Remodeling of the postsynaptic plasma membrane during neural development

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INTRODUCTION

The neuronal synapse underlies much of neural physiology, and therefore tremendous research attention has focused on characterizing the composition and organization of this unique cellular compartment (Sheng and Hoogenraad, 2007; Swulius et al., 2010; Bayes et al., 2011), with most of that attention focused on proteins. In contrast, little is known about the detailed lipid composition of synaptic terminals, which may serve key roles in the function of the neuronal synapse. Membranes or how this composition is altered during synaptic remodeling. Mammalian cells produce a remarkable diversity of membrane lipids, the extent of which has only recently become appreciated due to advances in mass spectrometry. A given cellular membrane can contain nearly 1000 distinct lipid species (Sampaio et al., 2011; Gerl et al., 2012; Atilla-Gokcumen et al., 2014), and these are actively turned over and trafficked to produce spatial (Surma et al., 2011) and temporal (Klose et al., 2012) lipid gradients between intracellular compartments (van Meer et al., 2008; Klose et al., 2013).

Neuronal membranes have a unique lipid composition, enriched in cholesterol, sphingolipids, and polyunsaturated lipids (Catman et al., 1969; Breckenridge et al., 1972). Establishment and maintenance of the unique synaptic membrane phenotype are important for neural physiology, as evidenced by the various neurodevelopmental abnormalities associated with lipid perturbations. For example, “essential” fats such as ω-3 polyunsaturated fatty acids (PUFAs) are not synthesized by mammalian cells and yet comprise...
up to 20 mol% of synaptic membranes (Cotman et al., 1969). Dietary restriction of such ω-3 PUFAs (most commonly docosahexaenoic acid [DHA]) in developing animals leads to disruptions in neurogenesis, altered neuronal signal transduction, and ultimately deficits in learning behaviors (Salem et al., 2001). These studies have also been extended to humans (Innis, 2007) and correlated with development of attention-deficit hyperactivity disorder (Stevens et al., 1995). Similarly, cholesterol is a critical factor for synapse development (Mauch et al., 2001), with genetic perturbations of its synthesis leading to profound neurodevelopmental disorders (Kanungo et al., 2013). Remarkably, certain features of these disorders can be corrected by dietary cholesterol supplementation (Elias et al., 1997), reinforcing the connection between diet, neuronal lipid composition, and synaptic function.

The lipid compositions and bulk structural properties of cellular membranes are key regulators of protein activity. Specific lipid moieties act as protein recruitment sites (Levenson, 2008), enzyme substrates (Czech, 2000), and/or allosteric regulators of synaptic proteins (Suh and Hille, 2008). In addition to functions controlled by individual lipids, lipid assembly into membranes results in bulk biological properties that have important physiological consequences, including 1) membrane curvature in neurotransmitter exocytosis (Pinot et al., 2014), 2) membrane fluidity for protein diffusion (Low-Nam et al., 2011), and 3) regulation of ion channels by membrane stiffness (Rosenhouse-Dantsker et al., 2012). A dynamic layer of organization is provided by functional membrane domains driven by interactions between lipids and proteins in the plane of the membrane. Such domains can affect cellular processes by selectively recruiting specific proteins (Levental et al., 2010), controlling their activity and interactions (Dart, 2010), and thereby regulating signal transduction, membrane traffic (Schuck and Simons, 2004), and protein localization (Diaz-Rohrer et al., 2014).

