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Review

# Synaptoneurolipidomics: lipidomics in the study of synaptic function

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**The brain is an exceptionally lipid-rich organ with a very complex lipid composition. Lipids are central in several neuronal processes, including membrane formation and fusion, myelin packing, and lipid-mediated signal transmission. Lipid diversity is associated with the evolution of higher cognitive abilities in primates, is affected by neuronal activity, and is instrumental for synaptic plasticity, illustrating that lipids are not static components of synaptic membranes. Several lines of evidence suggest that the lipid composition of synapses is unique and distinct from other neuronal subcompartments. Here, we delve into the nascent field of synaptoneurolipidomics, offering an overview of current knowledge on the lipid composition of synaptic junctions and technological advances that will allow us to study the impact on synaptic function.**

## Highlights

Synaptic transmission is mediated by specialized junctions that are characterized by a complex lipid and protein composition.

The protein composition of synapses is diverse, and it remains unclear whether the molecular diversity of lipids impacts circuit-specific differences in neurotransmission as well as differential predisposition to synaptic dysfunction in disease.

Recent work has shown that rather subtle changes in lipid content can influence synaptic function, but those that depend on molecular interactions between lipids and proteins remain unknown as technical limitations have precluded lipidomic studies at the level of individual synapses.

Novel technologies, including mass spectrometry imaging and chemical tagging, now exist to study lipid function with the necessary molecular and spatial detail to resolve the lipidome of single synaptic junctions.

## Synaptic membrane lipid structure and membrane organization

The primary cellular function of lipids is to form membranes essential for cellular compartmentalization. They also serve as energy-building blocks, storage molecules, and signaling molecules. Unlike metabolites, lipids enable the solubilization of hydrophobic proteins and provide crucial anchor points for protein immobilization at the outer and inner membrane leaflets through their polar or ionic headgroups. Lipids are small, chemically diverse, amphipathic molecules that assemble under aqueous conditions to complex membranes (Figure 1A,B). Due to different backbones, headgroups, and the number and length of fatty acyl groups, they create membranes of different thicknesses, shapes, and polarities (Figure 1A,C). The leaflets can be composed of different lipids: for example, the inner leaflet of the pre- and postsynaptic membrane is rich in anionic lipids carrying polyunsaturated fatty acyls, while the outer leaflets are rich in cholesterol and glycosphingolipids [1] (Figure 1C,D). The anionic lipid classes, such as phosphatidylinositols (PIs), phosphatidylglycerols, and phosphatidic acids (PAs), are important anchor points for intracellular signaling, whereas glycosphingolipids at the outer leaflet are crucial for cell–cell communications. **Membrane fluidity** (see Glossary) and its **phase** (Box 1) are likely driven by cholesterol on the outer side and the number of esterified polyunsaturated fatty acids on the inner side of the membrane (Figure 1A).

Neural information processing relies on the proper formation and maintenance of synaptic contacts; those between lipids and between lipids and proteins are especially crucial in neural circuits because they are the synaptic interface. In the following sections we provide first a general overview of lipid function at synaptic membranes. We then summarize the current knowledge about their lipid inventory before describing the involvement of lipids in sophisticated mechanisms of synaptic neurotransmission. We finally address recent technological advances and future challenges to establish a reliable synaptic lipid inventory in health and disease.

## Lipid function at synapses

The synaptic junction is the central building block of a chemical synapse that mediates cell–cell contact and signal transduction (Figure 2). Compelling evidence suggests a crucial role of lipids

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in synaptic neurotransmission where negatively charged lipids in the plasma membrane, including phosphatidylinositol phosphates (PIPs) and phosphatidylserine (PS), are essential for multiple steps of the synaptic vesicle cycle [1]. In addition, several studies show how lipids determine membrane shape, regulate ion channel activity and protein interactions, and control endocytosis machinery, as well as receptor activity [2–7]. Since most **lipid mediators** are direct or indirect products of polyunsaturated phospholipids, sufficient molar content of complex polyunsaturated lipids must be located at synaptic junctions to guarantee lipid mediator formation and neurotransmitter release. Intriguingly, several lines of evidence suggest that the lipid composition of synapses might be dynamic following the induction of **synaptic plasticity** [8,9]. Additionally, recent work indicates that the molecular diversity of synapses at the protein level is presumably vast [10,11]

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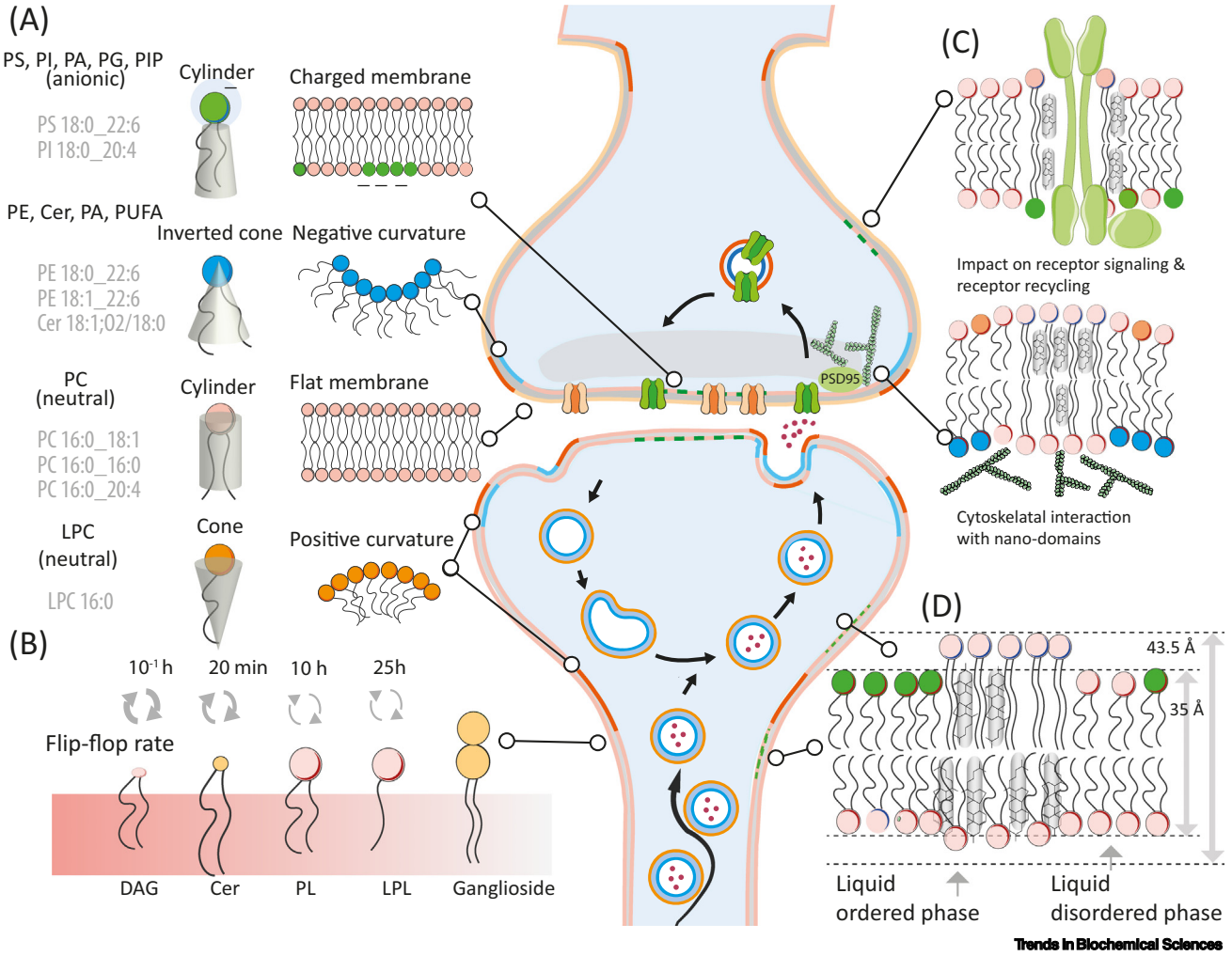


