

Remodeling of the postsynaptic plasma membrane during neural development

Karolina Tulodziecka^a, Barbara B. Diaz-Rohrer^a, Madeline M. Farley^b, Robin B. Chan^c, Gilbert Di Paolo^{c,†}, Kandice R. Levental^a, M. Neal Waxham^{b,*}, and Ilya Levental^{a,*}

^aDepartment of Integrative Biology and Pharmacology and ^bDepartment of Neurobiology and Anatomy, McGovern Medical School at the University of Texas Health Science Center at Houston, Houston, TX 77030; ^cDepartment of Pathology and Cell Biology, Taub Institute for Research on Alzheimer's Disease and the Aging Brain, Columbia University Medical Center, New York, NY 10032

ABSTRACT Neuronal synapses are the fundamental units of neural signal transduction and must maintain exquisite signal fidelity while also accommodating the plasticity that underlies learning and development. To achieve these goals, the molecular composition and spatial organization of synaptic terminals must be tightly regulated; however, little is known about the regulation of lipid composition and organization in synaptic membranes. Here we quantify the comprehensive lipidome of rat synaptic membranes during postnatal development and observe dramatic developmental lipidomic remodeling during the first 60 postnatal days, including progressive accumulation of cholesterol, plasmalogens, and sphingolipids. Further analysis of membranes associated with isolated postsynaptic densities (PSDs) suggests the PSD-associated postsynaptic plasma membrane (PSD-PM) as one specific location of synaptic remodeling. We analyze the biophysical consequences of developmental remodeling in reconstituted synaptic membranes and observe remarkably stable microdomains, with the stability of domains increasing with developmental age. We rationalize the developmental accumulation of microdomain-forming lipids in synapses by proposing a mechanism by which palmitoylation of the immobilized scaffold protein PSD-95 nucleates domains at the postsynaptic plasma membrane. These results reveal developmental changes in lipid composition and palmitoylation that facilitate the formation of postsynaptic membrane microdomains, which may serve key roles in the function of the neuronal synapse.

Monitoring Editor
Howard Riezman
University of Geneva

Received: Jun 15, 2016
Revised: Aug 3, 2016
Accepted: Aug 4, 2016

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; Swilius *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

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 (Cotman *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

This article was published online ahead of print in MBoC in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E16-06-0420>) on August 17, 2016.

[†]Present address: Denali Therapeutics, South San Francisco, CA 94080.

*Address correspondence to: Ilya Levental (ilya.levental@uth.tmc.edu), M. Neal Waxham (M.N.Waxham@uth.tmc.edu).

Abbreviations used: DHA, docosahexaenoic acid; PM, plasma membrane; PSD, postsynaptic density; PUFA, polyunsaturated fatty acid.

© 2016 Tulodziecka *et al.* This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (<http://creativecommons.org/licenses/by-nc-sa/3.0>).

"ASCB," "The American Society for Cell Biology," and "Molecular Biology of the Cell" are registered trademarks of The American Society for Cell Biology.

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 (Lemmon, 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 biophysical 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 non-raft 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 -amino-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 (Ejsing *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;

