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An overview of the synaptic vesicle lipid composition

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ABSTRACT

Chemical neurotransmission is the major mechanism of neuronal communication. Neurotransmitters are released from secretory organelles, the synaptic vesicles (SVs) via exocytosis into the synaptic cleft. Fusion of SVs with the presynaptic plasma membrane is balanced by endocytosis, thus maintaining the presynaptic membrane at steady-state levels. The protein machineries responsible for exo- and endocytosis have been extensively investigated. In contrast, less is known about the role of lipids in synaptic transmission and how the lipid composition of SVs is affected by dynamic exo-endocytotic cycling. Here we summarize the current knowledge about the composition, organization, and function of SV membrane lipids. We also cover lipid biogenesis and maintenance during the synaptic vesicle cycle.

1. Introduction

Our nervous system plays a central role in the organization, and regulation of all of our body functions in response to the different external and internal stimuli [1]. Neurons are the basic cellular units of our nervous system. They are highly interconnected and communicate with each other and with effector cells in a process termed neurotransmission. Neurotransmission occurs at the level of synapses, which are specialized cell-cell contact sites [2,3] where electrical information is converted into chemical information in the form of signalling molecules called neurotransmitters [4,5]. In presynaptic nerve terminals, these neurotransmitters are packed into secretory vesicles, called synaptic vesicles (SVs). Upon arrival of an electrical signal, SVs release their content by exocytosis into the synaptic cleft. The neurotransmitters then bind to receptors at the postsynaptic site where the chemical signal is reconverted into an electrical signal [2,6] (Fig. 1).

Neurotransmitter release by exocytosis is triggered by the local increase of intracellular calcium. In most synapses, it involves the fusion of SVs with a specialized region of the presynaptic plasma membrane termed active zones (AZs) [7,8]. To prevent presynaptic membrane expansion and SV depletion, exocytosis is tightly coupled with membrane retrieval by endocytosis [5]. These two processes are spatially segregated and temporally regulated, assuring the maintenance of synapse morphology and functionality. While we have detailed information of the protein machinery that regulates the synaptic vesicle cycle, much less is known about the functional role of the lipids involved in this process [9–12].

In this short review, we summarize the current knowledge about the composition and role of synaptic vesicle lipids, starting from their biogenesis up to their mature/final composition. We will focus on: (I) the role of lipids in neurotransmitter release, (II) their interaction with the proteins of the exo- and endocytosis machinery and (III) their regulatory role in synaptic vesicle recycling.

2. Synaptic vesicle biogenesis and lipid composition

Neurons contain two primary classes of regulated secretory vesicles, large dense core vesicles (LDCVs) and synaptic vesicles (SVs). LDCVs store neuropeptide transmitters whereas SVs exclusively contain "classical" neurotransmitters including a small group of low molecular weight metabolites [13–15]. While LDCVs are loaded with neuropeptides during their formation at the trans-Golgi network (TGN), SVs obtain their cargo by active transport across the membrane directly at synapse. While SVs also originate from the TGN, functional SVs can therefore be formed and regenerated within the presynaptic nerve terminal without being dependent on membrane traffic from the endoplasmic reticulum (ER) and the Golgi apparatus [13,16]. The different steps of selection and transport of SV lipids and proteins from the ER to

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the synapse are still elusive. One hypothesis is that SV proteins and lipids form a proto-SV domain on the ER and/or the Golgi membrane [5] (Fig. 2). Thus, proteins and lipids first diffuse freely into the ER and/or the Golgi apparatus, where they interact with different proteins and/or lipids of the proto-SV domain, restricting their diffusion and thereby forming microdomains. In this context, it is noteworthy to mention that lipids are not homogeneously distributed throughout the ER and Golgi apparatus [17]. Phosphatidylserine (PS), the most abundant anionic phospholipid (PL), is strongly enriched in the luminal leaflet on the ER [18], while it is exposed on the cytosolic side of the TGN [19,20]. Sphingolipids (SPs) and cholesterol (CHOL) are highly enriched in the TGN and the endosomal system [21,22], and CHOL is required for the formation of regulated secretory vesicles from the TGN [23]. The process of vesicle precursor formation from the TGN is poorly understood, but budding from the TGN generally requires different adaptor and coat proteins [24]. After scission from the TGN, synaptic vesicle precursors are directly transported along the axon to the nerve terminal using motor proteins and a cytoskeleton pathway made up of microtubules and actin [16,25] (Fig. 2).

