Regional changes in CNS and retinal glycerophospholipid profiles with age: a molecular blueprint

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Abstract We present here a quantitative molecular blueprint of the three major glycerophospholipid (GPL) classes, phosphatidycholine (PC), phosphatidylethanolamine (PE), and phosphatidylcholine (PS), in retina and six regions of the brain in C57Bl6 mice at 2, 10, and 26 months of age. We found an age-related increase in molecular species containing saturated and monoenoic FAs and an overall decrease in the longer-chain PUFAs across brain regions, with loss of DHA-containing molecular species as the most consistent and dramatic finding. Although we found very-long-chain PUFAs (VLC-PUFAs) (≥C28) in PC in the retina, no detectable levels were found in any brain region at any of the ages examined. All brain regions (except hippocampus and retina) showed a significant increase with age in PE plasmalogens. All three retina GPLs had di-PUFA molecular species (predominantly 44:12), which were most abundant in PS (∼30%). In contrast, low levels of di-PUFA GPL (1–2%) were found in all regions of the brain. This study provides a regional and age-related assessment of the brain’s lipidome with a level of detail, inclusion, and quantification that has not heretofore been published.—Hopiavuori, B. R., M.-P. Agbaga, R. S. Brush, M. T. Sullivan, W. E. Sonntag, and R. E. Anderson. Regional changes in CNS and retinal glycerophospholipid profiles with age: a molecular blueprint. J. Lipid Res. 2017, 58: 668–680.

Supplementary key words brain lipids • brain • fatty acid • phospholipids • phosphatidylycerol • chlorinated lipids • glycerophospholipid • LIPIDMASS ANALYSIS • MONO, monoenoic; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; VLC-PUFA, very long chain PUFAs

In the last few decades, neuroscientists have begun to identify and elucidate many unexpected and dynamic roles of lipid molecules in the brain (1). These changes include maintaining the biophysical properties of lipid rafts (2), regulating ion channel and receptor activities (3–12), protecting neurons from oxidant and other stresses (13–15), and regulating neuronal gene transcription (16–18) and neurotransmitter release (19). With more and more neuronal roles being identified each year for complex lipid molecules, FAs, and bioactive lipid derivatives, it is imperative that we have a molecular blueprint of which lipid classes are expressed in which regions of the brain and the molecular species they contain. By generating such a blueprint, we can begin a targeted approach to understanding the potentially multifaceted roles for these molecules in regulating and maintaining neuronal development, health, and function.

Since the early pioneering work of Folch-Pi (20–22), numerous studies with both human and animal postmortem brains under normal aging conditions, with various levels of cognitive impairment, and those with Alzheimer’s or other dementias. The primary focus of many of these studies was on bioactive lipids such as DHA and arachidonic acid (AA), with emphasis on the relative percent composition of n3 and n6 FAs, lipid modification of age-induced alterations in gene expression, and relative percent composition of certain lipid classes (3, 4, 16, 41–43). To our knowledge, there has been no thorough comparative analysis of the age-related variations.

Abbreviations: AA, arachidonic acid; FAME, FA methyl ester; GPL, glycerophospholipid; LIMSA, Lipid Mass Spectrum Analysis; MONO, monoenoic; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SAT, saturated; UOHSC, University of Oklahoma Health Sciences Center; VLC-PUFA, very long chain PUFAs

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changes in major glycerophospholipid (GPL) profiles in multiple regions of the brain and the retina. Like any other mammalian organ system, the brain changes as a consequence of age (44–46). There is some degree of evidence that components of the lipidome have been shown to change with age and have been linked to pathological degeneration and disease (41, 42, 47–49). Yet, the age-related lipid profiles of the various brain regions have not been adequately characterized, and, thus, it is important to determine whether significant age- and region-specific changes occur and, if so, to define the nature of those alterations.

We hypothesize, first, that the various regions of the CNS are composed of unique compositions of lipid molecules that depend on that region’s function, and, second, that the relative composition of these molecules changes differentially with age. We chose the aging mouse due to its close genetic proximity to humans, its reliable use in aging research due to its relatively short life span (50), and its malleability to genetic engineering. Using significant advances in the technology associated with lipidomic analysis, we combined traditional lipid biochemistry with new and cutting-edge technology to evaluate the regional lipid composition of the mouse brain with a level of detail, scrutiny, and inclusion that to date has not been done. In the present study, we present a detailed quantitative molecular analysis of the three major GPL classes, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), in retina, hippocampus, cerebellum, brainstem, cortex, white matter, and midbrain of 2-, 10-, and 26-month-old mice.

