The phospholipid composition of the human entorhinal cortex remains relatively stable over 80 years of adult aging

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Abstract Membrane lipid composition is altered in the brain during the pathogenesis of several age-related neurodegenerative diseases, including Alzheimer's disease. The entorhinal cortex is one of the first regions of the brain to display the neuropathology typical of Alzheimer's disease, yet little is known about the changes that occur in membrane lipids within this brain region during normal aging (i.e., in the absence of dementia). In the present study, the phospholipid composition of mitochondrial and microsomal membranes from human entorhinal cortex was examined for any changes over the adult lifespan (18–98 years). Overall, changes in several molecular phospholipids were seen with age in the entorhinal cortex across both membranes. The proportion of total phosphatidylcholine within the mitochondrial fraction increased within the entorhinal cortex with age, while total mitochondrial phosphatidylethanolamine decreased. Many mitochondrial phosphatidylethanolamines containing docosahexaenoic acid

increased with age; however, this did not translate into an overall age-related increase in total mitochondrial docosahexaenoic acid. The most abundant phospholipid present within the human brain, PC 16:0_18:1, also increased with age within the mitochondrial membranes of the entorhinal cortex. When compared to other regions of the brain, the phospholipid composition of the entorhinal cortex remains relatively stable in adults over the lifespan in the absence of dementia.

Keywords Lipids · DHA · Docosahexaenoic acid · Phosphatidylcholine · Phosphatidylethanolamine; mass spectrometry · Lipidomics

Abbreviations

AD	Alzheimer's disease
DHA	Docosahexaenoic acid
EC	Entorhinal cortex
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine

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Introduction

Phospholipids are essential components of the human brain, comprising a quarter of its dry mass (O'Brien and Sampson 1965). Phospholipids have a high capacity for structural variety within membranes, with different permutations of head group and fatty acids generating up to 10,000 theoretical molecular species (Yetukuri et al.

2008). Phospholipid composition is highly altered within many regions of the human brain in Alzheimer's disease (AD) (Kosicek and Hecimovic 2013), often within the earliest stages (Han 2005). The incidence of AD is highly correlated with advanced age, but despite this relationship, little is known about what changes occur to the phospholipid composition of the human brain over the adult lifespan.

The entorhinal cortex (EC) is located within the medial temporal lobe in the anterior portion of the parahippocampal gyrus and forms the interface between the hippocampal formation and associated neocortical regions. Superficial layers of the EC project to the dentate gyrus and hippocampus, forming the perforant pathway involved in encoding episodic memory (Gallagher and Koh 2011). The neurofibrillary tangles typical of AD are deposited within the EC in the earliest stages of the disease (Braak and Braak 1991). Alongside this neuropathology, the EC experiences significant atrophy that is detectable even in the preclinical stages of AD (Duara et al. 2015; Schröder and Pantel 2016), as well as a substantial loss in neuron number and density (Gómez-Isla et al. 1996; Price et al. 2001; von Gunten et al. 2005; von Gunten et al. 2006). In respect to phospholipids, Chan et al. (2012) observed numerous changes to phospholipid species in the EC with AD. Changes to the phospholipid composition of the entire parahippocampal gyrus with AD have also been reported, with alterations in several polyunsaturated fatty acids being observed (Skinner et al. 1993; Prasad et al. 1998).

Considerably less is known about what occurs to the EC during normal aging (i.e., in the absence of dementia). Several studies have reported substantial declines in cortical volume and/or thickness with age (Jiang et al. 2014; Fjell et al. 2014), but the rate of atrophy within this region is thought to be lower than other related regions such as the hippocampus (Raz et al. 2004; Raz et al. 2005). Recent studies have observed age-related alterations in the composition of phospholipids within the mitochondrial and microsomal membranes of the human prefrontal cortex (Norris et al. 2015) and hippocampus (Hancock et al. 2015), but to date, no studies have examined the changes occurring in the phospholipid composition of the EC during normal aging. Given the important role that the EC plays in the pathogenesis of AD, understanding any age-related changes occurring within this brain region could potentially lead to a

better understanding of the mechanisms underlying the development of AD.

