Title: Remodeling of the postsynaptic plasma membrane during neural development

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Running head: Development of synaptic membranes

Abbreviations:
PM – plasma membrane
PSD – post-synaptic density
PUFA – polyunsaturated fatty acid
DHA – docosahexaenoic acid

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 post-natal development and observe dramatic developmental lipidomic remodeling during the first 60 post-natal days, including progressive accumulation of cholesterol, plasmalogens, and sphingolipids. Further analysis of membranes associated with isolated post-synaptic densities (PSDs) suggests the PSD-associated post-synaptic 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 wherein palmitoylation of the immobilized scaffold protein PSD-95 nucleates domains at the post-synaptic plasma membrane. These results reveal developmental changes in lipid composition and palmitoylation that facilitate the formation of post-synaptic membrane microdomains, which may serve key roles in function of the neuronal synapse.

HIGHLIGHT SUMMARY
Neuronal synapses require precise regulation, particularly of membrane components. The composition and organization of synaptic membranes are dramatically remodeled during development, including accumulation of lipids associated with raft domains, and concomitant palmitoylation of PSD-95, suggesting recruitment of domains via scaffold lipidation.

INTRODUCTION

The neuronal synapse underlies much of neural physiology; 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 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 is important for neural physiology, as evidenced by the various neurodevelopmental abnormalities associated with lipid perturbations. For example, 'essential' fats like ω-3 polyunsaturated fatty acids are not synthesized by mammalian cells, 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 could 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 and Ferguson, 2000), 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 PIP_2 clusters as hotspots 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 themselves (Soni *et al.*, 2008), they can enhance raft formation by fluidizing non-raft regions and 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 AMPA- and NMDA-type glutamate receptors that gate post-synaptic 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. To date, there is 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 post-synaptic density (PSD) . The PSD is a dense web of scaffolds, signaling proteins, and receptors, many of which interact with the plasma membrane and play an important role in its organization . This physical connection between the post-synaptic membrane and its underlying PSD is mediated in large part by the scaffold protein PSD-95. PSD-95 is one of most abundant PSD proteins and interacts with the membrane via two post-translational 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 many other synaptic proteins involved in neuronal function and development (Fukata and Fukata, 2010).

Despite the clear functional connections between synaptic membranes neuronal function, there remains little detailed information about developmental changes in synaptic membrane composition and organization. Further, there is yet 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 post-natal development of the rat forebrain. We observe dramatic remodeling of synaptic membranes during the first weeks of post-natal development and correlate these effects to changes in the plasma membrane of the post-synaptic 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 yet 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 (Kusumi *et al.*, 2012).

Lipidomic analysis revealed clear accumulation of specific lipid subtypes in synaptic membranes during neural development(Fig. 1). Most notably, the canonical raft components cholesterol and sphingolipids (SLs) (Fiedler *et al.*, 1993; (Brown and London, 1998)(Fig. 1A), as well as the raft-associated phosphatidylethanolamine plasmalogen (PEp) (Pike *et al.*, 2002), were progressively enriched from 2 to 60 postnatal days, at the expense of phosphatidylcholine (PC)(Fig. 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 (Fig. 1C) leading to a progressive increase in lipid unsaturation that plateaued at P21

