

# Decreases in Phospholipids Containing Adrenic and Arachidonic Acids Occur in the Human Hippocampus over the Adult Lifespan

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**Abstract** One of the biggest risk factors for developing Alzheimer's disease is advanced age. Despite several studies examining changes to phospholipids in the hippocampus during the pathogenesis of Alzheimer's disease, little is known regarding changes to phospholipids in this region during normal adult aging. This study examined the phospholipid composition of the mitochondrial and microsomal membranes of the human hippocampus from post-mortem tissue of neurologically normal subjects aged between 18 and 104 years. Many of the age-related changes found were in low-to-moderately abundant phospholipids in both membrane fractions, with decreases with age being seen in many phospholipids containing either adrenic or arachidonic acid. The most abundant phospholipid of this type was phosphatidylethanolamine 18:0\_22:4, which decreased in both the mitochondrial and microsomal membranes by approximately 20 % from ages 20 to 100. Subsequent decreases with age were seen in total adrenic and arachidonic acid in the phospholipids of both membrane fractions, but not in either fatty acid specifically within the phosphatidylethanolamine class. Increases with age were seen in the hippocampus for mitochondrial phosphatidylserine 18:0\_22:6. This is the first report of changes to molecular phospholipids of the human hippocampus over

the adult lifespan, with this study also providing a comprehensive profile of the phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine phospholipids of the human hippocampus.

**Keywords** Mass spectrometry · Mitochondria · Microsome · Neurodegeneration · Lipidomics · DHA · ARA · Membrane · Aging

## Abbreviations

AD	Alzheimer's disease
ARA	Arachidonic acid
DHA	Docosahexaenoic acid
ChoGpl	Choline glycerophospholipids
EtnGpl	Ethanolamine glycerophospholipids
PakCho	Plasmanyl phosphatidylcholine
PakEtn	Plasmanyl phosphatidylethanolamine
PlsEtn	Plasmenyl phosphatidylethanolamine (ethanolamine plasmalogen)
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdSer	Phosphatidylserine
PUFA	Polyunsaturated fatty acid(s)

## Introduction

Age is the number one risk factor for several neurodegenerative diseases, and with an increasingly older population worldwide the prevalence of age-related pathologies is also rising [1]. Due to this, research into early diagnosis, prevention and treatment strategies for neurodegenerative diseases is becoming increasingly important. Changes in membrane phospholipids have recently been explored as potential serum biomarkers for the development of age-related

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neurodegenerative diseases such as Alzheimer's disease (AD), with several choline glycerophospholipids (ChoGpl) being identified as possible biomarkers of the disease [2, 3]. Nevertheless, little knowledge is currently available on the changes that occur to membrane phospholipids during normal aging in the human brain, and research into these changes could shed some light on the pathways underpinning both AD and other neurodegenerative diseases.

The hippocampus is one of the earliest sites where atrophy is exhibited in the development of AD [4, 5]. Less severe atrophy of this region is also seen during normal aging [6]. The presence of amyloid-beta deposits characteristic of AD within the medial temporal region is thought to accelerate atrophy within the hippocampus and other brain structures of the medial temporal region [7]. Additionally, the hippocampus is thought to be one of the initial sites of the development of neurofibrillary tangles [8]. The phospholipids of the hippocampus have been examined for their role in the pathogenesis of AD in several studies over the past few decades [9–14], but very few studies have examined the changes occurring in hippocampal phospholipids during normal aging [15, 16].

Phospholipids make up approximately half of the total lipids in neural membranes [17], and can have a large range of structural diversity depending on the combination of head group and fatty acids present. Different permutations of head group and fatty acids within a phospholipid can generate up to 10,000 possible individual molecular phospholipids [18]. Of particular importance in the human brain are long-chain polyunsaturated fatty acids (PUFA), which are present in high amounts in the brain, particularly within ethanolamine glycerophospholipids (EtnGpl) and phosphatidylserine (PtdSer) [19]. PUFA can be further subdivided into two classes: omega-6 fatty acids, with arachidonic acid (ARA) being the dominant fatty acid of this class in the brain, or omega-3 fatty acids, with the most abundant being docosahexaenoic acid (DHA). DHA has been found to increase significantly in the brain during childhood development [20], but it is currently unknown if such changes persist over the adult lifespan. Decreases in total phospholipids with age have been reported after age 60 in the hippocampus [16], alongside decreases in total ChoGpl and EtnGpl [15]. No age-related changes have previously been reported for the fatty acids of either ChoGpl or EtnGpl phospholipids in the human hippocampus [15]. Since publication of these studies newer methods using mass spectrometry to examine phospholipids at a molecular level have been developed [21], and any age-related changes to molecular phospholipids in the human hippocampus over the adult lifespan are yet to be determined.

The aim of this study was to examine changes in the human hippocampus during normal adult aging at the molecular level for the three most dominant phospholipid

classes: ChoGpl, EtnGpl and PtdSer. Additionally the hippocampal tissue was fractionated into a mitochondria-enriched fraction and microsomal (predominately endoplasmic reticulum, Golgi and plasma membrane) component in an attempt to isolate any age-related phospholipid changes to a particular subcellular compartment. We observed decreases across the adult lifespan in phospholipids containing either adrenic acid or ARA in both the mitochondrial and microsomal fractions, with the most abundant phospholipid of this type being phosphatidylethanolamine (PtdEtn) 18:0\_22:4. Analysis of fatty acids found that total adrenic acid and ARA decrease with age in both membrane fractions, but no age-related changes were seen for either fatty acid specifically within EtnGpl. Additionally, we saw increases with age in mitochondrial PtdSer 18:0\_22:6. This study identifies for the first time changes to molecular phospholipids within the hippocampus during normal adult aging, and provides a comprehensive profile of the ChoGpl, EtnGpl and PtdSer phospholipids present within the mitochondrial and microsomal membranes of this region within the human brain.

## Materials and Methods

### Materials

Methanol and chloroform of HPLC grade or higher were purchased from VWR International (QLD, Australia). Analytical grade ammonium acetate was obtained from Crown Scientific (NSW, Australia). Zirconium oxide beads (1.4 mm) were purchased from Sapphire Biosciences (NSW, Australia). Sucrose, Tris, dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) were purchased from Astral Scientific (NSW, Australia). BCA Protein Assay Kits were obtained from Thermo Fisher Scientific (VIC, Australia). Complete protease inhibitor cocktail and butylated hydroxytoluene (BHT) were purchased from Sigma Aldrich (NSW, Australia). Phospholipid internal standards were purchased from Avanti Polar Lipids through Auspep (VIC, Australia), and consisted of 20  $\mu$ M each of phosphatidylcholine (PtdCho) 19:0/19:0, PtdEtn 17:0/17:0, and PtdSer 17:0/17:0; and 10  $\mu$ M of lyso-PtdCho 17:0 and lyso-PtdEtn 14:0 in chloroform:methanol (2:1, v/v).