MATERIALS AND METHODS

Animals

C57Bl/6 mice of mixed sex and ages 2, 10, and 26 months were purchased from the National Institute on Aging (NIA) and acclimated in the University of Oklahoma Health Sciences Center (OUHSC) vivarium for at least 2 weeks (12 h ON, 12 h OFF, ~150 lux). The animals were fed Purina Lab irradiated 5053F lab diet (LabDiet®, Land O’ Lakes Inc., St. Louis, MO) ad libitum. The animals were monitored routinely for endo and ecto parasites; blood samples are taken quarterly from sentinel mice as part of our health monitoring of all rodents to exclude most bacteria and viruses. Animals were euthanized by cervical dislocation followed by decapitation. The following tissues were dissected, frozen, and stored at −80°C: retina, hippocampus, cerebellum, brainstem, cortex, white matter, and midbrain.

All procedures were performed in accordance with the Association for Research in Vision and Ophthalmic Statement for the Use of Animals in Ophthalmic and Vision Research and the UOHS Guidelines for Animals in Research. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the UOHS.

Tandem MS analysis of lipids

The methods have been described previously (51). Briefly, tissue was homogenized in 40% aqueous methanol and then diluted to a concentration of 1:40 with 2-propanol/methanol/chloroform (4:2:1 v/v/v) containing 20 mM ammonium formate and 1.0 µM PC (14:0/14:0), 1.0 µM PE (14:0/14:0), 0.33 µM PS (14:0/14:0), and 12.5 nM ceramide (d18:1/12:0) as internal standards. Samples were introduced into a triple-quadrupole mass spectrometer (TSQ Ultra, Thermo Scientific, Oakwood Village, OH) by using a chip-based nano-ESI source (Advion NanoMate, Advion, Ithaca, NY) operating in infusion mode. PC lipids were measured by using precursor ion scanning of m/z 184; PE lipids (including plasmalogens) were measured by using neutral loss scanning of m/z 141; and PS lipids were measured by using neutral loss scanning of m/z 185. All species detected for each group are represented as a relative percentage of the sum based on their response values. Abundances of lipid molecular species were calculated by using the Lipid Mass Spectrum Analysis (LIMSA) software (University of Helsinki, Helsinki, Finland). LIMSA was developed at the University of Helsinki for quantitative analysis of mass spectra of complex lipid samples. LIMSA can do peak finding, integration, assigning, isotope correction, and quantitation with internal standards. In this work, raw data from the instrument were exported into Excel, and LIMSA was used as an isotope correction algorithm. Specifically, the method used the integrated area of the first isotope peak and corrected for the isotope overlap by scaling and subtracting the calculated isotope pattern from subsequent peaks. LIMSA then calculated the isotope-corrected abundances by comparison to added internal standards [1.0 µM PC (14:0/14:0), 1.0 µM PE (14:0/14:0), 0.33 µM PS (14:0/14:0), and 12.5 nM ceramide (d18:1/12:0)].

2D-TLC and FAME determination of total PC, PE, and PS

Total lipids from tissues were extracted in chloroform-methanol-water (1:1:1) according to the method of Bligh and Dyer (52) as described in Martin et. al. (53). The total lipid extracts were concentrated and stored at −20°C under N₂ in a known volume of chloroform-methanol (2:1, v/v).

PC, PE, and PS lipid classes were isolated from the total lipid extracts by using high-performance TLC (HPTLC) plates (Analtech, Newark, DE) and a 2D, three-solvent method described previously (53–56). Lipid spots on the HPTLC plates were visualized under UV after staining with 2,7-dichlorofluorescein. The PC, PE, and PS spots were scraped from the plate for gas chromatographic analysis of FAs.