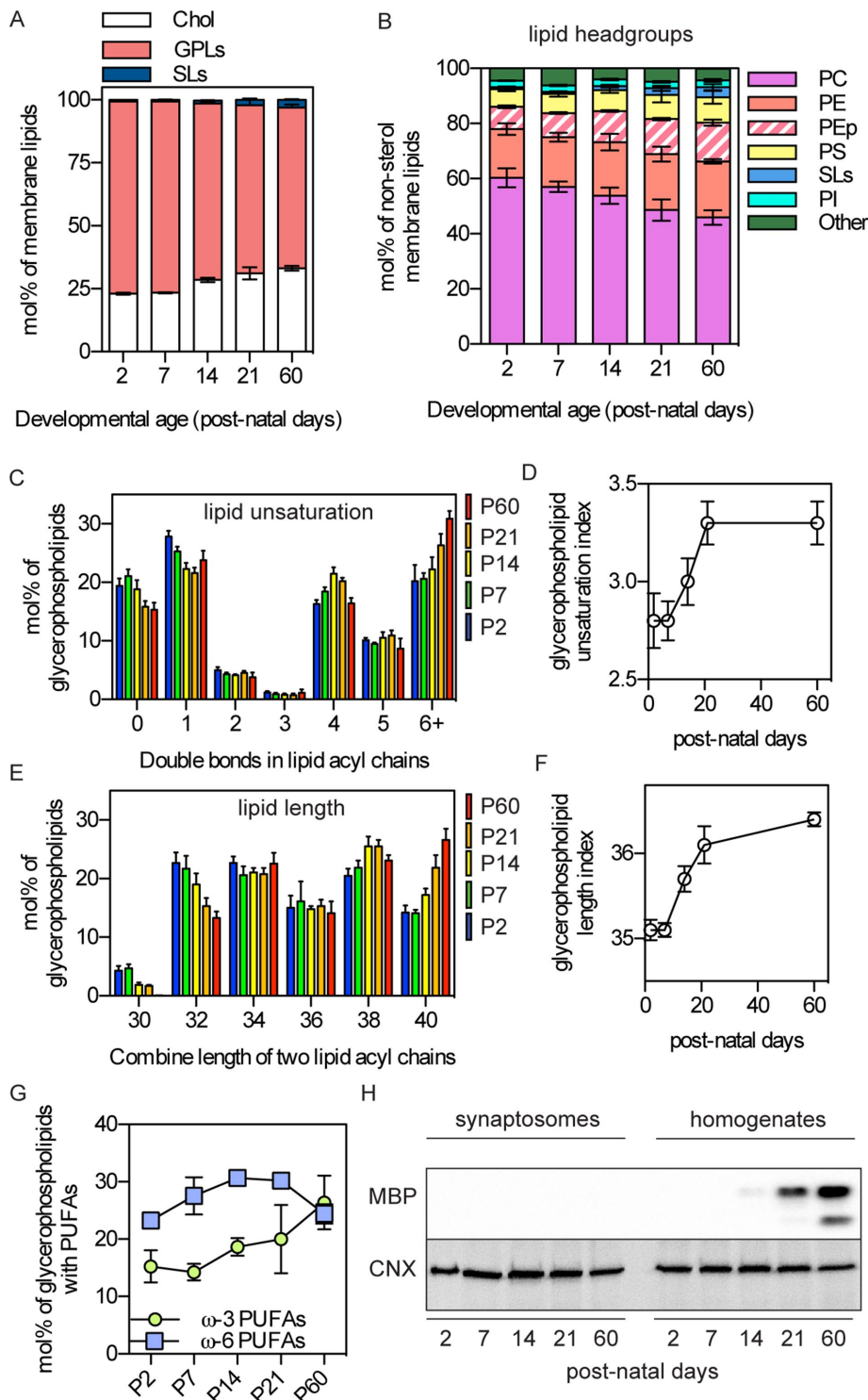


FIGURE 1: Synaptic membranes accumulate raft-promoting lipids during development. (A) Membranes of synaptosomes isolated from rat forebrains show increased abundance of cholesterol (Chol) and sphingolipids (SLs) during postnatal development, compensated for by a decrease in glycerophospholipids (GPLs). (B) The major nonsterol lipid class PC decreases, whereas PS and PE, and especially PEps, all increase during development. (C) Developmental accumulation in synaptosomal membranes of polyunsaturated lipid acyl chains at the expense of saturated and monounsaturated lipids. (D) Unsaturation index (defined as the weighted average of the number of double bonds) increases progressively until P21 and then plateaus. (E, F) Similar effects are seen in the length profile and index, with a developmental shift from shorter to longer lipids. (G) Progressive developmental accumulation of lipids containing ω -3 PUFAs, not observed for ω -6 PUFA-containing lipids. All data are average \pm SD from four independent preparations. (H) Lipidomic remodeling of synaptosomes did not result from contamination by

Figure 1A), 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 \sim 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, suggesting minimal contamination by myelin membranes (Figure 1H). Moreover, the observed remodeling is not a general effect

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.

of development on organismal lipidomes, as previous analyses of liver (Eberspacher *et al.*, 1977), jejunum (Meddings and Theisen, 1989), and intestinal microvilli (Schwarz *et al.*, 1985) lipids did not show trends similar to those observed here.