The lipid composition of SVs has been under investigation ever since protocols for purifying SVs were established in the seventies [26–28] and subsequently improved in the eighties [29–32]. However, it was not until the development of quantitative mass spectrometry techniques that it was possible to quantify membrane lipids of SVs more accurately and to resolve the composition of their fatty acyl chains [33–36]. Using electrospray ionization tandem mass spectrometry (ESI-MS/MS), Takamori et al. published a quantitative lipidomic analysis of mammalian synaptic vesicles [37]. Overall the results were comparable to previous measurements involving thin-layer chromatography (Table 1) [26,30]. Accordingly, mammalian synaptic vesicles contain high levels of cholesterol (CHOL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM) contributing 40 mol%, 17 mol%, 20 mol%, 6 mol% and 3.6 mol% respectively (Fig. 3A). Remarkable features include a very high contribution of CHOL, a low amount of total phosphatidylinositol (PIns) (contrasting with the higher amount of phosphatidylinositol-4,5-bisphosphate (PIns $(4,5)P_2$) in the plasma membrane [38,39]), and an unusually high degree of highly unsaturated fatty acyl chains. In SV, CHOL/PL molar ratio is around 0.8, in contrast to other cell organelles as ER, mitochondrion, late endosomes and plasma membrane with a CHOL/PL molar ratio of 0.15, 0.1, 0.5 and 1 respectively. Additionally, SVs present higher amount of PE compare to all the above cell organelles, while the rest of main PLs are quite similar [17,37].

Importantly, while SVs can be purified to very high homogeneity [37,40,41], other purified organelles and organellar membranes are heterogeneous and not pure, making the overall lipid composition of an organelle only of limited use [42].

Despite the progress in lipidomics [43–46], Takamori's work still remains the major reference for the synaptic vesicle lipid composition.

3. Function of synaptic vesicle lipids

Synaptic vesicle lipids play two main roles at synapses: recruit and regulate the components of the exo- and endocytotic machinery, and define membrane biophysical parameters such curvature, fluidity, and thickness.

3.1. Controlling membrane biophysical parameters

Membrane fusion and its reversal, membrane fission, are associated with major changes in membrane architecture involving transitions between flat and highly curved membrane states and non-bilayer intermediates (referred to as fusion stalks) (Fig. 4C) whose stability is greatly influenced by the physical properties of the participating membrane lipids [47]. Parameters affecting these properties include size and charge of the polar headgroups as well as structure, packaging and length of the hydrophobic tail domains. Moreover, the relative space requirement of the hydrophilic and hydrophobic domains (grossly characterized as cylindrical, cone- and inverted cone-shaped) (Fig. 4A) determine the propensity of the membrane lipids to form bilayers versus

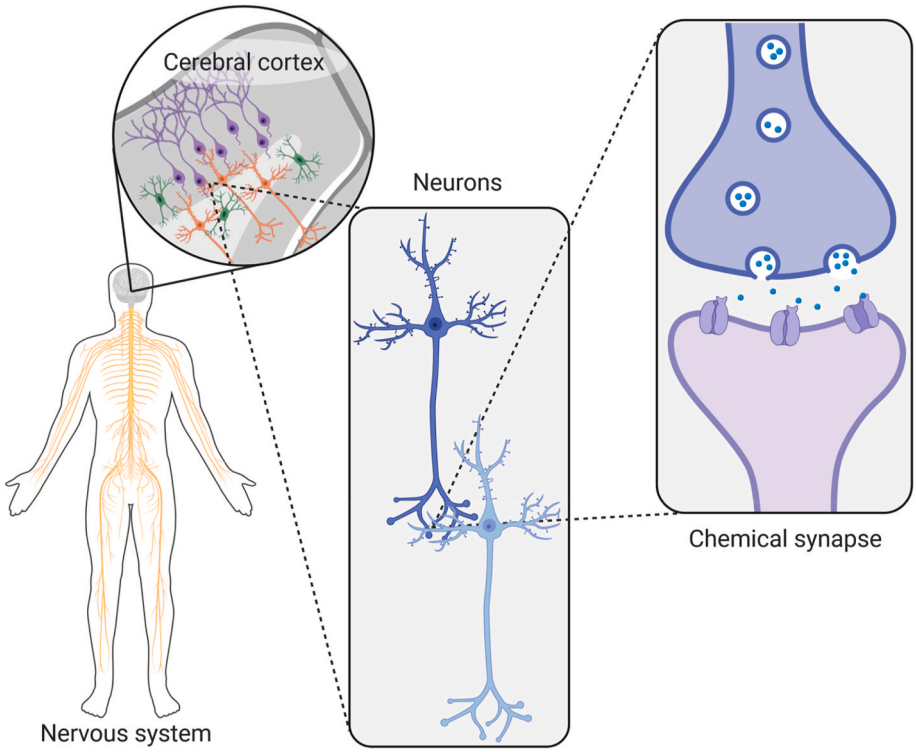

Fig. 1. Structure of the nervous system. Main components of the nervous system showing the synaptic transmission between neurons (created with BioRender.com).