Dichlorofluorescein-stained lipid spots were scraped from the TLC plates, and esterified FAs were hydrolyzed and converted to methyl esters for GC. Silica from each spot was added to a screw-top test tube, and a mixture of pentadecanoic acid (15:0) and heptadecanoic acid (17:0) was added as internal standards. FA methyl esters (FAMEs) were formed by heating in the presence of 2% sulfuric acid in methanol at 85°C for 1 h. FAMEs were extracted into hexane, dried under nitrogen, resuspended in nonane, and quantified by using an Agilent Technologies 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) with flame ionization detector (57).

Statistical analysis

Each value presented in the figures is the mean ± SD of four independent analyses. Two-way ANOVA with Tukey’s multiple comparisons test was performed on all molecular species comparisons. Total lipid-phosphorus values reported in supplemental Fig. S1 and supplemental Table S23 are mean values for each region at all ages (n = 12). After failing to pass a Brown-Forsythe test of equal SD between each region, raw data for this comparison were transformed to log₂[(Y = log(Y)] and subsequent P values and significance were derived from these transformed values. All analysis was performed by using GraphPad Prism (Version 6.07 for Windows, GraphPad Software, San Diego, CA). Error bars for all figures represent SD of the mean; multiplicity-adjusted P values are reported in the supplemental tables for each comparison. The
RESULTS

Analysis of PC, PE, and PS was performed in retina, hippocampus, cerebellum, brainstem, cortex, white matter, and midbrain of 2-, 10-, and 26-month-old mice. The relative percentages of each molecular species of PC, PE, and PS were compared for each age, and those present at 4% abundance or greater were graphed along with PE plasmalogens for each tissue (Figs. 1–7). The relative percentage of all molecular species in each GPL class and the statistical analysis of changes with age are presented in supplemental Tables S1–S21. In addition, we measured total nanomoles lipid-phosphorus per milligram of wet tissue weight for each region at each age (supplemental Table S22). After determining no age-related differences within the same tissue (except for a P = 0.048 for 2 vs. 26 months in white matter), we collapsed the age groups and compared each region. This revealed significant differences in total lipid-phosphorus between some of the regions (supplemental Fig. S1 and supplemental Table S23). PC, PE, and PS were quantified by 2D-TLC and are presented both as nanomoles per milligram of wet weight and as the relative mol% of each to the others (supplemental Tables S24–S26). There was a significant age-related loss of PC and a relative increase in PE in both white matter and cortex. There were no age-related changes in PS for any tissue.

Examination of the specific GPL classes revealed that each had a unique molecular composition that, in many cases, changed significantly with age. PC contained the largest percentage of shorter-chain saturated (SAT) and monoenoic (MONO) molecular species, whereas PE and PS were predominantly composed of species containing PUFAs. Only the retina contained a high percentage of di-PUFA species, primarily in PS. There were significant age-related changes in the molecular species composition across most regions, with the relative levels of SAT and MONO species increasing with age at the expense of those containing PUFA. There were also regional differences within the same GLC class, with the tissues most abundant in neurons and synapses (i.e., retina and hippocampus) containing the highest levels of PUFA species, whereas the tissues most abundant in myelin (i.e., white matter and brainstem) contained the highest levels of SAT and MONO species. These tissues high in myelinated fibers also contained the highest levels of total lipid-phosphorus per milligram of wet weight.

Retina

PC has a large amount of di-SAT (32:00, 16:0/16:0) and SAT/MONO (34:01, 16:0/18:1) molecular species, which are relatively low in PE and PS. Of all the tissues and classes analyzed, retinal PC is the only class to contain detectable levels of very-long-chain PUFA (VLC-PUFA; 28 carbons in length and typically containing both n3 and n6 PUFA). VLC-PUFA makes up approximately 4% of the total PC isolated from retina, and these levels seem to remain stable in the retina with age (Fig. 1 and supplemental Table S1).