(Fig. 1D). There was also a robust shift to lipids with longer acyl chains (Fig. 1E) that showed a similar developmental time course (Fig. 1F). A notable result was the developmental accumulation of lipids containing ω-3 polyunsaturated fatty acids, 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 (Fig. 1G). Lipids with ω-6 PUFAs did not show the same trend (Fig. 1G). ω-3 PUFAs like DHA have been 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 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 (Fig. 1H). Moreover, the observed remodeling is not a general effect 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 similar trends 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, while synaptosomes are a convenient synaptic preparation, lipids derived from these represent a mixture of pre- and post-synaptic 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 post-synaptic 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 (TEM) revealed that these structures are often associated with a membrane component (Fig. 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 (Fig. 2B). This result was confirmed by quantifying the protein-normalized total lipid content of PSD preparations, which also increased during development (Fig. 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 non-ionic 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), which 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 Methods); however, we expected similar detergent effects as classical lipid raft preparations. Consistently, PSD-PMs from adult rats (P60) were enriched in raft lipids (most notably cholesterol, but also glycosphingolipids) compared to their precursor synaptosomes (Fig. 2C). However, beyond this likely artifactual enrichment, we observed developmental trends in PC, cholesterol (Fig 2C), PE plasmalogens (PEp), sphingolipids (Fig. 2D), and long, polyunsaturated lipids (Figs. 2E-H) that quantitatively mirrored those observed in synaptosomes. Based on these similarities, we posit that the developmental changes observed in synaptosomes reflect, at least in part, compositional remodeling of the post-synaptic 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

For 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 have recently shown in computational studies (Lin *et al.*, 2016), 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 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 (Fig. 3A). Specifically, the disordered / non-raft phase dye (rhodamine-DOPE) was excluded from the circular patches, while the ordered / raft phase dye (naphthopyrene (Baumgart *et al.*, 2007)) was enriched in those domains. Qualitatively similar behavior has been 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. Notably, 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 (Fig. 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 (Fig. 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 has been 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; Fig. 3C) nor lipid packing (C-laurdan GP; Fig. 3D) of isolated synaptosomes were affected during development. Similarly, no significant developmental trends in these biophysical properties were observable in membranes reconstituted from lipids extracted from synaptosomes (Fig. 3E-F). Thus, developmental lipidomic remodeling was associated specifically with stabilization of membrane domains rather than broad changes to membrane fluidity and packing.

It is necessary to point out that these reconstituted membrane experiments do not necessarily reflect the native organization of synaptic membranes. Rather, 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 (10-100s of nanometers) and highly dynamic, and have been implicated in 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 suggests 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 – like PSD-95 when integrated into the densely cross-linked PSD matrix – the converse may apply, i.e. immobile, membrane-embedded palmitates may recruit/retain diffusible raft domains. Thus, one potential mechanism for the enrichment of raft domains in the post-

synaptic 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 (previously noted (Petralia *et al.*, 2005; (Swulius *et al.*, 2010)) increase in PSD-95 expression (Fig. 4A), but also an increase in PSD-95 palmitoylation levels independent of expression during development (Fig. 4A-B). As a control, we measured palmitoylation of calnexin, an ER protein not be expected to participate in PM organization, and observed no developmental changes in palmitoylation levels. Combined, the increased expression and palmitoylation yielded an ~80-fold increase in palmitoylated PSD-95 from P2 to P60 (Fig. 4C). These observations are consistent with the enhanced physical coupling between isolated PSDs and their overlying PMs (Fig. 2A-B) and a working model for palmitoylation-mediated recruitment of synaptic raft domains described below.

<u>*Hypothetical working model*</u>

Our observations prompt a tentative hypothetical model for the distinct composition and organization of the post-synaptic plasma membrane (Fig. 5). This membrane appears to be enriched in raft forming lipids (Fig. 1-2), which accumulate during early post-natal development to promote stable raft domains (Fig. 3). These changes are temporally coincident with a dramatic increase in the abundance of palmitoylated PSD-95 (Fig. 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 post-synaptic PM;

(A3) the two mechanisms above participate in a coordinated positive feedback wherein 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 wherein a palmitoylated, immobile scaffold and the unique lipid composition of the synapse cooperate to form a stable raft-like domain at the post-synaptic plasma membrane (Fig. 5B). One possible rationale for localizing a raft domain at the synapse may be to control the protein content of the post-synaptic membrane, e.g. by recruiting palmitoylated neurotransmitter receptors, and thereby regulate synaptic activity. We emphasize that although this model is consistent with all 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 while the mechanistic details and functional consequences of these effects remain to be resolved, we propose they are important contributors to synaptic physiology.