Therefore, the aim of this study was to establish a baseline on the age-related changes occurring to phospholipids of the EC in neurologically normal humans aged 18–98. The lipidomic workflow used allowed us to characterize any age-related changes to EC phospholipids at the fatty acyl/alkyl positional level. Additionally, the tissue was fractionated into mitochondria-enriched and microsomal membranes (plasma membrane and endomembranous system consisting of the endoplasmic reticulum, Golgi, etc.) to isolate any age-related changes to a particular subcellular compartment.

Methods

Brain tissue

Neurologically normal frozen postmortem brain tissue from the EC was obtained from the New South Wales Brain Tissue Resource Centre at the University of Sydney, Australia, with detailed demographics of the cohort being previously published (Norris et al. 2015). The status of donor brains was assessed by the brain tissue resource centre as outlined in Sutherland et al. (2016). All donors over the age of 60 underwent a modified Braak staging screen, with either stage I or II being observed in most of the elderly donors (Supplementary table S1). Due to the loss of one sample in this region, there were 35 samples in the EC cohort (11 female, 24 male) with an average age of 57.6 years (± 3.6 , range 18–98). No differences were observed between brain pH or postmortem interval between the two sexes, but females were significantly older than males (68.5 ± 6.9 years versus 52.7 ± 3.9 respectively, $p < 0.05$). All experiments were conducted with the approval of the Human Research Ethics Committee of the University of Wollongong (HE11/267).

Subcellular fractionation and lipids extraction

Subcellular fractionation followed that previously reported (Norris et al. 2015; Hancock et al. 2015). Briefly, 100 mg of frozen, pulverized tissue was homogenized by a bead homogenizer (FastPrep[®]-24, MP Biomedicals, NSW, Australia) at 6.0 m/s for 40 s using 1.4-mm zirconium oxide beads in 2 mL of an ice-cold 20 mM Tris buffer (pH 7.4) containing 250 mM sucrose, 2 mM

EDTA, 2 mM DTT, and complete protease inhibitor. Large cellular debris and nuclei were removed from each sample by a short centrifuge step (10 min, 1000×g). The supernatant was then sequentially centrifuged to obtain a mitochondria-enriched (35 min, 10,000×g, 4 °C) and microsomal (mixed membrane) pellet (40 min, 100,000×g, 4 °C). Total protein was determined for both the mitochondrial and microsomal fractions (BCA Protein Assay Kit, Thermo Fisher Scientific, VIC, Australia). Lipids were extracted from 75 µg of protein as described previously (Deeley et al. 2008) in the presence of internal standards (20 µM of PC 19:0/19:0, PE 17:0/17:0 and PS 17:0/17:0; 10 µM of lyso-PC 17:0 and lyso-PE 14:0). Extracted lipids were stored in chloroform: methanol (1 mL, 1:2 v/v with 0.01% butylated hydroxytoluene) at −20 °C until analysis.

Mass spectrometry

Nano-electrospray ionization mass spectrometry of lipid extracts was performed using a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP® 5500 SCIEX, MA, USA) equipped with an automated chip-based nanoelectrospray source (TriVersa Nanomate™, Advion Biosciences, NY, USA) using parameters and targeted ion scans described previously (Norris et al. 2015). Phospholipids were quantified from internal standards by Lipidview™ software (version 1.2, SCIEX, MA, USA). Briefly, phospholipids at the sum composition level (i.e., head group and sum fatty acid composition) were quantified from positive precursor ion (PC, m/z 184) and neutral loss (PE, 141 Da; PS, 185 Da) scans, and molecular phospholipids (i.e., head group and fatty acids) were determined from complementary negative precursor ion scans for fatty acids (Norris et al. 2015). This method cannot determine *sn* position of fatty acids (i.e., their position on the glycerol backbone), and so phospholipid structure was reported with an underscore separating the two fatty acids as recommended by Liebisch et al. (2013).