Fig. 1. Changes in GPL molecular species composition in retina with age. Looking at major molecular species (> 4% abundance) contained within GPLs: PC (left), PE (middle), and PS (right). Retinal tissue was taken at 2, 10, and 26 months of age. Statistics were calculated by using two-way ANOVA with Tukey’s multiple comparisons test. Full list of age-related changes for all molecular species detected can be found in supplemental Tables S1–S3.
Changes in CNS GPL lipidome by region and age

There were minimal significant age-related changes in the GPL molecular species composition of retina. There was a modest decrease in 32:00 and 38:06 and a concomitant increase in 40:06 in PC (Fig. 1 and supplemental Table S1). In PE, there was a small age-related decrease in 40:06 and compensatory increase in 44:12 (22:6n3/22:6n3). Notably, there was an age-related increase in the levels of PE plasmalogens with age (Fig. 1 and supplemental Table S2). PS shows a significant age-related increase in the shorter-chain FA-containing molecular species [34:01 and 38:04 (18:0/20:4n6)] and a subsequent decrease in 40:06 and 44:12 (Fig. 1 and supplemental Table S3).

The age-related changes in molecular species composition in retinal GPLs are small compared with the changes we found in all regions of the brain.

Hippocampus

PC is composed primarily of di-SAT and SAT/MONO species, with only approximately 18% of the species containing PUFA, with ~12% of 20:4n6- and ~6% of 22:6n3-containing species. In contrast, both PE and PS contain high levels of 40:06 (18:0/22:6n3), which make up ~60% of PS and ~30% of PE. Plasmalogens make up approximately 2.5% of hippocampal PE, which is among the lowest levels found in brain and similar to retina. PS contains only small amounts of 20:4n6-containing species (approximately 7%), whereas PE species contain almost 30% of 20:4n6.

There were small, but significant, age-related changes in several of the hippocampal GPL species, which, in general, were an increase in the SAT-containing species and reduction in the PUFA-containing species. In PC, there was a

Fig. 2. Changes in GPL molecular species composition in hippocampus with age. Looking at major molecular species (> 4% abundance) contained within GPLs: PC (left), PE (middle), and PS (right). Hippocampal tissue was taken at 2, 10, and 26 months of age. Statistics were calculated by using two-way ANOVA with Tukey’s multiple comparisons test. Full list of age-related changes for all molecular species detected can be found in supplemental Tables S4–S6.

Fig. 3. Changes in GPL molecular species composition in cerebellum with age. Looking at major molecular species (>4% abundance) contained within GPLs: PC (left), PE (middle), and PS (right). Cerebellar tissue was taken at 2, 10, and 26 months of age. Statistics were calculated by using two-way ANOVA with Tukey’s multiple comparisons test. Full list of age-related changes for all molecular species detected can be found in supplemental Tables S7–S9.
slight increase in 32:00 and 32:01 at the expense of 34:01, 36:04, and 38:04. There were no age-related changes in PC species containing 22:6n3 (Fig. 2 and supplemental Table S4). PE also had an age-related increase in the SAT- and MONO-containing molecular species (34:01 and 36:01), with a concomitant decrease in the PUFA-containing molecular species (38:04, 38:06, and 40:06). Of interest, hippocampus was the only tissue to show an age-related decline rather than an increase in the percent plasmalogens in PE (Fig. 2 and supplemental Table S5).

PS showed the greatest age-related increase in the shorter-chain SAT/MONO FA-containing species 36:01 (12% at 2 months vs. 22% at 26 months), with a parallel decrease in the 22:6n3 species 40:06 (63% at 2 months vs. 56% at 26 months) (Fig. 2 and supplemental Table S6).

Cerebellum

PC was primarily composed of di-SAT and SAT/MONO species, with the most predominant at 2 months of age being 34:01. Species containing 22:6n3 were ~19% of the total and those containing 20:4n6 were ~8%. The major molecular species in PE and PS was 40:06. However, PS also contained high levels of 36:01 (18:0/18:1). The combination of 36:01 and 40:06 was also found in PS from brainstem, white matter, and midbrain. Plasmalogens make up approximately 7% of cerebellar PE.

There were significant age-related changes in many of the GPL molecular species in the cerebellum. Although statistically significant (supplemental Table S7), the changes in PC were small and reflected a slight increase in the SAT and SAT/MONO species at the expense of those
containing PUFA. More dramatic changes were found in PE and PS. In PE, there was a decrease in the major 22:6n3-containing species and an increase in the SAT and SAT/MONO species. There was also an age-related increase in PE plasmalogens. PS had the greatest age-related changes, with an increase in 36:01 from 21 to 34% (2 vs. 26 months) and a decrease in 40:06 from 41 to 30%. For details on the cerebellum findings, see Fig. 3 and supplemental Tables S8 and S9.