### Brain Tissue

Neurologically normal frozen post mortem brain tissue from the hippocampus was obtained from the New South Wales Tissue Resource Centre at the University of Sydney. The demographics of the cohort have been previously described elsewhere [22]. Briefly, there were 36 samples in the cohort (12 females, 24 males) with an average age

of 59 years ( $\pm 4$ , range 18–104). There were no differences between brain pH or post mortem interval between the two sexes, but females were significantly older than males ( $71.4 \pm 7.0$  years *versus*  $52.7 \pm 3.9$  respectively,  $p < 0.05$ ). Samples were shipped on dry ice and stored at  $-80^\circ\text{C}$  prior to analysis. Brain tissue was pulverized on dry ice prior to being accurately weighed for subcellular fractionation. All experiments were conducted in accordance with the approval of the Human Research Ethics Committee of the University of Wollongong (HE11/267).

### Subcellular Fractionation

The method for the subcellular fractionation of tissue into mitochondrial and microsomal membranes were described previously [22]. Briefly, 100 mg of brain tissue was homogenized by a bead homogenizer (FastPrep<sup>®</sup>-24 instrument MP Biomedicals, NSW, Australia) at 6.0 m/s for 40 s using zirconium oxide beads in an ice-cold 20 mM Tris buffer (pH 7.4) containing 250 mM sucrose, 2 mM EDTA, 2 mM DTT and complete protease inhibitor. The homogenate was subjected to a three-step differential centrifugation to obtain a nuclei/large cellular debris pellet ( $1000\times g$ , 10 min), a mitochondria-enriched pellet ( $10,000\times g$ , 35 min) and a microsomal pellet ( $100,000\times g$ , 40 min; endomembranous system comprised of Golgi, endoplasmic reticulum and plasma membrane). All centrifugation steps were conducted at  $4^\circ\text{C}$ . Both the mitochondrial and microsomal pellets were resuspended in Milli-Q water and their total protein content was determined using the BCA assay.

### Lipid Extraction

Lipids were extracted from 75  $\mu\text{g}$  of total protein from the mitochondrial and microsomal fractions using a modified Folch method described previously [22]. Extracted lipids were stored in chloroform:methanol (1 mL, 1:2 v/v with 0.01 % BHT) at  $-20^\circ\text{C}$  until analysed.

### Mass Spectrometry and Lipid Analysis

Nano-electrospray ionization mass spectrometry of lipid extracts was performed using a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP<sup>®</sup> 5500 AB Sciex, MA, USA) equipped with an automated chip-based nano-electrospray source (TriVersa Nanomate<sup>™</sup>, Advion Biosciences, NY, USA) using parameters and targeted ion scans described previously [22]. Briefly, samples were diluted to approximately 10  $\mu\text{M}$  total phospholipid and spiked with 5 mM ammonium acetate. The samples were loaded onto a 96-well plate and centrifuged ( $2200\times g$ , 10 min) prior to direct infusion. Spray parameters were set at a gas pressure of 0.4 psi and a voltage of 1.2 and

1.1 kV for positive and negative ion mode respectively. *Brutto* phospholipids (i.e. head group and sum composition) were obtained from precursor ion scans for ChoGpl (184 m/z), and from neutral loss scans for EtnGpl (141 Da) and PtdSer (185 Da). Fatty acids were determined by complementary negative precursor ion scans. Lipidview<sup>™</sup> software (version 1.2, AB Sciex, MA, USA) was used to quantify phospholipids and determine molecular phospholipid composition using methods described by Norris *et al.* [22]. Processing settings in Lipidview<sup>™</sup> were set at a mass tolerance of 0.5 Da, with a minimum intensity of 0.1 % and a minimum signal-to-noise ratio of 10. Any phospholipid comprising less than 0.5 % of each phospholipid class across the cohort were removed from analysis. Molecular phospholipids (i.e. head group and fatty acid pair) were determined using data from complementary negative precursor ion scans. PtdCho or PtdEtn containing an odd-chain fatty acid were unable to be separated from their respective plasmalyl choline (PakCho) or plasmalyl ethanolamine (PakEtn) isobar using this method. Likewise, PakEtn and plasmalyl phosphatidylethanolamine (PlsEtn) isomers were also unable to be separated, and so no correction factor was applied. Phospholipids are reported using a nomenclature modified from that described by Liebisch *et al.* [23] for phospholipids with unknown *sn* positional fatty acids, and were normalized relative to total phospholipid detected in each class. Molecular phospholipids with significant changes in quantified amount (pmol/ $\mu\text{g}$  membrane protein) with age are included for comparison in the supplementary material (Tables S4-5).

### Statistical Analysis

Statistical analysis was performed using SPSS Statistics (version 19, IBM Corp., NY, USA) and R (version 3.1.1). The Wilcoxon signed-rank test was used for comparisons of phospholipid classes between mitochondria and microsomal fractions. To examine the relationship between age and phospholipids multiple linear regression was used with sex as a second independent variable, with significance set at a level of  $p < 0.05$ . Normality of the dependent variable was assessed by examining the histograms of the residuals and non-normal data was transformed where required. Positively skewed residuals were transformed using logarithmic or reciprocal transformations depending on the level of skew present, while negatively skewed data used the reflection of these transformations. Outliers were identified as being greater than three standard deviations from the mean of the standardized residuals, and were dealt with by either transformation of the dependant variable or by removal from the analysis. Influential data points were identified by a cook's distance of greater than one, and were removed from the analysis. Where the dependant variable was

transformed data is displayed in figures as untransformed scatter plots with transformed beta-coefficients and  $p$  value to allow direct comparison between phospholipids. As this was an exploratory study a correction for multiple comparisons was not applied. Further linear regression parameters are reported in the supplementary material for all analyses. Values reported in the results for ages 18 and 104 are predicted from the linear regression equation.

## Results

### Age-Related Changes to Total Protein Content in Subcellular Fractions

Statistically significant age-related declines were seen in the total protein content of the microsomal fraction of the hippocampus, with no changes with age found for either the whole tissue homogenate or mitochondrial fraction (Fig. 1, Table S1). Total protein decreased in the microsomal membranes from 2.5  $\mu\text{g}/\text{mg}$  wet weight of tissue to 1.8  $\mu\text{g}/\text{mg}$  wet weight of tissue from ages 18 to 104, representing a decrease of 27 %. A trend was seen for a decline in total protein with age within the whole tissue homogenate, but this failed to reach statistical significance ( $p = 0.055$ ).

### Major Phospholipid Classes of Mitochondria and Microsomal Fractions

The ratio of the three major phospholipid classes ChoGpl, EtnGpl and PtdSer showed a similar distribution between the mitochondria and microsomal membranes (Fig. 2).