MATERIALS AND METHODS

Isolation of synaptosomes and post-synaptic 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) *ad libitum* and sacrificed at P2, P7, P14, P21 and P60. Forebrains were harvested within 30 sec of decapitation and placed in ice cold isotonic sucrose solution (0.5 mM Hepes, pH 7.4, 0.32 M sucrose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 1μg/ml leupeptin) followed by disruption with 12 strokes of a motor driven glass/Teflon homogenizer at 900 rpm on ice. After clarifying the homogenate by centrifugation at 1,400 x g for 10 min, the supernatant was centrifuged at 13,800 x g for 10 min and the pellet was suspended in isotonic solution (0.5 mM Hepes, pH 7.4, 0.32 M sucrose, and 1μg/ml leupeptin) with 5 strokes in a glass/Teflon homogenzier. The synaptosomal preparation was obtained by layering the suspended samples on discontinuous sucrose density gradient (0.8 M/1.0 M/1.4 M) and centrifuging at 110,000 x g for 2 hr at 4°C. The synaptosomal fraction was recovered from the

interface between the 1.0 M 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 synaptosomses using 0.5% Triton X-100, followed by centrifugation on a discontinuous sucrose gradient (1.0 M/1.5 M/2.1 M) at 210,000 x g for 2 hr. The interface between the 1.5 M 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 M/1.5 M/2.1 M) at 210,000 x 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 x g for 20 min at 4°C and finally resuspended in 5 mM HEPES, pH 7.4. Samples were either 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, 25ug of protein (determined by BCA assay) from synaptosomal preparations were diluted in 1 ml of H_2O to lyse synaptosomes, then centrifuged at 60,000xg for 2 hours in 4°C to pellet the membranes, which were resuspended in 150 mM ammonium bicarbonate.

Lipidomics by electron spray ionization and tandem MS-MS
Lipidomics on synaptosome preparations were performed at Lipotype GmbH (Dresden, Germany) as described previously (Ejsing *et al.*, 2009; (Surma *et al.*, 2015). Briefly, membrane suspensions were spiked with internal standard lipid mixture, then extracted with chloroform/methanol 10:1 (v:v). After centrifugation, the lower, lipid-containing, organic phase was collected (1st step extract), and the remaining water phase was extracted again under the same conditions. Again the lower, organic phase was collected (2nd step extract). Extracts were dried in a speed vacuum concentrator. The 1st step extract underwent acetylation with acetyl chloride/chloroform 1:2 (v:v) 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) into a mass spectrometer instrument (Q Exactive, Thermo Scientific). Cer, DiHexCer, HexCer, lysolipids, and SM were monitored by negative ion mode FT MS. PA, PC, PE, PI, PS, and ether species were monitored by negative ion mode FT MSMS. Acetylated cholesterol was monitored by positive ion mode FT MS. SE, DAG, TAG and species were monitored by positive ion mode FT MSMS. Automated processing of acquired mass spectra, 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 5-fold 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 w-3 PUFAs cannot be explicitly identified. For our classification of w-3 and w-6 PUFAs, we focused on structures exclusives to those groups, namely 22:6 (DHA) and 20:5 (eicosapentaenoic) 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 x 100 mm) with mobile phase A (chloroform:methanol:1 M ammonium hydroxide, 89.9:10:0.1, v/v) and mobile phase B (chloroform:methanol:water:ammonium hydroxide, 55:39.9:5:0.1, v/v); 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 x 100 mm). Quantification of lipid species was accomplished using multiple reaction monitoring (MRM) transitions that were 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, D_7-cholesterol, CE 17:0, MG 17:0, 4ME 16:0 diether DG, D_5-TG 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) and 100 ug of protein per sample were used for the lipid extraction. Lipids were extracted using a slightly modified Folch procedure (Folch *et al.*, 1957). Briefly, 4 volumes of 2:1 (v/v) chloroform:methanol were added to the synaptosomes samples, which were vigorously shaken for 30 minutes, 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 2x by 1:1 (v/v) methanol:water, as above. The final organic phase was then dried under a nitrogen stream, then lipids were dissolved in 250ul of 2:1 (v/v) chloroform:methanol. To estimate lipid content, cholesterol concentration in the extracted lipid samples was calculated using Amplex red cholesterol assay (Thermo Fischer). Using the cholesterol content of the samples and the mol fraction obtained from lipidomics, the total lipid concentration was calculated.