Figure 1. Chemical diversity of lipids and the organization of excitatory synapses. (A) The chemical structure of a lipid determines physical membrane properties. The contributing lipid classes are sorted according to their abundance (top 10%), with each class's most abundant lipid species displayed below. (B) The headgroup polarity, fatty acyl chain length, and the number of fatty acyls determine the cross-leaflet movement besides the active transport. Depicted in the middle is a postsynapse with receptor recycling and lipid-protein interaction at the postsynaptic density. Below is the release of neurotransmitter and synaptic vesicle recycling at the presynapse depicted. (C) Lipid-ordered and disordered regions are essential as anchor points for transmembrane and cytoskeletal proteins the color code corresponds to A. (D) Chain length and saturation level determine membrane thickness and fluidity and are mainly determined in synapses by the esterified fatty acids: FA 16:0, 18:0, 18:1, 20:4, and 22:6. Abbreviations: Cer, ceramide; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid.

and this diversity is presumably crucial for neural circuit function. It is conceivable that lipid diversity equally contributes to circuit-specific properties of synaptic connectivity.

## Current knowledge about synaptic lipid inventory

In recent years, a few studies have tried to shed light on different aspects of lipids in neurobiology, such as the diversity of lipids noted earlier. Usually, in such investigations, the white matter or different brain regions were analyzed [12,13]. The challenge with omics data derived from such tissue samples is that they present a mixture of different regions, cell types, and extracellular matrices. Given that glial cells clearly outnumber neurons in most brain regions, the major contribution to the lipidome under study stems from glial cells and their metabolism. However, the results are often interpreted in such a way that they are connected to neuronal function or brain disorders.

By contrast, two recent studies contributed a lipid inventory of the hippocampus [9,13], a brain region that is intimately linked to learning and memory. Synaptic junctions isolated from hippocampal **heavy membranes** primarily consist of three lipid categories: sterols (STs), glycerophospholipids, and sphingolipids [9]. The machinery for lipid metabolism, including anabolism and catabolism, was also present at the synaptic membrane [9]. For example, the *de novo* sphingolipid synthesis initiating enzyme, serine palmitoyltransferase (SPTCL), was found in both of its isoforms. Also, as expected from the long-chain base profile of sphingolipids present in synaptic junctions, with LCB 18:1;O2 as the most prominent fatty acyl, CERS1 was the main ceramide (Cer) synthase detected. Besides these anabolic enzymes, catabolic enzymes such as ceramidases (SMPD1–3) or sialidases (NEU3 and 4) can be found in synaptic junctions. Overall, the main sphingolipid metabolic enzymes for the *de novo*, salvage, and sphingomyelin

### Box 1. Lipid phases in synaptic membranes

All observed membrane phase behaviors are derived from the chemical properties of the most abundant membrane lipids. This means the phase properties depend on ST > PC >PE > PS, and SM in synapses. A plausible perspective is that membrane proteins influence and potentially regulate the phase behaviors by lipid–lipid interactions, which, therefore, need to be understood first.

While the lamellar states depicted in the inlet are crucial for the structure of membrane bilayers, it is important to recognize the potential significance of other nonlamellar lipid phases. These phases, such as the cubic or hexagonal phase, might play a vital role in vesicular transport and the associated fission and fusion processes, thereby underscoring their relevance in biological processes.

The status of a phase is intricately linked to the structure of the lipids, including their length, degree of unsaturation, and the number and type of lipids present. Equally important are the interactions between these lipids at a given time. This underscores the crucial role of lipid structure and interaction in determining the phase status, thereby clarifying the factors influencing phase behavior. Bilayers (Figure I) can occur in solid (SO) liquid-ordered (LO) and liquid-disordered (LD) phase and be converted into each other.

The most abundant lipid species, cholesterol, does not form a bilayer, but together with bilayer-forming lipids such as PC, SM, and PE, cholesterol forms a highly ordered phase with high translational mobility. However, PC cholesterol-dominated membranes can transition to an LD phase depending on the degree of unsaturation in phospholipids and the number of cholesterol molecules in the leaflet.

In addition, both leaflets can influence each other in dependence on composition and membrane asymmetry. For example, models for the inner leaflet of the plasma membrane – comprising three-component mixtures of PC, PE, PS plus cholesterol, with one saturated and one unsaturated acyl chain – are homogeneous by themselves but separate into two phases when phase separation occurs in the opposite bilayer leaflet. At the synaptic membrane phase, behavior is mainly controlled by cholesterol (ST), PC, and PE and their degree of unsaturation. Notably, fatty acyl 18:1 esterified to different phospholipids is decreased in the synaptic membrane, whereas FA 22:6 (DHA) and FA 22:4 (AA) are not compared with heavy membranes. Due to their high amount these polyunsaturated fatty acids (PUFAs), are a further component to be considered in studies of synaptic phase behavior.

## Glossary

**Heavy membranes:** fraction that contains the plasma membrane, organelles, and that is enriched in synaptosomes. This crude membrane extract is further used for synapse purification using sucrose-density gradient ultracentrifugation.
**Lipid mediators:** bioactive lipids that are often derived from polyunsaturated fatty acids, and that are potent signaling molecules.
**Matrix-assisted laser desorption/ionization (MALDI):** soft ionization technique that enables MS analysis of many different biomolecular classes.
**MALDI-2:** next-generation ionization process with higher sensitivity and efficiency that use a two-step ionization.
**MALDI-MSI:** technical combination of MALDI as the soft ionization technique and label-free imaging to visualize the spatial distribution of molecules with a resolution of up to 1 μm.
**Mass spectrometry imaging (MSI):** label-free imaging is a technique to visualize the molecular content of cells and tissue slices.
**Membrane fluidity:** refers to the viscosity of the lipid bilayer of the cell.
**Nanodiscs:** solubilized particles consisting of a lipid bilayer (with or without embedded proteins) stabilized by a polymer, which can be a protein or a synthetic polymer.
**Nanodomain:** macromolecular protein and lipid complexes found in the cell membrane.
**Nano-secondary ion mass spectrometry imaging (NanoSIMS):** measures, visualizes, and quantifies the distribution of elements and their stable isotopes with a resolution of up to 50 nm.
**Phase:** the phase behavior of a membrane depends upon the relative fluidity and temperature-dependent mobility changes of the lipid molecules. Lipid membranes can exist in liquid or solid phases.
**Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2):** polar anionic glycerophospholipid with multiple negative charges.
**Postsynapse:** part of the neuron that is located on a dendrite or soma, with receptors for neurotransmitters.
**Presynapse:** part of the neuron that is located on the axon and contains vesicles with neurotransmitters.
**SIMPLEX:** simple method for extraction of metabolites, proteins, and