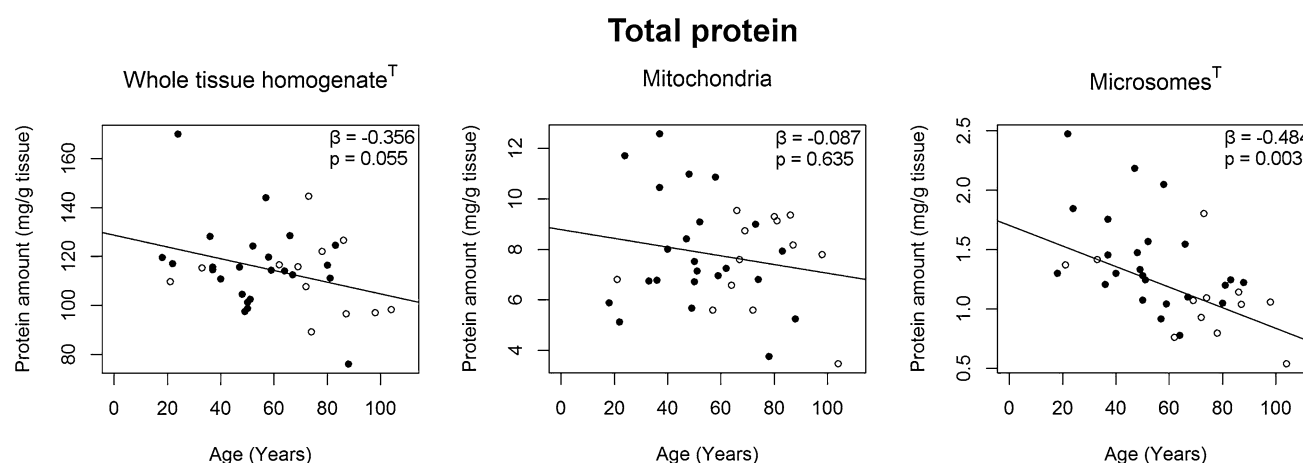
Overall, ChoGpl comprised around 43 %, EtnGpl 36 %, and PtdSer 21 % of total phospholipids measured in each fraction with some small differences in phospholipid content between the two membrane fractions. A slightly higher percentage of EtnGpl was present in the mitochondria ( $p < 0.001$ ), while the microsomes contained more ChoGpl ( $p < 0.001$ ). There were no differences in the percent abundance of PtdSer between the two membrane fractions. The percent contribution of total ChoGpl, EtnGpl, PtdSer were also examined for any age-related changes in the mitochondrial and microsomal fractions, with no age-related changes seen.

The quantified amount of each phospholipid class (as  $\text{pmol}/\mu\text{g}$  membrane protein) between the two membrane fractions was also analysed, with the microsomal fraction found to contain higher amounts of ChoGpl ( $p < 0.001$ ) and PtdSer ( $p = 0.005$ ). Total quantified phospholipids were also higher in the microsomes than in the mitochondria ( $p = 0.001$ ). Regression of quantified phospholipids ( $\text{pmol}/\mu\text{g}$  membrane protein) with age showed no statistically significant age related changes for any phospholipid class in either membrane fraction, including total quantified phospholipids.

### Changes in Mitochondrial Phospholipids with Age

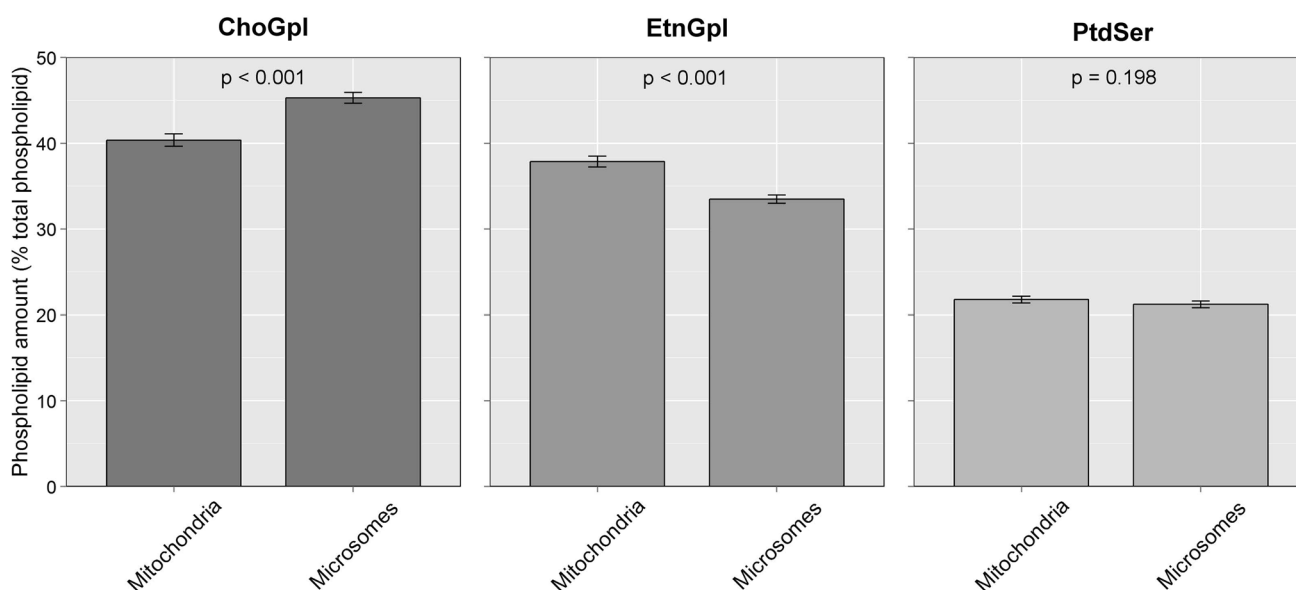
#### ChoGpl

Three mitochondrial ChoGpl changed with age in the human hippocampus (Fig. 3, Table S2). PtdCho 16:0\_18:1, the most abundant phospholipid present in mitochondrial membranes, increased from 40.8 % of total mitochondrial



**Fig. 1** Linear regression of age against total protein amount ( $\text{mg}/\text{g}$  wet tissue) measured in whole tissue homogenate and subcellular fractions derived from normal human hippocampus. Total protein was measured using BCA assay as described in methods and materials. Regression model was adjusted for sex: males (black circle), females

(white circle). <sup>T</sup> Indicates dependant variable transformed for linear regression, with transformed beta-coefficient and  $p$  value reported on original scatterplot for comparison. Regression tables including 95 % confidence interval are available in the supplementary material (Table S1)



**Fig. 2** Percent composition of the three major phospholipid classes within the mitochondrial (*left*) and microsomal (*right*) fractions in normal human hippocampus. Values are the mean  $\pm$  SEM for entire cohort. The microsomal membranes showed significantly higher

amounts of total ChoGpl compared to mitochondria ( $p < 0.001$ , Wilcoxon signed-rank test), while the mitochondria contained more EtnGpl ( $p < 0.001$ , Wilcoxon signed-rank test). There were no differences between fractions for PtdSer

ChoGpl at age 18–42.5 % at age 104, representing a 4 % increase from its initial abundance. PtdCho 16:0\_18:2 also increased in abundance with age, but this phospholipid is only present in low-to-moderate amounts within mitochondrial membranes (Figure S1). The only mitochondrial ChoGpl to decrease with age was PtdCho 18:0\_20:4, which decreased in abundance by 26 % from ages 18 to 104.