GUVs were prepared by electrofomation using Teflon chambers with platinum wires in 100mM 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 analog (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, synaptosomes lipids were reconstituted into multilamellar vesicles. Briefly, dried lipid films were dispersed in 100 ul of 10 mM Hepes, 150 mM NaCl (pH 7.4) and subjected to 10 freeze-thaw cycles. For membrane packing measurements, the environment-sensitive dye C-Laurdan (TPProbes) was added at 0.1% of total lipid concentration and the generalized polarization calculate as previously (Sezgin *et al.*, 2012). For fluidity measurements, diphenylhexatriene (DPH) (Sigma-Aldrich) was used as decribed (Lentz, 1993).

Acyl-biotinyl exchange (ABE)

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 v/v), then centrifuged at 13,000 g for 5 minutes 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 minutes, then pellets were dissolved in 100 µl 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) in 2SB for 1 hour at 37°C, followed by two more methanol-chloroform precipitation steps, as described above. In order to release thioester-linked palmitoyl moieties, proteins were treated with 0.4 M hydroxylamine (Sigma) 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 re-dissolved in 100 µl 2SB. Volumes of samples were adjusted to 1ml by conjugation buffer (CB: 0.2% Triton X-100, 150 mM NaCl, 50 mM Tris pH 7.4). At this step, 100 µl aliquots were taken as the 'input' (In) fraction, and the protein concentration was measured using BCA Protein Assay Kit (Pierce). 900 µl of samples were applied to streptavidin–agarose beads (Thermo Fisher) and incubated for an hour at room temperature to bind biotinylated proteins. Followed incubation, streptavidin-agarose resin was washed 3 times in CB. Proteins were eluted from the resin for 15 minutes at 37C in 100 µl of CB solution supplemented in 1% β-mercaptoethanol. The eluate from this step represents the palmitoylated (pal) pool of proteins and was compared to whole protein pool (in) by Western blot.

Western blotting

Protein samples were mixed with 4x Laemmli sample buffer (Bio-Rad) and heated to 95°C for 3 minutes. 20 µg of protein sample was loaded to 10% polyacrylamide gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transfer on PVDF (Millipore) membrane using Mini Trans-Blot® system

(Bio-Rad). Membranes were probed with PSD-95 rabbit polyclonal antibody 1:1000 (Thermo Fisher) and developed via immunofluorescence using anti-rabbit secondary antibody coupled with Alexa-555® (Life Technologies) and visualized on Bio-Rad ChemiDoc MP Imaging System. Signals were quantified using Fiji software densitometry plug-in.