A variety of membrane domains have been associated with aspects of neuronal physiology, including phosphatidylinositol (4,5)-bisphosphate clusters as hot spots for neuronal exocytosis (van den Bogaart et al, 2011), ankyrin-based domains at nodes of Ranvier (Bennett and Healy, 2009), and tetraspanin webs in cell adhesions (Levy and Shoham, 2005). In addition to these, prominent membrane domains known as lipid rafts have been widely implicated in neuronal signaling (Allen et al., 2007). Rafts are nanoscopic lipid and protein assemblies whose formation is driven by preferential interactions between cholesterol, sphingolipids, and certain proteins (Lingwood and Simons, 2010). These domains comprise a major fraction of the plasma membrane (Levental et al., 2009; Owen et al., 2012) and have been implicated in nearly every aspect of membrane physiology (Lingwood and Simons, 2010; Simons and Gerl, 2010). The potential relevance of raft domains in neuronal signaling is highlighted by the enrichment of raft lipids (cholesterol and glycosphingolipids) in synaptic membranes, as well as their high levels of polyunsaturated lipids (Cotman et al., 1969; Breckenridge et al., 1972). Although polyunsaturated lipids are usually not raft components (Soni et al., 2008), they can enhance raft formation by fluidizing nonraft regions, thereby increasing the propensity for domain separation (Wassall and Stillwell, 2008; Georgieva et al., 2015; Levental et al., 2016). Rafts are believed to regulate synaptic signal transduction by their recruitment and regulation of neurotransmitter receptors (Allen et al., 2007). In particular, both the α-aminoado-3-hydroxy-5-methyl-4-isoxazolepropionic acid– and N-methyl-d-aspartate–type glutamate receptors that gate postsynaptic signal transmission in excitatory synapses have been reported to localize to raft domains (Suzuki et al., 2001; Hering et al., 2003). Domain residence appears to be important for surface expression of the receptors and therefore their activity and ability to support a functional synapse (Hering et al., 2003).

The putative involvement of rafts in synaptic function prompts the question of how the distinct membrane phenotype of the neuronal synapse is established and maintained. There has been little insight into how a given membrane composition is maintained at a specific cellular location in spite of the rapid lateral diffusion. One clue is provided by previous observations of a direct association between synaptic rafts and the neuronal specialization called the postsynaptic density (PSD; Suzuki et al., 2011). The PSD is a dense web of scaffolds, signaling proteins, and receptors, many of which interact with the plasma membrane (PM) and play an important role in its organization. This physical connection between postsynaptic membrane and its underlying PSD is mediated in large part by the scaffold protein PSD-95. PSD-95 is one of the most abundant PSD proteins and interacts with the membrane via two posttranslational palmitoylation modifications on its N-terminus, which serve as hydrophobic anchors for membrane attachment. This palmitoylation is a major regulator of the localization and activity of PSD-95, as well as of many other synaptic proteins involved in neuronal function and development (Fukata and Fukata, 2010).

Despite the clear functional connections between synaptic membranes and neuronal function, there is little detailed information about developmental changes in synaptic membrane composition and organization. Further, there is no convincing explanation for how the unique lipid composition at the synapse is established and maintained or how perturbations thereof lead to synaptic dysfunction. Here we investigate the compositional and biophysical dynamics of synaptic membranes during postnatal development of the rat forebrain. We observe dramatic remodeling of synaptic membranes during the first weeks of postnatal development and correlate these effects with changes in the plasma membrane of the postsynaptic terminus. Finally, we postulate a mechanism to explain our observations based on recruitment of lipid raft domains to the PSD-PM via palmitoylation of the immobilized scaffold PSD-95. These investigations yield the first detailed insights into the developmental plasticity of synaptic membranes and suggest protein–lipid interactions that may facilitate the functional organization of the neuronal synapse.

RESULTS AND DISCUSSION

Developmental remodeling of synaptic membrane composition

Although the composition of synaptic membranes is crucial for neuronal function, there has been no systematic investigation of the developmental dynamics of the lipid composition of synaptic membranes. We prepared synapse-enriched membranes (synaptosomes) from rat forebrains at various developmental ages and analyzed their lipid composition by shotgun lipidomics. This technology involves generation of molecular ions by “soft” electrospray ionization (ESI) and assignment of specific structure by tandem mass spectrometry (MS-MS), which permits comprehensive analysis of even highly complex samples (i.e., containing hundreds or thousands of different molecules) without chromatographic separation (Eising et al., 2009). Internal standards that control for extraction, ionization, and detection efficiency are used to quantify minute (picomole) quantities of lipids with high fidelity and repeatability (Surma et al., 2015).

Lipidomic analysis revealed clear accumulation of specific lipid subtypes in synaptic membranes during neural development (Figure 1). Most notably, the canonical raft components cholesterol and sphingolipids (Fiedler et al., 1993; Brown and London, 1998;
As well as the raft-associated phosphatidylethanolamine (PE) plasmalogen (PEp; Pike et al., 2002), were progressively enriched from 2 to 60 postnatal days at the expense of phosphatidylcholine (PC) (Figure 1B). These results are consistent with previous implications of cholesterol in synaptogenesis in vitro (Mauch et al., 2001) and plasmalogen lipids in neural development and dysfunction (Braverman and Moser, 2012). Phosphatidylserine (PS) also accumulated through development, consistent with its role in neuronal survival (Akbar et al., 2005).