Regression of quantified phospholipids (pmol phospholipid/ $\mu$ g total protein) with age was also performed, with no mitochondrial ChoGpl showing any statistically significant age-related changes.

### EtnGpl

Age-related changes were seen in several mitochondrial EtnGpl (Fig. 4, Table S2). PtdEtn 18:0\_22:4 was the most abundant mitochondrial EtnGpl to decrease with age, declining by 13 % in abundance over the 86 year period. This phospholipid is the third-most abundant mitochondrial EtnGpl, comprising approximately 8 % of total mitochondrial EtnGpl (Figure S1) and 3 % of total mitochondrial phospholipid. Two other mitochondrial EtnGpl decreased with age: PtdEtn 16:0\_22:4 and Pak 16:1\_20:2/PlsEtn 16:0\_20:2 (Fig. 4, Table S2). PtdEtn 16:0\_22:4 is a moderately abundant mitochondrial EtnGpl, which contributes up to 1.5 % of mitochondrial EtnGpl (Figure S1) and 0.6 % of total mitochondrial phospholipid. This phospholipid decreased by 20 % in abundance from ages 18 to 104 (Fig. 4, Table S2). The remaining phospholipid to decrease

with age, Pak 16:1\_20:2/PlsEtn 16:0\_20:2, is only of low abundance in the mitochondrial membranes, comprising just over 0.4 % of total mitochondrial phospholipid.

Three mitochondrial EtnGpl increased in percent abundance over the 86 year period: PtdEtn 18:1\_22:6, lyso PtdEtn 22:6 and lyso-PtdEtn 18:1. PtdEtn 18:1\_22:6 is a moderately abundant phospholipid in the mitochondrial membranes, comprising approximately 1 % of total mitochondrial phospholipids (Figure S1). PtdEtn 18:1\_22:6 increased from 2.3 % of total mitochondrial EtnGpl to 2.9 % from ages 18 to 104, representing an increase in abundance of 28 % (Fig. 4, Table S2). The two other lyso-PtdEtn showing increases with age, lyso-PtdEtn 22:6 and lyso-PtdEtn 18:1, comprised less than 1.7 % of total mitochondrial EtnGpl when combined (Figure S1).

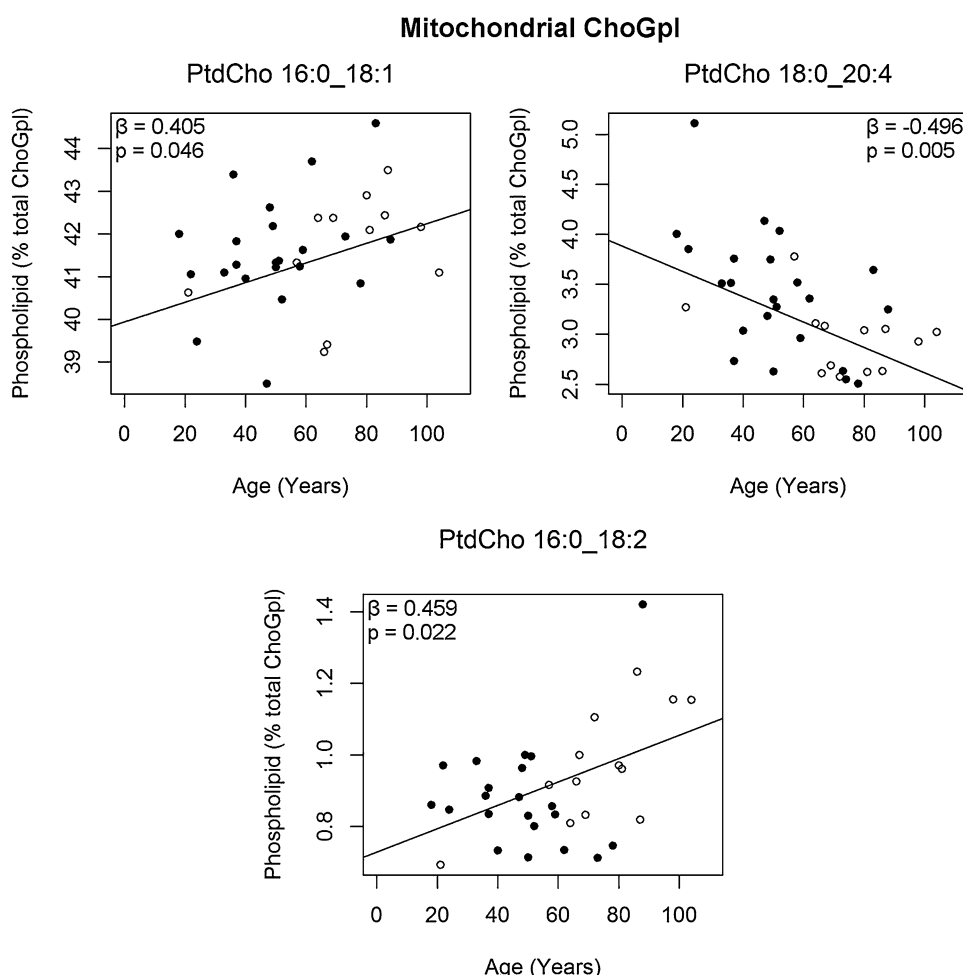
Analysis of the quantified phospholipids (pmol phospholipid/ $\mu$ g membrane protein) showed similar trends for all mitochondrial EtnGpl identified as changing in their percent abundance with age, but only a single mitochondrial EtnGpl reached statistical significance: PakEtn 16:1\_20:2/PlsEtn 16:0\_20:2 (Table S4). PakEtn 16:1\_20:2/PlsEtn decreased with age from 1.2 pmol/ $\mu$ g membrane protein to 0.3 pmol/ $\mu$ g membrane protein from ages 18 to 104.

### PtdSer

Within the mitochondrial membranes three PtdSer phospholipids changed in percent abundance with age (Fig. 5, Table S2). The most abundant phospholipid to show an



**Fig. 3** Mitochondrial ChoGpl changing significantly with age (as percent of total ChoGpl) in normal human hippocampus ( $n = 33$ – $36$ ). Regression model was adjusted for sex: males (*black circle*), females (*white circle*). Regression tables including 95 % confidence interval are available in the supplementary material (Table S2)



age-related change was PtdSer 18:0\_22:6, which increased from 36.8 % of total mitochondrial PtdSer to 46.8 % from ages 18 to 104. The remaining two phospholipids all decreased with age in the mitochondrial membranes. PtdSer 18:0\_22:4, a moderately abundant mitochondrial phospholipid, declined by 18 % over the 86 year period (Fig. 5, Table S2). The low abundance mitochondrial phospholipid PtdSer 18:0\_20:4 decreased by 41 % over the 86 year period.

