of hydrophobic ion
 1049 adsorption in bilayer lipid membranes taking into account their lateral interaction and charge
 1050 discreteness, *Biofizika*, 28 (1983) 61-66.

1051 [138] G.W. Kemble, T. Danieli, J.M. White, Lipid-anchored influenza hemagglutinin promotes
 1052 hemifusion, not complete fusion, *Cell*, 76 (1994) 383-391.

1053 [139] G.B. Melikyan, J.M. White, F.S. Cohen, GPI-anchored influenza hemagglutinin induces
 1054 hemifusion to both red blood cell and planar bilayer membranes, *J. Cell Biol.*, 131 (1995) 679-
 1055 691.

1056 [140] L.V. Chernomordik, J. Zimmerberg, M.M. Kozlov, Membranes of the world unite!, *J. Cell Biol.*,
 1057 175 (2006) 201-207.

1058 [141] P.K. Tarafdar, H. Chakraborty, M.J. Bruno, B.R. Lentz, Phosphatidylserine-dependent catalysis
 1059 of stalk and pore formation by synaptobrevin jmr-tmd peptide, *Biophys. J.*, 109 (2015) 1863-
 1060 1872.

1061 [142] A. Grafmuller, J. Shillcock, R. Lipowsky, The fusion of membranes and vesicles: pathway and
 1062 energy barriers from dissipative particle dynamics, *Biophys. J.*, 96 (2009) 2658-2675.

1063 [143] M.M. Kozlov, L.V. Chernomordik, Membrane tension and membrane fusion, *Curr. Opin. Struct.*
 1064 *Biol.*, 33 (2015) 61-67.

1065 [144] H.T. McMahon, M.M. Kozlov, S. Martens, Membrane curvature in synaptic vesicle fusion and
 1066 beyond, *Cell*, 140 (2010) 601-605.

1067 [145] S. Wang, Y. Li, C. Ma, Synaptotagmin-1 C2B domain interacts simultaneously with SNAREs and
 1068 membranes to promote membrane fusion, *eLife*, 5 (2016).

1069 [146] M.M. Kozlov, V.S. Markin, [Possible mechanism of membrane fusion], *Biofizika*, 28 (1983) 242-
 1070 247.

1071 [147] Y. Xu, F. Zhang, Z. Su, J.A. McNew, Y.K. Shin, Hemifusion in SNARE-mediated membrane fusion,
 1072 *Nat. Struct. Mol. Biol.*, 12 (2005) 417-422.

1073 [148] X. Lu, F. Zhang, J.A. McNew, Y.K. Shin, Membrane fusion induced by neuronal SNAREs transits
 1074 through hemifusion, *J. Biol. Chem.*, 280 (2005) 30538-30541.

1075 [149] L. Shi, Q.T. Shen, A. Kiel, J. Wang, H.W. Wang, T.J. Melia, J.E. Rothman, F. Pincet, SNARE
 1076 proteins: one to fuse and three to keep the nascent fusion pore open, *Science*, 335 (2012) 1355-
 1077 1359.

1078 [150] C.G. Giraudo, C. Hu, D. You, A.M. Slovic, E.V. Mosharov, D. Sulzer, T.J. Melia, J.E. Rothman,
 1079 SNAREs can promote complete fusion and hemifusion as alternative outcomes, *J. Cell Biol.*, 170
 1080 (2005) 249-260.

1081 [151] J.L. Wong, D.E. Koppel, A.E. Cowan, G.M. Wessel, Membrane hemifusion is a stable
 1082 intermediate of exocytosis, *Dev. Cell*, 12 (2007) 653-659.

1083 [152] M. Oelkers, H. Witt, P. Halder, R. Jahn, A. Janshoff, SNARE-mediated membrane fusion
 1084 trajectories derived from force-clamp experiments, *Proc. Nat'l Acad. Sci. U S A*, 113 (2016)
 1085 13051-13056.

1086 [153] M.H. Abdulreda, A. Bhalla, E.R. Chapman, V.T. Moy, Atomic force microscope spectroscopy
 1087 reveals a hemifusion intermediate during soluble N-ethylmaleimide-sensitive factor-attachment
 1088 protein receptors-mediated membrane fusion, *Biophys. J.*, 94 (2008) 648-655.

1089 [154] W.-D. Zhao, E. Hamid, W. Shin, P.J. Wen, E.S. Krystofiak, S.A. Villarreal, H.-C. Chiang, B. Kachar,
 1090 L.-G. Wu, Hemi-fused structure mediates and controls fusion and fission in live cells, *Nature*,
 1091 534 (2016) 548-552.

1092 [155] G.A. Zampighi, L.M. Zampighi, N. Fain, S. Lanzavecchia, S.A. Simon, E.M. Wright, Conical
1093 electron tomography of a chemical synapse: vesicles docked to the active zone are hemi-fused,
1094 *Biophys. J.*, 91 (2006) 2910-2918.

1095 [156] T. Salditt, S. Aeffner, X-ray structural investigations of fusion intermediates: Lipid model
1096 systems and beyond, *Sem. Cell Dev. Biol.*, 60 (2016) 65-77.

1097 [157] L.V. Chernomordik, M.M. Kozlov, Mechanics of membrane fusion, *Nat. Struct. Mol. Biol.*, 15
1098 (2008) 675-683.

1099 [158] M. Fuhrmans, G. Marelli, Y.G. Smirnova, M. Muller, Mechanics of membrane fusion/pore
1100 formation, *Chem. Phys. Lipids*, 185 (2015) 109-128.

1101 [159] Y. Kozlovsky, L.V. Chernomordik, M.M. Kozlov, Lipid intermediates in membrane fusion:
1102 formation, structure, and decay of hemifusion diaphragm, *Biophys. J.*, 83 (2002) 2634-2651.

1103 [160] H.J. Risselada, H. Grubmuller, How SNARE molecules mediate membrane fusion: recent
1104 insights from molecular simulations, *Curr. Opin. Struct. Biol.*, 22 (2012) 187-196.

1105 [161] H.J. Risselada, C. Kutzner, H. Grubmuller, Caught in the act: visualization of SNARE-mediated
1106 fusion events in molecular detail, *Chembiochem*, 12 (2011) 1049-1055.

1107 [162] H.J. Risselada, G. Bubnis, H. Grubmuller, Expansion of the fusion stalk and its implication for
1108 biological membrane fusion, *Proc. Nat'l Acad. Sci. U S A*, 111 (2014) 11043-11048.

1109 [163] J. Rohrbough, K. Broadie, Lipid regulation of the synaptic vesicle cycle, *Nat. Rev. Neurosci.*, 6
1110 (2005) 139-150.

1111 [164] B. Weinhausen, S. Aeffner, T. Reusch, T. Salditt, Acyl-chain correlation in membrane fusion
1112 intermediates: x-ray diffraction from the rhombohedral lipid phase, *Biophys. J.*, 102 (2012)
1113 2121-2129.

1114 [165] C.A. Helm, J.N. Israelachvili, P.M. McGuiggan, Role of hydrophobic forces in bilayer adhesion
1115 and fusion, *Biochemistry*, 31 (1992) 1794-1805.

1116 [166] V.A. Frolov, A.V. Shnyrova, J. Zimmerberg, Lipid polymorphisms and membrane shape, *Cold
1117 Spring Harb. Perspect. Biol.*, 3 (2011) a004747.

1118 [167] M.M. Kamal, D. Mills, M. Grzybek, J. Howard, Measurement of the membrane curvature
1119 preference of phospholipids reveals only weak coupling between lipid shape and leaflet
1120 curvature, *Proc. Nat'l Acad. Sci. U S A*, 106 (2009) 22245-22250.

1121 [168] L.J. Breckenridge, W. Almers, Final steps in exocytosis observed in a cell with giant secretory
1122 granules, *Proc. Nat'l Acad. Sci. U S A*, 84 (1987) 1945-1949.

1123 [169] N. Vardjan, J. Jorgacevski, R. Zorec, Fusion pores, SNAREs, and exocytosis, *Neuroscientist*, 19
1124 (2013) 160-174.

1125 [170] K. Lollike, N. Borregaard, M. Lindau, The exocytotic fusion pore of small granules has a
1126 conductance similar to an ion channel, *J. Cell Biol.*, 129 (1995) 99-104.

1127 [171] M.J. Curran, F.S. Cohen, D.E. Chandler, P.J. Munson, J. Zimmerberg, Exocytotic fusion pores
1128 exhibit semi-stable states, *J. Membr. Biol.*, 133 (1993) 61-75.

1129 [172] M. Lindau, High resolution electrophysiological techniques for the study of calcium-activated
1130 exocytosis, *Biochim. Biophys.*, 1820 (2012) 1234-1242.

1131 [173] N. Takahashi, H. Kasai, Exocytic process analyzed with two-photon excitation imaging in
1132 endocrine pancreas, *Endocr.J.*, 54 (2007) 337-346.

1133 [174] K.A. Myers, C. Janetopoulos, Recent advances in imaging subcellular processes, *F1000Res*, 5
1134 (2016).

1135 [175] I. Hafez, K. Kisler, K. Berberian, G. Dernick, V. Valero, M.G. Yong, H.G. Craighead, M. Lindau,
1136 Electrochemical imaging of fusion pore openings by electrochemical detector arrays, *Proc. Nat'l*
1137 *Acad. Sci. U S A*, 102 (2005) 13879-13884.

1138 [176] P.E. MacDonald, S. Obermuller, J. Vikman, J. Galvanovskis, P. Rorsman, L. Eliasson, Regulated
1139 exocytosis and kiss-and-run of synaptic-like microvesicles in INS-1 and primary rat beta-cells,
1140 *Diabetes*, 54 (2005) 736-743.

1141 [177] D.C. Hodgkin, The Banting Memorial Lecture 1972. The structure of insulin, *Diabetes*, 21 (1972)
1142 1131-1150.

1143 [178] A. Oliva, J. Farina, M. Llabres, Development of two high-performance liquid chromatographic
1144 methods for the analysis and characterization of insulin and its degradation products in
1145 pharmaceutical preparations, *J. Chromatogr. B B*, 749 (2000) 25-34.