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 organellar 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 (Swulius *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 to the 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

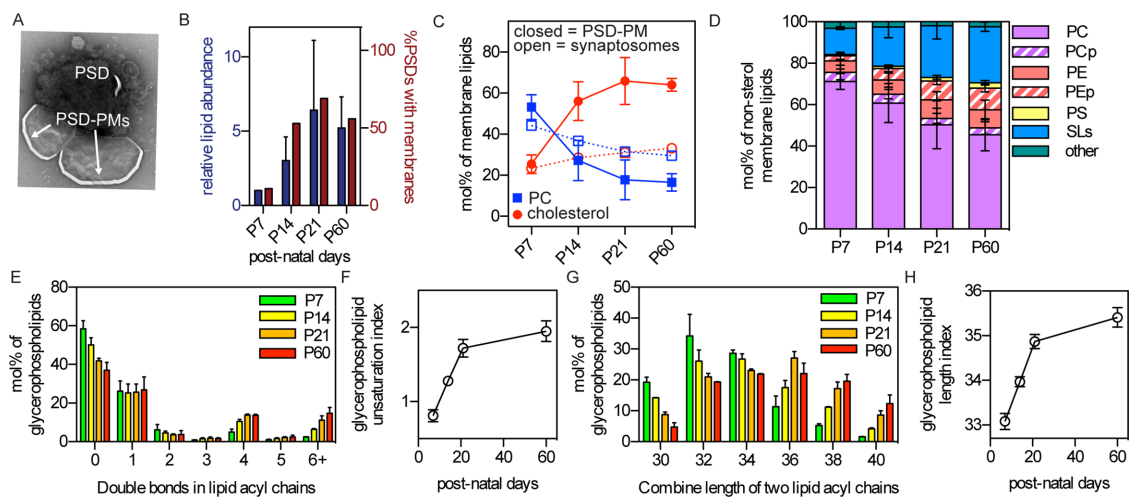


FIGURE 2: Characterization of PM associated with PSD. (A) Transmission electron microscopy image of isolated PSD, highlighting its associated PM. (B) Relative abundance of lipids in isolated protein-normalized PSD preparations (blue) and the percentage of PSDs exhibiting physically associated membranes both increase during development. (C–H) Lipidomic development of this PSD-PM mirrors the effects observed in synaptosomes, including (C) dramatic enrichment of cholesterol at the expense of PC and (D) gradual accumulation of sphingolipids, PE, and PE plasmalogens. (E, F) Similarly, PSD-PMs mirror synaptosomal membrane trends in (E, F) lipid unsaturation and (G, H) length. Average \pm SD from two experiments for all except P2, which produced only one usable preparation.

unilamellar vesicles (GUVs) doped with a trace amount of fluorescent lipids for microscopic visualization. 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 lipid composition of neurons and other sensory membranes (e.g., the rod outer segments of the retina; Fliesler 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; Rosenhouse-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

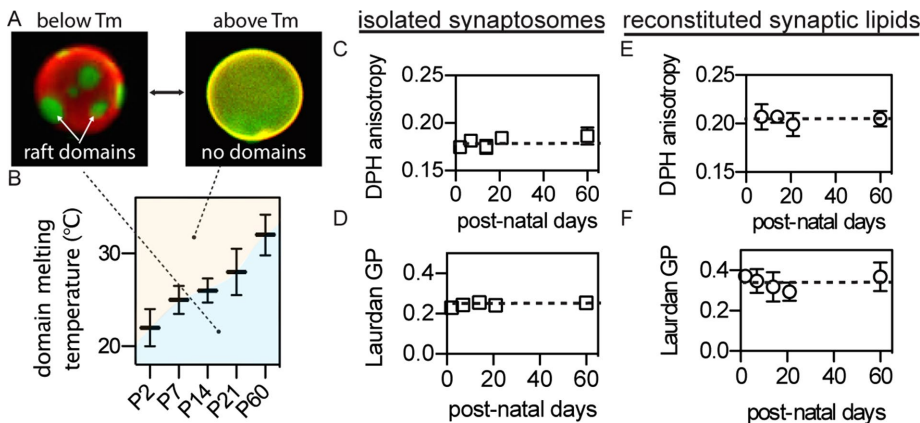


FIGURE 3: Developmental stabilization of domain formation in reconstituted synaptic membranes. (A) Lipids extracted from synaptosome membranes and reconstituted into model membranes (GUVs) showed robust, temperature-dependent domain formation, with microscopic, circular domains below the melting temperature (T_m) and uniform appearance above T_m . (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 \pm SD from three or four independent isolation and/or reconstitution trials.

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).