The MS-MS technique also allowed detailed insights into the developmental changes in the acyl chain compositions of synaptic lipids. For example, there was a clear shift in acyl chain unsaturation, with fully saturated lipids decreasing and polyunsaturated lipids increasing (Figure 1C), leading to a progressive increase in lipid unsaturation that plateaued at postnatal day 21 (P21; Figure 1D). There was also a robust shift to lipids with longer acyl chains (Figure 1E) that showed a similar developmental time course (Figure 1F). A notable result was the developmental accumulation of lipids containing ω-3 PUFAs, the most abundant of which is the fish oil component DHA. Lipids containing such ω-3 PUFAs gradually doubled from ~10% of all phospholipids at P2 to almost 20% at P60 (Figure 1G). Lipids with ω-6 PUFAs did not show the same trend (Figure 1G). ω-3 PUFAs like DHA were previously implicated as key players in neuronal development both in vitro and in vivo. Although the mechanisms underlying these effects have not been elucidated, PUFA-containing lipids affect a variety of membrane properties, including membrane stiffness (Pinot et al., 2014), fluidity (Hashimoto et al., 1999), and domain formation (Shaikh et al., 2004; Georgieva et al., 2015), suggesting physical remodeling of synaptic membranes in neural development.

An important point was that these lipidomic changes were not a function of increased developmental myelination. All synaptosomal preparations analyzed showed undetectable levels of myelin basic protein (MBP), suggesting minimal contamination by myelin membranes (Figure 1H). Moreover, the observed remodeling is not a general effect of myelin. Western blotting showed undetectable levels of myelin basic protein (MBP) in any synaptosomal preparations, in contrast to significant accumulation of this myelin marker in crude brain homogenates starting at P14. CNX, calnexin.
Developmental changes to synaptosomes are mirrored in the PSD-PM
The robust developmental changes observed in the lipidomes of synaptosomes reveal comprehensive compositional remodeling of synaptic membranes. However, although synaptosomes are a convenient synaptic preparation, lipids derived from these represent a mixture of presynaptic and postsynaptic plasma membranes, synaptic vesicle membranes, and some contamination from other organelar membranes, including mitochondria and endoplasmic reticulum (Wilhelm et al., 2014). To attempt to spatially specify the developmental changes measured in synaptosomes, we took advantage of the physical association between the postsynaptic PM and the PSD. A major methodological advantage afforded by the density and stability of PSDs is that they can be efficiently isolated for detailed characterization of PSD-enriched fractions (Swilulius et al., 2010). Imaging of isolated PSDs by negative-stain transmission electron microscopy revealed that these structures are often associated with a membrane component (Figure 2A). The presence of a PSD-associated plasma membrane (PSD-PM) was dependent on the age of the rats, with a progressive increase in PSDs bearing a PSD-PM during development (Figure 2B). This result was confirmed by quantifying the protein-normalized total lipid content of PSD preparations, which also increased during development (Figure 2B). These results suggest that the physical coupling between PSDs and their overlying PM is reinforced during developmental synaptic remodeling.

Lipidomic analysis of the PM associated with PSDs also revealed robust developmental changes. It is important to point out that PSD isolation involves extraction steps with the nonionic detergent Triton X-100 (0.5%) and that this treatment almost certainly affects the lipid composition of the PSD-PM. Indeed, similar conditions have historically been used to extract membrane fractions, called detergent-resistant membranes (DRMs), that are putatively enriched in lipid rafts (Lingwood and Simons, 2007). The PSD-PMs analyzed here are not simply synaptosome DRMs, as DRMs float to the top of sucrose gradients, whereas PSDs accumulate at a high-density interface (see Materials and Methods); however, we expected similar detergent effects as for classical lipid raft preparations. Consistently, PSD-PMs from adult rats (P60) were enriched in raft lipids (most notably cholesterol, but also glycosphingolipids) compared with their precursor synaptosomes (Figure 2C). However, beyond this likely artifactual enrichment, we observed developmental trends in PC, cholesterol (Figure 2C), PEps, sphingolipids (Figure 2D), and long, polyunsaturated lipids (Figure 2, E–H) that quantitatively mirrored those observed in synaptosomes. On the basis of these similarities, we posit that the developmental changes observed in synaptosomes reflect, at least in part, compositional remodeling of the postsynaptic PM overlying the PSD. The accumulation of raft lipids at the PSD-PM is consistent with previous observations of direct association between membrane rafts and PSDs (Suzuki et al., 2011).