1146 [179] L.J. Breckenridge, W. Almers, Currents through the fusion pore that forms during exocytosis of
1147 a secretory vesicle, *Nature*, 328 (1987) 814-817.

1148 [180] J. Del Castillo, B. Katz, La base "quantale" de la transmission neuro-musculaire, in: CNRS (Ed.)
1149 *Microphysiologie comparée des éléments excitables*, CNRS, Gif-sur-Yvette, 1957.

1150 [181] B. Ceccarelli, W.P. Hurlbut, A. Mauro, Turnover of transmitter and synaptic vesicles at the frog
1151 neuromuscular junction, *J. Cell Biol.*, 57 (1973) 499-524.

1152 [182] S.O. Rizzoli, R. Jahn, Kiss-and-run, collapse and 'readily retrievable' vesicles, *Traffic*, 8 (2007)
1153 1137-1144.

1154 [183] A. Oleinick, I. Svir, C. Amatore, 'Full fusion' is not ineluctable during vesicular exocytosis of
1155 neurotransmitters by endocrine cells, *Proc. Math. Phys. Eng. Sci.*, 473 (2017) 20160684.

1156 [184] A. Gucek, J. Jorgacevski, P. Singh, C. Geisler, M. Lisjak, N. Vardjan, M. Kreft, A. Egner, R. Zorec,
1157 Dominant negative SNARE peptides stabilize the fusion pore in a narrow, release-unproductive
1158 state, *Cell Mol. Life Sci.*, 73 (2016) 3719-3731.

1159 [185] L.A. Sombers, H.J. Hanchar, T.L. Colliver, N. Wittenberg, A. Cans, S. Arbault, C. Amatore, A.G.
1160 Ewing, The effects of vesicular volume on secretion through the fusion pore in exocytotic
1161 release from PC12 cells, *J. Neurosci.*, 24 (2004) 303-309.

1162 [186] J. Jorgacevski, M. Fosnaric, N. Vardjan, M. Stenovec, M. Potokar, M. Kreft, V. Kralj-Iglic, A. Iglic,
1163 R. Zorec, Fusion pore stability of peptidergic vesicles, *Mol Membr Biol*, 27 (2010) 65-80.

1164 [187] J. Jorgacevski, M. Potokar, S. Grilc, M. Kreft, W. Liu, J.W. Barclay, J. Buckers, R. Medda, S.W.
1165 Hell, V. Parpura, R.D. Burgoyne, R. Zorec, Munc18-1 tuning of vesicle merger and fusion pore
1166 properties, *J. Neurosci.*, 31 (2011) 9055-9066.

1167 [188] A.H. Kunding, M.W. Mortensen, S.M. Christensen, V.K. Bhatia, I. Makarov, R. Metzler, D.
1168 Stamou, Intermembrane docking reactions are regulated by membrane curvature, *Biophys. J.*,
1169 101 (2011) 2693-2703.

1170 [189] J.M. Hernandez, A.J. Kreutzberger, V. Kiessling, L.K. Tamm, R. Jahn, Variable cooperativity in
1171 SNARE-mediated membrane fusion, *Proc. Nat'l Acad. Sci. U S A*, 111 (2014) 12037-12042.

1172 [190] P. Gipson, Y. Fukuda, R. Danev, Y. Lai, D.H. Chen, W. Baumeister, A.T. Brunger, Morphologies of
1173 synaptic protein membrane fusion interfaces, *Proc. Nat'l Acad. Sci. U S A*, 114 (2017) 9110-
1174 9115.

1175 [191] A. Reetz, M. Solimena, M. Matteoli, F. Folli, K. Takei, P. De Camilli, GABA and pancreatic beta-
1176 cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like
1177 microvesicles suggests their role in GABA storage and secretion, *EMBO J.*, 10 (1991) 1275-1284.

1178 [192] P.E. MacDonald, M. Braun, J. Galvanovskis, P. Rorsman, Release of small transmitters through
1179 kiss-and-run fusion pores in rat pancreatic β cells, *Cell Metab.*, 4 (2006) 283-290.

1180 [193] M. Braun, A. Wendt, J. Karanauskaite, J. Galvanovskis, A. Clark, P.E. MacDonald, P. Rorsman,
1181 Corelease and differential exit via the fusion pore of GABA, serotonin, and ATP from LDCV in rat
1182 pancreatic beta cells, *J. Gen. Physiol.*, 129 (2007) 221-231.

1183 [194] S.T. Hanna, G.M. Pigeau, J. Galvanovskis, A. Clark, P. Rorsman, P.E. MacDonald, Kiss-and-run
1184 exocytosis and fusion pores of secretory vesicles in human β -cells, *Eur. J. Physiol.*, 457 (2008)
1185 1343.

1186 [195] D.S. Koh, J.H. Cho, L. Chen, Paracrine interactions within islets of Langerhans, *J. Mol. Neurosci.*,
1187 48 (2012) 429-440.

1188 [196] S. Barg, A. Gucek, How Kiss-and-Run Can Make Us Sick: SOX4 Puts a Break on the Pore,
1189 *Diabetes*, 65 (2016) 1791-1793.

1190 [197] G. Miesenbock, D.A. De Angelis, J.E. Rothman, Visualizing secretion and synaptic transmission
1191 with pH-sensitive green fluorescent proteins, *Nature*, 394 (1998) 192-195.

1192 [198] D. Perrais, I.C. Kleppe, J.W. Taraska, W. Almers, Recapture after exocytosis causes differential
1193 retention of protein in granules of bovine chromaffin cells, *J. Physiol.*, 560 (2004) 413-428.

1194 [199] N. Takahashi, T. Kishimoto, T. Nemoto, T. Kadowaki, H. Kasai, Fusion pore dynamics and insulin
1195 granule exocytosis in the pancreatic islet, *Science*, 297 (2002) 1349-1352.

1196 [200] Z. Lavagnino, J. Dwight, A. Ustione, T.-U. Nguyen, T.S. Tkaczyk, D.W. Piston, Snapshot
1197 Hyperspectral Light-Sheet Imaging of Signal Transduction in Live Pancreatic Islets, *Biophys. J.*,
1198 111 (2016) 409-417.

1199 [201] J.W. Taraska, W. Almers, Bilayers merge even when exocytosis is transient, *Proc. Nat'l Acad.*
1200 *Sci. U S A*, 101 (2004) 8780-8785.

1201 [202] M. Lindau, W. Almers, Structure and function of fusion pores in exocytosis and ectoplasmic
1202 membrane fusion, *Curr. Opin. Cell Biol.*, 7 (1995) 509-517.

1203 [203] M. Lindau, O. Nusse, J. Bennett, O. Cromwell, The membrane fusion events in degranulating
1204 guinea pig eosinophils, *J. Cell Sci.*, 104 (1993) 203-210.

1205 [204] R.B. Sutton, D. Fasshauer, R. Jahn, A.T. Brunger, Crystal structure of a SNARE complex involved
1206 in synaptic exocytosis at 2.4 Å resolution, *Nature*, 395 (1998) 347-353.

1207 [205] W. Xiao, M.A. Poirier, M.K. Bennett, Y.K. Shin, The neuronal t-SNARE complex is a parallel four-
1208 helix bundle, *Nat. Struct. Biol.*, 8 (2001) 308-311.

1209 [206] F. Zhang, Y. Chen, D.H. Kweon, C.S. Kim, Y.K. Shin, The four-helix bundle of the neuronal target
1210 membrane SNARE complex is neither disordered in the middle nor uncoiled at the C-terminal
1211 region, *J. Biol. Chem.*, 277 (2002) 24294-24298.

1212 [207] Y. Zhang, Z. Su, F. Zhang, Y. Chen, Y.K. Shin, A partially zipped SNARE complex stabilized by the
1213 membrane, *J. Biol. Chem.*, 280 (2005) 15595-15600.

1214 [208] J. Shin, X. Lou, D.H. Kweon, Y.K. Shin, Multiple conformations of a single SNAREpin between
1215 two nanodisc membranes reveal diverse pre-fusion states, *Biochem. J.*, 459 (2014) 95-102.

1216 [209] S.J. An, W. Almers, Tracking SNARE complex formation in live endocrine cells, *Science*, 306
1217 (2004) 1042-1046.

1218 [210] Y. Zhao, Q. Fang, A.D. Herbst, K.N. Berberian, W. Almers, M. Lindau, Rapid structural change in
1219 synaptosomal-associated protein 25 (SNAP25) precedes the fusion of single vesicles with the
1220 plasma membrane in live chromaffin cells, *Proc. Nat'l Acad. Sci. U S A*, 110 (2013) 14249-14254.

1221 [211] N. Takahashi, H. Hatakeyama, H. Okado, J. Noguchi, M. Ohno, H. Kasai, SNARE conformational
1222 changes that prepare vesicles for exocytosis, *Cell Metab.*, 12 (2010) 19-29.

1223 [212] N. Takahashi, W. Sawada, J. Noguchi, S. Watanabe, H. Ucar, A. Hayashi-Takagi, S. Yagishita, M.
1224 Ohno, H. Tokumaru, H. Kasai, Two-photon fluorescence lifetime imaging of primed SNARE
1225 complexes in presynaptic terminals and beta cells, *Nat. Commun.*, 6 (2015) 8531.

1226 [213] A. Stein, G. Weber, M.C. Wahl, R. Jahn, Helical extension of the neuronal SNARE complex into
1227 the membrane, *Nature*, 460 (2009) 525-528.

1228 [214] M. Bowen, A.T. Brunger, Conformation of the synaptobrevin transmembrane domain, *Proc.*
1229 *Nat'l Acad. Sci. U S A*, 103 (2006) 8378-8383.

1230 [215] Y. Chen, Y. Xu, F. Zhang, Y.K. Shin, Constitutive versus regulated SNARE assembly: a structural
1231 basis, *EMBO J.*, 23 (2004) 681-689.

