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POLYUNSATURATED FATTY ACID BIOSTATUS, PHOSPHOLIPASE A₂ ACTIVITY AND BRAIN WHITE MATTER MICROSTRUCTURE ACROSS ADOLESCENCE

ROBERT K. MCNAMARA^a, PHILIP R. SZESZKO^{b,c,1}, STEFAN SMESNY^d, TOSHIKAZU IKUTA^{b,c,2}, PAMELA DEROSSE^{b,c}, FRÉDÉRIC M. VAZ^e, BERKO MILLEIT^f, UTA-CHRISTINA HIPLER^f, CORNELIA WIEGAND^f, JANA HESSE^f, G. PAUL AMMINGER^g, ANIL K. MALHOTRA^{b,c}, and BART D. PETERS^{b,c,*}

^aDepartment of Psychiatry, University of Cincinnati College of Medicine, Cincinnati, OH 45219, USA

^bDivision of Psychiatry Research, Zucker Hillside Hospital, Glen Oaks, NY 11004, USA

^cCenter for Psychiatric Neuroscience, Feinstein Institute for Medical Research, Manhasset, NY 11030, USA

^dDepartment of Psychiatry, University Hospital Jena, D-07743 Jena, Germany

^eLaboratory Genetic Metabolic Diseases, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands

^fDepartment of Dermatology, University Hospital Jena, Erfurter Straße 35, D-07743 Jena, Germany

^gOrygen, the National Centre of Excellence in Youth Mental Health, Parkville, Australia; Centre for Youth Mental Health, The University of Melbourne, Parkville VIC 3052, Australia

Abstract

Adolescence is a period of major brain white matter (WM) changes, and membrane lipid metabolism likely plays a critical role in brain WM myelination. Long-chain polyunsaturated fatty acids (LC-PUFAs) are essential components of cell membranes including oligodendrocytes, and LC-PUFA release and turnover in membranes is regulated by phospholipase A₂ enzymes. To investigate the role of membrane lipid metabolism in healthy WM myelination across adolescence, the present study examined the relationship between membrane LC-PUFA biostatus, phospholipase A₂ activity, and brain WM microstructure in healthy subjects aged 9–20 years ($n = 30$). Diffusion tensor imaging (DTI) was performed to measure average fractional anisotropy (FA) and diffusivity (indices sensitive to WM myelination) of nine major cerebral WM tracts. Blood samples were collected to measure erythrocyte membrane fatty acid concentrations and plasma intracellular phospholipase A₂ activity (*inPLA*₂). Plasma *inPLA*₂ activity showed a significant U-curved association with WM radial diffusivity, and an inverted U-curved association with WM FA, independent of age. A significant positive linear correlation was observed between docosahexaenoic acid concentration and axial diffusivity in the corpus callosum. These findings suggest that there may be optimal physiological *inPLA*₂ activity levels associated with healthy

*Correspondence to: B. D. Peters, Department of Child and Adolescent Psychiatry, VU University Medical Center, 1105 AZ Amsterdam, The Netherlands; Arkin Youth and Family, Baarsjesweg 224, 1058 AA Amsterdam, The Netherlands, bpeters1@nshs.edu.

¹Present addresses: James J. Peters Veterans Administration Medical Center, Bronx, NY 10468, USA; Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

²Present address: Department of Communication Sciences and Disorders, University of Mississippi, University, MS 38677, USA

WM myelination in late childhood and adolescence. Myelination may be mediated by cleavage of docosahexaenoic acid from membrane phospholipids by *in*PLA₂. These findings have implications for our understanding of the role of LC-PUFA homeostasis in myelin-related neurodevelopmental disorders.

Keywords

white matter; myelin; polyunsaturated fatty acids; phospholipase; adolescence; development

INTRODUCTION

Adolescence is a period of major brain white matter (WM) changes. Significant growth of the brain's WM tracts, as visualized using diffusion tensor imaging (DTI), occurs during this age period and is associated with higher order cognitive development (Brauer et al., 2011; Lebel and Beaulieu, 2011; Fjell et al., 2012; Peters et al., 2012, 2014a). For example, development of frontotemporal connections within the arcuate fasciculus has been associated with increases in working memory and language performance (Brauer et al., 2011; Peters et al., 2012) and development of the cingulum bundle with increases in executive functioning (Peters et al., 2014a) and cognitive control (Fjell et al., 2012).