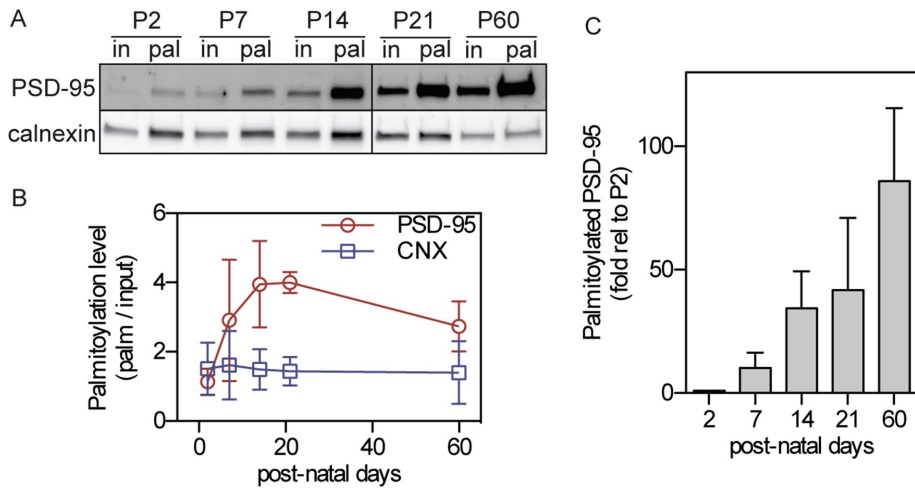


FIGURE 4: Abundance of palmitoylated PSD-95 increases dramatically during development. (A) Palmitoylation of PSD-95 and calnexin (CNX; used as control for assay efficiency) assayed by acyl-biotinyl exchange reveals a dramatic increase in palmitoylated PSD-95 not observed for CNX. (B) Normalizing the palmitoylated signal (palm) by input reveals relative palmitoylation levels. PSD-95 becomes progressively more palmitoylated until P21, whereas CNX shows no increased palmitoylation. (C) The relative abundance of palmitoylated PSD-95 increases by ~80-fold from P2 to P60. This is due to both the effect on palmitoylation level shown in B and a robust increase in PSD-95 expression during development.

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

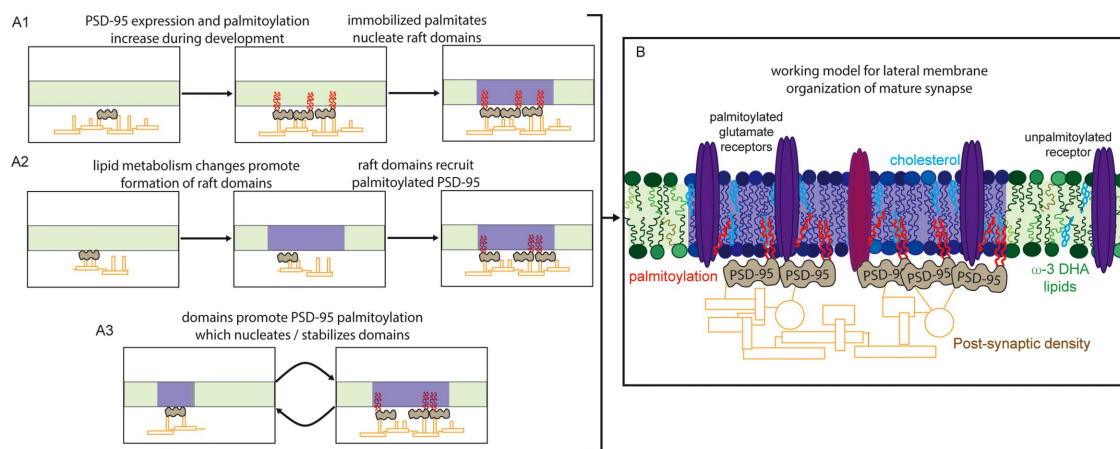


FIGURE 5: Hypothetical model of postsynaptic membrane organization. The developmental changes in synaptic lipidomes and membrane biophysical properties are conceptualized in a model for the lateral organization of the postsynaptic PM. This organization could come about by several possible routes, including the following: (A1) enhanced PSD-95 expression/palmitoylation nucleates membrane domains (purple rectangles); (A2) an established membrane domain recruits palmitoylated (red squiggles) PSD-95; or (A3) there is coordinated positive feedback between PSD-95 palmitoylation and membrane domain formation. (B) These mechanisms converge on a model in which palmitoylated PSD-95 anchors a stable raft-like membrane domain via its posttranslational palmitoylation. This domain may be used to selectively recruit protein components of the postsynaptic membrane, namely palmitoylated neurotransmitter receptors.