Reconstitution of synaptic lipids reveals developmental enhancement of raft domain stability
From the standpoint of membrane organization, the changes in the composition of synaptic membranes suggested a trend toward more stable raft domains during development. Most direct was the accumulation of canonical raft-forming lipids, including cholesterol, sphingolipids, and plasmalogens. More indirectly, polyunsaturated lipids have also been implicated in enhancing raft stability (Shaikh et al., 2004; Georgieva et al., 2015), and we recently confirmed in model membranes and cultured mast cells (Levental et al., 2016) that DHA supplementation stabilizes lipid raft domains. To determine the biophysical effects of the developmental lipidomic remodeling, we reconstituted synaptic membranes in a model membrane system. To this end, we extracted synaptosomal lipids using standard protocols (Folch et al., 1957) and used them to produce giant
unilamellar vesicles (GUVs) doped with a trace amount of fluorescent lipids. These synaptic lipid model membranes showed clear separation into coexisting fluid domains, evidenced by circular domains that selectively include certain fluorescent lipids (Figure 3A). Specifically, disordered/nonraft-phase dye (rhodamine–dioleoyl phosphatidylethanolamine) was excluded from the circular patches, whereas ordered/raft-phase dye (naphthopyrene; Baumgart et al., 2007) was enriched in those domains. Qualitatively similar behavior was observed in intact, isolated PMS (Levental and Levental, 2015a) and reconstituted brush border membranes (Dietrich et al., 2001) and is consistent with the formation of raft-like membrane domains in biomimetic membranes. Of note, crude cellular lipids do not phase separate at any experimentally accessible temperature. Thus these observations reveal that synaptic membranes have the capacity for raft domain formation.

Further, we observed significant differences between the preparations at different developmental ages. These differences were quantified by measuring the temperatures at which coexisting fluid domains appear in the microscopic vesicles (Figure 3), a widely used proxy for the stability of raft domains (Veatch et al., 2008; Levental et al., 2009; Runas and Malmstadt, 2015). There was a progressive increase in raft domain stability during development, evidenced by microscopic domains persisting to higher temperatures in membranes reconstituted from P60 synaptosomes (Figure 3B). These results are fully consistent with the accumulation of raft-promoting lipids during development and support the hypothesis that raft domains are recruited to mature synaptic membranes. This hypothesis is supported by extensive labeling of the raft glycolipid GM1 in isolated PSD-PMs (Swulius et al., 2012) and synaptosomes (Cole et al., 2010).

It was previously hypothesized that the unusual rod lipid composition of neurons and other sensory membranes (e.g., the rod outer segments of the retina; Fliers and Anderson, 1983) is necessary to achieve distinct bulk membrane properties (e.g., fluidity) that sustain the unique demands of neuronal processes (Salem et al., 2001), namely extremely fast signal transduction. We tested whether developmental lipidomic dynamics had an effect on these properties and observed that neither membrane fluidity (measured by anisotropy of DPH; Figure 3C) nor lipid packing (C-laurdan GP; Figure 3D) of isolated synaptosomes was affected during development. Similarly, no significant developmental trends in these biophysical properties were observable in membranes reconstituted from lipids extracted from synaptosomes (Figure 3, E–F). Thus developmental lipidomic remodeling was associated specifically with stabilization of membrane domains rather than broad changes to membrane fluidity and packing.