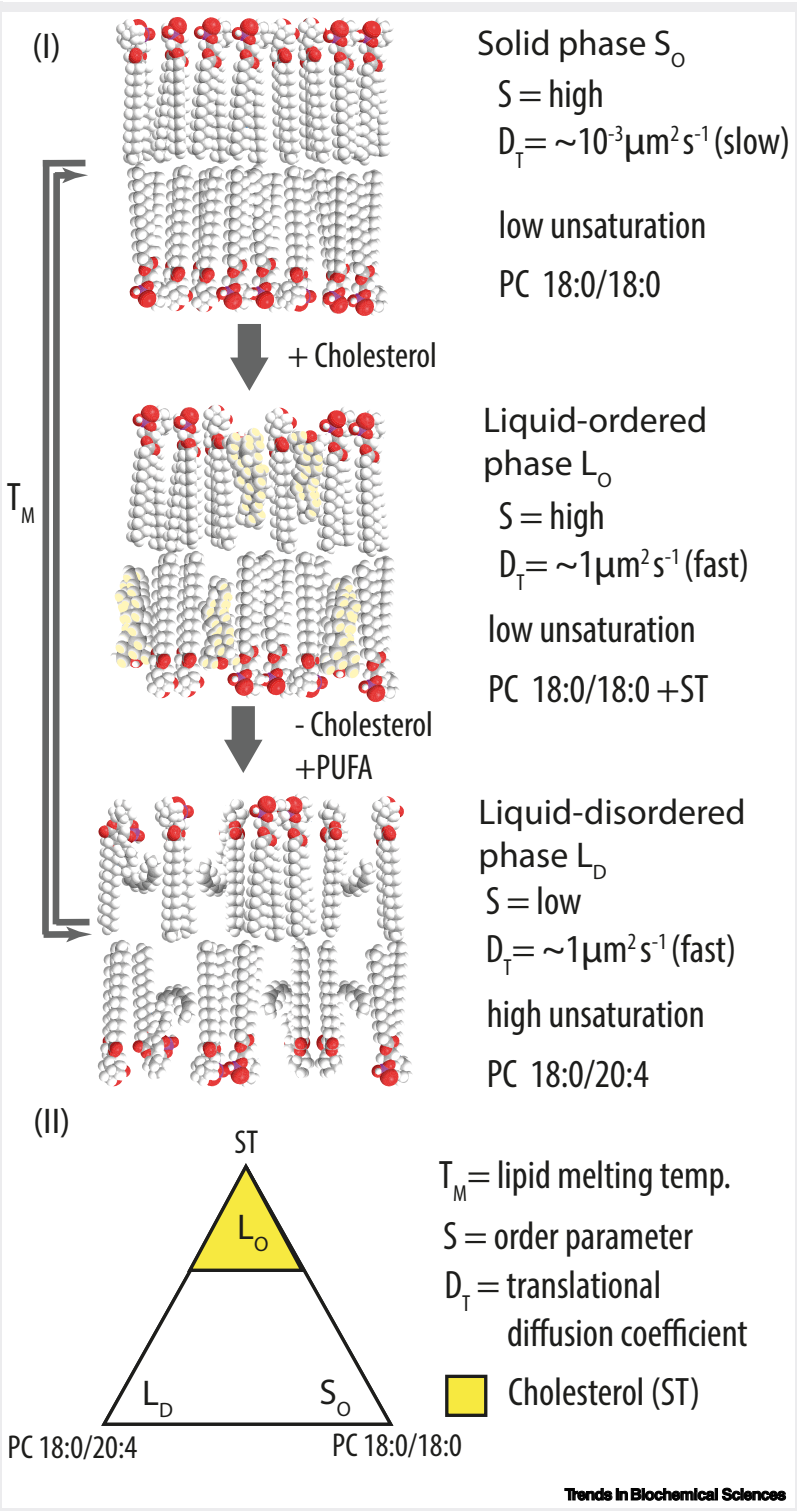


Figure I. Synaptic phase behavior. (I) Transition between different phases. (II) Phase diagram. Abbreviations: LD, liquid disordered; LO, liquid ordered; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acid; SO, solid; ST, sterol.

lipids from one sample, reduces sample amount for multiomics experiments.

**Soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) complex:** catalyzes the fusion of lipid membranes; for example, during the exocytosis of small molecules at the synaptic cleft.

**Spine:** most abundant type of synapse in the mammalian brain. A dendritic spine is a membranous protrusion that typically receives input from a single axon.

**Styrene–maleic acid lipid particle (SMALP):** a specific type of lipid nanodisc.

**Synaptic plasticity:** refers to the use and activity-dependent modification of synaptic strength and input.

**Synaptoneurolipidomics:** deals with the quantitative assessment of synaptic membrane composition by assessment of their lipid inventory and lipid metabolism.