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 PSDs was then subjected to a second detergent extraction with 0.5% Triton X-100 plus 75 mM KCl 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 PSDs 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 synaptosomal 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 (Ejising *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 nano-flow 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 exclusives 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 Rx-Sil column (inner diameter 2.1 × 100 mm) with mobile phase A (chloroform:methanol:1 M ammonium hydroxide, 89.9:10:0.1, vol/vol/vol) and mobile phase B (chloroform:methanol:water:ammonium hydroxide, 55:39.9:5:0.1, 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 isocratic 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; D₅-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 lipids were dissolved in 250 μ l 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.

GUVs 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 GUVs at various temperatures. All GUVs 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 μ l 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-biotinyl exchange

ABE was performed as previously described (Wan *et al.*, 2007), with minor modifications. Briefly, 500 μ g of synaptosomal fractions were precipitated with chloroform-methanol-water (1:2:2 vol/vol) and then centrifuged at 13,000 $\times g$ 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 μ l of 2% SDS buffer (2SB; 2% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4). Proteins were then subjected to alkylation with 20 mM *N*-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 biotinylation reagent 0.4 mM HPDP-biotin (Thermo Scientific). Proteins were subjected to two further methanol-chloroform precipitation rounds and redissolved in 100 μ l of 2SB. Volumes of samples were adjusted to 1 ml by conjugation buffer (CB; 0.2% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.4). At this step, 100- μ l aliquots were taken as the “input” fraction, and the protein concentration was measured using BCA Protein Assay Kit (Pierce). Samples of 900 μ l were applied to streptavidin–agarose beads (Thermo Fisher) and incubated for 1 h at room temperature to bind biotinylated proteins. After incubation, streptavidin–agarose resin was washed three times in CB. Proteins were eluted from the resin for 15 min at 37°C in 100 μ l of CB solution supplemented in 1% β -mercaptoethanol. The

eluate from this step represents the palmitoylated pool of proteins and was compared with whole protein pool by Western blot.

Western blotting

Protein samples were mixed with 4 \times Laemmli sample buffer (Bio-Rad, Hercules, CA) and heated to 95°C for 3 min. A 20- μ g sample of protein was loaded onto a 10% polyacrylamide gel and separated by SDS-PAGE. Proteins were transferred onto 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.

ACKNOWLEDGMENTS

We thank Bowen Zhou and Sanjid Shahriar of Columbia University and Michal Surma and Christian Klose of Lipotype for help with the lipidomic analyses. G.D.P. is funded by National Institutes of Health Grant R01 NS056049. The other authors are funded by the Cancer Prevention and Research Institute of Texas (R1215), the National Institute of General Medical Sciences, National Institutes of Health (1R01GM114282), and the UT BRAIN Seed Program (362640). M.N.W. also acknowledges an endowment from the William W. Heless III Professorship. M.M.F. acknowledges support from a Ruth Kirschstein NRSA award from the National Institutes of Health/National Institute of Neurological Disorders and Stroke (F31NS084671).

REFERENCES

- Akbar M, Calderon F, Wen Z, Kim HY (2005). Docosahexaenoic acid: a positive modulator of Akt signaling in neuronal survival. *Proc Natl Acad Sci USA* 102, 10858–10863.
- Allen JA, Halverson-Tamboli RA, Rasenick MM (2007). Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci* 8, 128–140.
- Atilla-Gokcumen GE, Muro E, Relat-Goberna J, Sasse S, Bedigian A, Coughlin ML, Garcia-Manyes S, Eggert US (2014). Dividing cells regulate their lipid composition and localization. *Cell* 156, 428–439.
- Baumgart T, Hunt G, Farkas ER, Webb WW, Feigenson GW (2007). Fluorescence probe partitioning between Lo/Ld phases in lipid membranes. *Biochim Biophys Acta* 1768, 2182–2194.
- Bayes A, van de Lagemaat LN, Collins MO, Croning MD, Whittle IR, Choudhary JS, Grant SG (2011). Characterization of the proteome, diseases and evolution of the human postsynaptic density. *Nat Neurosci* 14, 19–21.
- Bennett V, Healy J (2009). Membrane domains based on ankyrin and spectrin associated with cell–cell interactions. *Cold Spring Harb Perspect Biol* 1, a003012.
- Braverman NE, Moser AB (2012). Functions of plasmalogen lipids in health and disease. *Biochim Biophys Acta* 1822, 1442–1452.
- Breckenridge WC, Gombos G, Morgan IG (1972). The lipid composition of adult rat brain synaptosomal plasma membranes. *Biochim Biophys Acta* 266, 695–707.
- Brown DA, London E (1998). Structure and origin of ordered lipid domains in biological membranes. *J Membr Biol* 164, 103–114.
- Chan RB, Oliveira TG, Cortes EP, Honig LS, Duff KE, Small SA, Wenk MR, Shui G, Di Paolo G (2012). Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. *J Biol Chem* 287, 2678–2688.
- Cohen RS, Blomberg F, Berzins K, Siekevitz P (1977). The structure of postsynaptic densities isolated from dog cerebral cortex. I. Overall morphology and protein composition. *J Cell Biol* 74, 181–203.
- Cole AA, Dosemeci A, Reese TS (2010). Co-segregation of AMPA receptors with G(M1) ganglioside in synaptosomal membrane subfractions. *Biochem J* 427, 535–540.
- Cotman C, Blank ML, Moehl A, Snyder F (1969). Lipid composition of synaptic plasma membranes isolated from rat brain by zonal centrifugation. *Biochemistry* 8, 4606–4612.