**Brainstem**

Major GPL molecular species in the brainstem closely resemble those found in the cerebellum with the exception of an interesting shift in PS from 40:06 as the dominant species to 36:01 as the most abundant. Plasmalogens make up approximately 9% of brainstem PE. There were significant age-related changes in all three GPL classes, with changes in PE and PS being greater than those in PC. All glycerolipid classes again showed an age-related increase in shorter chain di-SAT- and SAT/MONO-containing molecular species, with an associated reduction in the PUFA-containing molecular species. PC showed the same type of age-related changes noted previously in the cerebellum, with the most notable being a significant increase in 34:01 and a decrease in 40:06. PE had significant age-related changes in all major molecular species, with increases in 34:01 and 36:01 and a large decrease in 40:06 (28% at 2 months to 19% at 26 months). Similar to the other tissues except the hippocampus, there was a significant age-related increase in brainstem plasmalogens. For details on the brainstem findings, see supplemental Tables S10 and S11.

The molecular species in brainstem PS were quite different from PS in any of the other tissues in that the major species is the SAT/MONO 36:01, which was 37% at 2 months and increased to 49% at 26 months. The species 40:06, the predominant species in the other regions, was
Cortex

The majority of PC is made up of di-SAT (29%) and SAT/MONO (40%) molecular species, with only ~8% containing 22:6n3. As found in other tissues, the largest molecular species in PE is 40:06, followed by 38:04. Unlike most other tissues, the level of PE plasmalogens was quite low (3%). The most surprising finding was the very large amount of 40:06 in PS (75% at 2 months), which was greater than that found in hippocampus (63%). Although there were significant age-related changes that favored an increase in SAT and SAT/MONO species at the expense of PUFA species, the magnitude of these changes was similar to the small changes noted in the retina and hippocampus, compared with the larger changes found in the other brain regions. For details on the cortex findings, see Fig. 5 and supplemental Tables S13–S15.

White matter

In general, the GPL in white matter contained more SAT-containing molecular species compared with most of the other regions. In addition, although the overall amounts of PUFA were low, it was interesting to note that, in white matter, the major PUFA present was 20:4n6 instead of the more typical 22:6n3. PC composition of white matter was similar to that seen in the brainstem and cerebellum, with high levels of 32:00, 34:01, and 36:01 (combined 63% at 2 months) and low levels of PUFA species containing 22:6n3 (~5%). PE contained equal amounts (18%) of 38:04 (18/0/20:4n6) and 40:6 (18/0/22:6n3), which was not the case in the other regions. White matter contained the highest percent of PE plasmalogens of all brain regions analyzed (12%). PS in white matter more closely resembled that of the brainstem, with 36:01 as the most abundant molecular species over 40:06 (36 and 27%, respectively). There were small, but significant, age-related changes in PC. The di-SAT species 32:00 and 34:00 were reduced with age, and the SAT/MONO species 34:01 and 36:01 were increased. The changes in PE were of greater magnitude, with 36:01 increasing from 12 to 16% and 40:06 decreasing from 18 to 11%. The PE plasmalogens also increased with age. The largest changes were in PS, where 36:01 increased from 36 to 53% with age, whereas 40:06 decreased from 27 to 14%. For details on the white matter findings, see Fig. 6 and supplemental Tables S16–S18.

Midbrain

Midbrain contains a lipid composition similar to that of the cerebellum and brainstem. PC is made up of di-SAT (32:00) and SAT/MONO (34:01 and 36:01) molecular species and low levels of species containing 22:6n3 (12%). The PE molecular species in 2-month-old midbrain were 38:04 (15%) and 40:06 (31%). There were small age-related changes, with increases in the SAT/MONO species and decreases in the 22:6n3-containing species. Plasmalogens make up ~7% of midbrain PE and increased slightly, but significantly, with age. We found that 40:06 was the most abundant molecular species in midbrain PS (47% at 2 months of age), with 36:01 present at 22%.

There were statistically significant, but minor, age-related changes in the PC molecular species (±1%), with the more saturated species increasing with age. PE showed similar minor changes with age. However, PS, as found in most of the other regions, underwent dramatic age-related changes, with 36:01 increasing from 22 to 32% and 40:06 decreasing from 47 to 35%. For details on the midbrain findings, see Fig. 7 and supplemental Tables S19–S22.