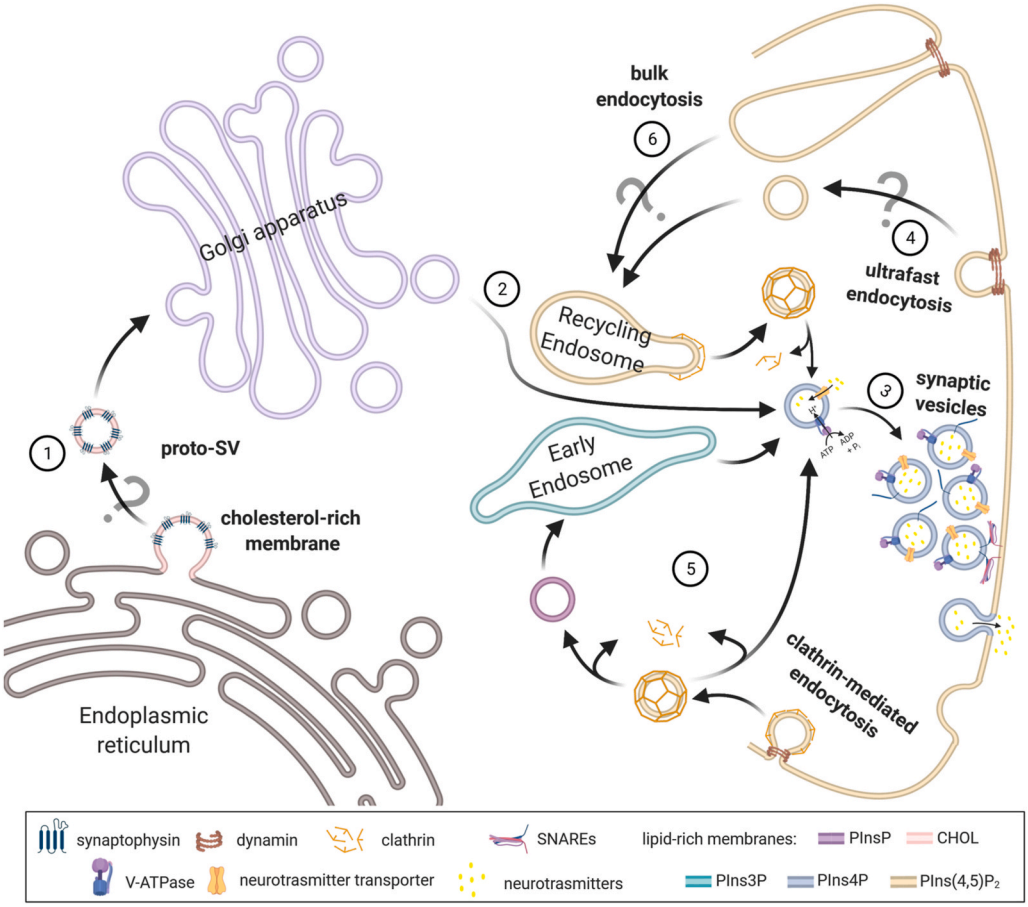

Fig. 2. Overview of synaptic vesicle biogenesis.
Proto-synaptic vesicles (proto-SV) could be generated in the endoplasmic reticulum (ER) in cholesterol/Synaptophysin specific domains. Then, proto-SVs bud and fuse with the Golgi apparatus (1). Here they are sorted and subsequently bud from the trans
-Golgi network (TGN) and transported to the synaptic terminal (2). The new SVs filled with neurotransmitter remain and dock at the active zone (3). After fusion, the endocytosis machinery removes SV-sized chunks, and recycles the lipid-protein components of the SVs through different pathways: ultrafast endocytosis (4), Clathrin-mediated endocytosis (CME) (5), and bulk endocytosis (6), generating a new uncoated synaptic vesicle. Question marks mean processes not well characterized. (created with BioRender.com).

Table 1
Lipid composition of synaptic vesicles (SVs).