One of these mitochondrial PtdSer also declined in quantified amount (pmol phospholipid/ $\mu$ g membrane protein) with age: PtdSer 18:0\_20:4 (Table S4). A further two mitochondrial PtdSer also decreased in quantified amount with age, PtdSer 18:0\_18:1 and PtdSer 18:0\_20:2.

## Changes in Microsomal Phospholipids with Age

### ChoGpl

Two microsomal ChoGpl changed with age (Fig. 6, Table S3). Decreases with age were seen in PtdCho 18:0\_18:1,

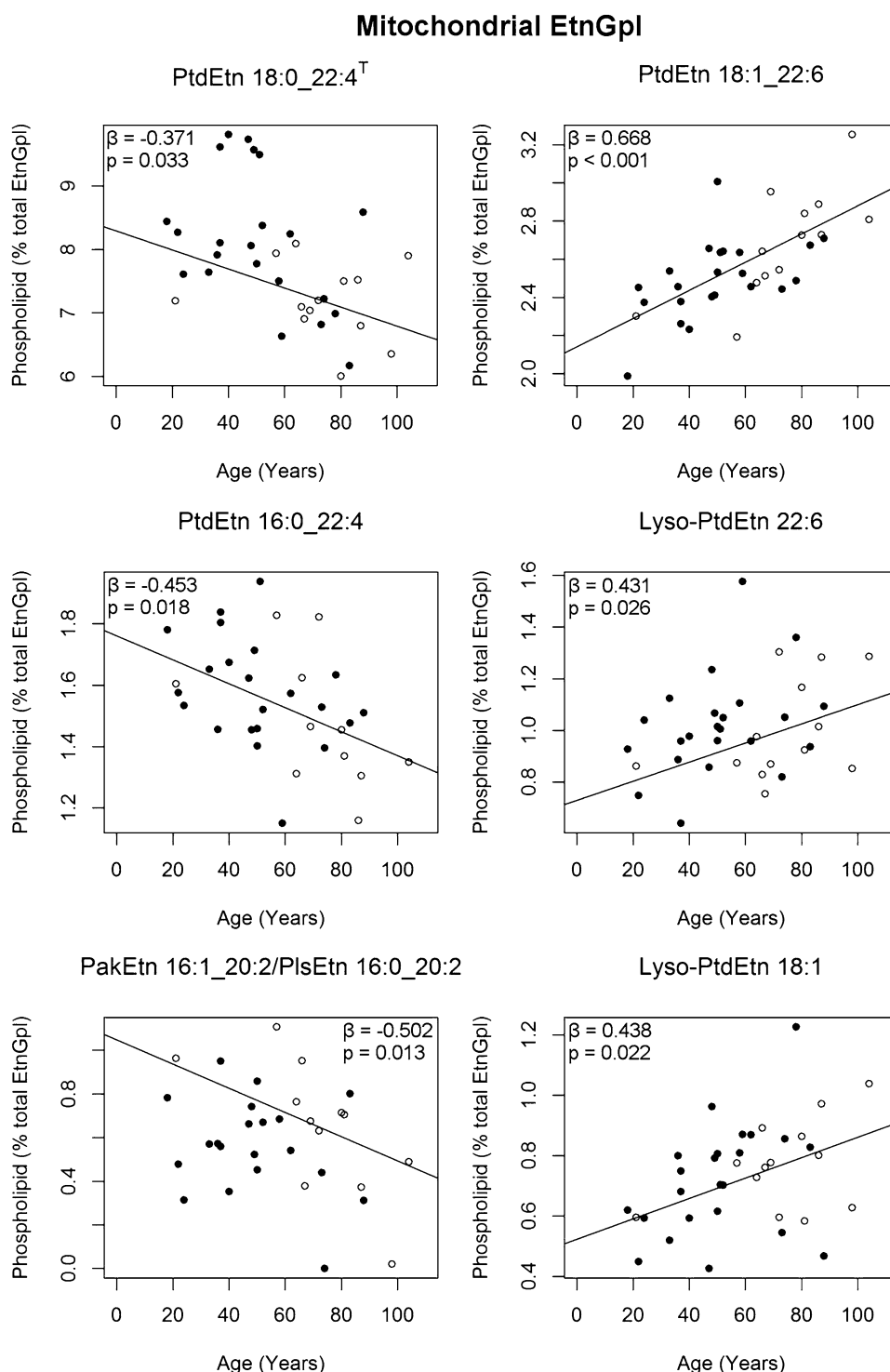
the third most abundant microsomal ChoGpl. PtdCho 18:0\_18:1 comprises 10 % of total microsomal ChoGpl (Figure S2) and 4 % of total microsomal phospholipids, and declined by 17 % from ages 18 to 104 (Fig. 6, Table S3). An age-related increase of 11 % in abundance was found for PtdCho 15:0\_16:0/PakCho 16:0\_16:0 (Fig. 6, Table S3), but this phospholipid comprises only 0.2 % of total microsomal phospholipid.

PtdCho 15:0\_16:0/PakCho 16:0\_16:0 also increased in quantified amount with age within the microsomal membranes, more than doubling from 0.76 pmol/ $\mu$ g membrane protein at age 18 to 1.7 pmol/ $\mu$ g membrane protein by age 104 (Table S5).

### EtnGpl

Three microsomal EtnGpl changed with age in their percent composition (percent of total microsomal EtnGpl) in the hippocampus (Fig. 7, Table S3). The most abundant microsomal EtnGpl showing age-related changes was PtdEtn 18:0\_22:4, which decreased by 19 % in abundance across the 86 year period. PtdEtn 18:0\_22:4 is the