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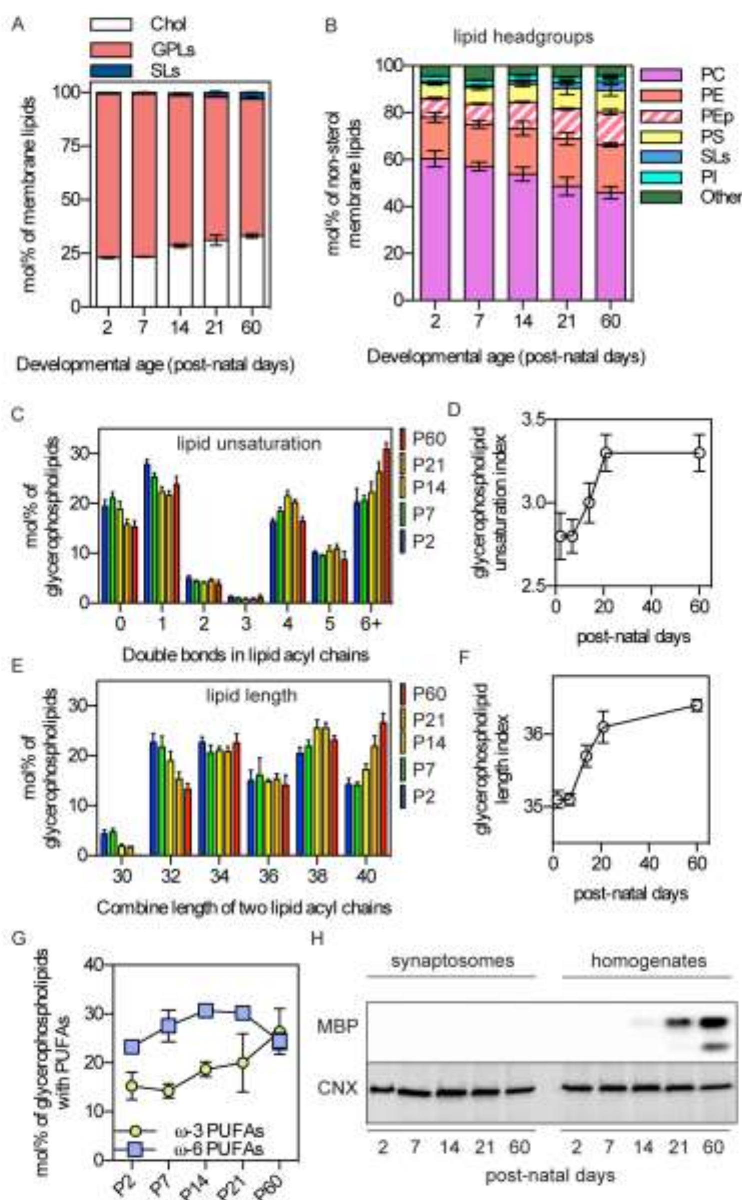

Fig. 1 – Synaptic membranes accumulate raft-promoting lipids during development. (A) Membranes of synaptosomes isolated from rat forebrains show increased abundance of cholesterol (Chol) and sphingolipids (SLs) during post-natal development, compensated for by a decrease in glycerophospholipids (GPLs). (B) The major non-sterol lipid class phosphatidylcholine (PC) decreases, while phosphatidylserine (PS) and –ethanolamin (PE), and especially PE plasmalogens (PEp) 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 number of double bonds) increases progressively until P21, 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 polyunsaturated fatty acids (PUFAs), not observed for ω-6 PUFA-containing lipids. All data are average +/- SD from N=4 independent preparations. (H) Lipidomic remodeling of synaptosomes did not result from contamination by 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.