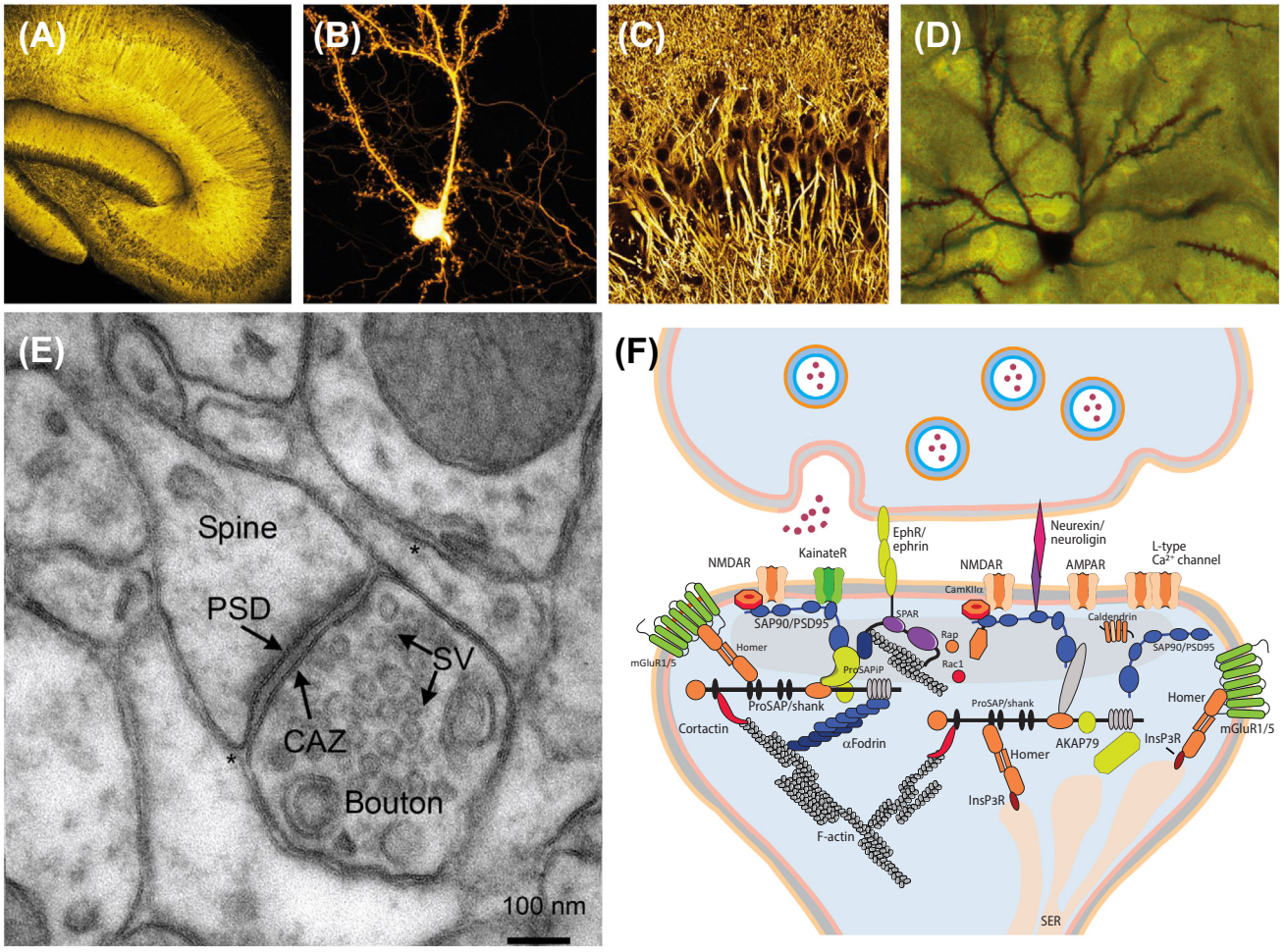


Figure 2. Dendritic spine synapses in the rodent hippocampus. Overview and spatial relationship of synaptic connectivity in a brain region that is relevant for learning and memory. (A) Immunofluorescence staining of hippocampal neurons with an antibody directed the dendritic marker protein MAP2. (B) A single pyramidal neuron of the CA1 region of the hippocampus filled with biocytin. Such a neuron can accommodate up to 15 000 spine synapses. (C) 3D reconstruction with IMARIS of the synaptic layer structure of the CA1 region. (D) Golgi-cox staining of a pyramidal neuron visualizes dendritic protrusions called spine synapses. (E) An electron micrograph shows multiple pre- and postsynaptic structures, as well as an astrocytic endfeet (*) in close contact with synapses. Note the presence of numerous synaptic vesicles in the presynaptic boutons. (F) Topology of synaptic junctions. Synaptic transmembrane proteins are anchored to protein components of the PSD. Spines are rich in F-actin and some spines contain smooth endoplasmic reticulum (SER). Abbreviations: CAZ, cytomatrix at the active zone; PSD, postsynaptic density; SV, synaptic vesicle. Scalebar: 100 nm.

(SM) hydrolysis pathways are present, allowing the local production of the entire bandwidth of sphingolipids. A similar enzyme inventory can also be found for glycerophospholipids and STs [9]. Additionally, the main enzymes involved in fatty acid synthesis, desaturation, and beta-oxidation are detectable at synaptic junctions [9].

Within these lipid categories of STs, glycerophospholipids, and sphingolipids, the top ten lipid classes account for almost 100% of membrane lipids, with the following percentages: STs (32%), phosphatidylcholine (PC, 28%), phosphatidylethanolamine (PE, 22%), PS (9%), PI (3%), SM (4%), cardiolipins (CLs, 1%), Cers (1%), PA (0.8), and galactosylceramides (GalCers, 1%) [9,14–17]. Initially described through classical biochemical experiments, the concentration of most lipid classes has been corroborated by modern lipidomics mass spectrometry (MS)

workflows, providing detailed insights into their fatty acyl and double bond composition [9]. Compared with heavy membranes, in synaptic junctions, Cers show an increased concentration while cholesterol slightly decreases, indicating a significant shift in membrane composition [9]. In addition, synaptic membranes exhibit decreased proportions of simple glycosphingolipids like sulfatides and GalCers, displaying distinct lipidome compositions of pre-and postsynaptic compartments in comparison to whole brain samples [13]. Fatty acids ranging from 14 to 24 carbons with 0–6 double bonds are typical in the brain, but synaptic environments also feature 12-carbon and very long chain fatty acids up to 26 carbons. In hippocampus rodent-derived synapses, the most common fatty acids are 16:0 < 18:0 < 18:1 < 20:4 < 22.6 < 22:4, with arachidonic acid (AA, 20:4) and docosahexaenoic acid (DHA, 22:6) being pivotal in inflammation-related signaling [18]. Notably, long-chain fatty acids (20–26 carbons) are reduced except for AA and DHA, which are constant compared with hippocampus sections [9,13]. With lower levels of monounsaturated and very long chain fatty acids, as well as a higher proportion of DHA and AA, hippocampal synaptic membranes acquire a thinner, more fluid nature for an effective vesicular signaling. Therefore, higher fluidity is further supported by a lower cholesterol level.

However, DHA- and AA-derived lipids are also crucial sources of various lipid-derived mediators, such as oxidized fatty acids and endocannabinoids, which have been proven to directly influence synaptic plasticity. These bioactive lipids, along with their metabolic pathways and, to some extent, their receptors, are present in synapses and in the synaptic cytoplasm [9,19,20].

Recent studies have highlighted that AA-derived lipids such as 2-arachidonoylglycerol (2-AG) and leukotriene (LT)B4/A4 impact synaptic function. Where 2-AG, an endocannabinoid, was shown to affect long-term synaptic plasticity [9,20], the inhibition of oxylipin production, specifically LTB4/A4, has been demonstrated to directly increase the expression of glutamate/N-methyl-d-aspartate receptors in hippocampal CA1 synapses [19]. More than 416 membrane lipids have been identified in synaptic junctions, yet only 15 lipid species constitute 75% of the total membrane content [9]. Overall, the synaptic membrane lipidome composition seems to be controlled by only 4% of all present lipids; we predict the other 96% of membrane lipids are likely to fine-tune the synaptic membrane and its signaling components.