- Czech MP (2000). PIP2 and PIP3: complex roles at the cell surface. *Cell* 100, 603.
- Dart C (2010). Lipid microdomains and the regulation of ion channel function. *J Physiol* 588, 3169–3178.
- Delint-Ramirez I, Fernandez E, Bayes A, Kicsi E, Komiyama NH, Grant SG (2010). In vivo composition of NMDA receptor signaling complexes differs between membrane subdomains and is modulated by PSD-95 and PSD-93. *J Neurosci* 30, 8162–8170.
- Diaz-Rohrer BB, Levental KR, Simons K, Levental I (2014). Membrane raft association is a determinant of plasma membrane localization. *Proc Natl Acad Sci USA* 111, 8500–8505.
- Dietrich C, Bagatolli LA, Volovyk ZN, Thompson NL, Levi M, Jacobson K, Gratton E (2001). Lipid rafts reconstituted in model membranes. *Biophys J* 80, 1417–1428.
- Eberspacher BE, Organisciak DT, Massaro EJ (1977). Alterations in lipid composition of liver cell plasma membranes during development. *J Exp Zool* 199, 289–296.
- Ejsing CS, Sampaio JL, Surendranath V, Duchoslav E, Ekroos K, Klemm RW, Simons K, Shevchenko A (2009). Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proc Natl Acad Sci USA* 106, 2136–2141.
- Elias ER, Irons MB, Hurley AD, Tint GS, Salen G (1997). Clinical effects of cholesterol supplementation in six patients with the Smith-Lemli-Opitz syndrome (SLOS). *Am J Med Genet* 68, 305–310.
- Fiedler K, Kobayashi T, Kurchalia TV, Simons K (1993). Glycosphingolipid-enriched, detergent-insoluble complexes in protein sorting in epithelial cells. *Biochemistry* 32, 6365–6373.
- Fliessler SJ, Anderson RE (1983). Chemistry and metabolism of lipids in the vertebrate retina. *Prog Lipid Res* 22, 79–131.
- Folch J, Lees M, Stanley GHS (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226, 497–509.
- Fukata Y, Dimitrov A, Boncompain G, Vielemeyer O, Perez F, Fukata M (2013). Local palmitoylation cycles define activity-regulated postsynaptic subdomains. *J Cell Biol* 202, 145–161.
- Fukata Y, Fukata M (2010). Protein palmitoylation in neuronal development and synaptic plasticity. *Nat Rev Neurosci* 11, 161–175.
- Georgieva R, Chachaty C, Hazarsova R, Tessier C, Nuss P, Momchilova A, Staneva G (2015). Docosahexaenoic acid promotes micron scale liquid-ordered domains. A comparison study of docosahexaenoic versus oleic acid containing phosphatidylcholine in raft-like mixtures. *Biochim Biophys Acta* 1848, 1424–1435.
- Gerl MJ, Sampaio JL, Urban S, Kalvodova L, Verbavatz JM, Binnington B, Lindemann D, Lingwood CA, Shevchenko A, Schroeder C, et al. (2012). Quantitative analysis of the lipidomes of the influenza virus envelope and MDCK cell apical membrane. *J Cell Biol* 196, 213–221.
- Hashimoto M, Hossain S, Yamasaki H, Yazawa K, Masumura S (1999). Effects of eicosapentaenoic acid and docosahexaenoic acid on plasma membrane fluidity of aortic endothelial cells. *Lipids* 34, 1297–1304.
- Hering H, Lin CC, Sheng M (2003). Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. *J Neurosci* 23, 3262–3271.
- Innis SM (2007). Dietary (n-3) fatty acids and brain development. *J Nutr* 137, 855–859.
- Kanungo S, Soares N, He M, Steiner RD (2013). Sterol metabolism disorders and neurodevelopment—an update. *Dev Disabil Res Rev* 17, 197–210.
- Klose C, Surma MA, Gerl MJ, Meyenhofer F, Shevchenko A, Simons K (2012). Flexibility of a eukaryotic lipidome—insights from yeast lipidomics. *PLoS One* 7, e35063.
- Klose C, Surma MA, Simons K (2013). Organellar lipidomics—background and perspectives. *Curr Opin Cell Biol* 25, 406–413.
- Lemmon MA (2008). Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol* 9, 99–111.
- Lentz BR (1993). Use of fluorescent probes to monitor molecular order and motions within liposome bilayers. *Chem Phys Lipids* 64, 99–116.
- Levental I, Byfield FJ, Chowdhury P, Gai F, Baumgart T, Janmey PA (2009). Cholesterol-dependent phase separation in cell-derived giant plasma-membrane vesicles. *Biochem J* 424, 163–167.
- Levental I, Grzybek M, Simons K (2010). Greasing their way: lipid modifications determine protein association with membrane rafts. *Biochemistry* 49, 6305–6316.
- Levental KR, Levental I (2015a). Giant plasma membrane vesicles: models for understanding membrane organization. *Curr Top Membr* 75, 25–57.
- Levental KR, Levental I (2015b). Isolation of giant plasma membrane vesicles for evaluation of plasma membrane structure and protein partitioning. *Methods Mol Biol* 1232, 65–77.