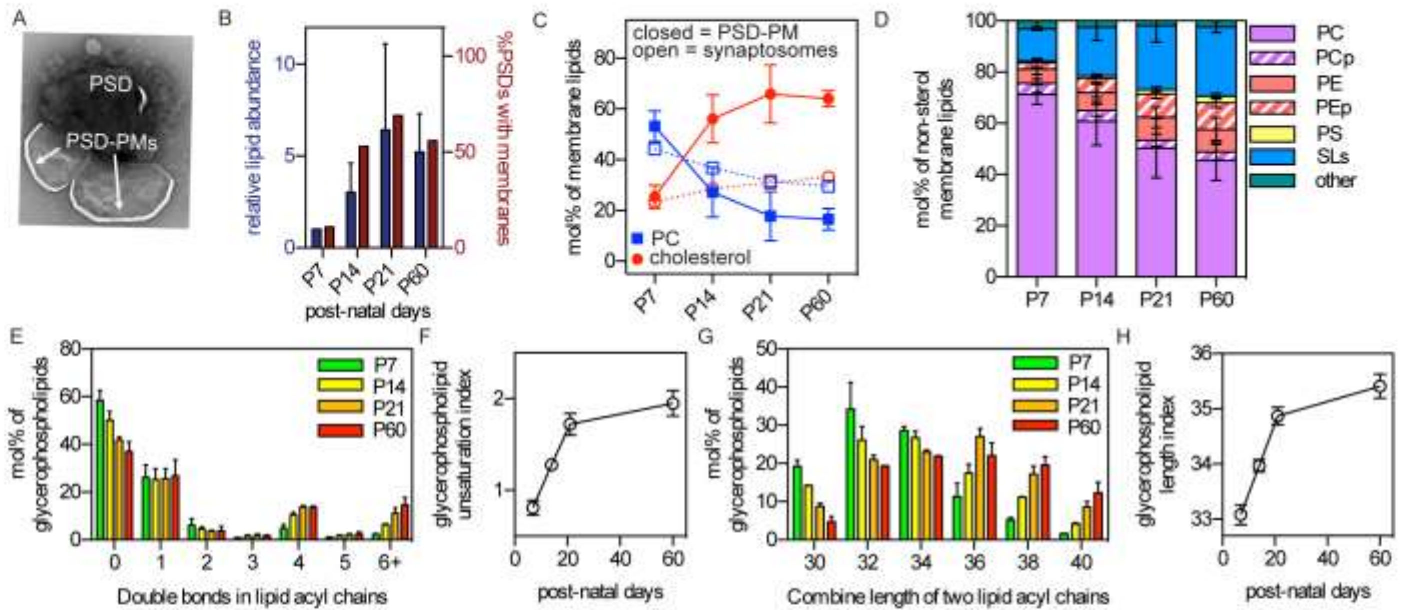

Fig. 2 – Characterization of PM associated with PSD. (A) Transmission electron microscopy (TEM) image of isolated post-synaptic density (PSD) highlighting its associated plasma membrane (PM). (B) Relative abundance of lipids in isolated protein-normalized PSD preparations (blue) and the percentage of 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 +/- SD from N=2 experiments for all except P2, which only produced one usable preparation.

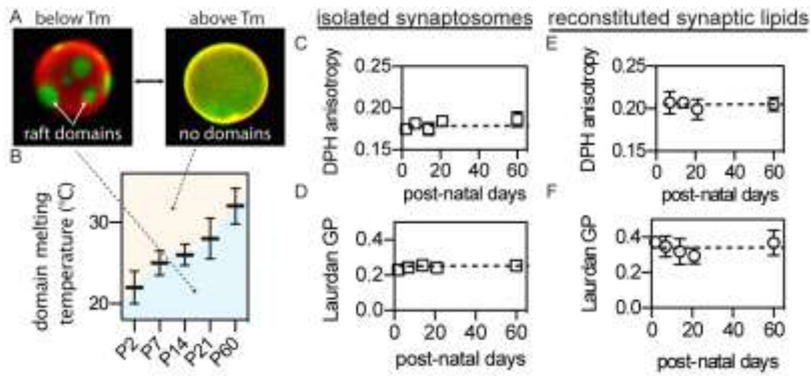

Fig. 3 – Developmental stabilization of domain formation in reconstituted synaptic membranes. (A) Lipids extracted from synaptosome membranes and reconstituted into model membranes (GUVs) showed robust, temperature-dependent domain formation, with microscopic, circular domains below the melting temperature (T_m) and uniform appearance above T_m. (B) Increased domain stability is evidenced by higher domain melting temperature during development. (C and E) DPH anisotropy probing membrane fluidity and (D and 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 ± st. dev. from 3-4 independent isolation and/or reconstitution trials.