The mass spectrometry method used in this study was also unable to distinguish alkyl and plasmenyl ether isobars, and so these are reported as being monounsaturated alkyl ether phospholipids. Due to this, no correction factor for PE-plasmenyl ethers was applied (Berry and Murphy 2004), and any

PE-plasmenyl ethers will be underestimated by approximately 29% (Mitchell et al. 2007). Small amounts of saturated odd-chain acyl isobars were detected alongside all reported alkyl ether phospholipids. The exact proportion of each isobar could not be determined quantitatively, and so these isobars were reported as being potentially either molecular phospholipid.

Statistical analysis

Statistical analysis was performed using SPSS Statistics (version 19, IBM Corp., NY, USA) and R (v. 3.1.1). The Wilcoxon signed-rank test was used to compare the amount within phospholipid classes between the two membrane fractions. Linear regression was performed between phospholipids and age with sex as a second independent variable, with a significance level, was set at $p < 0.05$. Influential data points were identified by a Cook's distance of greater than one and were removed from the analysis. Normality of the dependent variable was assessed by examining histograms of the residuals. Outliers were identified as being more than three standard deviations from the mean by examining histograms of the residuals and were first treated by transformation of the dependent variable or by removal from analysis if transformation was unsuccessful or violated the assumptions of normality. Values reported for ages 20 and 100 are derived from the slope of the regression line. Parameters for all statistically significant linear regression results are included in the supplementary material (Table S2–4).

Results

Phospholipid composition of mitochondrial and microsomal membranes

Examination of the phospholipid composition of the mitochondrial and microsomal membranes revealed a similar profile of molecular phospholipids between the two fractions (Fig. 1). Over half of the phospholipid present within each class was made up of only two to four molecular phospholipids. These highly abundant molecular phospholipids did not differ significantly in amount between the two membrane fractions. Indeed, most of the differences present between the

Fig. 1 Phospholipids detected within PC (**a**), PS (**b**), and PE (**c**) in the mitochondrial (dark gray) and microsomal (white) membranes of the human entorhinal cortex (as a percent of total phospholipid

within each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort $\pm$ SEM

mitochondrial and microsomal membranes were small and occurred in molecular phospholipids comprising less than 5% of each phospholipid class.

Small, statistically significant differences were seen between the two membranes in the proportion of total phospholipid within each class (Fig. 2a, Table S2). Mitochondrial membranes contained more PE than microsomal ($29.1 \pm 0.4\%$ vs. $32.1 \pm 0.5\%$; $p < 0.001$), while the microsomes had more PC ($49.9 \pm 0.6\%$ vs. $51.7 \pm 0.6\%$; $p < 0.005$). No differences were observed between the two membrane fractions for total PS ($18.1 \pm 0.5\%$ vs. $19.2 \pm 0.4\%$).

Changes in mitochondrial phospholipid with age

Two phospholipid classes changed in abundance with age within the mitochondrial membranes (Fig. 2b). PC increased by 12% over the 80-year period, while PE declined by 11%. Several mitochondrial molecular phospholipids also changed in abundance with age within PC and PE only (Table S3). An age-related increase was observed in the most abundant phospholipid of the mitochondrial fraction, PC 16:0_18:1, which increased from 42% of total mitochondrial PC at age 20 to 44% at age 100 (Fig. 3). Due to the high abundance of

Fig. 2 Phospholipid classes within the mitochondrial and microsomal membranes of the entorhinal cortex. **a** The percent composition of each class within the mitochondrial (*left*) and microsomal (*right*) fractions. Values shown are the mean for the entire cohort. **b** Two phospholipid classes changed significantly with age within the mitochondrial membranes ($n = 35$), mitochondrial PC (*left*) and mitochondrial PE (*right*). Regression model was adjusted for sex: males (*black circle*), females (*white circle*), and parameters are available in Table S2

this phospholipid, this represents one of the largest age-related changes for any single mitochondrial phospholipid in the human EC. PC 16:0_18:2 was the only other PC to change with age in this fraction, increasing from 0.7 to 1.3% from ages 20 to 100.