- Levental KR, Lorent JH, Lin X, Skinkle AD, Surma MA, Stockenbojer EA, Gorfe AA, Levental I (2016). Polyunsaturated lipids regulate membrane domain stability by tuning membrane order. *Biophys J* 110, 1800–1810.
- Levy S, Shoham T (2005). Protein-protein interactions in the tetraspanin web. *Physiology* 20, 218–224.
- Lingwood D, Simons K (2007). Detergent resistance as a tool in membrane research. *Nat Protoc* 2, 2159–2165.
- Lingwood D, Simons K (2010). Lipid rafts as a membrane-organizing principle. *Science* 327, 46–50.
- Low-Nam ST, Lidke KA, Cutler PJ, Roovers RC, van Bergen en Henegouwen PM, Wilson BS, Lidke DS (2011). ErbB1 dimerization is promoted by domain co-confinement and stabilized by ligand binding. *Nat Struct Mol Biol* 18, 1244–1249.
- Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, Otto A, Pfrieger FW (2001). CNS synaptogenesis promoted by glia-derived cholesterol. *Science* 294, 1354–1357.
- Meddings JB, Theisen S (1989). Development of rat jejunum: lipid permeability, physical properties, and chemical composition. *Am J Physiol* 256, G931–G940.
- Owen DM, Williamson DJ, Magenau A, Gaus K (2012). Sub-resolution lipid domains exist in the plasma membrane and regulate protein diffusion and distribution. *Nat Commun* 3, 1256.
- Petralia RS, Sans N, Wang YX, Wenthold RJ (2005). Ontogeny of postsynaptic density proteins at glutamatergic synapses. *Mol Cell Neurosci* 29, 436–452.
- Pike LJ, Han X, Chung KN, Gross RW (2002). Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry* 41, 2075–2088.
- Pinot M, Vanni S, Pagnotta S, Lacas-Gervais S, Payet LA, Ferreira T, Gautier R, Goud B, Antony B, Barelli H (2014). Polyunsaturated phospholipids facilitate membrane deformation and fission by endocytic proteins. *Science* 345, 693–697.
- Rosenhouse-Dantsker A, Mehta D, Levitan I (2012). Regulation of ion channels by membrane lipids. *Compr Physiol* 2, 31–68.
- Runas KA, Malmstadt N (2015). Low levels of lipid oxidation radically increase the passive permeability of lipid bilayers. *Soft Matter* 11, 499–505.
- Salem N Jr, Litman B, Kim HY, Gawrisch K (2001). Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids* 36, 945–959.
- Sampaio JL, Gerl MJ, Klose C, Ejsing CS, Beug H, Simons K, Shevchenko A (2011). Membrane lipidome of an epithelial cell line. *Proc Natl Acad Sci USA* 108, 1903–1907.
- Schuck S, Simons K (2004). Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. *J Cell Sci* 117, 5955–5964.
- Schwarz SM, Hostetler B, Ling S, Mone M, Watkins JB (1985). Intestinal membrane lipid composition and fluidity during development in the rat. *Am J Physiol* 248, G200–G207.
- Sezgin E, Grzybek M, Buhl T, Dirxk R, Gutmann T, Coskun U, Solimena M, Simons K, Levental I, Schwillie P (2015). Adaptive lipid packing and bioactivity in membrane domains. *PLoS One* 10, e0123930.
- Sezgin E, Kaiser HJ, Baumgart T, Schwillie P, Simons K, Levental I (2012). Elucidating membrane structure and protein behavior using giant plasma membrane vesicles. *Nat Protoc* 7, 1042–1051.
- Shaikh SR, Dumaual AC, Castillo A, LoCascio D, Siddiqui RA, Stillwell W, Wassall SR (2004). Oleic and docosahexaenoic acid differentially phase separate from lipid raft molecules: a comparative NMR, DSC, AFM, and detergent extraction study. *Biophys J* 87, 1752–1766.
- Sheng M, Hoogenraad CC (2007). The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu Rev Biochem* 76, 823–847.
- Simons K, Gerl MJ (2010). Revitalizing membrane rafts: new tools and insights. *Nat Rev Mol Cell Biol* 11, 688–699.
- Soni SP, LoCascio DS, Liu Y, Williams JA, Bittman R, Stillwell W, Wassall SR (2008). Docosahexaenoic acid enhances segregation of lipids between: 2H-NMR study. *Biophys J* 95, 203–214.
- Stevens LJ, Zentall SS, Deck JL, Abate ML, Watkins BA, Lipp SR, Burgess JR (1995). Essential fatty acid metabolism in boys with attention-deficit hyperactivity disorder. *Am J Clin Nutr* 62, 761–768.
- Suh BC, Hille B (2008). PIP2 is a necessary cofactor for ion channel function: how and why? *Annu Rev Biophys* 37, 175–195.
- Surma MA, Herzog R, Vasilj A, Klose C, Christinat N, Morin-Rivron D, Simons K, Masoodi M, Sampaio JL (2015). An automated shotgun lipidomics platform for high throughput, comprehensive, and quantitative analysis of blood plasma intact lipids. *Eur J Lipid Sci Technol* 117, 1540–1549.