DISCUSSION

The goals of this study in testing our initial hypothesis were to provide a novel and all-inclusive molecular blueprint of the age-related changes in the composition of the three major GPL classes in the retina and specific brain regions. The significance of the work done here is that it provides a high level of detail regarding every detectable molecular species of the three major brain and retina GPLs in a single source. The supplemental tables provide detailed compositional information on the retina and brain lipidome in its detectable entirety. Our study confirms that the various regions of the brain contain unique compositions of lipid molecules and that this composition changes in a molecule- and region-specific manner with age.

The most prevalent and consistent findings were as follows: 1) age-dependent increases in SAT-containing molecular species at the expense of those containing PUFA, especially DHA; 2) dramatic differences in the molecular species composition of the three GPL classes in each region; and 3) large regional differences in the molecular species composition of each GPL class, with gray matter-dominant tissues having higher levels of PUFA-containing molecular species.

PUFA-containing GPLs are reduced in myelin-rich regions, whereas SATs are elevated, suggesting the need for unique molecular compositions depending on regional function

The molecular species for each GPL class in the different regions of 2-month-old brain and retina are shown in Fig. 8, arranged in descending order from lowest to highest levels of myelin. Within each GPL class, there was a pronounced change in the relative molecular species composition between the different tissues, with the levels of PUFA-containing species dramatically lower in the tissues containing the highest levels of myelin. This is most evident when cortex and white matter are compared, where the sum of 38:06 and 40:06 ranged from 49% (PE) and 76% (PS) in cortex to 24% and 33%, respectively, in white matter. Alterations in the lipid composition of the same class by region may be indicative of the membrane fluidity needed by that region to function properly. The high incorporation of DHA and the other PUFA in regions like the retina, cortex, hippocampus, and cerebellum may result in more "plastic" membranes, allowing for improved information processing and synaptic function (58–64). Conversely, the increased levels of SAT-containing species in the myelin-rich tissues supports their role in providing...
insulation for the nerve fibers. Understanding the possible influence of these specific molecules on neural membranes and the known functional outputs of the regions in which they are located will lead to a better understanding of the dramatic compositional and functional differences we observe in the brain as a whole. Because these molecular species change with age, any influence they may have on cellular function may also change.

DHA is an essential, life-giving FA that we cannot make

The major PUFAs in the brain and retina are DHA (22:6n3) and AA (20:4n6), both of which are essential FAs because they cannot be synthesized by any vertebrate, but by only the lower forms of invertebrates (e.g., Caenorhabditis elegans). Mammals obtain DHA and AA from their diet or from hepatic conversion of shorter-chain PUFAs, such as linoleic (18:2n6) and linolenic (18:3n3) acids (60, 65). Although the levels of DHA and, to some extent, AA are relatively low in the blood, the retina and brain are able to take them up and incorporate them into the various molecular species reported in the supplemental tables. This enrichment of DHA and AA in retina and brain lipids begins in utero and is essential for normal brain and retina development and function. Once incorporated into these organs, DHA and AA are tenaciously retained and cannot be depleted by removing all dietary sources of n3 and n6 PUFA. Thus, any age-related changes in DHA- and AA-containing molecular species are due to specific events in brain and retina, and are not due to dietary restrictions, which should not have a demonstrable effect on the brain and retina FA compositions measured in this study.

A study of more than 6,000 individuals over the age of 65 indicated that increasing DHA levels with a high fish diet had a protective effect on cognitive decline (66, 67). Work by Bazan (13, 14) demonstrated that this is likely, in part, due to DHA serving as a precursor for bioactive lipid derivatives like neuroprotectant-D1, which has been shown to have an extensive and beneficial role in both brain and retina neurons and may protect against age-related cognitive decline and other neurodegenerative diseases.