These reconstituted membrane experiments do not necessarily reflect the native organization of synaptic membranes. Instead, they suggest that lipidomic changes are associated with biophysical consequences, most notably stabilization of lipid raft domains. In vivo, such domains are postulated to be small (ten to hundreds of nanometers) and highly dynamic and have been associated with a tremendous diversity of cell functions by modulation of signaling and trafficking at the plasma membrane. Consistently, these raft domains have been implicated as key determinants of neuronal signaling, including regulation of neurotransmitter receptors (Allen et al., 2007) and other ion channels (Dart, 2010; Rosehouse-Dantsker et al., 2012), as well as synaptogenesis (Hering et al., 2003). Although our data tentatively suggest that synaptic lipid composition supports raft domains that become more stable with development, these in vitro observations will need to be validated in more physiologically relevant settings.

Developmental regulation of abundance of palmitoylated PSD-95 mirrors lipidomic and biophysical changes

What are the molecular mechanisms underlying the broad lipidomic and biophysical remodeling of synaptic membranes during development? To rationalize the recruitment of raft lipids to the PSD-PM, we focused on palmitoylation of the scaffold protein PSD-95, which mediates the physical connection between this membrane and its underlying PSD. For diffusible membrane proteins, palmitoylation is a primary mechanism for their recruitment to raft domains (Delint-Ramirez et al., 2010; Levental et al., 2010). However, for a relatively immobile protein, such as PSD-95 when integrated into the densely cross-linked PSD matrix, the converse may apply; that is, immobile, membrane-embedded palmitates may recruit/retain diffusible raft domains. Thus one potential mechanism for the enrichment of raft domains in the postsynaptic PM is developmental accumulation of immobile palmitoylated proteins, with PSD-95 being a prime candidate. To evaluate this possibility, we quantified PSD-95 expression and palmitoylation levels during development by an acyl-biotinyl exchange (ABE) approach (Wan et al., 2007). Indeed, we observed a dramatic (and previously noted; Petralia et al., 2005; Swulius et al., 2010) increase in PSD-95 expression (Figure 4A) but also an increase in PSD-95 palmitoylation levels independent of expression during development (Figure 4, A–B). As a control, we measured palmitoylation of calnexin, an endoplasmic reticulum protein not expected to participate in PM organization, and observed no developmental changes in palmitoylation levels. Combined, the increased expression and palmitoylation yielded ~80-fold increase in palmitoylated PSD-95 from P2 to P60 (Figure 4C).

**FIGURE 3**: Developmental stabilization of domain formation in reconstituted synaptic membranes. (A) Lipids extracted from synaptosome membranes and reconstituted into model membranes (GUVs) showed robust, temperature-dependent domain formation, with microscopic, circular domains below the melting temperature (Tm) and uniform appearance above Tm. (B) Increased domain stability is evidenced by higher domain melting temperature during development. (C, E) DPH anisotropy probing membrane fluidity and (D, F) laurdan GP probing membrane order showed no significant effects of development in either (C, D) isolated synaptosomes or (E, F) reconstituted synaptic membranes. Average ± SD from three or four independent isolation and/or reconstitution trials.
These observations are consistent with the enhanced physical coupling between isolated PSDs and their overlying PMs (Figure 2, A and B), and a working model for palmitoylation-mediated recruitment of synaptic raft domains is described next.

**Hypothetical working model**

Our observations prompt a tentative hypothetical model for the distinct composition and organization of the postsynaptic plasma membrane (Figure 5). This membrane appears to be enriched in raft-forming lipids (Figures 1 and 2), which accumulate during early postnatal development to promote stable raft domains (Figure 3). These changes are temporally coincident with a dramatic increase in the abundance of palmitoylated PSD-95 (Figure 4), the major physical linker between the immobilized PSD and its overlying PM. We envision several plausible scenarios that could account for these observations:

A1) Increased expression of a palmitoylated protein immobilized by its integration into the PSD (i.e., PSD-95) nucleates/stabilizes raft domains at the PSD-PM.

A2) Changes in lipid metabolism/synthesis produce lipids that facilitate stable raft domains, which in turn recruit palmitoylated PSD-95 to the postsynaptic PM.

A3) The two foregoing mechanisms participate in a coordinated positive feedback in which raft domains recruit palmitoylated PSD-95 (or promote its palmitoylation by a raft-resident palmitoyl transferase; Fukata et al., 2013). PSD-95 integrates into the PSD and recruits more rafts via its palmitoyl modifications.