## Lipids are crucially involved in synaptic neurotransmission

As we explore in the following sections, lipids directly or indirectly control synaptic function via interaction with membrane proteins, including ion channels and G-protein-coupled receptors (GPCRs), as well as several steps of neurotransmitter release [1]. Additionally, the metabolism of these lipids impacts synaptic function. In addition, the positioning of synaptic proteins influences their signaling capacity. Lipid rafts are sphingolipid and cholesterol-enriched membrane microdomains that serve as hubs for a variety of signal transduction pathways. Palmitoylated signaling proteins accumulate at lipid rafts in dendrites [21]. Dendritic **spines** are the postsynaptic sites of most excitatory synapses, found along the dendrites of neurons (Figure 2D,E). The enrichment of the postsynaptic membrane in cholesterol and sphingolipids and an abundance of palmitoylated proteins in spine synapses suggest that highly confined **nanodomains** reminiscent of lipid rafts exist in this type of synapse. Reversible palmitoylation translocate otherwise soluble proteins to the plasma membrane and enables docking to lipid rafts [21]. This in turn could impact activity-dependent changes in protein organization at synapses, thereby controlling synaptic physiology and function.

### Synaptic lipidome impacts surface mobility of transmembrane proteins

It is known since many years that compartmentalization of lipids in the synaptic membrane controls the localization of synaptic proteins which in turn is critical for their function [22,23].

Membrane thickness and fluidity are determined by lipids such as cholesterol, sphingolipids, and glycerophospholipids lipids that supposedly cluster proteins at nano- and microdomains at synaptic junctions [2]. Lipid composition determines membrane fluidity and thereby the mobility and lateral diffusion of membrane proteins [24]. Surface mobility of ion channels, foremost voltage-dependent calcium channels [25] and ionotropic glutamate receptors [26], plays a key role in synaptic neurotransmission and is a bona fide mechanism of synaptic plasticity [27]. Lateral diffusion of synaptic membrane proteins in and out of the synapse appears to be controlled at least in part by the lipid composition of the plasma membrane [28]. The exchange of glutamate receptors between synaptic and extrasynaptic sites is highly dynamic and the exchange rates are surprisingly different for miscellaneous synaptic transmembrane proteins [29]. Moreover, single-particle tracking studies revealed that the mobility of lipid-anchored proteins clearly differs between synaptic and extrasynaptic membrane regions [30], suggesting substantial differences in membrane fluidity or type of lipid anchor. However, these differences might also reflect their anchorage to the specialized synaptic cytoskeleton, the cytomatrix of the active zone beneath the presynaptic membrane and its counterpart the postsynaptic density (Figure 2E,F).

## Synaptic lipids serve highly specialized functions

Although several studies have suggested that the synaptic lipid bilayer exhibits a heterogeneous spatial organization of membrane lipids and associated proteins, the lack of tools to resolve the spatial organization of lipids in the postsynaptic membrane has precluded a more detailed test of this hypothesis (Boxes 2 and 3). This is unfortunate because the profound impact of subtle lipid–protein interactions on various facets of synaptic function has become obvious in many studies published in recent years. Space limitations do not allow us to cover all published work but we want to showcase just a few examples of recent work that demonstrates the necessity to expand the tool box beyond classical biochemical purification [9] for the study of synaptic lipids to break new ground.

The significance of anionic synaptic lipids as crucial membrane components that serve as anchor points for proteins is well established [31,32]. Most studies focused on membrane–protein interactions at the postsynaptic membrane [20,26], while the existence of lipid nanodomains at the presynaptic membrane has recently attracted substantial interest. In particular, the central role of the charged phospholipid **phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)** and its synthesis, which might be regulated locally, for synaptic vesicle fusion and exocytosis has recently become much clearer. For example, the calcium sensor synaptotagmin 1, which is an integral membrane protein of synaptic vesicles, couples vesicle fusion to the endocytic retrieval of synaptic vesicle membrane by promoting the local activity-dependent formation of PI(4,5)

### Box 2. Lipid nanodiscs

The study of membrane proteins in their isolated form is more complex than for soluble proteins because their recombinant expression is usually of low yield, and their isolation requires a solubilization step. Traditionally, detergents have been used to strip away the membrane lipids and isolate membrane proteins in micelles. However, the lipid bilayer of the membrane influences the structure, stability and function of membrane proteins. The reconstitution of solubilized membrane proteins into liposomes re-introduces a membrane environment, but liposomes are not compatible with all types of biochemical and biophysical assays. Moreover, the original lipids surrounding the membrane protein of interest are lost. To address these problems, several agents have been developed that can stabilize disc-like particles with lipid bilayers and embedded proteins. Although the nomenclature slightly varies in the literature, we will here refer to all of these bilayer-filled discs as lipid nanodiscs. Nanodiscs can be formed by using a membrane scaffolding protein, by the lipid-binding protein saposin A [83], or by applying styrene–maleic acid (SMA) amphipathic polymers [84]. While the protein scaffolds need prior detergent solubilization of the membrane protein of interest, SMA and its derivatives can be applied to isolated membrane fractions or even whole mammalian cells for direct solubilization of membrane proteins into so-called native lipid nanodiscs. These techniques have led to increased structural and functional insights into membrane proteins, including those occurring at the synaptic membrane.

Box 3. MSI

MSI enables the label-free imaging of many different molecular classes throughout tissues and cells. Lipids are one of the most widely studied analytes using MSI and contemporary methods can image up to many hundreds of different lipid species in a single experiment. MSI is most widely performed using ionization techniques such as MALDI and SIMS, which can often detect and image hundreds of different lipids in a single experiment. MALDI is a soft ionization technique capable of imaging many intact lipids such as phospholipids, sphingolipids, glycerolipids, and sterols. It has been used to study how lipid composition varies throughout brain tissue [85] and even individual cells. Recent developments now enable spatial resolution as low as 1 μm that can achieve subcellular resolution for lipid imaging [67]. Techniques such as MALDI-MSI are being deployed for single-cell lipidomics, capable of resolving the lipid profiles of many different cells within a single experiment, and can be combined with fluorescence microscopy. Such approaches have revealed how sphingolipids determine cell fate of fibroblasts [71] and the role of lipids in defining cellular heterogeneity [73]. SIMS is a more energetic ionization technique that can lead to more extensive lipid fragmentation (i.e., detection of only lipid-class specific fragments) but offers spatial resolutions as low as ~50 nm. This makes it a powerful technique for performing MSI at subcellular resolution, but at the cost of lipid coverage. However, combined with recent developments in gas cluster ion sources that now enable detection of many intact lipid species at resolutions down to 1 μm, SIMS offers a powerful method for lipid imaging at cellular and subcellular resolutions. Techniques such as NanoSIMS offer spatial resolutions as low as 50 nm, and when combined with stable isotope labelling, can reveal changes in molecular turnover within individual organelles, including for lipids [63,86].

P2 at presynaptic sites [33]. Thus, highly localized lipid production and signaling maintains the essential homeostatic balance between exo- and endocytosis at presynaptic terminals.

A recent report showed a highly specific role of membrane lipid–protein interaction preceding the cell adhesion contact in synaptogenesis [34]. It appears that interactions of the synapse defective protein (SYD)-1 with plasma membrane PI(4,5)P2 is necessary for presynaptic assembly of cell adhesion molecules before initiating the cell–cell contact [34]. The rare late endosomal signaling lipid phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2] then directs the axonal cotransport of synaptic vesicle and active zone proteins in precursor vesicles that are important for assembly of the **presynapse** [35]. In neuronal development, these vesicles are transported to nascent synaptic junctions by concurrent detection of PI(3,5)P2 and the Arf-like GTPase ARL8 via kinesin KIF1A [35].