Six mitochondrial PEs increased in abundance over the 80-year period, with four of these containing a 22:6 fatty acid: PE 16:0_22:6, PE 18:1_22:6, PE O-18:1_22:6, and PE 17:0_22:6/O-18:0_22:6 (Fig. 4). The most abundant of this type, PE 16:0_22:6, increased

by 17% from ages 20 to 100. The remaining two PEs to increase with age in this fraction were PE 16:0_18:1 by 25% and PE 16:0_20:1 by 30%.

Changes in microsomal phospholipid with age

There were no age-related changes seen in the total amount of any phospholipid class within the microsomal membranes. A total of eight molecular phospholipids changed with age within the EC microsomes (Table S4).

Fig. 3 Mitochondrial PCs changing significantly with age (as a percent of total PC) in normal human entorhinal cortex ($n = 30$ –35). Regression model was adjusted for sex: males (*black circle*), females (*white circle*). Regression parameters are available in the Table S3

Fig. 4 Mitochondrial PEs changing significantly with age (as a percent of total PE) in normal human entorhinal cortex ($n = 30\text{--}35$). Regression model was adjusted for sex: males (black circle), females (white circle). Regression parameters are available in Table S3

Three PCs increased with age: PC 18:1_18:1 by 4%, PC 14:0_16:0 by 23%, and PC 15:0_16:0/O-16:0_16:0 by 38% from ages 20 to 100 (Fig. 5). Within PE, both PE 18:1_22:6 and PE 16:0_22:5 increased with age while PE 16:0_20:1 decreased over the 80-year period. In contrast to the mitochondrial membranes, two microsomal PS phospholipids changed with age: PS 18:0_22:4 decreased by 20% from ages 20 to 100, whereas PS 18:1_20:1 increased by 180% (Fig. 6). While a seemingly large age-related increase was reported PS 18:1_20:1, this phospholipid is of low abundance in microsomal membranes making up less than 0.7% of total PS (Fig. 1).

Discussion

Overall, several changes occurred to molecular phospholipids within the mitochondrial and microsomal membrane of the EC with age; however, these were fewer in number compared to that recently reported

for the dorsolateral prefrontal cortex (Norris et al. 2015) and hippocampus (Hancock et al. 2015). At present, no other studies of age-related changes in phospholipid composition of the EC exist, preventing any comparisons from being made.

The phospholipid that underwent the largest age-related increase within the EC was mitochondrial PC 16:0_18:1, the most abundant phospholipid present within this membrane fraction (Fig. 1). This phospholipid was also previously found to increase with age within the mitochondria of the prefrontal cortex (Norris et al. 2015) and hippocampus (Hancock et al. 2015). Increased amounts of this phospholipid can attenuate the oxidation of polyunsaturated fatty acids within model membranes (Cortie and Else 2015), a mechanism that may support longevity (Dei et al. 2002; Montine et al. 2011; Guest et al. 2014). Additionally, PC 18:1/16:0 is enriched in the protrusion tips of PC12 and neuro-2A cultures as well as in the neuronal synapses of mouse brain (Kuge et al. 2014), indicating that this phospholipid could be important for

Microsomal phosphatidylcholine

Microsomal phosphatidylethanolamine

Fig. 5 Microsomal PCs (top) and PEs (bottom) changing significantly with age (as a percent of total PC and PE respectively) in normal human entorhinal cortex ($n = 30$ – 35). Regression model

was adjusted for sex: males (*black circle*), females (*white circle*). Regression parameters are available in Table S4

establishing and maintaining neural connections. However, the lipidomics method used in the present study was unable to separate these two isomers (PC 16:0/18:1 versus PC 18:1/16:0), and therefore understanding the

role that PC 18:1/16:0 may play in the aging brain will require further investigation.