	Takamori et al.	Deutsch et Kelly	Nagy et al.
Phosphatidylcholine (PC)	~17	~32	~25
Phosphatidylethanolamine (PE)	~20	~20	~20
Phosphatidylserine (PS)	~6	~6	~10
Phosphatidylinositol (PIns)	~1	~2	~4
Sphingomyelin (SM)	~4	~3	~5
Cholesterol (CHOL)	~40	~33	~33

Values of the main SV lipids are calculated by inference considering data from Takamori et al. [37], Deutsch et Kelly [30], and Nagy et al. [26] and expressed by mol percent.

hexagonal phases in an aqueous environment and thus their propensity to support non-bilayer intermediates during fusion and fission [47–49] (Fig. 4C).

CHOL, the most abundant SV lipid, has a major impact on physical properties of the membrane such as membrane fluidity, thickness and curvature, which are parameters critically influencing the energy landscape of membrane fusion and budding pathways [23,50–52]. Similarly, the SV PLs contribute to the spontaneous curvature of the membrane due to the differences in their molecular shape [46]. Thus, cylinder shaped PLs, as PC and PS (Fig. 4A), assemble into planar bilayers [53], while cone-shaped PLs, such as PE, can facilitate membrane fusion by promoting negative curvature in the cytosolic membrane leaflets during exocytosis (Fig. 4A and C) [47,48,54]. Indeed, SV recycling involves transient and rapid major changes in the membrane curvature, particularly when considering that SVs belong to the smallest and thus most highly curved membrane vesicles in mammalian cells.

Although less abundant, other SV lipids have been suggested to play a role in exo- and endocytosis because of their molecular shape. Thus, phosphatidic acid (PA) and diacylglycerol (DAG) (Figs. 3A and 4A), produced by phospholipase D (PLD) and phospholipase C (PLC) respectively (Fig. 4B), might promote synaptic vesicle exocytosis by influencing the spontaneous curvature of membranes [55], similar to PE [46,56]. Indeed, PA presents local accumulation at the plasma membrane at the AZs [57]. By the contrary, ceramide (Fig. 4A) has been suggested to modulate endocytosis, although this hypothesis is in conflict with observations were reduced ceramide content showed only a mild reduction in synaptic vesicle pool size and a minor endocytosis impairment but a relatively strong defect in synaptic transmission. This suggests that ceramide reduction causes exocytosis defects rather than affecting SV endocytosis and recycling [58–60]. Additionally, phosphoinositides (PIs), gangliosides, and lyso-PC, which present inverted cone-shaped lipids (Fig. 4A) [46,53], would promote fusion favoring positive curvature on the luminal/extracellular membrane leaflet [56,61,62] (Fig. 4C).

Finally, it is noteworthy to mention that lipids have diverse fatty acyl chains, which vary in saturation and length. Poly-unsaturated fatty acids (PUFAs) are enriched in the presynaptic plasma membrane [63,64] (Fig. 3B), and are particularly abundant in synaptic vesicles [37]. They stabilize the membrane of synaptic vesicles, which is highly curved because of their small diameter minimizing the exposure of hydrophobic patches. Additionally, PUFA-containing lipids directly facilitate membrane fusion because their acyl chains have high conformational flexibility and thus probably lower the energy barriers of the highly curved transition states [65].