Another recent study by Xu *et al*. [36] reported an example of a unique type of lipid signaling. The authors showed that the adaptor protein AP-3 targets the phospholipid flippase ATP8A1 to a subfraction of synaptic vesicles and this results in the recruitment of synapsin by the cytoplasmically oriented PS translocated by ATP8A1. Synapsin thereby increases the number of synaptic vesicles available for release via exocytosis [36]. This prevents depletion by rapid and synchronous synaptic vesicle release during high-frequency action potential firing.

## Activity-dependent lipid metabolism and synaptic function

Synaptic plasticity, the use-dependent modification of synaptic strength, is a tightly regulated cellular mechanism that is essential for the formation of memory. A complex interplay of multiple signaling pathways and molecular players drives the underlying processes, which act on different time scales. The best studied lipids in the context of synaptic plasticity are phosphoinositides that are important for structural changes in synapses following the induction of long-term potentiation (LTP) [37,38]. Recent studies contribute to the mounting evidence for physiological relevant alterations in lipid synthesis and metabolism at synaptic sites that are intimately linked to synaptic plasticity. Environmental enrichment of mouse housing conditions directly affects endocannabinoid catabolism and respective plasticity at spine synapses [9]. Contextual fear conditioning, a learning paradigm in mice, induces palmitoylation of dozens of synaptic proteins within just 1 h [39]. Similar results were obtained following the induction of LTP [39]. One of the palmitoylated proteins was the lipid phosphatase PRG-1, an integral membrane protein that directly regulates the membrane insertion of AMPA receptors and thereby might regulate synaptic strength [40].

Following LTP induction the type III phosphatidylinositol 4-kinase PI4KIIIα associates with the dendritic plasma membrane and causes a local increase in the levels of PI4P [8]. This increase is necessary for trafficking of the AMPA receptor to synaptic membranes during LTP induction, and interruption of this process causes severe impairment of LTP and long-term memory [8]. In several studies, phospholipase (PL)A1 activity has been shown to modulate synaptic plasticity and gene knockout of the PLA1 isoform DDHD2 in mice results in reduced saturated free fatty acid in various memory tasks that present with severe memory deficits [41]. Although the emerging picture from these studies is still rather sketchy, and many other lipids might be involved in synaptic plasticity, collectively this work suggests an intimate causal link between activity-dependent lipid metabolism and synaptic plasticity.

## Recent technological advances in spatial lipidomics

**Synaptoneurolipidomics** strives to understand how lipids interact with the embedded membrane proteins and how protein composition is related to and influenced by lipid composition. The existence of >40 different lipids known to modulate signaling and/or to influence membrane geometry in neurons, synapses, and synaptic vesicles, some of which were described earlier, demands a systematic large-scale study of lipid abundance and functional regulation in neuronal subcompartments. Comparison of neuronal lipidomes in different animal species revealed that lipid diversity rapidly expanded in primates, linking the complexity of the brain lipidome to the evolution of higher cognitive brain functions [42]. Moreover, analysis of cell-type specific lipid profiles in mice revealed that neurons are particularly enriched in cholesterol and Cer [13]. However, as noted earlier, studying brain lipids has largely been performed by analysis of entire brain regions without prior subcellular fractionation and isolation of neuronal subcompartments [13,37]. Thus, although it is known from early work that synapses are enriched in certain lipid species [1], the lack of detailed knowledge about their lipid composition precludes a deeper appreciation of membrane dynamics of synaptic junctions, as well as the consequences of lipid–protein interactions for synapse formation, organization, and function.

### Lipid nanodiscs for studying lipid–protein interactions, lipid species, and lipid–lipid exchange

Towards the latter point, advances in lipid **nanodiscs** have expanded our ability to probe membrane biology in synapses (Figure 3A). Lipid particles stabilized by macromolecules, here referred to as lipid nanodiscs (Box 2), have been used since the early 2000s to facilitate research on the structure and function of integral membrane proteins. While the majority of studies so far are protein-centered and do not analyze interactions with lipids [43], insights into their function at the synapse have been investigated such as the membrane saturation of amyloid β oligomers at synapses [44].

In addition, complexes such as v- and t-**SNAREs** (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) that are central for the fusion of synaptic vesicles with the presynaptic membrane were investigated. By using membrane scaffold protein (MSP)-stabilized lipid nanodiscs (with various amounts of the v-SNARE VAMP2 embedded) as fusion partners for liposomes (with embedded t-SNAREs), the fusion complex was trapped in its nascent fusion pore state, the first step in vesicle fusion where release of vesicle content already takes place. It turned out that one v-SNARE is sufficient for formation of such a pore, but that three v-SNARE-t-SNARE complexes are necessary for efficient release [45]. The glycine receptor (GlyR) is an ionotropic receptor that leads to an influx of chloride ions upon interaction with the inhibitory neurotransmitter glycine. Interestingly, different conformational states were detected by performing cryo-electron microscopy (EM) of **styrene–maleic acid lipid particles (SMALPs)** containing the GlyR, in complex with the full agonist glycine and the partial agonists taurine and GABA [46].

Cryo-EM structural studies on lipid nanodiscs have enabled the identification of protein–lipid interactions [47], but this remains a challenging task. The large heterogeneity amongst lipid species