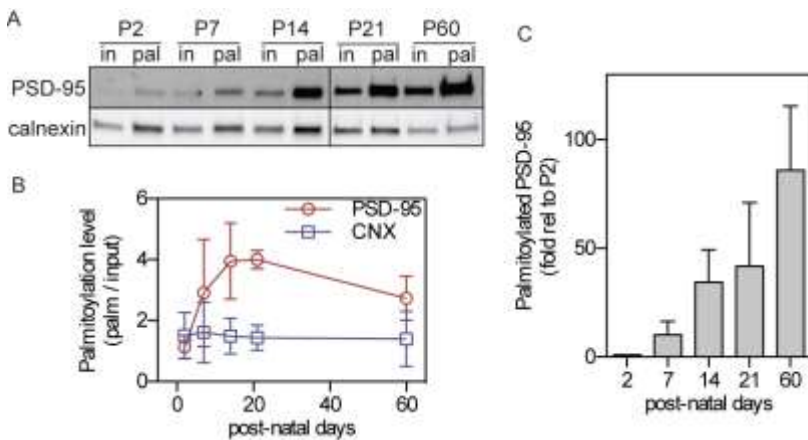

Fig 4 – Abundance of palmitoylated PSD-95 increases dramatically during development. (A) Palmitoylation of PSD-95 and calnexin (CNX; used as control for assay efficiency) assayed by acyl-biotinyl exchange reveals a dramatic increase in palmitoylated (pal) PSD-95 not observed for CNX. (B) Normalizing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

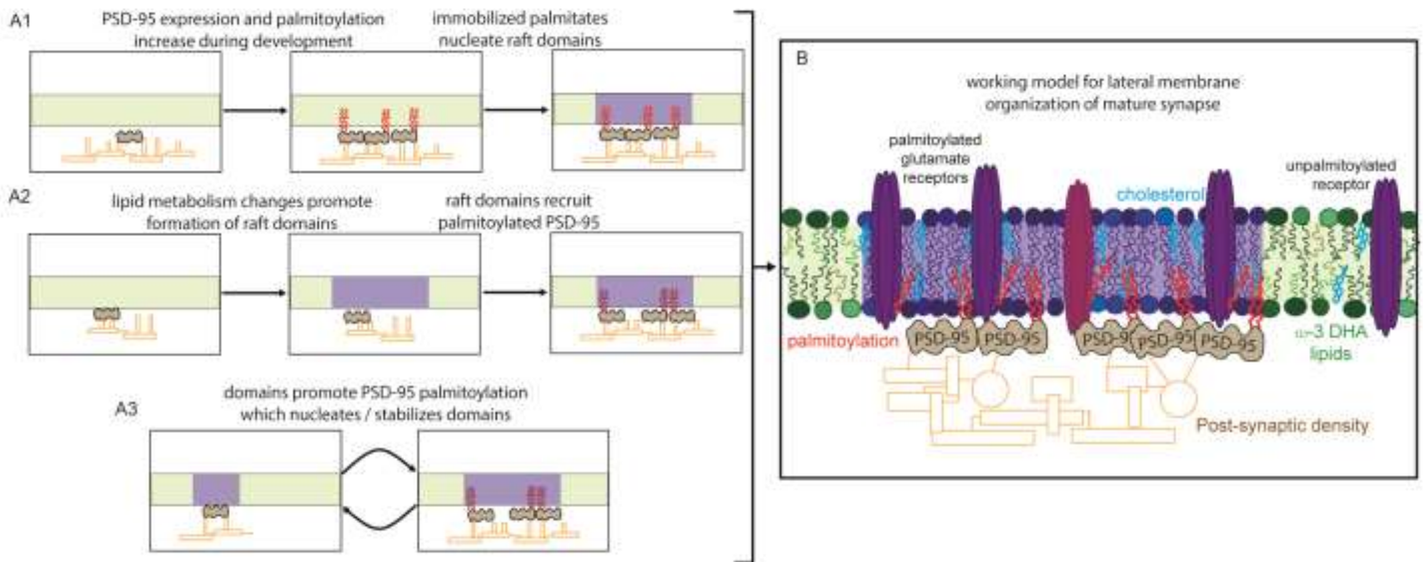

Fig 5 – Hypothetical model of post-synaptic membrane organization. The developmental changes in synaptic lipidomes and membrane biophysical properties are conceptualized in a model for the lateral organization of the post-synaptic PM. This organization could come about by several possible routes, including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