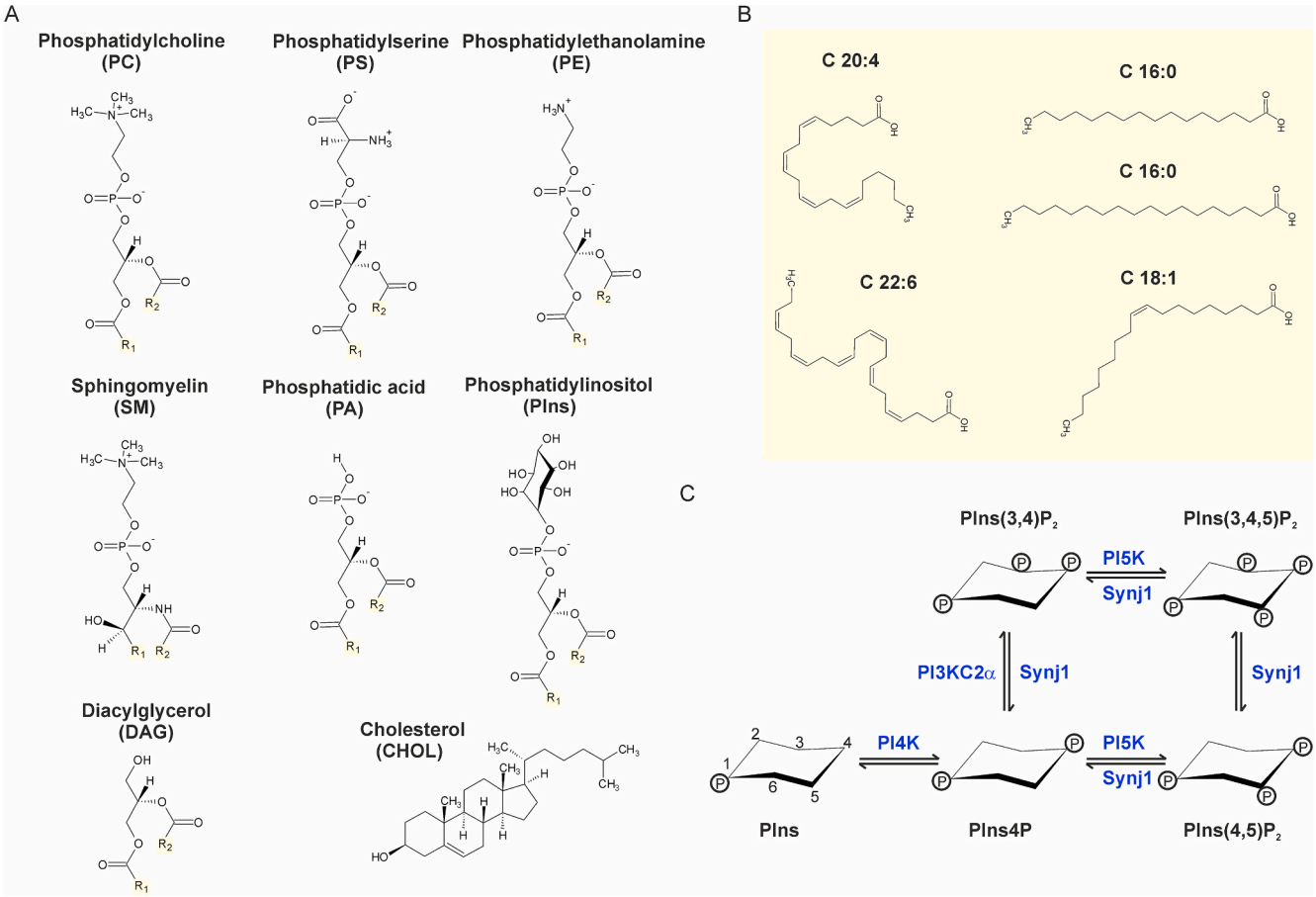

Fig. 3. Major synaptic vesicle lipid structures.
(A) Synaptic vesicle membrane consists primarily of phospholipids (PLs), of which the most abundant are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cholesterol (CHOL). PLs can present different lipid chains in positions R_1 and R_2 (in yellow) that define their hydrophobic portion. (B) Fatty acid chains vary in length, saturation state and configuration, which define the shape and flexibility of a phospholipid. (C) Phosphorylated head groups of different phosphoinositides (PIs) involved in synaptic vesicle biogenesis and recycling. These lipids are interconverted by specific kinases and phosphatases at the synapse (created with ACD/ChemSketch, version 2020.1.2, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2020). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Recruiting and regulating the exo- and endocytotic machinery

Presynaptic terminals are specialized for rapid exo-endocytotic vesicle recycling, thus continuously undergoing major remodeling of the membranes. Therefore lipid-protein interactions must be precisely regulated in space and time to allow for proper synaptic vesicle docking, priming, fusion at the AZ and subsequent endocytosis.

The contribution of lipids to the spatial organization of SV release sites is still not fully understood. Intriguingly, the propensity to undergo phase separation is viewed as one of the main parameters determining the formation and stability of local domains with a specific protein and lipid composition. For instance, it is proposed that CHOL self-aggregates into domains together with SPs, as SM, forming tightly clustered lipid platforms called rafts [66]. Although the existence of lipid rafts remains controversial [67], CHOL and SPs domains are involved in neuronal protein localization [68] and neurotransmission [51,52]. Interestingly, CHOL binds to Synaptophysin [69], one of the major protein constituents of SVs [37]. It is tempting to speculate that Synaptophysin and CHOL spontaneously form a proto-SV domain in as early as at the exit site of the ER (Fig. 2), at which other synaptic vesicle proteins would be clustered. Indeed, it is known that Synaptophysin interacts with Synaptobrevin-2 (Syb-2), controlling its targeting to synaptic vesicles [70]. In support of this hypothesis, changes in CHOL concentration can affect the rate of SV release and exocytosis [71–73]. Furthermore, CHOL also plays an important role in SV endocytosis. Studies in the *Drosophila* neuromuscular junction demonstrated that depletion of CHOL from the synaptic vesicle membrane, but not from the presynaptic plasma membrane, severely affect synaptic vesicle retrieval [74]. Most likely, depletion of CHOL causes missorting of Synaptotagmin 1 and Synaptophysin during endocytosis [69].