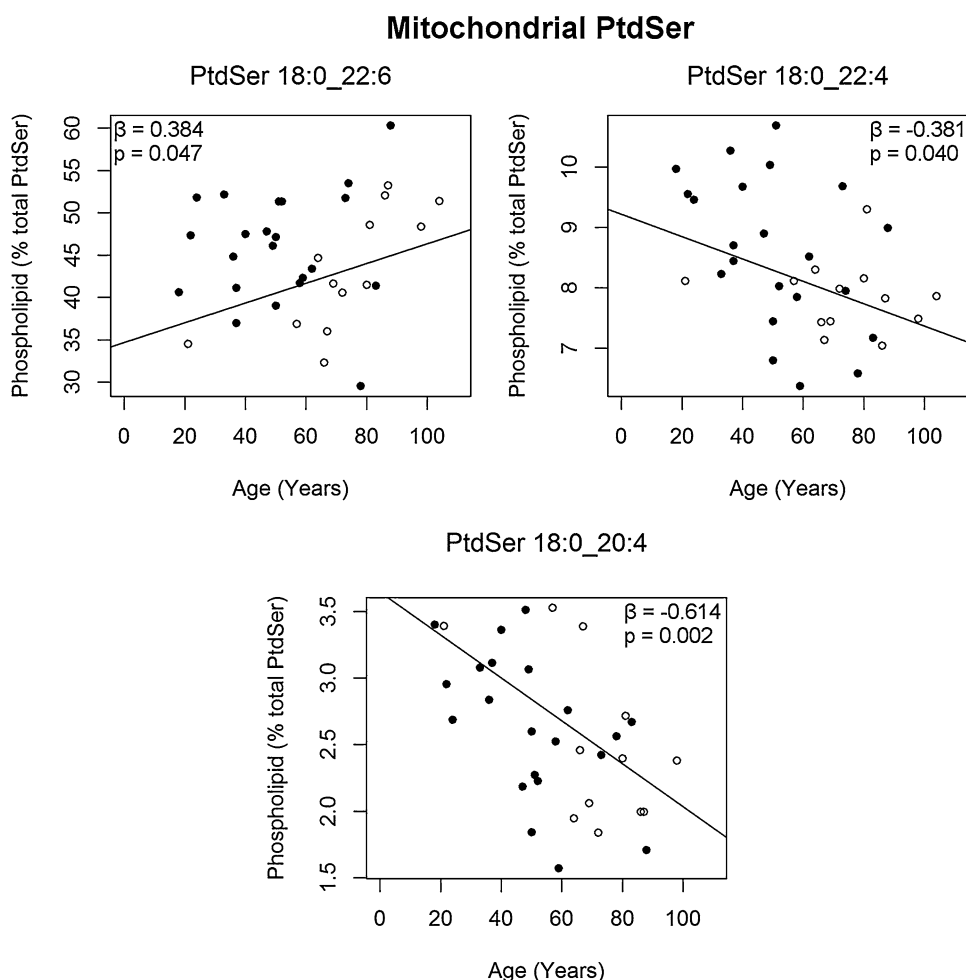
**Fig. 4** Mitochondrial EtnGpl changing significantly with age (as percent of total EtnGpl) in normal human hippocampus ( $n = 32$ – $36$ ). Regression model was adjusted for sex: males (black circle), females (white circle). <sup>T</sup> Indicates dependant variable transformed for linear regression, with transformed beta-coefficient and  $p$  value reported on original *scatterplot* for comparison. Regression tables including 95 % confidence interval are available in the supplementary material (Table S2)



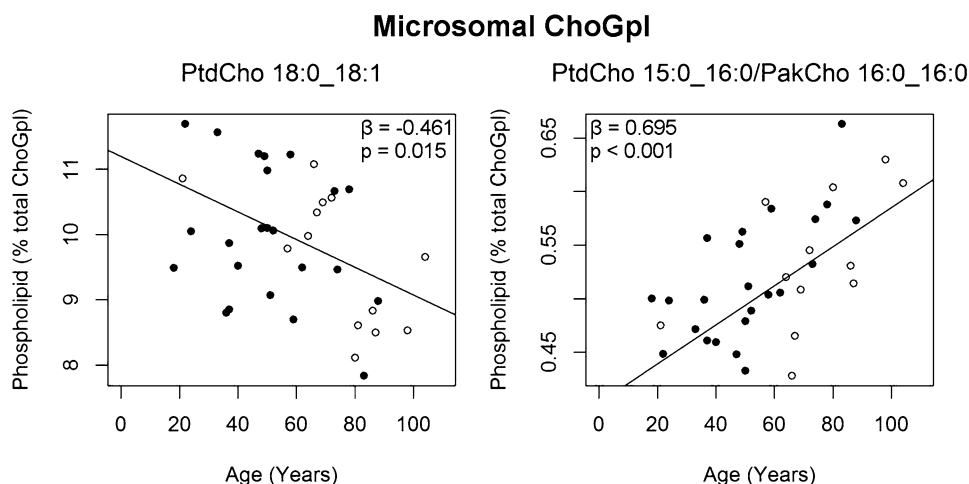
third most abundant microsomal EtnGpl, comprising 8 % of total microsomal EtnGpl (Figure S2) and 6 % of total microsomal phospholipid. The two remaining microsomal EtnGpl increased significantly in percent abundance with age. PtdEtn 18:1\_22:6, a moderately abundant microsomal EtnGpl, increased from 1.9 % of microsomal EtnGpl to 2.5 % from ages 18 to 104, representing an increase of

29 % in abundance (Fig. 7, Table S3). PtdEtn 15:0\_22:6/PakEtn 16:0\_22:6 increased by 76 % across the 86 year period, but this phospholipid is only of low abundance in microsomal membranes (Figure S2). PtdEtn 15:0\_22:6/PakEtn 16:0\_22:6 also increased with age in quantified amount, rising from 0.74 pmol/ $\mu$ g membrane protein at age 18–1.5 pmol/ $\mu$ g membrane protein at age 104 (Table S5).

**Fig. 5** Mitochondrial PtdSer changing significantly with age (as percent of total PtdSer) in normal human hippocampus ( $n = 33$ – $35$ ). Regression model was adjusted for sex: males (*black circle*), females (*white circle*). Regression tables including 95 % confidence interval are available in the supplementary material (Table S2)



**Fig. 6** Microsomal ChoGpl changing significantly with age (as percent of total ChoGpl) in normal human hippocampus ( $n = 36$ ). Regression model was adjusted for sex: males (*black circle*), females (*white circle*). Regression tables including 95 % confidence interval are available in the supplementary material (Table S3)