Within the present work, a number of phospholipids containing a 22:6 fatty acid increased with age within

Fig. 6 Microsomal PSs changing significantly with age (as a percent of total PS) in normal human entorhinal cortex ($n = 30$ – 35). Regression model was adjusted for sex: males (*black circle*), females (*white circle*). Regression parameters are available in Table S4

Microsomal phosphatidylserine

the EC, including mitochondrial PE 16:0_22:6, PE O-18:1_22:6, and PE 17:0_22:6/O-18:0_22:6, as well as both mitochondrial and microsomal PE 18:1_22:6 (Tables S2–3). Despite these changes to individual phospholipids with age, no significant changes were seen with age in total 22:6 (data not shown). The 22:6 fatty acid within these phospholipids can be putatively identified as docosahexaenoic acid (DHA), the principal omega-3 fatty acid present in the brain. DHA is important in the developing brain, accumulating in the earliest months of life (Martínez and Mougan 1998). DHA has a number of pro-cell survival roles within cell membranes, including the production of anti-inflammatory D-series resolvins and neuroprotectins (Weylandt et al. 2012). DHA is known to be severely reduced in many regions of the brain affected by AD pathology (Söderberg et al. 1991; Martín et al. 2010; Igarashi et al. 2011; Cunnane et al. 2012; Fabelo et al. 2014); however, there are very few studies of this type looking specifically at the changes to the EC. Only a single study has examined the EC for changes in its phospholipids in AD, finding no changes in either total PC, PE or PS (Chan et al. 2012). Two further studies examined the parahippocampal gyrus (Skinner et al. 1993; Prasad et al. 1998), but only Prasad et al. (1998) observed a loss of DHA with AD within the PE class of phospholipids. Although the current evidence is relatively sparse, if the loss of PE-DHA in the EC and greater parahippocampal gyrus is indeed involved in the progression of AD, then the increase in PE-DHA during normal aging could be protective against further development of AD neuropathology; however, much work remains to be done to explore this theory.

Considering that decreases in DHA with AD have been documented in many affected regions of the brain, the question remains as to how to maintain general levels of DHA within the brain over the lifespan. The inefficient conversion of the long-chain omega-3 fatty acids DHA and eicosapentaenoic acid (EPA, 20:5 n-3) from shorter chain precursors in humans suggests that both should be considered to essential dietary fatty acids (Parletta et al. 2013). But supplementation with DHA and/or EPA in the healthy elderly, elderly with mild cognitive impairment, and elderly with neurodegenerative disease have shown mixed results regarding improvement or retention of cognitive status (Sydenham et al. 2012; Janssen and Kiliaan 2014; Burckhardt et al. 2016). More recently it has been

suggested that dietary consumption of seafood as a source of DHA may be more important for prevention of AD than supplementation, with APOE ϵ 4 carriers who consumed seafood at least once per week showing a lower burden of neuritic plaques and neurofibrillary tangles at death (Morris et al. 2016). However, the discrepancies between intervention studies could also be the product of an alteration to DHA metabolism even in normal, healthy adults with advanced age (Astarita et al. 2010; Plourde et al. 2011). Needless to say, much work remains to be done in this area to determine the best way of maintaining DHA levels within the brain over the lifespan.

In summary, although many molecular phospholipids changed with age within the mitochondrial and microsomal membranes of the EC, the number of molecular phospholipids affected was far fewer than those reported for other brain regions. PC 16:0_18:1, a phospholipid which may be involved in the growth and maintenance of neuronal synapses as well as the attenuation of peroxidation within the membrane, showed substantial age-related increases within the mitochondrial membranes. We also observed an increase in several DHA-containing PE phospholipids within the mitochondrial membranes of the EC, but no age-related change in total DHA levels was observed. Despite this, the maintenance of PE-DHA levels within the entorhinal cortex over the adult lifespan could be neuroprotective. Overall, these findings suggest that the phospholipid composition of the EC remains relatively stable in adults over the course of normal aging without dementia.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

All experiments were conducted with the approval of the Human Research Ethics Committee of the University of Wollongong (HE11/267).

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