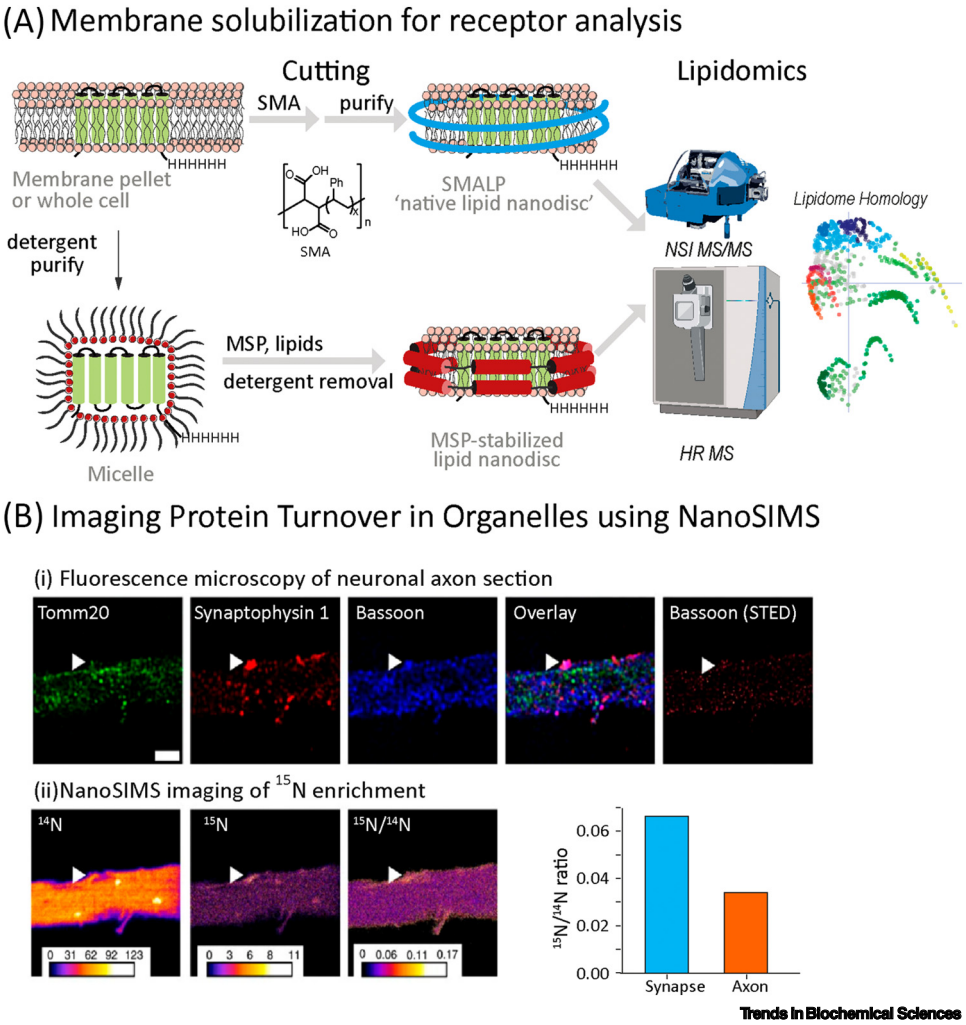


Figure 3. Spatial lipidomics with nanodiscs and mass spectrometry. (A) Workflows of lipid nanodisc purification by styrene–maleic acid (SMA) (top) or membrane scaffold protein (MSP) (bottom) lipid nanodisc with subsequent shotgun lipidomics analysis. (B) Correlated light microscopy and Nano-SIMS to image protein turnover in organelles of a neuronal axon section. (i) Immunostaining of a rat primary neuron (left to right) for TOMM20 (mitochondrial marker, green, confocal), synaptophysin 1 (synaptic vesicle marker, red, confocal), and active zone marker (bassoon, blue confocal). Image second from right shows an overlay of the three confocal images. Arrows indicate a synapse, where all three labels are colocalized. Right hand panel shows STED microscopy of Bassoon staining. (ii) NanoSIMS imaging neuronal axon section after culture with 15N-labelled leucine for 3 days. Panel shows the ion images of 14N, 15N and 15N/14N ratio (left to right), where elevated 15N/14N ratio is observed in the synapse. Right hand plot compares 15N/14N ratio for synapse and axon regions. Scale bar = 2 µm. Image adapted, with permission, from [62]. Abbreviations: HR MS, high-resolution mass spectrometry; MS/MS, tandem mass spectrometry; Nano-SIMS, nano-secondary ion mass spectrometry imaging; NSI, SIMS, secondary ion mass spectrometry; SMALP, styrene–maleic acid lipid particle; STED, stimulated emission depletion microscopy.

complicates the determination of the precise nature of lipids in structural studies. With tandem MS on intact proteins in SMALPs it was possible to identify an ether phospholipid bound to rhodopsin [48] that had been observed in crystal structures previously [49].

Lipidomics can be used to identify the exact lipid species in a protein–lipid nanodisc sample. Of course, this is most relevant if the protein is isolated in a lipid nanodisc with native membrane composition. In a study on water channel aquaporin Z (AqpZ), Schmidt *et al*. catalogued the

annular lipids from SMALP-purified AqpZ tetramers while evaluating direct affinities of lipids by intact protein MS, revealing an interaction site for CL [50]. By lipidomics, as well as thin-layer chromatography (TLC) analysis, phosphatidyl ethanolamine, phosphatidyl glycine, and CL were identified as annular lipids in SMA- and diisobutylene–maleic acid (DIBMA)-based lipid discs containing the intramembrane protease GlpG [51,52].

Lipid–lipid exchange between nanodiscs also creates opportunities for novel methodological avenues. MSP-stabilized nanodiscs embedding a membrane protein of interest, and subjected these to lipid exchange with nanodiscs incorporate a very specific lipid mixture [53]. Some lipid classes are depleted during exchange, whereas others remained unchanged in class, but their average tail length and average tail saturation changed. These results point towards interactions of membrane proteins with specific lipids. In light of these findings, it is plausible that the isolation of membrane proteins of the pre- and postsynaptic membrane within lipid nanodiscs allows the identification of the annular shell of lipids surrounding these proteins. This, in turn, creates a higher resolution of the spatial organization of the synaptic lipidome.

### MS imaging for understanding lipid metabolism and membrane composition

Besides the chemical nanodisc toolbox, recent advances in **MS imaging (MSI)** technology may enable the study of lipid metabolism within the synapse and the surrounding cellular and tissue environment (Box 3). MSI is the core technology that has enabled the field of spatial lipidomics that is now widely applied to both biological tissues and single cells [54,55]. MSI allows for label-free imaging of many different lipid species by virtue of detecting their mass-to-charge values at different spatial coordinates across a sample. Given the small size of the synapse, only secondary ion mass spectrometry (SIMS)-based approaches offer the spatial resolution for synapse analysis [56]. SIMS uses a focused ion beam to sputter and ionize material from the sample which is then detected using MS [57].

SIMS methods, such Nano-secondary ion mass spectrometry imaging (**NanoSIMS)**, offer a spatial resolution down to ~50 nm when using atomic-based beams but as a consequence of the energetic sputtering extensive molecular fragmentation is widely observed. This results largely in the detection of elements and molecular fragments. For lipids this often means only detection of class-identifying fragments such as the phosphocholine headgroup fragment at *m/z* 184. Recent developments in giant gas cluster beams allow for significantly enhanced detection of intact lipids but are often limited in spatial resolution of ≥1 μm [58]. NanoSIMS offers the highest spatial resolution of any MSI technique (up to ~50 nm) but the extensive fragmentation means only atomic and diatomic fragments are detected [59].

However, when coupled with stable isotope labeling, localized enrichment of isotope ratios (e.g., $^{13}C$ and $^{15}N$) can provide insight into biomolecular synthesis and turnover. For example, NanoSIMS has been used to visualize sphingolipid-enriched domains within fibroblasts [60] using $^{15}N$ labeled sphingolipids and imaging of 15N labeled cholesterol and sphingomyelin binding proteins in Chinese hamster ovary cells [61]. Combining NanoSIMS with super-resolution optical microscopy (Figure 3B) has also been used to study protein turnover within different organelles of cultured hippocampal neurons [62]. Enriched $^{15}N$ signals in the synapse were observed providing evidence for higher protein turnover than surrounding organelles. NanoSIMS has also been used to quantitatively map the distribution of $^{13}C$-labeled drugs within PC12 vesicles [56]. While not at the single organelle level, other studies have used conventional SIMS approaches such as: time of flight (TOF)-SIMS to study lipids within various cellular systems, such as PC and vitamin E within single neurons derived from induced pluripotent stem cells (iPSCs) [63]; investigating the effect of n-3 and n-6 18:2 fatty acid supplementation on catecholamine release dynamics,

vesicle consent, and membrane lipid composition in single PC12 cells [64]; and alterations in lipid composition of neurons and neurites upon treatment with tetrodotoxin and bicuculline which can induce changes in synaptic activity [64].