## PtdSer

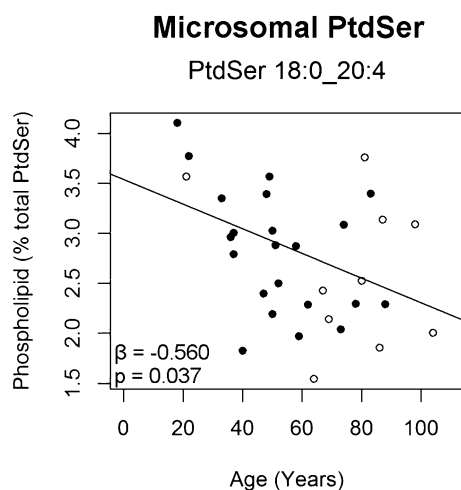
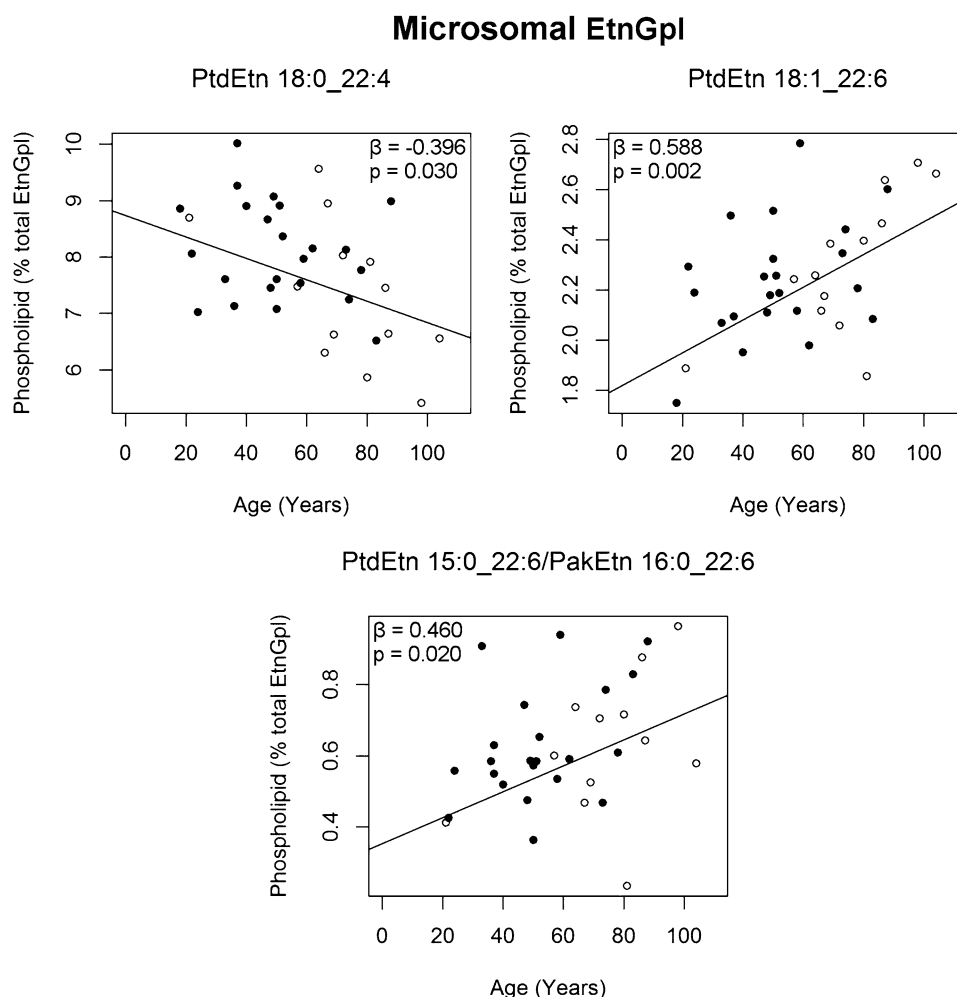
Only a single PtdSer phospholipid changed in its percent composition with age in the microsomal membranes: PtdSer 18:0\_20:4 (Fig. 8, Table S3). PtdSer 18:0\_20:4 decreased

from 3.3 % of microsomal PtdSer to 2.3 % over the 86 year period studied, representing a loss of one-third in abundance.

There were no microsomal PtdSer that changed significantly in quantified amount over the 86 year period (pmol phospholipid/ $\mu$ g membrane protein).



**Fig. 7** Microsomal EtnGpl changing significantly with age (as percent of total EtnGpl) in normal human hippocampus ( $n = 34$ – $36$ ). Regression model was adjusted for sex: males (*black circle*), females (*white circle*). Regression tables including 95 % confidence interval are available in the supplementary material (Table S3)



**Fig. 8** Microsomal PtdSer changing significantly with age (as percent of total PtdSer) in normal human hippocampus ( $n = 32$ ). Regression model was adjusted for sex: males (*black circle*), females (*white circle*). Regression tables including 95 % confidence interval are available in the supplementary material (Table S3)

## Discussion

Examination of the mitochondrial and microsomal membranes of the human hippocampus showed that many phospholipids underwent significant changes during normal aging. These changes were not limited to any single class of phospholipid or to a single membrane fraction. Rather, the age-related changes in the hippocampus occurred in a wide range of phospholipids in both the mitochondria and microsomal membranes, with many of these changes occurring in low-to-moderately abundant phospholipids. In particular, phospholipids containing a 22:4 fatty acid underwent consistent age-related decreases in both membrane fractions across the 86 year period studied, while those containing a 22:6 fatty acid (DHA) increased across the adult lifespan.

PtdEtn 18:0\_22:4 is the third most abundant EtnGpl in both the mitochondria and microsomes (Figure S1 and S2), and a decrease of approximately 13 and 19 % in percent abundance with age was found in both membranes fractions respectively (Figs. 4 and 7, Tables S2–S3). Several

other phospholipids with a 22:4 fatty acid also declined in percent abundance with age in the mitochondrial membranes: PtdEtn 16:0\_22:4 and PtdSer 18:0\_22:4 declined by 20 and 18 % respectively from ages 18 to 104 (Figs. 4 and 5, Table S2). The 22:4 fatty acid can be putatively assigned as being adrenic acid, an omega-6 PUFA, based on its synthesis via the elongation and desaturation pathway [24]. To our knowledge, no omega-3 isomer of this fatty acid has been detected previously in human brain [9, 12, 25, 26]. Adrenic acid is the product of elongation of the omega-6 PUFA 20:4 (arachidonic acid, ARA) by two carbons, and can undergo  $\beta$ -oxidation within peroxisomes to form ARA. Two isomers of 20:4 can exist: an omega-3 and an omega-6 isomer (ARA) which differ only by the position of their double bonds. Since we cannot determine double bond position with the mass spectrometry method used in the present study, we cannot definitively establish which 20:4 isomer is present. However, previous studies of fatty acids within the phospholipids of human hippocampus that used gas chromatography have not detected any omega-3 20:4, so we can assume that the 20:4 detected is primarily ARA [9, 11, 15, 16]. Many phospholipids containing ARA also decreased with age in the mitochondrial and microsomal membranes. Common between both membrane fractions was a decrease in the percent abundance of PtdSer 18:0\_20:4 with age, by 41 and 32 % in mitochondrial and microsomal membranes respectively (Figs. 5 and 8, Tables S2–S3). Age-related decreases were also seen in mitochondrial PtdCho 18:0\_20:4, which declined by 26 % over the 86 year period (Fig. 6, Table S2).