1232 [216] C.S. Kim, D.H. Kweon, Y.K. Shin, Membrane topologies of neuronal SNARE folding
1233 intermediates, *Biochemistry*, 41 (2002) 10928-10933.

1234 [217] D.H. Kweon, C.S. Kim, Y.K. Shin, The membrane-dipped neuronal SNARE complex: a site-
1235 directed spin labeling electron paramagnetic resonance study, *Biochemistry*, 41 (2002) 9264-
1236 9268.

1237 [218] J. Tong, P.P. Borbat, J.H. Freed, Y.K. Shin, A scissors mechanism for stimulation of SNARE-
1238 mediated lipid mixing by cholesterol, *Proc. Nat'l Acad. Sci. U S A*, 106 (2009) 5141-5146.

1239 [219] Z. Wu, O.D. Bello, S. Thiagarajan, S.M. Auclair, W. Vennekate, S.S. Krishnakumar, B.
1240 O'Shaughnessy, E. Karatekin, Dilatation of fusion pores by crowding of SNARE proteins, *eLife*, 6
1241 (2017).

1242 [220] R. Mendelsohn, G. Mao, C.R. Flach, Infrared reflection-absorption spectroscopy: principles and
1243 applications to lipid-protein interaction in Langmuir films, *Biochim. Biophys. Acta*, 1798 (2010)
1244 788-800.

1245 [221] Z.D. Schultz, I.W. Levin, Vibrational spectroscopy of biomembranes, *Annu. Rev. Anal. Chem.*, 4
1246 (2011) 343-366.

1247 [222] S.E. Glassford, B. Byrne, S.G. Kazarian, Recent applications of ATR FTIR spectroscopy and
1248 imaging to proteins, *Biochim. Biophys. Acta*, 1834 (2013) 2849-2858.

1249 [223] R.M.A. Azzam, N.M. Bashara, *Ellipsometry and polarized light.*, North-Holland Publishing Co,
1250 Amsterdam, New York, Oxford, 1977.

1251 [224] S.A. Tatulian, P. Hinterdorfer, G. Baber, L.K. Tamm, Influenza hemagglutinin assumes a tilted
1252 conformation during membrane fusion as determined by attenuated total reflection FTIR
1253 spectroscopy, *EMBO J.*, 14 (1995) 5514-5523.

1254 [225] I. Martin, E. Goormaghtigh, J.M. Ruysschaert, Attenuated total reflection IR spectroscopy as a
1255 tool to investigate the orientation and tertiary structure changes in fusion proteins, *Biochim.*
1256 *Biophys. Acta*, 1614 (2003) 97-103.

1257 [226] Y. Li, L.K. Tamm, Structure and plasticity of the human immunodeficiency virus gp41 fusion
1258 domain in lipid micelles and bilayers, *Biophys. J.*, 93 (2007) 876-885.

1259 [227] Y. Shai, ATR-FTIR studies in pore forming and membrane induced fusion peptides, *Biochim.*
1260 *Biophys. Acta*, 1828 (2013) 2306-2313.

1261 [228] H.J. Sharpe, T.J. Stevens, S. Munro, A comprehensive comparison of transmembrane domains
1262 reveals organelle-specific properties, *Cell*, 142 (2010) 158-169.

1263 [229] S. Neumann, D. Langosch, Conserved conformational dynamics of membrane fusion protein
1264 transmembrane domains and flanking regions indicated by sequence statistics, *Proteins*, 79
1265 (2011) 2418-2427.

1266 [230] P.Y. Chou, G.D. Fasman, Empirical predictions of protein conformation, *Annu. Rev. Biochem.*,
1267 47 (1978) 251-276.

1268 [231] K. Lin, V.A. Simossis, W.R. Taylor, J. Heringa, A simple and fast secondary structure prediction
1269 method using hidden neural networks, *Bioinformatics*, 21 (2005) 152-159.

1270 [232] T.Z. Sen, R.L. Jernigan, J. Garnier, A. Kloczkowski, GOR V server for protein secondary structure
1271 prediction, *Bioinformatics*, 21 (2005) 2787-2788.

1272 [233] D. Langosch, J.M. Crane, B. Brosig, A. Hellwig, L.K. Tamm, J. Reed, Peptide mimics of SNARE
1273 transmembrane segments drive membrane fusion depending on their conformational plasticity,
1274 *J. Mol. Biol.*, 311 (2001) 709-721.

1275 [234] B. Hastoy, P.A. Scotti, A. Milochau, Z. Fezoua-Boubegtiten, J. Rodas, R. Megret, B. Desbat, M.
1276 Laguerre, S. Castano, D. Perrais, P. Rorsman, R. Oda, J. Lang, A central small amino acid in the
1277 vamp2 transmembrane domain regulates the fusion pore in exocytosis, *Sci. Rep.*, 7 (2017) 2835.

1278 [235] W. Yassine, A. Milochau, S. Buchoux, J. Lang, B. Desbat, R. Oda, Effect of monolayer lipid
1279 charges on the structure and orientation of protein VAMP1 at the air-water interface, *Biochim.*
1280 *Biophys. Acta*, 1798 (2010) 928-937.

1281 [236] W. Yassine, N. Taib, S. Federman, A. Milochau, S. Castano, W. Sbi, C. Manigand, M. Laguerre, B.
1282 Desbat, R. Oda, J. Lang, Reversible transition between alpha-helix and beta-sheet conformation
1283 of a transmembrane domain, *Biochim. Biophys. Acta*, 1788 (2009) 1722-1730.

1284 [237] Y. Hua, R.H. Scheller, Three SNARE complexes cooperate to mediate membrane fusion, *Proc.*
1285 *Nat'l Acad. of Sci. USA*, 98 (2001) 8065-8070.

1286 [238] R. Mohrmann, H. de Wit, M. Verhage, E. Neher, J.B. Sorensen, Fast vesicle fusion in living cells
1287 requires at least three SNARE complexes, *Science*, 330 (2010) 502-505.

1288 [239] Y. Zhang, Y.K. Shin, Transmembrane organization of yeast syntaxin-analogue Sso1p,
1289 *Biochemistry*, 45 (2006) 4173-4181.

1290 [240] J.F. Ellena, B. Liang, M. Wiktor, A. Stein, D.S. Cafiso, R. Jahn, L.K. Tamm, Dynamic structure of
1291 lipid-bound synaptobrevin suggests a nucleation-propagation mechanism for trans-SNARE
1292 complex formation, *Proc. Nat'l Acad. Sci. U S A*, 106 (2009) 20306-20311.

1293 [241] T. Werner, W.B. Church, Kink characterization and modeling in transmembrane protein
1294 structures, *J. Chem. Inf. Model.*, 53 (2013) 2926-2936.

1295 [242] A.E. Blanchard, M.J. Arcario, K. Schulten, E. Tajkhorshid, A highly tilted membrane
1296 configuration for the prefusion state of synaptobrevin, *Biophys. J.*, 107 (2014) 2112-2121.

1297 [243] D.H. Kweon, C.S. Kim, Y.K. Shin, Insertion of the membrane-proximal region of the neuronal
1298 SNARE coiled coil into the membrane, *J. Biol. Chem.*, 278 (2003) 12367-12373.

1299 [244] M.W. Hofmann, K. Weise, J. Ollesch, P. Agrawal, H. Stalz, W. Stelzer, F. Hulsbergen, H. de
1300 Groot, K. Gerwert, J. Reed, D. Langosch, De novo design of conformationally flexible
1301 transmembrane peptides driving membrane fusion, *Proc. Nat'l Acad. Sci. U S A*, 101 (2004)
1302 14776-14781.

1303 [245] J. Ollesch, B.C. Poschner, J. Nikolaus, M.W. Hofmann, A. Herrmann, K. Gerwert, D. Langosch,
1304 Secondary structure and distribution of fusogenic LV-peptides in lipid membranes, *Eur Biophys.*
1305 *J.*, 37 (2008) 435-445.

1306 [246] M. Dhara, A. Yarzagaray, M. Makke, B. Schindeldecker, Y. Schwarz, A. Shaaban, S. Sharma, R.A.
1307 Bockmann, M. Lindau, R. Mohrmann, D. Bruns, v-SNARE transmembrane domains function as
1308 catalysts for vesicle fusion, *eLife*, 5 (2016).

1309 [247] J. Bigay, B. Antonny, Curvature, lipid packing, and electrostatics of membrane organelles:
1310 defining cellular territories in determining specificity, *Dev. Cell*, 23 (2012) 886-895.

1311 [248] J.G. Kay, M. Koivusalo, X. Ma, T. Wohland, S. Grinstein, Phosphatidylserine dynamics in cellular
1312 membranes, *Mol. Biol. Cell*, 23 (2012) 2198-2212.

1313 [249] G.D. Fairn, N.L. Schieber, N. Ariotti, S. Murphy, L. Kuerschner, R.I. Webb, S. Grinstein, R.G.
1314 Parton, High-resolution mapping reveals topologically distinct cellular pools of
1315 phosphatidylserine, *J. Cell Biol.*, 194 (2011) 257-275.

1316 [250] R. Laage, D. Langosch, Dimerization of the synaptic vesicle protein synaptobrevin (vesicle-
1317 associated membrane protein) II depends on specific residues within the transmembrane
1318 segment, *Eur. J. Biochem.*, 249 (1997) 540-546.

1319 [251] R. Laage, J. Rohde, B. Brosig, D. Langosch, A conserved membrane-spanning amino acid motif
1320 drives homomeric and supports heteromeric assembly of presynaptic SNARE proteins, *J. Biol.*
1321 *Chem.*, 275 (2000) 17481-17487.

1322 [252] M.E. Bowen, D.M. Engelman, A.T. Brunger, Mutational analysis of synaptobrevin
1323 transmembrane domain oligomerization, *Biochemistry*, 41 (2002) 15861-15866.

1324 [253] S. Takamori, M. Holt, K. Stenius, E.A. Lemke, M. Gronborg, D. Riedel, H. Urlaub, S. Schenck, B.
1325 Brugger, P. Ringler, S.A. Muller, B. Rammner, F. Grater, et al., Molecular anatomy of a trafficking
1326 organelle, *Cell*, 127 (2006) 831-846.