All of these mechanisms converge on a working model for a mature synapse in which a palmitoylated, immobile scaffold and the unique lipid composition of the synapse cooperate to form a stable raft-like domain at the postsynaptic plasma membrane (Figure 5B). One possible rationale for localizing a raft domain at the synapse may be to control the protein content of the postsynaptic membrane, for example, by recruiting palmitoylated neurotransmitter receptors that regulate synaptic activity. We emphasize that although this model is consistent with all of our observations, it remains largely hypothetical because of the experimental limitations associated with imaging membrane lipid organization/composition.
at the level of a single synapse. Moreover, PSD-95 is unlikely to be the sole mediator of the connection between the PSD and its PM, as a number of other palmitoylated proteins are abundant at the synapse (Fukata and Fukata, 2010). Ultimately, our observations reveal robust developmental plasticity of synaptic membrane composition and physical organization, and although the mechanistic details and functional consequences of these effects remain to be resolved, we propose that they are important contributors to synaptic physiology.

MATERIALS AND METHODS

Isolation of synaptosomes and postsynaptic densities

Subcellular fractions were obtained from rat brains by using a slight modification of published protocol (Cohen et al., 1977). Mixed-gender Sprague Dawley rats were fed standard chow (Harlan Tech, Indianapolis, IN) ad libitum and killed at P2, P7, P14, P21, and P60. Forebrains were harvested within 30 s of decapitation and placed in ice-cold isotonic sucrose solution (0.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 0.32 M sucrose, 1 mM MgCl₂, 0.5 mM CaCl₂, and 1 μg/ml leupeptin) followed by disruption with 12 strokes of a motor-driven glass/Teflon homogenizer at 900 rpm on ice. After clarification of the homogenate by centrifugation at 1400 × g for 10 min, the supernatant was centrifuged at 13,800 × g for 10 min, and the pellet was suspended in isotonic solution (0.5 mM HEPES, pH 7.4, 0.32 M sucrose, and 1 μg/ml leupeptin) with five strokes in a glass/Teflon homogenizer. The synaptosomal preparation was obtained by layering the suspended samples on discontinuous sucrose density gradient (0.8/1.0/1.4 M) and centrifuging at 110,000 × g for 2 h at 4°C. The synaptosomal fraction was recovered from the interface between the 1.0 and 1.4 M sucrose and either stored as frozen aliquots at −80°C for subsequent analysis or processed further for preparation of PSDs.

PSDs were prepared by solubilizing the synaptosomes using 0.5% Triton X-100, followed by centrifugation on a discontinuous sucrose gradient (1.0/1.5/2.1 M) at 210,000 × g for 2 h. The interface between the 1.5 and 2.1 M sucrose-containing PSWs was then subjected to a second detergent extraction with 0.5% Triton X-100 plus 75 mM KC1 for 15 min at 4°C and a second round of centrifugation on a sucrose gradient (1.0/1.5/2.1 M) at 210,000 × g for 20 min at 4°C. The PSWs were collected from the 1.5/2.1 M interface, diluted in 5 mM HEPES, pH 7.4, pelleted at 210,000 × g for 20 min at 4°C, and resuspended in 5 mM HEPES, pH 7.4. Samples were used immediately or made into aliquots containing 20% glycerol and frozen at −80°C.

For lipidomics, membranes were isolated from synaptic vesicle preparations by hypotonic lysis. Specifically, 25 μg of protein (determined by bicinchoninic acid [BCA] assay) from synaptosomal preparations was diluted in 1 ml of H₂O to lyse synaptosomes and then centrifuged at 60,000 × g for 2 h in 4°C to pellet the membranes, which were resuspended in 150 mM ammonium bicarbonate.

Lipidomics by electron spray ionization and MS-MS

Lipidomics on synaptosome preparations were performed at Lipotype GmbH (Dresden, Germany) as described previously (Eising et al., 2009; Surma et al., 2015). Briefly, membrane suspensions were spiked with internal standard lipid mixture and then extracted with chloroform/methanol 10:1 (vol/vol). After centrifugation, the lower, lipid-containing, organic phase was collected (first-step extract), and the remaining water phase was extracted again under the same conditions. Again, the lower, organic phase was collected (second-step extract). Extracts were dried in a speed vacuum concentrator. The first-step extract underwent acetylation with acetyl chloride/chloroform 1:2 (vol/vol) mixture to derivatize cholesterol. All liquid-handling steps were performed using a Hamilton STARlet robotic platform.