Loss of the molecular species that contain DHA with age was the most consistent finding across brain regions. DHA has been studied extensively in brain health and function as a neuroprotectant and has been linked to many important neural processes that span from early development to death (68). The highest concentrations of DHA molecular species exist in PE and PS and are enriched in synaptic membranes, followed by mitochondrial membranes, and finally microsomal membranes (60, 69). DHA enriched membranes are thin due to DHA’s 3D conformation via its six methylene-interrupted cis double bonds (70). The fact that DHA undergoes rapid interconversion between various states of torsion results in membranes with increased permeability, compression, fusion, and flipping properties (62, 63, 71). The presence of DHA has also been shown to drive the generation of cholesterol-depleted domains (64), and, by favoring insertion into cholesterol-rich lipid raft domains, DHA promotes activities such as neurotransmitter release, second messenger signaling, resistance to oxidative stress, and even gene regulation (58, 61). In other words, loss of DHA-containing molecular species with age results in more rigid, less fluid neural membranes. We would predict that noted age-related declines in cognitive function could be due, at least in part, to changes in the synaptic apparatus due to reduced DHA molecular species.

Increased brain accumulation of DHA from diet in rodents resulted in higher levels of both presynaptic and postsynaptic proteins critical for neurotransmission, including synaptin-3, PSD-95, and synapsin-1 (72). Indeed, VanGuilder et al (73) demonstrated that synaptosomes isolated from young, adult, and aged Wistar rats had significant decreases with age in SNAP25, synaptophysin, synaptic vesicle glycoprotein 2B, SV2-related protein, Homer 1, and synaptoporin, all of which are critical for neurotransmission. Finally, electron microscopy of synaptic vesicle membranes isolated from these animals appeared to lose their structural and morphological integrity with age (74). These findings support the concept that initial changes in the lipidome could be driving the loss of membrane stability and integrity that subsequently deregulates the protein machinery necessary for synaptic transmission.

Another hypothesis for DHA’s high incorporation into neural membranes was proposed by Crawford et al. (59) in a paper describing a quantum theory for DHA’s role in the brain, in that its unique molecular structure allows for quantum transfer of its π-electrons between neural membranes as a form of intercellular communication. DHA is also involved in the regulation and biosynthesis of PS in that high levels of DHA correlate with increased biosynthesis of PS (75), which has been linked to a positive effect on neuronal function and survival via the PI 3-kinase/Akt pathway (76).

Given these multifaceted and important roles for DHA (77), it is problematic that we are reporting a consistent age-related loss of the 40:06 molecular species from PC in both the cerebellum (P < 0.0001) and brainstem (P < 0.0001). In addition, significant age-related reduction of 40:06 was observed in PE in retina and in every brain region except cortex, and significant reduction of 40:06 from PS was observed in every brain region, as well as in retina. We found that 38:06 (16:0 + 22:6n3), another relevant DHA-containing molecular species, was significantly reduced with age in PC in retina, brainstem, and white matter, and in PE in retina and every brain region except

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**Fig. 8.** Percent composition of major molecular species across region and glycerolipid class at 2 months of age. Ordered from top to bottom with increasing white matter content. Values of the major molecular species chosen are expressed as percentages with other molecular species detected summed as “other.” Significant age-related changes in all molecular species for all three classes of glycerolipids can be found in supplemental Tables S1–S21.
cortex. These age-related changes could have profound effects on synaptic function and cognitive ability.

Although immensely important in neural function for the retina, VLC-PUFAs were not detected in the rodent brain, except for trace amounts during embryogenesis