1327 [254] R. Roy, R. Laage, D. Langosch, Synaptobrevin transmembrane domain dimerization-revisited,
1328 *Biochemistry*, 43 (2004) 4964-4970.

1329 [255] A.E. Kroch, K.G. Fleming, Alternate interfaces may mediate homomeric and heteromeric
1330 assembly in the transmembrane domains of SNARE proteins, *J. Mol. Biol.*, 357 (2006) 184-194.

1331 [256] K.G. Fleming, D.M. Engelman, Computation and mutagenesis suggest a right-handed structure
1332 for the synaptobrevin transmembrane dimer, *Proteins*, 45 (2001) 313-317.

1333 [257] J. Han, K. Pluhackova, T.A. Wassenaar, R.A. Bockmann, Synaptobrevin Transmembrane Domain
1334 Dimerization Studied by Multiscale Molecular Dynamics Simulations, *Biophys. J.*, 109 (2015)
1335 760-771.

1336 [258] J. Han, K. Pluhackova, R.A. Bockmann, Exploring the Formation and the Structure of
1337 Synaptobrevin Oligomers in a Model Membrane, *Biophys. J.*, 110 (2016) 2004-2015.

1338 [259] M. Stangl, D. Schneider, Functional competition within a membrane: Lipid recognition vs.
1339 transmembrane helix oligomerization, *Biochim. Biophys. Acta*, 1848 (2015) 1886-1896.

1340 [260] J.J. Flanagan, I. Mukherjee, C. Barlowe, Examination of sec22 homodimer formation and role in
1341 snare-dependent membrane fusion, *J. Biol. Chem.*, 290 (2015) 10657-10666.

1342 [261] V.A. Klyachko, M.B. Jackson, Capacitance steps and fusion pores of small and large-dense-core
1343 vesicles in nerve terminals, *Nature*, 418 (2002) 89-92.

1344 [262] A. Albillos, G. Dernick, H. Horstmann, W. Almers, G. Alvarez de Toledo, M. Lindau, The
1345 exocytotic event in chromaffin cells revealed by patch amperometry, *Nature*, 389 (1997) 509-
1346 512.

1347 [263] A. Chanturiya, L.V. Chernomordik, J. Zimmerberg, Flickering fusion pores comparable with
1348 initial exocytotic pores occur in protein-free phospholipid bilayers, *Proc. Nat'l Acad. Sci. U S A*,
1349 94 (1997) 14423-14428.

1350 [264] C. Nanavati, V.S. Markin, A.F. Oberhauser, J.M. Fernandez, The exocytotic fusion pore modeled
1351 as a lipidic pore, *Biophys. J.*, 63 (1992) 1118-1132.

1352 [265] C.W. Chang, C.W. Chiang, M.B. Jackson, Fusion pores and their control of neurotransmitter and
1353 hormone release, *J. Gen. Physiol.*, 149 (2017) 301-322.

1354 [266] F.W. Tse, A. Iwata, W. Almers, Membrane flux through the pore formed by a fusogenic viral
1355 envelope protein during cell fusion, *J. Cell Biol.*, 121 (1993) 543-552.

1356 [267] W. Almers, F.W. Tse, Transmitter release from synapses: does a preassembled fusion pore
1357 initiate exocytosis?, *Neuron*, 4 (1990) 813-818.

1358 [268] L.V. Chernomordik, V.A. Frolov, E. Leikina, P. Bronk, J. Zimmerberg, The pathway of membrane
1359 fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion, and lipidic fusion
1360 pore formation, *J. Cell Biol.*, 140 (1998) 1369-1382.

1361 [269] V.S. Markin, J.P. Albanesi, Membrane fusion: stalk model revisited, *Biophys. J.*, 82 (2002) 693-
1362 712.

1363 [270] M. Schick, Membrane fusion: the emergence of a new paradigm, *J. Stat. Phys.*, 142 (2011)
1364 1317-1323.

1365 [271] H. Gratkowski, J.D. Lear, W.F. DeGrado, Polar side chains drive the association of model
1366 transmembrane peptides, *Proc. Nat'l Acad. of Sci. USA*, 98 (2001) 880-885.

1367 [272] J.D. Lear, Z.R. Wasserman, W.F. DeGrado, Synthetic amphiphilic peptide models for protein ion
1368 channels, *Science*, 240 (1988) 1177-1181.

1369 [273] R. Jahn, H. Grubmüller, Membrane fusion, *Curr. Opin. Cell Biol.*, 14 (2002) 488-495.

1370 [274] S. Sharma, M. Lindau, The mystery of the fusion pore, *Nat. Struct. Mol. Biol.*, 23 (2016) 5-6.

1371 [275] A.N. Ngatchou, K. Kisler, Q. Fang, A.M. Walter, Y. Zhao, D. Bruns, J.B. Sørensen, M. Lindau, Role
1372 of the synaptobrevin C terminus in fusion pore formation, *Proc. Nat'l Acad. Sci. U S A*, 107
1373 (2010) 18463-18468.

1374 [276] R. Sinha, S. Ahmed, R. Jahn, J. Klingauf, Two synaptobrevin molecules are sufficient for vesicle
1375 fusion in central nervous system synapses, *Proc. Nat'l Acad. Sci. U S A*, 108 (2011) 14318-14323.

1376 [277] J.A. McNew, M. Sogaard, N.M. Lampen, S. Machida, R.R. Ye, L. Lacomis, P. Tempst, J.E.
1377 Rothman, T.H. Sollner, Ykt6p, a prenylated SNARE essential for endoplasmic reticulum-Golgi
1378 transport, *J. Biol. Chem.*, 272 (1997) 17776-17783.

1379 [278] J. Koehler Leman, M.B. Ulmschneider, J.J. Gray, Computational modeling of membrane
1380 proteins, *Proteins*, 83 (2015) 1-24.

1381 [279] S. Sharma, B.N. Kim, P.J. Stansfeld, M.S. Sansom, M. Lindau, A coarse grained model for a lipid
1382 membrane with physiological composition and leaflet asymmetry, *PLoS One*, 10 (2015)
1383 e0144814.

1384 [280] C.W. Chang, C.W. Chiang, J.D. Gaffaney, E.R. Chapman, M.B. Jackson, Lipid-anchored
1385 synaptobrevin provides little or no support for exocytosis or liposome fusion, *J. Biol. Chem.*, 291
1386 (2016) 2848-2857.

1387 [281] S. Chang, K. Reim, M. Pedersen, E. Neher, N. Brose, H. Taschenberger, Complexin stabilizes
1388 newly primed synaptic vesicles and prevents their premature fusion at the mouse calyx of held
1389 synapse, *J. Neurosci.*, 35 (2015) 8272-8290.

1390 [282] X. Han, M.B. Jackson, Electrostatic interactions between the syntaxin membrane anchor and
1391 neurotransmitter passing through the fusion pore, *Biophys. J.*, 88 (2005) L20-22.

1392 [283] X. Han, C.T. Wang, J. Bai, E.R. Chapman, M.B. Jackson, Transmembrane segments of syntaxin
1393 line the fusion pore of Ca²⁺-triggered exocytosis, *Science*, 304 (2004) 289-292.

1394 [284] D.H. Kweon, B. Kong, Y.K. Shin, Hemifusion in synaptic vesicle cycle, *Front. Mol. Neurosci.*, 10
1395 (2017) 65.

1396 [285] J.A. Szule, J.R. Coorssen, Comment on "Transmembrane segments of syntaxin line the fusion
1397 pore of Ca²⁺-triggered exocytosis", *Science*, 306 (2004) 813; author reply 813.

1398 [286] H. Bao, M. Goldschen-Ohm, P. Jeggle, B. Chanda, J.M. Edwardson, E.R. Chapman, Exocytotic
1399 fusion pores are composed of both lipids and proteins, *Nat. Struct. Mol. Biol.*, 23 (2016) 67-73.

1400 [287] C. Peters, M.J. Bayer, S. Buhler, J.S. Andersen, M. Mann, A. Mayer, Trans-complex formation by
1401 proteolipid channels in the terminal phase of membrane fusion, *Nature*, 409 (2001) 581-588.

1402 [288] S. Couoh-Cardel, Y.C. Hsueh, S. Wilkens, L. Movileanu, Yeast v-atpase proteolipid ring acts as a
1403 large-conductance transmembrane protein pore, *Sci. Rep.*, 6 (2016) 24774.

1404 [289] A. Bodzēta, M. Kahms, J. Klingauf, The presynaptic v-ATPase reversibly disassembles and
1405 thereby modulates exocytosis but is not part of the fusion machinery, *Cell reports*, 20 (2017)
1406 1348-1359.

1407 [290] L. Chen, M.S. Lau, D.K. Banfield, Multiple ER-Golgi SNARE transmembrane domains are
1408 dispensable for trafficking but required for SNARE recycling, *Mol. Biol. Cell*, 27 (2016) 2633-
1409 2641.

1410 [291] M. Pieren, Y. Desfougeres, L. Michailat, A. Schmidt, A. Mayer, Vacuolar SNARE protein
1411 transmembrane domains serve as nonspecific membrane anchors with unequal roles in lipid
1412 mixing, *J. Biol. Chem.*, 290 (2015) 12821-12832.

1413 [292] H. Xu, M. Zick, W.T. Wickner, Y. Jun, A lipid-anchored SNARE supports membrane fusion, *Proc.*
1414 *Nat'l Acad. of Sci. USA*, 108 (2011) 17325-17330.

1415 [293] P. Zhou, T. Bacaj, X. Yang, Z.P. Pang, T.C. Sudhof, Lipid-anchored SNAREs lacking
1416 transmembrane regions fully support membrane fusion during neurotransmitter release,
1417 *Neuron*, 80 (2013) 470-483.

1418 [294] E. Grote, M. Baba, Y. Ohsumi, P.J. Novick, Geranylgeranylated SNAREs are dominant inhibitors
1419 of membrane fusion, *J. Cell Biol.*, 151 (2000) 453-466.