Extracts in acquisition mixtures were infused with a robotic nanoflow ion source (TriVersa NanoMate; Advion Biosciences, Ithaca, NY) into a mass spectrometer instrument (Q Exactive; Thermo Scientific). Ceramide, dihexosylceramide, hexosylceramide, lysolipids, and sphingomyelin (SM) were monitored by negative-ion-mode Fourier transform (FT) MS. PA, PC, PE, phosphatidylinositol, PS, and ether species were monitored by negative-ion-mode FT MS-MS. Acetylated cholesterol was monitored by positive-ion-mode FT MS. SE, diacylglycerol, triacylglycerol species were monitored by positive-ion-mode FT MS-MS. Automated processing of acquired mass spectra and identification and quantification of detected molecular lipid species were performed by LipidXplorer software. Only lipid identifications with a signal-to-noise ratio >5, an absolute abundance of at least 1 pmol, and a signal intensity fivefold higher than in corresponding blank samples were considered for further data analysis.

Identification of ω-3 PUFAs

ESI-MS identifies lipids solely based on molecular weight; therefore the location of double bonds is not specified and ω-3 PUFAs cannot be explicitly identified. For our classification of ω-3 and ω-6 PUFAs, we focused on structures exclusive to those groups, namely 22:6 (DHA) and 20:5 (eicosapentaenoic acid) for the former and 20:4 (arachidonic acid) and 22:4 (adrenic acid) for the latter. These comprise the large majority of all lipid-incorporated PUFAs.

The PSD-PM samples were analyzed with a slightly different method because of residual detergents remaining from the extraction. The procedure has been previously described (Chan et al., 2012). Lipid extracts spiked with internal standards were analyzed using a 6490 Triple Quadrupole LC/MS system (Agilent Technologies, Santa Clara, CA). Glycerophospholipids and sphingolipids were separated by HPLC using an Agilent Zorbax RxSil column (inner diameter 2.1 × 100 mm) with mobile phase A (chloroform:methanol:1 M ammonium hydroxide, 89:10:0.1, vol/vol/vol) and mobile phase B (chloroform:methanol:water:ammonium hydroxide, 55:39:5:0.1, vol/vol/vol/vol); 95% A for 2 min, linear gradient to 30% A over 18 min and held for 3 min, and linear gradient to 95% A over 2 min and held for 6 min. Sterols and glycerolipids were separated with reverse-phase HPLC using an isotropic mobile phase with an Agilent Zorbax Eclipse XDB-C18 column (4.6 × 100 mm). Quantification of lipid species used multiple reaction monitoring transitions developed in earlier studies (Chan et al., 2012) in conjunction with referencing of appropriate internal standards: PA, 14.0/14.0; PC, 14.0/14.0; PE, 14.0/14.0; PI, 12.0/13.0; PS, 14.0/14.0; SM, d18:1/12:0; D₇-cholesterol, CE, 17:0; monoacylglycerol, 17:0; 4-methyl, 16:0, diether DAG; D₃-TAG, 16:0/18:0/16:0 (Avanti Polar Lipids, Alabaster, AL).

Lipid extraction and reconstitution into model membranes

The protein concentration of the synaptosome preparations was calculated by BCA assay (Thermo Scientific, Waltham, MA), and 100 μg of protein per sample was used for the lipid extraction. Lipids were extracted using a slightly modified Folch procedure (Folch et al., 1957). Briefly, four volumes of 2:1 (vol/vol) chloroform:methanol were added to the synaptosomes samples, which were vigorously shaken for 30 min and then centrifuged for 5 min at 13,000 × g. The aqueous phase and protein interphase
were discarded, and the organic (lipid-containing) phase was transferred to a new vial. The organic phase was washed twice by 1:1 (vol/vol) methanol:water as described. The final organic phase was then dried under a nitrogen stream, and then liquids were dissolved in 250 μl of 2:1 (vol/vol) chloroform:methanol. To estimate lipid content, cholesterol concentration in the extracted lipid samples was calculated using Amplex red cholesterol assay (Thermo Fisher). The total lipid concentration was calculated from the cholesterol content of the samples and the mole fraction obtained from lipidomics.