PIns(4,5)P_2 was shown to be present in CHOL-rich raft domains in neuronal AZs [62], but super-resolution microscopy experiments suggested that the majority of the inner leaflet plasma membrane PIns(4,5)P_2 clusters independently of CHOL [75] forming nanodomains [76]. Importantly, many membrane proteins are clustered with lipids forming specific lipid-protein nanodomains via segregation of proteins and lipids. Segregation is mediated through both protein-lipid electrostatic interactions, and hydrophobic interactions between proteins transmembrane domains and the hydrophobic tails of the membrane lipids [77–80]. PIns(4,5)P_2 is located in the presynaptic plasma membrane, forming PIns(4,5)P_2 nanodomains which regulate the synaptic vesicle priming and fusion events [81,82]. In addition to its role in forming nanodomains, PIns(4,5)P_2 recruits several proteins involved in regulated exocytosis [83], such as Synaptotagmin [84], Syntaxin [85], Ca^{2+}-dependent activator protein for secretion (CAPS) [86], and members of the PLD signaling pathway [81,87–90].

Moreover, other proteins involved in synaptic vesicle docking, priming, fusion and subsequent recycling are reported to bind negative charged lipids, particularly PLs [91]. For instance, PS binds to the synaptic vesicle protein Synaptotagmin 1, the main calcium sensor in

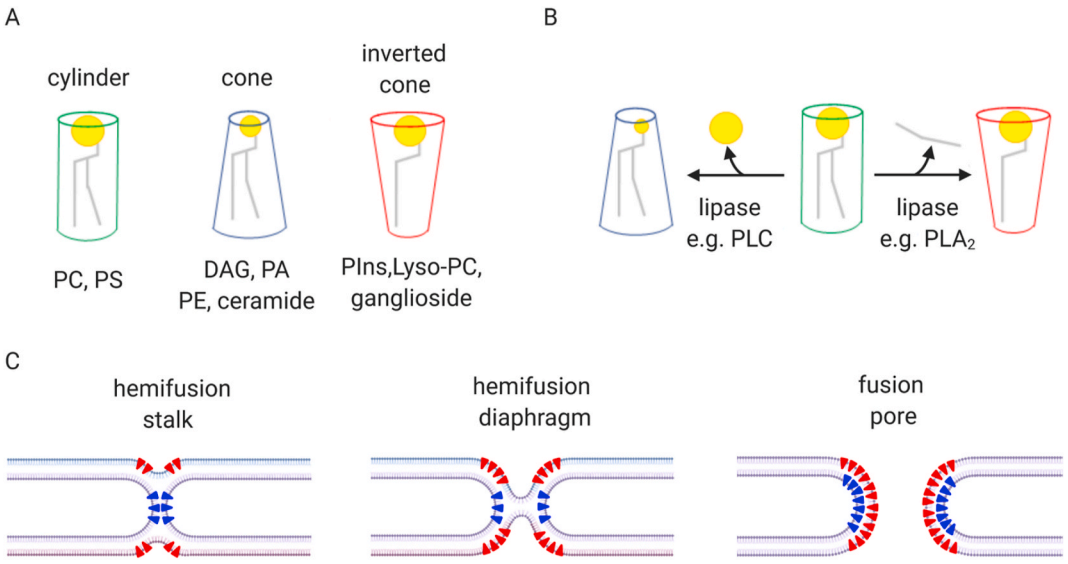

Fig. 4. Membrane lipids influence membrane curvature.
(A) The geometry of the membrane lipids depends on the area of the head group and the hydrophobic lipid chains and it is defined as: cone-shaped, cylindrical and inverted cone geometry. (B) Lipase activity can remove the head group or fatty acyl chain of lipid interconverting the geometry of lipid membrane. (C) Cone-shaped lipids (blue) and inverse cone-shaped lipids (red) promote and stabilize negative and positive curvature respectively (created with BioRender.com). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

synchronous neurotransmitter release [92], and other regulatory proteins of neuronal exocytosis such as Rabphilin, DOC2 and Munc13 via their C2 domains [93]. Additionally, PS plays an essential role in the interaction of the SNARE protein Synaptobrevin 2 with membranes, mainly due to its negative charges [94,95]. Therefore, PS is an important structural component of the exocytosis machinery and is required for efficient synaptic transmission [96].