1420 [295] J.A. McNew, T. Weber, F. Parlati, R.J. Johnston, T.J. Melia, T.H. Sollner, J.E. Rothman, Close is
1421 not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces
1422 force to membrane anchors, *J. Cell Biol.*, 150 (2000) 105-117.

1423 [296] C.W. Chang, E. Hui, J. Bai, D. Bruns, E.R. Chapman, M.B. Jackson, A structural role for the
1424 synaptobrevin 2 transmembrane domain in dense-core vesicle fusion pores, *J. Neurosci.*, 35
1425 (2015) 5772-5780.

1426 [297] M.E. Graham, R.D. Burgoyne, Comparison of cysteine string protein (Csp) and mutant alpha-
1427 SNAP overexpression reveals a role for csp in late steps of membrane fusion in dense-core
1428 granule exocytosis in adrenal chromaffin cells, *J. Neurosci.*, 20 (2000) 1281-1289.

1429 [298] R.J. Fisher, J. Pevsner, R.D. Burgoyne, Control of fusion pore dynamics during exocytosis by
1430 Munc18, *Science*, 291 (2001) 875-878.

1431 [299] E. Oh, M.A. Kalwat, M.J. Kim, M. Verhage, D.C. Thurmond, Munc18-1 regulates first-phase
1432 insulin release by promoting granule docking to multiple syntaxin isoforms, *J. Biol. Chem.*, 287
1433 (2012) 25821-25833.

1434 [300] D. Zhu, L. Xie, N. Karimian, T. Liang, Y. Kang, Y.C. Huang, H.Y. Gaisano, Munc18c mediates
1435 exocytosis of pre-docked and newcomer insulin granules underlying biphasic glucose stimulated
1436 insulin secretion in human pancreatic beta-cells, *Mol. Metab.*, 4 (2015) 418-426.

1437 [301] S.A. Mandic, M. Skelin, J.U. Johansson, M.S. Rupnik, P.O. Berggren, C. Bark, Munc18-1 and
1438 Munc18-2 proteins modulate beta-cell Ca^{2+} sensitivity and kinetics of insulin exocytosis
1439 differently, *J. Biol. Chem.*, 286 (2011) 28026-28040.

1440 [302] S.H. White, W.C. Wimley, Membrane protein folding and stability: physical principles, *Annu.*
1441 *Rev. Biophys. Biomol. Struct.*, 28 (1999) 319-365.

1442 [303] W. Dowhan, Molecular basis for membrane phospholipid diversity: why are there so many
1443 lipids?, *Annu. Rev. Biochem.*, 66 (1997) 199-232.

1444 [304] E. Sezgin, I. Levental, S. Mayor, C. Eggeling, The mystery of membrane organization:
1445 composition, regulation and roles of lipid rafts, *Nat. Rev. Mol. Cell. Biol.*, 18 (2017) 361-374.

1446 [305] I.M. Hafez, P.R. Cullis, Roles of lipid polymorphism in intracellular delivery, *Adv. Drug. Deliv.*
1447 *Rev.*, 47 (2001) 139-148.

1448 [306] S. Manno, Y. Takakuwa, N. Mohandas, Identification of a functional role for lipid asymmetry in
1449 biological membranes: Phosphatidylserine-skeletal protein interactions modulate membrane
1450 stability, *Proc. Nat'l Acad. Sci. U S A*, 99 (2002) 1943-1948.

1451 [307] P.A. Janmey, P.K. Kinnunen, Biophysical properties of lipids and dynamic membranes, *Trends*
1452 *Cell Biol.*, 16 (2006) 538-546.

1453 [308] J. Israelachvili, Intermolecular and surface forces, Academic Press 1992.

1454 [309] C. Salaun, D.J. James, L.H. Chamberlain, Lipid rafts and the regulation of exocytosis, *Traffic*, 5
1455 (2004) 255-264.

1456 [310] S. Aeffer, T. Reusch, B. Weinhausen, T. Salditt, Energetics of stalk intermediates in membrane
1457 fusion are controlled by lipid composition, *Proc. Nat'l Acad. Sci. U S A*, 109 (2012) E1609-1618.

1458 [311] S.T. Yang, A.J. Kreutzberger, J. Lee, V. Kiessling, L.K. Tamm, The role of cholesterol in
1459 membrane fusion, *Chem. Phys. Lipids*, 199 (2016) 136-143.

1460 [312] A.A. Spector, M.A. Yorek, Membrane lipid composition and cellular function, *J. Lipid Res.*, 26
1461 (1985) 1015-1035.

1462 [313] A.J. Kreutzberger, V. Kiessling, L.K. Tamm, High cholesterol obviates a prolonged hemifusion
1463 intermediate in fast SNARE-mediated membrane fusion, *Biophys. J.*, 109 (2015) 319-329.

1464 [314] S. Dhanvantari, Y.P. Loh, Lipid raft association of carboxypeptidase e is necessary for its
1465 function as a regulated secretory pathway sorting receptor, *J. Biol. Chem.*, 275 (2000) 29887-
1466 29893.

1467 [315] E. Stuken, A. Porat, F. Shimron, E. Fass, D. Kaloyanova, B. Brugger, F.T. Wieland, Z. Elazar, J.B.
1468 Helms, Intra-Golgi protein transport depends on a cholesterol balance in the lipid membrane, *J.*
1469 *Biol. Chem.*, 278 (2003) 53112-53122.

1470 [316] A. Ridsdale, M. Denis, P.Y. Gougeon, J.K. Ngsee, J.F. Presley, X. Zha, Cholesterol is required for
1471 efficient endoplasmic reticulum-to-Golgi transport of secretory membrane proteins, *Mol. Biol.*
1472 *Cell*, 17 (2006) 1593-1605.

1473 [317] Y. Wang, C. Thiele, W.B. Huttner, Cholesterol is required for the formation of regulated and
1474 constitutive secretory vesicles from the trans-Golgi network, *Traffic*, 1 (2000) 952-962.

1475 [318] M.C. Gondré-Lewis, H.I. Petrache, C.A. Wassif, D. Harries, A. Parsegian, F.D. Porter, Y.P. Loh,
1476 Abnormal sterols in cholesterol-deficiency diseases cause secretory granule malformation and
1477 decreased membrane curvature, *J. Cell Sci.*, 119 (2006) 1876-1885.

1478 [319] N. Wang, C. Kwan, X. Gong, E.P. de Chaves, A. Tse, F.W. Tse, Influence of cholesterol on
1479 catecholamine release from the fusion pore of large dense core chromaffin granules, *J.*
1480 *Neurosci.*, 30 (2010) 3904-3911.

1481 [320] A. Tse, A.K. Lee, L. Yan, F.W. Tse, Influence of cholesterol on cellular signaling and fusion pore
1482 kinetics, *J. Mol. Neurosci.*, 48 (2012) 395-401.

1483 [321] M.A. Churchward, T. Rogasevskaia, J. Hofgen, J. Bau, J.R. Coorssen, Cholesterol facilitates the
1484 native mechanism of Ca^{2+} -triggered membrane fusion, *J. Cell Sci.*, 118 (2005) 4833-4848.

1485 [322] J. Vanderkooi, S. Fischkoff, B. Chance, R.A. Cooper, Fluorescent probe analysis of the lipid
1486 architecture of natural and experimental cholesterol-rich membranes, *Biochemistry*, 13 (1974)
1487 1589-1595.

1488 [323] R.A. Cooper, M.H. Leslie, S. Fischkoff, M. Shinitzky, S.J. Shattil, Factors influencing the lipid
1489 composition and fluidity of red cell membranes in vitro: production of red cells possessing more
1490 than two cholesterol per phospholipid, *Biochemistry*, 17 (1978) 327-331.

1491 [324] J.M. Crane, L.K. Tamm, Role of cholesterol in the formation and nature of lipid rafts in planar
1492 and spherical model membranes, *Biophys. J.*, 86 (2004) 2965-2979.

1493 [325] D. Scherfeld, N. Kahya, P. Schwille, Lipid dynamics and domain formation in model membranes
1494 composed of ternary mixtures of unsaturated and saturated phosphatidylcholines and
1495 cholesterol, *Biophys. J.*, 85 (2003) 3758-3768.

1496 [326] J. Pan, T.T. Mills, S. Tristram-Nagle, J.F. Nagle, Cholesterol perturbs lipid bilayers
1497 nonuniversally, *Phys. Rev. Lett.*, 100 (2008) 198103.

1498 [327] J.H. Ipsen, O.G. Mouritsen, M.J. Zuckermann, Theory of thermal anomalies in the specific heat
1499 of lipid bilayers containing cholesterol, *Biophys. J.*, 56 (1989) 661-667.

1500 [328] M.B. Sankaram, T.E. Thompson, Modulation of phospholipid acyl chain order by cholesterol. A
1501 solid-state ²H nuclear magnetic resonance study, *Biochemistry*, 29 (1990) 10676-10684.

1502 [329] T. Baumgart, S.T. Hess, W.W. Webb, Imaging coexisting fluid domains in biomembrane models
1503 coupling curvature and line tension, *Nature*, 425 (2003) 821-824.

1504 [330] F. Julicher, R. Lipowsky, Domain-induced budding of vesicles, *Phys. Rev. Lett.*, 70 (1993) 2964-
1505 2967.

1506 [331] A.J. Garcia-Saez, S. Chiantia, P. Schwille, Effect of line tension on the lateral organization of
1507 lipid membranes, *J. Biol. Chem.*, 282 (2007) 33537-33544.

1508 [332] M.A. Hanson, V. Cherezov, M.T. Griffith, C.B. Roth, V.P. Jaakola, E.Y. Chien, J. Velasquez, P.
1509 Kuhn, R.C. Stevens, A specific cholesterol binding site is established by the 2.8 Å structure of the
1510 human beta2-adrenergic receptor, *Structure*, 16 (2008) 897-905.