Due to these age-related declines in mitochondrial and microsomal phospholipids containing the omega-6 fatty acids adrenic acid or ARA additional regressions were conducted to check for changes in fatty acid content with age in the hippocampus (Tables S6–S7). Decreases with age in ARA were seen in the combined phospholipids of the mitochondrial membranes (Table S6). When phospholipids containing either ARA or adrenic acid were pooled together an even greater loss with age was revealed in the combined mitochondrial phospholipids. In the microsomal membranes, only the pooled ARA and adrenic acid declined with age in the combined phospholipids (Table S7). Together, these results indicate that there is a significant loss of long chain omega-6 fatty acids in both membrane fractions within the hippocampus over the adult lifespan. We can only speculate on what is driving this loss of long chain omega-6 PUFA with age within the mitochondrial and microsomal membranes of the hippocampus. Under the mitochondrial free radical theory of ageing, PUFA such as ARA and adrenic acid are theorized to be lost with age due to damage by reactive oxygen species; but if this were the case we would also expect to see a loss of phospholipids containing DHA with age. Chronic, low-grade

inflammation has also been suggested as a driving force in aging [27], and ARA is involved in the production of many pro-inflammatory eicosanoids after cleavage from phospholipids by phospholipase A<sub>2</sub>. Ultimately the cause of this loss of long chain omega-6 PUFA from the hippocampus with age remains to be determined in future studies.

Despite significant age-related losses of PtdEtn 18:0\_22:4 in both membrane fractions (Figs. 6 and 7) there were no changes seen with age to either EtnGpl-adrenic acid or to pooled EtnGpl-ARA and EtnGpl-adrenic acid in either the mitochondrial or microsomal membranes (Tables S6–S7). Instead most of the age-related declines in adrenic acid and ARA were seen in PtdSer, with mitochondrial and microsomal PtdSer-adrenic acids declining significantly over the 86 year period, alongside considerable losses of mitochondrial PtdSer-ARA. In the mitochondria this loss of PtdSer-adrenic acid and PtdSer-ARA were driven by declines in PtdSer 18:0\_22:4 and PtdSer 18:0\_20:4 respectively (Fig. 5, Table S2), while in the microsomal membranes there were losses with age of PtdSer 18:0\_22:4 (Fig. 8, Table S3). These results emphasize that age-related changes to individual phospholipids are not necessarily reflected in the total contribution of a given fatty acid within a class of phospholipid. Previous studies using combinations of thin-layer chromatography and gas chromatography to examine the changes with age to phospholipids and their fatty acids as separate entities would have not seen these age-related changes.

Interestingly we saw large increases in mitochondrial PtdSer 18:0\_22:6 over the adult lifespan in the human hippocampus (Fig. 5, Table S2). This is a similar finding to what we have reported previously in the mitochondrial and microsomal human prefrontal cortex [22]. 22:6 can be putatively classified as the omega-3 docosahexaenoic acid (DHA), a fatty acid found in high levels in EtnGpl and PtdSer phospholipids within the human brain [19]. The inclusion of DHA within PtdSer phospholipids is thought to be neuroprotective, promoting neural survival and longevity [28]. An inability to synthesize DHA results in severe brain abnormalities and death within the first few years of life [29]. Several other low-abundance phospholipids containing DHA also increased with age in both membrane fractions, including mitochondrial and microsomal PtdEtn 18:1\_22:6 (Figs. 4 and 7, Tables S2–S3), mitochondrial lyso-PtdEtn 22:6 (Fig. 4, Table S2) and microsomal PtdEtn 15:0\_22:6/PakEtn 16:0\_22:6 (Fig. 7, Table S3). Increases in mitochondrial PtdSer-DHA were also seen with age in the human hippocampus (Tables S6), but this age-related increase in DHA was not reflected in combined mitochondrial phospholipids. This suggests that the increase in DHA across the adult lifespan within the hippocampus is not as extensive as that seen in the human prefrontal cortex [22], but that this smaller increase in mitochondrial PtdSer-DHA

still may exhibit a neuroprotective effect leading to increased longevity.

The largest change in any single phospholipid occurred in mitochondrial PtdCho 16:0\_18:1, the predominate phospholipid present in this membrane fraction (Figure S1). Mitochondrial PtdCho 16:0\_18:1 increased from 40.8 % of total mitochondrial ChoGpl to 42.5 % from ages 18 to 104, representing a 4 % increase in initial abundance over the 86 year period (Fig. 3, Table S2). The role of this particular molecular phospholipid is largely unknown; however, its high abundance could suggest a structural or scaffolding role in cellular membranes. Some evidence points to a role for PtdCho 16:0\_18:1 in modulating membrane fluidity [30, 31], as well as participating in the formation of lipid raft domains [32]. Our previous study of the prefrontal cortex also found age-related changes for mitochondrial PtdCho 16:0\_18:1 [22].

Two previous studies have examined changes to the phospholipids of the human hippocampus during normal aging. Similar to the present study, Söderberg *et al.* [16] reported decreases in total protein concentration (mg/g of wet tissue) in the human hippocampus with age. However, unlike the present study Söderberg *et al.* [16] did not perform a fractionation step and so was unable to pinpoint the loss of protein with age to a specific subcellular fraction (Fig. 1). Both studies by Söderberg *et al.* [15, 16] reported decreases with age in the concentration of total phospholipids (mg/g wet tissue) in the hippocampus from ages 50–60 years, a finding that was not seen in the present study. Between the two studies by Söderberg *et al.* there were conflicting results for age-related changes to phospholipid classes, with Söderberg *et al.* [16] reporting no changes in concentration to either total ChoGpl or EtnGpl with age in the hippocampus, while Söderberg *et al.* [15] found age-related decreases in both total ChoGpl and EtnGpl. In the present study no changes with age were seen for any phospholipid class, either in percent abundance or in quantified amount. Söderberg *et al.* [15] also examined changes to hippocampal ChoGpl and EtnGpl fatty acids with no age-related changes with age being found, which again does not agree with the present study. This study has the advantage of using mass spectrometry which is able to examine molecular phospholipids, unlike thin layer chromatography combined with gas chromatography which measures phospholipids their fatty acids as separate entities. This may explain why these age-related changes have not been found by previous studies in the human hippocampus. Additionally, both studies by Söderberg *et al.* [15, 16] examined the phospholipid content of whole tissue, which could also explain the differences between their findings and the current study.

In summary, there were several phospholipids containing either adrenic acid or ARA that decreased with age in the mitochondrial and microsomal membranes of the

human hippocampus. The most abundant phospholipid of this type was PtdEtn 18:0\_22:4 in both the mitochondrial and microsomal membrane fractions, which decreased by approximately 13–18 % in abundance from ages 20 to 100. Analysis of total fatty acids found that both adrenic acid and ARA decreased with age in the combined phospholipids in both membrane fractions, but no age-related changes were seen specifically in the EtnGpl class for either fatty acid. Similarly to our previous study of the human prefrontal cortex [22], large age-related increases were seen in mitochondrial PtdSer 18:0\_22:6. The present study details a comprehensive profile of the ChoGpl, EtnGpl and PtdSer phospholipids present in the mitochondrial and microsomal membranes of the human hippocampus, and identifies for the first time changes to molecular phospholipids in this brain region during normal adult aging.

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