Finally, in addition to their impact on the membrane physical properties, PA and DAG play also a role as regulatory lipids. DAG, for instance, is essential for the priming of synaptic vesicles at the AZs, owing to the activation of protein kinase C (PKC) and Munc 13 [97,98], while PA would facilitate the functional interactions among SNARE and SNARE chaperones [99].

4. Synaptic vesicle recycling

Neurotransmitter release occurs by exocytosis of SVs that results in the insertion of proteins and lipids into the presynaptic plasma membrane. To maintain presynaptic membrane homeostasis, synapses possess fast and efficient mechanisms for SV endocytosis and recycling, thus counterbalancing major perturbance and remodeling caused by the continuous incorporation of SV membranes into the presynaptic plasma membrane [100]. Fast recycling and turnover of SVs is essential to sustain neuronal activity when considering that SVs are only slowly replaced by the *novo* synthesis in the cell body and delivered by axonal transport [100,101].

The site of synaptic vesicle exocytosis is a spatially organized protein matrix containing the core machinery for the synaptic vesicle exocytosis, and thereby neurotransmitter release, called active zone (AZ) [7]. Due to difficulties in preparing presynaptic plasma membranes and AZ to a sufficient degree of purity the membrane lipid composition of SV release sites cannot be quantitatively determined but only inferred from studies on non-neuronal cells and imaging studies using microscopic probes (see Refs. [62,102] for a more details review). Generally, specific lipids are known to be enriched in the plasma membrane of neuronal cells and to contribute to all steps of SV exocytosis: docking, priming and fusion between the SVs and the plasma membrane [56,61,62]. At the presynaptic membrane, CHOL and SPs, mainly gangliosides, cluster in raft domains [61,102,103], and PA presents local accumulation [57]. Additionally, lipids of the plasma membrane of the eukaryotic cells are asymmetrically distributed, with SPs and PC predominantly on the outer and, PE and PS on the cytosolic layer. The differential distribution of lipids between the cytosolic and the outer leaflets of plasma membranes is maintained thanks to active and energy-dependent lipid transports by lipid translocases [104,105].

Especially relevant is the membrane distribution of PIs at the synapses. PIs show only a very low abundance in synaptic vesicles, but they play an essential role in the synaptic vesicle cycle [38,83], acting as recruiting and/or signaling factors [61,91]. During synaptic vesicle exo- and endocytosis, PIs undergo specific changes in their phosphorylation status which were proposed to correlate with the different stages of the synaptic vesicle cycle [106]. Indeed, different phosphoinositide phosphatases and phosphokinases were found in synaptic vesicles and synaptic boutons [37,107]. PIns is found in recycling endosomes. It is phosphorylated to phosphatidylinositol-4-phosphate (PIns(4)P) by type II PIns 4-kinase (PI4KII) and presumably by phosphatidylinositol 4-kinase alpha (PI4Kα), which activity is tightly associated with secretory granule membranes and synaptic vesicles [108–110]. PIns(4)P is proposed to be the prevalent PI on the synaptic vesicle membrane prior to exocytosis. Subsequently, PIns(4)P is converted to PIns(4,5)P_2 by PIns phosphate kinase type I (PIPKIγ) [111], at the level of the plasma membrane (Fig. 3C), where it is a necessary cofactor for PLD and PLC, which produce PA and DAG respectively during exocytosis [89,112]. As mentioned above, PIns(4,5)P_2 plays a central role in synaptic vesicle exocytosis and recycling.

At the synapse, there are at least three different pathways of SV recycling: ultrafast endocytosis, Clathrin-mediated endocytosis (CME), and bulk endocytosis, which differ with respect to speed, maintenance of SV identity, and likely molecular components (Fig. 2) [101]. How the precise lipid composition of SVs is maintained throughout consecutive rounds of exo-/endocytosis and different endocytosis pathways remains elusive [38,87,113]. Key questions remain unsolved: are the lipid composition between SV and the presynaptic plasma membrane different and if so, how is this difference being maintained? To date it is difficult to answer these questions, as the lipid composition of the plasma membrane is not yet well characterized as mentioned above. Nevertheless, lipids play an essential role preserving the integrity and fidelity of the SV uniformity [62]. It is believed that PIs of the synaptic