1511 [333] J. Fantini, F.J. Barrantes, How cholesterol interacts with membrane proteins: an exploration of
1512 cholesterol-binding sites including CRAC, CARC, and tilted domains, *Front . Physiol.*, 4 (2013) 31.

1513 [334] H. Li, Z. Yao, B. Degenhardt, G. Teper, V. Papadopoulos, Cholesterol binding at the cholesterol
1514 recognition/ interaction amino acid consensus (CRAC) of the peripheral-type benzodiazepine
1515 receptor and inhibition of steroidogenesis by an HIV TAT-CRAC peptide, *Proc. Nat'l Acad. Sci. U*
1516 *S A*, 98 (2001) 1267-1272.

1517 [335] C.J. Baier, J. Fantini, F.J. Barrantes, Disclosure of cholesterol recognition motifs in
1518 transmembrane domains of the human nicotinic acetylcholine receptor, *Sci. Rep.*, 1 (2011) 69.

1519 [336] J. Chang, S.A. Kim, X. Lu, Z. Su, S.K. Kim, Y.K. Shin, Fusion step-specific influence of cholesterol
1520 on SNARE-mediated membrane fusion, *Biophys. J.*, 96 (2009) 1839-1846.

1521 [337] C. Enrich, C. Rentero, A. Hierro, T. Grewal, Role of cholesterol in SNARE-mediated trafficking on
1522 intracellular membranes, *J. Cell Sci.*, 128 (2015) 1071-1081.

1523 [338] J.J. Hulce, A.B. Coggnetta, M.J. Niphakis, S.E. Tully, B.F. Cravatt, Proteome-wide mapping of
1524 cholesterol-interacting proteins in mammalian cells, *Nat. Meth.*, 10 (2013) 259-264.

1525 [339] T. Lang, D. Bruns, D. Wenzel, D. Riedel, P. Holroyd, C. Thiele, R. Jahn, SNAREs are concentrated
1526 in cholesterol-dependent clusters that define docking and fusion sites for exocytosis, *EMBO J.*,
1527 20 (2001) 2202-2213.

1528 [340] J.R. Coorssen, R.P. Rand, Effects of cholesterol on the structural transitions induced by
1529 diacylglycerol in phosphatidylcholine and phosphatidylethanolamine bilayer systems, *Biochem.*
1530 *Cell Biol.*, 68 (1990) 65-69.

1531 [341] F.R. Maxfield, G. van Meer, Cholesterol, the central lipid of mammalian cells, *Curr. Opin. Cell*
1532 *Biol.*, 22 (2010) 422-429.

1533 [342] B.S. Stratton, J.M. Warner, Z. Wu, J. Nikolaus, G. Wei, E. Wagnon, D. Baddeley, E. Karatekin, B.
1534 O'Shaughnessy, Cholesterol increases the openness of snare-mediated flickering fusion pores,
1535 *Biophys. J.*, 110 (2016) 1538-1550.

1536 [343] N. Najafinobar, L.J. Mellander, M.E. Kurczy, J. Dunevall, T.B. Angerer, J.S. Fletcher, A.S. Cans,
1537 Cholesterol alters the dynamics of release in protein independent cell models for exocytosis,
1538 *Sci. Rep.*, 6 (2016) 33702.

1539 [344] S.J. Singer, G.L. Nicolson, The fluid mosaic model of the structure of cell membranes, *Science*,
1540 175 (1972) 720-731.

1541 [345] M. Bretou, O. Jouannot, I. Fanget, P. Pierobon, N. Larochette, P. Gestraud, M. Guillon, V.
1542 Emiliani, S. Gasman, C. Desnos, A.M. Lennon-Dumenil, F. Darchen, Cdc42 controls the dilation of
1543 the exocytotic fusion pore by regulating membrane tension, *Mol. Biol. Cell*, 25 (2014) 3195-
1544 3209.

1545 [346] A. Diz-Munoz, D.A. Fletcher, O.D. Weiner, Use the force: membrane tension as an organizer of
1546 cell shape and motility, *Trends Cell Biol.*, 23 (2013) 47-53.

1547 [347] J.R. Monck, G. Alvarez de Toledo, J.M. Fernandez, Tension in secretory granule membranes
1548 causes extensive membrane transfer through the exocytotic fusion pore, *Proc. Nat'l Acad. Sci. U*
1549 *S A*, 87 (1990) 7804-7808.

1550 [348] J.C. Shillcock, R. Lipowsky, Tension-induced fusion of bilayer membranes and vesicles, *Nat.*
1551 *Mater.*, 4 (2005) 225-228.

1552 [349] T.T. Kliesch, J. Dietz, L. Turco, P. Halder, E. Polo, M. Tarantola, R. Jahn, A. Janshoff, Membrane
 1553 tension increases fusion efficiency of model membranes in the presence of SNAREs, *Sci. Rep.*, 7
 1554 (2017) 12070.

1555 [350] P.J. Wen, S. Grenklo, G. Arpino, X. Tan, H.S. Liao, J. Heureaux, S.Y. Peng, H.C. Chiang, E. Hamid,
 1556 W.D. Zhao, W. Shin, T. Nareoja, E. Evergren, et al., Actin dynamics provides membrane tension
 1557 to merge fusing vesicles into the plasma membrane, *Nat. Commun.*, 7 (2016) 12604.

1558 [351] T.J. Melia, T. Weber, J.A. McNew, L.E. Fisher, R.J. Johnston, F. Parlati, L.K. Mahal, T.H. Söllner,
 1559 J.E. Rothman, Regulation of membrane fusion by the membrane-proximal coil of the t-SNARE
 1560 during zippering of SNAREpins, *J. Cell Biol.*, 158 (2002) 929-940.

1561 [352] W. Stelzer, B.C. Poschner, H. Stalz, A.J. Heck, D. Langosch, Sequence-specific conformational
 1562 flexibility of SNARE transmembrane helices probed by hydrogen/deuterium exchange, *Biophys.*
 1563 *J.*, 95 (2008) 1326-1335.

1564 [353] P. Agrawal, S. Kiihne, J. Hollander, M. Hofmann, D. Langosch, H. de Groot, A solid-state NMR
 1565 study of changes in lipid phase induced by membrane-fusogenic LV-peptides, *Biochim. Biophys.*
 1566 *Acta*, 1798 (2010) 202-209.

1567 [354] P. Agrawal, S. Kiihne, J. Hollander, F. Hulsbergen, M. Hofmann, D. Langosch, H. de Groot, Solid
 1568 state NMR investigation of the interaction between biomimetic lipid bilayers and de novo
 1569 designed fusogenic peptides, *Chembiochem*, 8 (2007) 493-496.

1570 [355] S.E. Kahn, R.L. Hull, K.M. Utzschneider, Mechanisms linking obesity to insulin resistance and
 1571 type 2 diabetes, *Nature*, 444 (2006) 840-846.

1572 [356] T.M. Frayling, N.J. Timpson, M.N. Weedon, E. Zeggini, R.M. Freathy, C.M. Lindgren, J.R. Perry,
 1573 K.S. Elliott, H. Lango, N.W. Rayner, B. Shields, L.W. Harries, J.C. Barrett, et al., A common variant
 1574 in the FTO gene is associated with body mass index and predisposes to childhood and adult
 1575 obesity, *Science*, 316 (2007) 889-894.

1576 [357] G. Boden, Interaction between free fatty acids and glucose metabolism, *Curr. Opin. Clin. Nutr.*
 1577 *Metab. Care*, 5 (2002) 545-549.

1578 [358] T.P. Williams, R. McGee, Jr., The effects of membrane fatty acid modification of clonal
 1579 pheochromocytoma cells on depolarization-dependent exocytosis, *J. Biol. Chem.*, 257 (1982)
 1580 3491-3500.

1581 [359] M.B. Hoppa, S. Collins, R. Ramracheya, L. Hodson, S. Amisten, Q. Zhang, P. Johnson, F.M.
 1582 Ashcroft, P. Rorsman, Chronic palmitate exposure inhibits insulin secretion by dissociation of
 1583 Ca^{2+} channels from secretory granules, *Cell Metab.*, 10 (2009) 455-465.

1584 [360] K. Pinnick, M. Neville, A. Clark, B. Fielding, Reversibility of metabolic and morphological
1585 changes associated with chronic exposure of pancreatic islet beta-cells to fatty acids, *J. Cell*
1586 *Biochem.*, 109 (2010) 683-692.

1587 [361] H.N. Ginsberg, Diabetic dyslipidemia: basic mechanisms underlying the common
1588 hypertriglyceridemia and low HDL cholesterol levels, *Diabetes*, 45 Suppl 3 (1996) S27-30.

1589 [362] M. Hao, W.S. Head, S.C. Gunawardana, A.H. Hasty, D.W. Piston, Direct effect of cholesterol on
1590 insulin secretion: a novel mechanism for pancreatic beta-cell dysfunction, *Diabetes*, 56 (2007)
1591 2328-2338.

1592 [363] J.S. Bogan, Y. Xu, M. Hao, Cholesterol accumulation increases insulin granule size and impairs
1593 membrane trafficking, *Traffic*, 13 (2012) 1466-1480.

1594 [364] M. Hao, J.S. Bogan, Cholesterol regulates glucose-stimulated insulin secretion through
1595 phosphatidylinositol 4,5-bisphosphate, *J. Biol. Chem.*, 284 (2009) 29489-29498.

1596 [365] L.R. Brunham, J.K. Kruit, T.D. Pape, J.M. Timmins, A.Q. Reuwer, Z. Vasanji, B.J. Marsh, B.
1597 Rodrigues, J.D. Johnson, J.S. Parks, C.B. Verchere, M.R. Hayden, Beta-cell ABCA1 influences
1598 insulin secretion, glucose homeostasis and response to thiazolidinedione treatment, *Nat. Med.*,
1599 13 (2007) 340-347.