- Surma MA, Klose C, Klemm RW, Ejsing CS, Simons K (2011). Generic sorting of raft lipids into secretory vesicles in yeast. *Traffic* 12, 1139–1147.
- Suzuki T, Ito J, Takagi H, Saitoh F, Nawa H, Shimizu H (2001). Biochemical evidence for localization of AMPA-type glutamate receptor subunits in the dendritic raft. *Brain Res Mol Brain Res* 89, 20–28.
- Suzuki T, Zhang J, Miyazawa S, Liu Q, Farzan MR, Yao WD (2011). Association of membrane rafts and postsynaptic density: proteomics, biochemical, and ultrastructural analyses. *J Neurochem* 119, 64–77.
- Swulius MT, Farley MM, Bryant MA, Waxham MN (2012). Electron cryotomography of postsynaptic densities during development reveals a mechanism of assembly. *Neuroscience* 212, 19–29.
- Swulius MT, Kubota Y, Forest A, Waxham MN (2010). Structure and composition of the postsynaptic density during development. *J Comp Neurol* 518, 4243–4260.
- van den Bogaart G, Meyenberg K, Risselada HJ, Amin H, Willig KI, Hubrich BE, Dier M, Hell SW, Grubmuller H, Diederichsen U, et al. (2011). Membrane protein sequestering by ionic protein-lipid interactions. *Nature* 479, 552–555.
- van Meer G, Voelker DR, Feigenson GW (2008). Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* 9, 112–124.
- Veatch SL, Cicuta P, Sengupta P, Honerkamp-Smith A, Holowka D, Baird B (2008). Critical fluctuations in plasma membrane vesicles. *ACS Chem Biol* 3, 287–293.
- Wan J, Roth AF, Bailey AO, Davis NG (2007). Palmitoylated proteins: purification and identification. *Nat Protoc* 2, 1573–1584.
- Wassall SR, Stillwell W (2008). Docosahexaenoic acid domains: the ultimate non-raft membrane domain. *Chem Phys Lipids* 153, 57–63.
- Wilhelm BG, Mandad S, Truckenbrodt S, Krohnert K, Schafer C, Rammner B, Koo SJ, Classen GA, Krauss M, Haucke V, et al. (2014). Composition of isolated synaptic boutons reveals the amounts of vesicle trafficking proteins. *Science* 344, 1023–1028.