Another important class of FAs are the VLC-PUFAs (28 carbons) and very-long-chain saturated FAs (primarily 28:0 and 30:0). These FAs are synthesized exclusively by ELOVL4, a condensing enzyme that catalyzes the rate-limiting first step in their biosynthesis from C-26 precursors (78). ELOVL4 is expressed in retina (78–82) and brain (83–87), as well as in other tissues including skin (88–91), testes (92), and Meibomian glands (93). In the retina, the major product is VLC-PUFA, which is found exclusively in the sn-1 position of PC (94). Because the brain expresses ELOVL4 and has such high levels of PUFA, we anticipated finding VLC-PUFA in brain PC. However, we were surprised to find no VLC-PUFA in PC in any brain region at any of the three ages we examined. Because Poulos and coworkers (32) had reported finding VLC-PUFA in neonatal rat brain, we dissected whole brain from postnatal day 1 (P1) rats, isolated PC by TLC, and examined methyl esters by GC/MS, as Poulos and coworkers had done. We were unable to detect even the smallest amounts of any VLC-PUFA, despite easy detection of VLC-PUFA in PC from retina as a positive control. We also examined the upper phase of the Folch extract, as well as the protein interface, for any potentially protein-bound VLC-PUFA, but again we found none. The highest embryonic expression of Elovl4 in mouse brain (95) is during late embryonic development, with a rapid loss of Elovl4 mRNA from P1–P30. We dissected hippocampus from embryonic day 18.15 (E18.5) rat brains as well as hippocampus, cortex, cerebellum, and whole brain from the E18.5 embryonic mouse. In the total lipid extracts of each of these regions, we did not find any detectable levels of VLC-PUFA, except for possibly trace amounts of a single VLC-PUFA peak. It is possible that, early in embryonic development, these molecules exist in the brain at very low abundance beyond the bounds of quantitative detection. If so, they could have an important, but transient, role in brain development, perhaps acting as precursors for other bioactive derivatives, as has been reported for DHA (13, 96, 97). Interestingly, we found significant amounts of 28:0 and 30:0, both products of ELOVL4, in sphingolipids in all regions of the brain and in the retina. These findings will be discussed in a subsequent paper.

**Plasmalogens are neuroprotective molecules that influence numerous dynamic cellular functions, and their loss results in both retinal and brain pathologies of a severe nature**

PE is the only lipid class in which we detected significant amounts of plasmalogens. Plasmalogens are unique PE lipid molecules that contain an ether-linked alk-l-enyl chain with a cis double bond, termed a “vinyl-ether linkage,” at the sn-1 position instead of the typical ester-linked FAs found in other GPL molecules (98–100). It is the presence of this vinyl ether double bond that makes these plasmalogens so uniquely sensitive to acid, mercury cations, and reactive oxygen species (15, 101–108) and, as a result, important for the aging organism. Patients with an inability to synthesize plasmalogens are left with a wide variety of pathologies, including severe mental retardation, hypotonicity, adrenal dysfunction, cataracts, deafness, facial dysmorphism, chondrodyplasia, and very early mortality, often within the first year of life (109). The brain region with the highest percent of ether-PE plasmalogens is white matter (<12%), with cortex being the lowest (<3%); retina was the lowest of all tissues measured (2.5%).

Plasmalogens have been shown to play a unique role in maintaining the biophysical properties of the membranes in which they are expressed. Their presence appears to facilitate membrane fluidity, and they have been suggested to play a role in membrane fusion and perhaps in mediating vesicle fusion (110). Plasmalogens have also been reported to increase or decrease certain protein kinase C-mediated responses in various models, which are well-known contributors to learning and memory circuitry in the hippocampus (111–116). Thus, plasmalogens are important for neurotransmission (117–119). The hippocampus is uniquely sensitive to an age-related decline in function, with reports of short-term memory loss as a consequence of normal aging (120–124). These hippocampal-mediated effects are even more profound in patients with various forms of cognitive impairment, and the hippocampal pathologies of Alzheimer’s disease have been a significant focus in the field for the last several decades. Transient ischemia is another risk factor for older individuals and is a cause of vascular-related dementias over time due to continuous oxidative stress. Plasmalogens have been shown to have a protective role in response to cellular oxidative stress during ischemia-reperfusion injury (103, 125). The hippocampus was the only brain region to show a significant, albeit small, age-related reduction in PE plasmalogens, whereas every other tissue demonstrated the opposite response. Given the well-documented neuroprotective roles of both DHA and plasmalogens, and the significant loss of both from the hippocampus alone, their reduction could be important in the context of age-related changes in cognition.

**Lipids are dynamic and influential molecules that deserve our attention as neuroscientists**

Piomelli et al. (126) stated in 2007 that, “Neuroscientists have a problem with fat.” As a whole, the field of neuroscience has neglected lipids, presuming that these critical components of the cell were not dynamic, but meant solely for membrane structure and axon insulation. With the incredible advances in our understanding of both the nervous system as a whole and having the tools for precise measurement of lipid species, we are now able to address questions whose answers were heretofore not attainable. The molecular blueprint we present here, we hope, will provide a template for scientists to ask specific questions targeting individual lipid molecules and uncover their region-specific or ubiquitous functions in the nervous system.
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