1600 [366] J.K. Kruit, N. Wijesekara, J.E. Fox, X.Q. Dai, L.R. Brunham, G.J. Searle, G.P. Morgan, A.J. Costin,
1601 R. Tang, A. Bhattacharjee, J.D. Johnson, P.E. Light, B.J. Marsh, et al., Islet cholesterol
1602 accumulation due to loss of ABCA1 leads to impaired exocytosis of insulin granules, *Diabetes*, 60
1603 (2011) 3186-3196.

1604 [367] F. Xia, L. Xie, A. Mihic, X. Gao, Y. Chen, H.Y. Gaisano, R.G. Tsushima, Inhibition of cholesterol
1605 biosynthesis impairs insulin secretion and voltage-gated calcium channel function in pancreatic
1606 beta-cells, *Endocrinology*, 149 (2008) 5136-5145.

1607 [368] Y. Xu, D.K. Toomre, J.S. Bogan, M. Hao, Excess cholesterol inhibits glucose-stimulated fusion
1608 pore dynamics in insulin exocytosis, *J. Cell. Mol. Med.*, (2017).

1609 [369] V. Chandra, O. Albagli-Curiel, B. Hastoy, J. Piccand, C. Randriamampita, E. Vaillant, H. Cave, K.
1610 Busiah, P. Froguel, M. Vaxillaire, P. Rorsman, M. Polak, R. Scharfmann, RFX6 regulates insulin
1611 secretion by modulating Ca²⁺ homeostasis in human beta cells, *Cell Rep.*, 9 (2014) 2206-2218.

1612 [370] H. Hatakeyama, T. Kishimoto, T. Nemoto, H. Kasai, N. Takahashi, Rapid glucose sensing by
1613 protein kinase A for insulin exocytosis in mouse pancreatic islets, *J. Physiol.*, 570 (2006) 271-
1614 282.

1615 [371] A. Tengholm, Cyclic AMP dynamics in the pancreatic beta-cell, *Ups. J. Med. Sci.*, 117 (2012)
1616 355-369.

1617 [372] M. Dubois, P. Vacher, B. Roger, D. Huyghe, B. Vandewalle, J. Kerr-Conte, F. Pattou, N.
1618 Moustaid-Moussa, J. Lang, Glucotoxicity inhibits late steps of insulin exocytosis, *Endocrinology*,
1619 148 (2007) 1605-1614.

1620 [373] X. Kong, D. Yan, X. Wu, Y. Guan, X. Ma, Glucotoxicity inhibits cAMP-protein kinase A-
1621 potentiated glucose-stimulated insulin secretion in pancreatic beta-cells, *J. DiabD*, 7 (2015) 378-
1622 385.

1623 [374] B. Roger, J. Papin, P. Vacher, M. Raoux, A. Mulet, M. Dubois, J. Kerr-Conte, B.H. Voy, F. Pattou,
1624 G. Charpentier, J.C. Jonas, N. Moustaid-Moussa, J. Lang, Adenylyl cyclase 8 is central to
1625 glucagon-like peptide 1 signalling and effects of chronically elevated glucose in rat and human
1626 pancreatic beta cells, *Diabetologia*, 54 (2011) 390-402.

1627 [375] M.Z. Khaldi, Y. Guiot, P. Gilon, J.C. Henquin, J.C. Jonas, Increased glucose sensitivity of both
1628 triggering and amplifying pathways of insulin secretion in rat islets cultured for 1 wk in high
1629 glucose, *Am J. Physiol. Endocrinol. Metab.*, 287 (2004) E207-217.

1630 [376] X.Q. Dai, J. Kolic, P. Marchi, S. Sipione, P.E. Macdonald, SUMOylation regulates Kv2.1 and
1631 modulates pancreatic beta-cell excitability, *J. Cell Sci.*, 122 (2009) 775-779.

1632 [377] X.Q. Dai, G. Plummer, M. Casimir, Y. Kang, C. Hajmrle, H.Y. Gaisano, J.E. Manning Fox, P.E.
1633 MacDonald, SUMOylation regulates insulin exocytosis downstream of secretory granule docking
1634 in rodents and humans, *Diabetes*, 60 (2011) 838-847.

1635 [378] M. Ferdaoussi, X. Dai, M.V. Jensen, R. Wang, B.S. Peterson, C. Huang, O. Ilkayeva, N. Smith, N.
1636 Miller, C. Hajmrle, A.F. Spigelman, R.C. Wright, G. Plummer, et al., Isocitrate-to-SEN1 signaling
1637 amplifies insulin secretion and rescues dysfunctional beta cells, *J. Clin. Invest.*, 125 (2015) 3847-
1638 3860.

1639 [379] M. Ferdaoussi, J. Fu, X. Dai, J.E. Manning Fox, K. Suzuki, N. Smith, G. Plummer, P.E. MacDonald,
1640 SUMOylation and calcium control syntaxin-1A and secretagogin sequestration by tomosyn to
1641 regulate insulin exocytosis in human ss cells, *Sci. Rep.*, 7 (2017) 248.

1642 [380] M. Ferdaoussi, P.E. MacDonald, Toward Connecting Metabolism to the Exocytotic Site, *Trends*
1643 *Cell Biol.*, 27 (2017) 163-171.

1644 [381] Z. Ling, D.G. Pipeleers, Prolonged exposure of human beta cells to elevated glucose levels
1645 results in sustained cellular activation leading to a loss of glucose regulation, *J. Clin. Invest.*, 98
1646 (1996) 2805-2812.

1647 [382] Z. Ling, R. Kiekens, T. Mahler, F.C. Schuit, M. Pipeleers-Marichal, A. Sener, G. Kloppel, W.J.
1648 Malaisse, D.G. Pipeleers, Effects of chronically elevated glucose levels on the functional
1649 properties of rat pancreatic beta-cells, *Diabetes*, 45 (1996) 1774-1782.

1650 [383] E. Roche, F. Assimacopoulos-Jeannet, L.A. Witters, B. Perruchoud, G. Yaney, B. Corkey, M.
1651 Asfari, M. Prentki, Induction by glucose of genes coding for glycolytic enzymes in a pancreatic
1652 beta-cell line (INS-1), *J. Biol. Chem.*, 272 (1997) 3091-3098.

1653 [384] L.F. Waanders, K. Chwalek, M. Monetti, C. Kumar, E. Lammert, M. Mann, Quantitative
1654 proteomic analysis of single pancreatic islets, *Proc. Nat'l Acad. of Sci. USA*, 106 (2009) 18902-
1655 18907.

1656 [385] H.Y. Gaisano, C.G. Ostenson, L. Sheu, M.B. Wheeler, S. Efendic, Abnormal expression of
1657 pancreatic islet exocytotic soluble N-ethylmaleimide-sensitive factor attachment protein
1658 receptors in Goto-Kakizaki rats is partially restored by phlorizin treatment and accentuated by
1659 high glucose treatment, *Endocrinology*, 143 (2002) 4218-4226.

1660 [386] B. Torrejon-Escribano, J. Escoriza, E. Montanya, J. Blasi, Glucose-dependent changes in SNARE
1661 protein levels in pancreatic beta-cells, *Endocrinology*, 152 (2011) 1290-1299.

1662 [387] H. Lu, Y. Yang, E.M. Allister, N. Wijesekara, M.B. Wheeler, The identification of potential factors
1663 associated with the development of type 2 diabetes: a quantitative proteomics approach, *Mol.*
1664 *Cell. Prot.*, 7 (2008) 1434-1451.

1665 [388] T. Daraio, L.K. Bombek, M. Gosak, I. Valladolid-Acebes, M.S. Klemen, E. Refai, P.O. Berggren, K.
1666 Brismar, M.S. Rupnik, C. Bark, SNAP-25b-deficiency increases insulin secretion and changes
1667 spatiotemporal profile of Ca²⁺ oscillations in beta cell networks, *Sci. Rep.*, 7 (2017) 7744.

1668 [389] C.G. Ostenson, H. Gaisano, L. Sheu, A. Tibell, T. Bartfai, Impaired gene and protein expression
1669 of exocytotic soluble N-ethylmaleimide attachment protein receptor complex proteins in
1670 pancreatic islets of type 2 diabetic patients, *Diabetes*, 55 (2006) 435-440.

1671 [390] E. Ottosson-Laakso, U. Krus, P. Storm, R.B. Prasad, N. Oskolkov, E. Ahlqvist, J. Fadista, O.
1672 Hansson, L. Groop, P. Vikman, Glucose-induced changes in gene expression in human pancreatic
1673 islets - causes or consequences of chronic hyperglycemia, *Diabetes*, (2017). doi: 10.2337/db17-
1674 0311. [Epub ahead of print]

1675 [391] P.M. Visscher, M.A. Brown, M.I. McCarthy, J. Yang, Five years of GWAS discovery, *Am. J. Hum.*
1676 *Genet.*, 90 (2012) 7-24.

1677 [392] M.I. McCarthy, Genomics, type 2 diabetes, and obesity, *N. Engl. J. Med.*, 363 (2010) 2339-2350.

1678 [393] A. Bonnefond, P. Froguel, Rare and common genetic events in type 2 diabetes: what should
1679 biologists know?, *Cell Metab.*, 21 (2015) 357-368.

1680 [394] M. van de Bunt, J.E. Manning Fox, X. Dai, A. Barrett, C. Grey, L. Li, A.J. Bennett, P.R. Johnson,
1681 R.V. Rajotte, K.J. Gaulton, E.T. Dermitzakis, P.E. MacDonald, M.I. McCarthy, et al., Transcript
1682 expression data from human islets links regulatory signals from genome-wide association
1683 studies for type 2 diabetes and glycemic traits to their downstream effectors, *PLoS Genet.*, 11
1684 (2015) e1005694.

1685 [395] A. Ragvin, E. Moro, D. Fredman, P. Navratilova, O. Drivenes, P.G. Engstrom, M.E. Alonso, E. de
1686 la Calle Mustienes, J.L. Gomez Skarmeta, M.J. Tavares, F. Casares, M. Manzanares, V. van
1687 Heyningen, et al., Long-range gene regulation links genomic type 2 diabetes and obesity risk
1688 regions to HHEX, SOX4, and IRX3, *Proc. Nat'l Acad. Sci. U S A*, 107 (2010) 775-780.