plasma membrane, particularly PIns(4,5)P_2, are prime candidates for regulating both timing and spatial segregation of endocytosis. Most endocytosis processes occur at specific membrane domains of the presynaptic plasma membrane, called periactive zone [114,115], which are in close proximity of the AZ. This allows for a fast vesicle reformation while keeping identity and integrity of the vesicle itself [56,87,102,116] (Fig. 2). Endocytosis occur after full collapse and lateral diffusion of the components of the synaptic vesicles [117–119], which will be subsequently reconstituted *de novo* directly from the plasma membrane [120,121] or via endosomal intermediates [122,123].

As mentioned, the endocytosis machinery is tightly regulated by PIs, which help to define endocytic zones [106,124]. Plasma membrane PIns(4,5)P_2 at the presynaptic AZs has a prominent role because it binds to many endocytic factors [125,126]. PIns(4,5)P_2, in most cases also PIns(3,4,5)P_3, recruits endocytic clathrin adaptors, as AP-2, AP180/CALM, epsin, and many other endocytic factors which control the fission process [39,126], as Amphiphysin and Dynamin, which are involved in the scission of the Ω-shaped Clathrin coated pits [127,128]. Thus, the reformed Clathrin-coated vesicles (CCV) are highly enriched in PIns(4,5)P_2 that tightly crosslink the Clathrin cage with the vesicles [129]. The PIns phosphatase Synaptojanin 1 hydrolizes PIns(4,5)P_2 in PIns(4)P [130–132], and subsequently the co-chaperon Auxilin associated on the CCV via its interaction with PIns(4)P will recruit Hsc70 promoting uncoating [133–135]. At this point SVs are fully reformed for a direct recycling. Interestingly, Synaptojanin 1 also dephosphorylates PIns(3)P, PIns(4)P, PIns(5)P and, to a lesser extent, PIns(3,5)P_2 in addition to the well characterized PIns(4,5)P_2 dephosphorylation [136,137]. These additional phosphatase activities might be required for the recycling of synaptic vesicles via different mechanisms. Indeed, different phosphorylation states of PIs were reported to play a role in AZ organization [75]. Furthermore, recycling via the endosomal pathway requires the presence of PIns(3)P, PIns(3,5)P_2 and PIns(3,4)P_2 [39], and might be involved in the conversion of recycling intermediates into mature synaptic vesicles [56].

Thus, PIs metabolizing enzymes modify the lipid composition of the vesicle membrane during synaptic vesicle recycling, conferring an ‘‘endosome-like identity’’, which might lead the fusion with an endosomal intermediate compartment [138] and subsequent SV generation. Here, PI4KIIα, an integral membrane associated kinase [110], was speculated to resynthesize PIns(4)P for the generation of functional new synaptic vesicles [108,110,139,140].

Although there is a consensus that CME plays a central role in SV recycling, there are two additional endocytic pathways: ultrafast endocytosis and bulk endocytosis. However, the molecular mechanism underlying the generation of endocytic vacuoles and subsequent SV formation in both processes remain largely unexplored and elusive [100]. Still, endocytosis at the synapse occurs through a combination of common steps that drive membrane deformation, budding and fission. This membrane reshaping is influenced by the composition and the geometries of lipids in opposing leaflets [141].

5. Concluding remarks

Despite the recent advances in understanding the synaptic protein machinery, our knowledge of the role of SV lipids lags behind. A key open question is how neurons ensure the presynaptic membrane homeostasis and, as a result, maintain the SV lipid composition. This lack of knowledge is largely due to the complexity and difficulties associated with the study of lipids and their metabolizing enzymes. Nevertheless, the development of new techniques to monitor the activity of the lipid-metabolizing enzymes and the consumption-generation of select lipid species, together with the development of more sensitive and sophisticated methods for lipid analysis, should enable us to unravel both, the SV lipid composition and homeostasis. Advances in lipid mass spectrometry [22,142–144] allowed a detailed monitoring of the lipid composition of the SVs, but lipid composition at the active zone is still unknown and elusive because of experimental limitations [62,102]. Nevertheless, minor changes in the membrane-lipid homeostasis in the AZs cause major changes in the function of membrane proteins and membranes themselves, leading to a number of different neurological pathologies [56].

Synaptic lipid biology will strongly benefit from the development of new methodologies [145] that would allow us to unravel spatially and temporally the precise pathways responsible to generate and maintain the integrity and functionality of the SVs.

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