GVUs were prepared by electroformation using Teflon chambers with platinum wires in 100 mM sucrose solution as described previously (Sezgin et al., 2015). Miscibility transition temperature was calculated as previously described (Levental and Levental, 2015b). Briefly, a fluorescent lipid analogue (FAST-DIO (Thermo Scientific); 0.1% of the total lipids) was used to visualize phase separation in GVUs at various temperatures. All GVUs phase separated into coexisting liquid domains at low temperature, and these phases mixed into a single, uniformly fluorescent phase at higher temperatures. At least 50 vesicles per temperature were counted, and the fraction of phase separated vesicles as a function of temperature was used to calculate (from a sigmoidal fit) the temperature at which 50% of vesicles were phase separated, which was termed \( T_m \), or the domain melting temperature.

For measurements of bulk membrane physical properties, synaptosome lipids were reconstituted into multilamellar vesicles. Briefly, dried lipid films were dispersed in 100 μl of 10 mM HEPES and 150 mM NaCl (pH 7.4) and subjected to 10 freeze–thaw cycles. For membrane packing measurements, the environment-sensitive dye C-laurdan (TPProbes, Gimhae, South Korea) was added at 0.1% of total lipid concentration, and generalized polarization was calculated as previously (Sezgin et al., 2012). For fluidity measurements, DPH (Sigma-Aldrich, St. Louis, MO) was used as described (Lentz, 1993).

**Acyl-biotinylation exchange**

ABE was performed as previously described (Wan et al., 2007), with minor modifications. Briefly, 500 μg of synaptosomal fractions were precipitated with chloroform-methanol-water (1:2:2 vol/vol) and then centrifuged at 13,000 × g for 5 min to induce phase separation. The aqueous and organic phases were discarded, and the protein interphase was washed with methanol. Precipitated protein films were air-dried for 10 min, and then pellets were dissolved in 100 μl of 2% SDS buffer (25B; 2% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4). Proteins were then subjected to alkylation with 20 mM ethylmaleimide (Sigma-Aldrich) in 2SB for 1 h at 37°C, followed by two more methanol-chloroform precipitation steps as described. To release thioester-linked palmitoyl moieties, proteins were treated with 0.4 M hydroxylamine (Sigma-Aldrich), and the newly generated free cysteines were labeled with biotin using thiol-reactive biotinyl reagent 0.4 mM HPDP-biotin (Thermo Scientific). Proteins were then subjected to dialysis against 2SB. Volumes of samples were air-dried for 10 min, and then pellets were dissolved in 100 μl of 2SB. Protein samples were mixed with 4× Laemmli sample buffer (Bio-Rad, Hercules, CA) and heated to 95°C for 3 min. A 20-μg sample of protein was loaded onto a 10% polyacrylamide gel and separated by SDS-PAGE. Proteins were transferred on a polyvinylidene fluoride (Millipore, Billerica, MA) membrane using a Mini Trans-Blot system (Bio-Rad). Membranes were probed with PSD-95 rabbit polyclonal antibody (Thermo Fisher) at 1:1000, developed via immunofluorescence using anti-rabbit secondary antibody coupled with Alexa 555 (Life Technologies, Carlsbad, CA), and visualized on a Bio-Rad ChemiDoc MP Imaging System. Signals were quantified using Fiji software densitometry plug-in.

**Western blotting**

Protein samples were mixed with 4× Laemmli sample buffer (Bio-Rad, Hercules, CA) and heated to 95°C for 3 min. A 20-μg sample of protein was loaded onto a 10% polyacrylamide gel and separated by SDS-PAGE. Proteins were transferred on a polyvinylidene fluoride (Millipore, Billerica, MA) membrane using a Mini Trans-Blot system (Bio-Rad). Membranes were probed with PSD-95 rabbit polyclonal antibody (Thermo Fisher) at 1:1000, developed via immunofluorescence using anti-rabbit secondary antibody coupled with Alexa 555 (Life Technologies, Carlsbad, CA), and visualized on a Bio-Rad ChemiDoc MP Imaging System. Signals were quantified using Fiji software densitometry plug-in.

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