1689 [396] S.K. Thomsen, A.L. Gloyn, The pancreatic beta cell: recent insights from human genetics,
1690 *Trends Endocrinol. Metab.*, 25 (2014) 425-434.

1691 [397] C. Fuchsberger, J. Flannick, T.M. Teslovich, A. Mahajan, V. Agarwala, K.J. Gaulton, C. Ma, P.
1692 Fontanillas, L. Moutsianas, D.J. McCarthy, M.A. Rivas, J.R. Perry, X. Sim, et al., The genetic
1693 architecture of type 2 diabetes, *Nature*, 536 (2016) 41-47.

1694 [398] A.S. Dimas, V. Lagou, A. Barker, J.W. Knowles, R. Magi, M.F. Hivert, A. Benazzo, D. Rybin, A.U.
1695 Jackson, H.M. Stringham, C. Song, A. Fischer-Rosinsky, T.W. Boesgaard, et al., Impact of type 2
1696 diabetes susceptibility variants on quantitative glycemic traits reveals mechanistic
1697 heterogeneity, *Diabetes*, 63 (2014) 2158-2171.

1698 [399] J. Fadista, P. Vikman, E.O. Laakso, I.G. Mollet, J.L. Esguerra, J. Taneera, P. Storm, P. Osmark, C.
1699 Ladenvall, R.B. Prasad, K.B. Hansson, F. Finotello, K. Uvebrant, et al., Global genomic and
1700 transcriptomic analysis of human pancreatic islets reveals novel genes influencing glucose
1701 metabolism, *Proc. Nat'l Acad. Sci. U S A*, 111 (2014) 13924-13929.

1702 [400] E. Zeggini, M.N. Weedon, C.M. Lindgren, T.M. Frayling, K.S. Elliott, H. Lango, N.J. Timpson, J.R.
1703 Perry, N.W. Rayner, R.M. Freathy, J.C. Barrett, B. Shields, A.P. Morris, et al., Replication of
1704 genome-wide association signals in UK samples reveals risk loci for type 2 diabetes, *Science*, 316
1705 (2007) 1336-1341.

1706 [401] M.I. McCarthy, G.R. Abecasis, L.R. Cardon, D.B. Goldstein, J. Little, J.P. Ioannidis, J.N.
1707 Hirschhorn, Genome-wide association studies for complex traits: consensus, uncertainty and
1708 challenges, *Nat. Rev. Genet.*, 9 (2008) 356-369.

- 1709 [402] S. Deng, M. Vatamaniuk, X. Huang, N. Doliba, M.M. Lian, A. Frank, E. Velidedeoglu, N.M. Desai,
1710 B. Koeberlein, B. Wolf, C.F. Barker, A. Naji, F.M. Matschinsky, et al., Structural and functional
1711 abnormalities in the islets isolated from type 2 diabetic subjects, *Diabetes*, 53 (2004) 624-632.
- 1712 [403] M. Masini, L. Marselli, M. Bugliani, L. Martino, P. Masiello, P. Marchetti, V. De Tata,
1713 Ultrastructural morphometric analysis of insulin secretory granules in human type 2 diabetes,
1714 *Acta Diabetol.*, 49 Suppl 1 (2012) S247-252.
- 1715 [404] M.J. Betts, R.B. Russell, Amino acid properties and consequences of substitutions, in: M.R.
1716 Barnes, I.C. Gray (Eds.) *Bioinformatics for Geneticists*, John Wiley & Sons, New York, 2003.
- 1717 [405] E. Fdez, M. Martinez-Salvador, M. Beard, P. Woodman, S. Hilfiker, Transmembrane-domain
1718 determinants for SNARE-mediated membrane fusion, *J. Cell Sci.*, 123 (2010) 2473-2480.
- 1719 [406] Z. Wu, S.M. Auclair, O. Bello, W. Vennekate, N.R. Dudzinski, S.S. Krishnakumar, E. Karatekin,
1720 Nanodisc-cell fusion: control of fusion pore nucleation and lifetimes by SNARE protein
1721 transmembrane domains, *Sci. Rep.*, 6 (2016) 27287.

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FIGURES AND LEGENDS

FIGURE 1

Figure 1: Insulin exocytosis in rodent pancreatic β -cells. Note the presence of immature, mainly pro-insulin containing secretory granules (white arrows) and of mature secretory granules (arrowheads) characterized by a denser core due to proinsulin conversion to insulin and subsequent crystallisation. A vesicle fused with the plasma membrane is visible on the upper left with the typical omega configuration (Ω) of the fusion pore, already considerably enlarged here. Note that the core is less dense than in non-fused mature granules indicating partial solvation of the insulin hexamers.

FIGURE 2

Figure 2: Exocytosis proceeds through distinct steps. **A**, General view of SNARE-mediated exocytosis. The v-SNARE (VAMP2) on the vesicle forms a complex with the t-SNAREs (syntaxin 1, SNAP-25) on the plasma membrane followed by zippering of the complex and final opening of a fusion pore. **B and C**, detailed membrane centred view of final steps during exocytosis. **B**: Membrane dimpling at future contact sites, hemifusion of apposed bilayer sheets and eventually radial extension of the stalk to a diaphragm. **C**, Generation of stalk or hemidiaphragm may result in fusion pore opening and expansion, which is a reversible process up to a certain radius of the pore.

FIGURE 3

Figure 3: Reduction of insulin secretion through the increase of kiss and run event. A. Fate of vesicles during full fusion (left) or kiss-and-run events (right). Note that in kiss-and-run exocytosis, insulin remains trapped inside the granule. Incubation with Ruthenium red (5 min) stains the plasma membrane but not the vesicular membrane that have never reached the cell surface beforehand. Membranes positive for Ruthenium Red appear more electron dense (thicker and darker). Vesicles that have undergone kiss-and-run exocytosis will be positive for both insulin and Ruthenium red. **B.** Electron micrographs of islets incubated with Ruthenium red. Sox4mut β -cells contain more vesicles positive for both insulin and ruthenium red (arrowhead) than control cells [40]. Abbreviations: M, mitochondrion; PM, plasma membrane; G, Golgi apparatus; ER., endoplasmic reticulum; Ins. Ves., Insulin Vesicle. Scale bar: 1µm.

1762

1763 **Fig. 4 Transmembrane domains of VAMP/synaptobrevin (R-SNARE) and syntaxin (Q-**

1764 **SNARE). A.** General architecture of VAMP/Synaptobrevin (VAMP) and Syntaxin (STX) with cytosolic

1765 N-terminal regulatory domain (not present in all isoforms), SNARE motif, juxtamembrane domain

1766 (JMD) and transmembrane domain (TMD). **B and C:** Peptide acid sequence of the TMDs in VAMP (B)

1767 and syntaxin (C) isoforms. Only the isoforms with a clearly identifiable TMD are displayed. Note that

1768 the R-SNARE Ykt6 [277] as well as the Q-SNAREs STX9/19 and STX11 do not possess a predictable

1769 TMD sequence but multiple cysteines that may be prenylated. Classification of amino acids

1770 according to Betts and Russell [404] is given on the image high-lighting C_β-branched (in yellow),

1771 hydrophobic, tiny (blue) or polar amino (red dot) acids. Note that Threonine, although slightly polar,

1772 has been classified along with isoleucine and valine according to its nature as a C_β-branched amino

1773 acid (and thus form α-helices with difficulty). The following human protein sequences were used:

1774 NP_055046 (VAMP1, isoform 1), NP_055047 (VAMP2, isoform 1), NP_004772 (VAMP3), NP_003753

1775 (VAMP4), NP_006625 (VAMP5), NP_001138621 (VAMP7), NP_003752 (VAMP8), NP_004883.3

1776 (SEC22B), NM_052874.4 (STX1), AAH47496.1 (STX2), AAH07429 (STX3), AAH02436 (STX4),

1777 AAH12137 (STX5), AAH09944 (STX6), AAH11975 (STX7), NP_004844 (STX8), AAC05087 (STX10),

1778 NP_803173 (STX12), AAC05647 (STX16).

FIGURE 5

Fig. 5 Synopsis of mutational analysis of transmembrane domains of VAMP2 and syntaxin

1. Given are the TMDs of VAMP2 and syntaxin 1 (STX1) with colour codes as in Fig. 4 except for orange background which denotes charged residues. Note that only most prominent results of different studies are depicted. Black bars and grey bars, homodimerisation sites in VAMP2 based on *in vitro* and *in silico* data [251, 254-257, 405] where grey bars indicate cholesterol sensitive changes [218], blue or red arrows denote increased fusion or accelerated kinetics vs decreased flux or slower kinetics, respectively. Arrows between VAMP2 and syntaxin 1 sequence indicate contact sites derived from crystallography of wild-type proteins [213]. The numbers (1)-(6) correlate with functional effects as follows: (1) fusion pore conductance in nanodisc-cell fusion (multiple point mutations) [406]; (2) amperometric foot amplitude and pore conductances (single and multiple point mutations) [281, 296]; (3) amperometric prespike foot amplitude and rise time using “en bloc” mutations [246]; (4) hormone secretion and fusion pore kinetics in clonal INS-1 β -cells (single and double point mutations) [234]; (5) polar solvent accessibility (blue circles) [286]; and (6) amperometric determination of foot current in pheochromocytoma PC12 cells (single point mutations) [282, 283]. Note the absence of in-vitro structural determinations of mutants except for (4).

Fig. 6 SNARE TMD and lipids in hemifusion prior to fusion pore opening. The combined action of cholesterol in bending and rigidifying the membrane and the structural dynamics of VAMP2 TMD (kinked at G₁₀₀) on membrane properties may initiate the lipid rearrangement (initiation point) and the opening of the fusion pore. Note that this represents a static 2D view of a swift 3D event. The morphology of the membrane may define subregions where different actors (protein/lipids) would have complementary functions on membrane structure and lipid rearrangement. Beyond *in-silico* modelling, temporal and spatial characterisations of these intermediate steps are not established and remain technically challenging.