Other MSI techniques such as **matrix-assisted laser desorption ionization (MALDI)** also offer powerful approaches for imaging lipids within biological tissues. Unlike SIMS-based methods, MALDI, along with many other soft ionization technologies, offers significant benefits by providing fuller information regarding the lipid composition across the sample and can detected hundreds of intact lipids within a single MSI experiment, while providing information regarding their identification using accurate MS and tandem MS approaches [65]. The rise of postionization technologies such as **MALDI-2** [66] has dramatically expanded the sensitivity of MSI for many lipids, providing increases of several orders of magnitude for many lipids classes such as glycosphingolipids and sterols. Recent developments in MALDI-based approaches employing new ion source designs now enable rich spatial lipidomics data to be acquired at pixel sizes as low as 1 μm [67,68]. However, as MALDI required application of a suitable matrix to the sample, great care must be taken to ensure this does not distort the spatial distributions of analytes within the sample.

**MALDI-MSI** has been widely applied to studying lipids within neuronal tissue [55], including investigating the lipid composition of amyloid plaques [69], among many others. Besides tissue imaging MALDI-MSI also finds increasing use for high-throughput single cell lipidomics studies where the profiles of hundreds to thousands of individual cells can be acquired in a single experiment. This approach was used for studying lipid heterogeneity amongst neurons and astrocytes derived from rat brain tissue [70]. Other examples of single cell lipidomics used MALDI-MSI include studying the role of sphingolipids in defining subpopulations of fibroblasts [71], mapping lipid distributions throughout the soma and processes of single neuronal cells [72], and investigating lipidomic changes occurring during differentiation of monocytes to M0 macrophages and heterogeneity among Vero B4 cells at 2-μm sizes [73]. Critically, the latter studies also begin to provide insight into subcellular localization of lipids which is clearly a direction single cell MSI is heading towards. MSI thus provides exciting capabilities that can enhance the field of synaptic neurolipidomics by enabling localized analysis of lipids from tissue and cells. However, while the spatial resolution of MSI techniques that allow for broad detection of intact lipids still requires improvements to enable analysis of synaptic structures within neuronal cells, the field is rapidly advancing in this area. With these developments, new applications are likely to emerge, such as the ability to investigate the synaptic lipidome of different neuronal subtypes and synapse diversity within different brain regions.

## Towards a reliable lipid inventory of synaptic junctions

During the past decade, MS has evolved into a state-of-the-art technology for lipid analysis. The technical progress regarding sensitivity, speed, and resolution in MS, combined with advances in systems biology, the accessibility of lipid databases and search engines [56], along with the availability of lipid standards for quantification allow us to address intriguing questions related to lipid function and regulation [74].

However, it remains a challenge to identify and quantify lipids accurately [75]. Many laboratories over-report the depth of the elucidated structures or even report lipids that can hardly be found in mammalian systems [76,77]. To assess such flaws, it is crucial to report the concentration of lipids to assess if the reported membrane lipidome divagates from known membrane compositions [78]. Most importantly, such information is essential to foster data-driven judgments of changing membrane thickness, degree of saturation, fluidity, and enzymatic activity, including

identifying molecules serving as precursors for lipid signaling [9,79]. Such information would stay hidden if a relative quantification is used.

Because synapses must be purified from brain homogenates and the number of biological samples is limited, extraction procedures should be applied to access multiple molecular classes related to lipid metabolism, such as enzymes, lipid building blocks, and complex lipids. These multiomics protocols, **SIMPLEX** or MPLEX, support lipidomics experiments by reducing the amount of biological material and delivering proteomics- and metabolomics-based experimental evidence to support observed lipidome changes [80]. In the future, although isolation of synapses form iPSC-derived neuronal cultures could potentially reduce variability due to the complexity of sample preparation from brain tissue, this approach has the downside that it will not be suitable to address synapse diversity.

The lipidome should not be a report of a compilation of lipid features, which are uncharacterized ions in the gas phase derived from liquid–liquid extraction presumably containing lipids, but rather annotated molecules with a fine granular resolved molecular structure [9] that should be based on experimental evidence and at least contain information about the length of fatty acyls and the number of double bonds.

## Concluding remarks

The rapid technological advances and the development of novel tools to study the role of lipids in synaptic function likely enable faster progress than in previous years. This includes the use of an isotope lipid building block to study the turnover of lipid molecules at synapses applying NanoSIMS to clarify how fast lipids are synthesized and degraded at the pre- and **postsynapse**. The field still suffers from the lack of reliable lipidomics search engines, and without time-consuming manual intervention by experts, the false-discovery rate of identified lipids is high. Different initiatives, such as the lipidomics standard initiative and the global ring trials of the International Lipidomics Society aim for an increased density of knowledge in lipidomics experiments and definition of reference values of lipids [75]. Tools that increase the comparability across data sets, such as GOSLIN and LIPIDSPACE, are essential building blocks of synaptoneurolipidomics because they enhance the comparability across experiments and species [81,82]. Finally, pinpointing the positions of fatty acyl chains and double bonds in complex lipids including analysis at the isomer level will enhance now our understanding of lipid diversity within the synapse lipidome and establish a connection between lipid and protein function, which are often isomer-specific (see Outstanding questions).

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### Declaration of interests

No interests are declared.

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## Outstanding questions

A direct test of how individual lipids contribute to membrane organization or functioning in living cells is still lacking. Specific modulation of the composition of cellular membranes cannot be achieved with common pharmacological treatments. Therefore, there is still a void in molecular tools to locally and temporally manipulate membrane composition without affecting downstream pathways. Optical manipulation of lipid biosynthesis might be an interesting future direction to manipulate lipid levels with high spatiotemporal precision.

The stoichiometry of individual lipids and synaptic transmembrane proteins is currently unknown but is essential to determine the capacity of lipids to alter protein function. Therefore, both quantitative lipidomics and quantitative proteomics are needed to elucidate the protein copy number at the synapse.

What does the complexity of the lipidome reveal about synaptic function? Only relatively highly abundant lipids contribute to synaptic membrane composition in comparison to the more complex lipidome of entire cells or tissue. This suggests a high level of control over synapse lipid complexity. Is lipid diversity sacrificed for a more stable lipidome composition at the synapse, and how does this relate to synaptic function?

Major building blocks of lipids are fatty acyls, which are used not only for β-oxidation but also to generate signaling molecules. This ability strongly depends on the presence of complex lipid precursors in high quantity. A precise, high-resolution structural picture of membrane composition is needed to estimate the potential of synapses to produce these molecules. New MS and computational sciences approaches might be important tools for this analysis.

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