Changes in brain WM tracts as measured using DTI are presumably driven by growth of the myelin sheath and/or axon diameter (Paus, 2010). In DTI, the magnetic resonance signal is made sensitive to the diffusion of water molecules. Commonly used DTI indices are fractional anisotropy (FA), radial diffusivity (RD) and axial diffusivity (AD). FA reflects the degree of diffusion anisotropy along the neural tracts, i.e. whether water diffusion is restricted more along one axis than along the other two axes. AD is the diffusivity along the principal axis and RD the diffusivities in the two minor axes (please also see the glossary). FA is positively associated with the degree of myelination, but is also associated with other microstructural features such as neural fiber coherence and diameter (Basser and Pierpaoli, 1996; Beaulieu, 2002). Basic data suggest that RD is more sensitive to myelin-related processes and AD more sensitive to axon-related processes (Song et al., 2002), though neither of these DTI measures are specific to these processes (Paus, 2010) and they do not directly measure degree of myelination.

Membrane lipid metabolism likely plays a critical role in myelination. Myelin is formed from the membranes of oligodendrocytes and consists of ~70% lipids, with phospholipids and cholesterol accounting for the largest proportion of membrane lipids in mammals (Sastry, 1985; Baumann and Pham-Dinh, 2001). Polyunsaturated fatty acids (PUFAs) are important components of the phospholipid bilayers of cell membranes including those of oligodendrocytes. There is active turnover of PUFAs in cellular membranes, which is regulated by intracellular phospholipase A₂ (*in*PLA₂), a group of enzymes catalyzing the cleavage of fatty acids from the sn-2 position of phospholipids (Smesny et al., 2014). Each PLA₂ enzyme specifically catalyzes the hydrolysis of the center (sn-2)- ester bond of substrate phospholipids and supplies PUFAs downstream to cyclooxygenase and lipoxygenase that in turn transform fatty acids into a group of biomediators called

eicosanoids (please see appendix A for additional information on *inPLA₂*). The biosynthesis of long-chain PUFAs (LC-PUFAs) is controlled by the fatty acid desaturase (FADS) gene cluster (Ameur et al., 2012), and we have recently demonstrated that FADS haplotype is associated with brain WM development during adolescence (Peters et al., 2014b). This finding suggests that deficient LC-PUFA biosynthesis, and/or dietary intake, during adolescence could compromise healthy WM development.

The notion that LC-PUFA homeostasis may play a role in healthy WM myelination could have implications for those mental disorders that are characterized by LC-PUFA deficits, altered *inPLA₂* activity, and disrupted WM development (Versace et al., 2010; Adisetiyo et al., 2014; Peters and Karlsgodt, 2015). For example, decreased LC-PUFA levels have been associated with decreased WM integrity in first-episode schizophrenia (Peters et al., 2009, 2013), increased *inPLA₂* activity with brain WM abnormalities in schizophrenia (Smesny et al., 2010), and increased levels of lipid peroxidation markers with decreased WM integrity in bipolar disorder (Versace et al., 2014).

To further characterize the relationship between membrane LC-PUFA homeostasis and brain WM during a critical phase of brain development, in the absence of any influence of mental illness, the present study directly examined the relationship among erythrocyte membrane LC-PUFA concentrations, plasma *inPLA₂* activity, and brain WM microstructure (assessed using DTI) in healthy youth. We hypothesized that (1) erythrocyte membrane LC-PUFA concentrations would show positive associations with WM tract FA, and (2) plasma *inPLA₂* activity would show positive associations with WM tract FA, while acknowledging that excessive *inPLA₂* activity can have detrimental WM effects (Smesny et al., 2010).

The results showed a significant U-curved association between plasma *inPLA₂* activity and WM radial diffusivity, paralleled by an inverted U-curved association between plasma *inPLA₂* activity and WM fractional anisotropy, independent of age. While LC-PUFA concentrations did not show significant associations with WM fractional anisotropy, docosahexaenoic acid concentration showed a significant positive linear correlation with AD in the corpus callosum (CC). These findings suggest that there may be optimal physiological *inPLA₂* activity levels associated with healthy WM myelination in late childhood and adolescence. Furthermore, myelination may be mediated by cleavage of docosahexaenoic acid at the sn-2 position of membrane phospholipids by *inPLA₂*.

EXPERIMENTAL PROCEDURES

Participants

Healthy individuals between the ages of 8 and 20 years were recruited through local advertisements and by word of mouth. All procedures were carried out with the adequate understanding of the subjects and written informed consent was obtained from participants or if the participant was a minor, from a parent or guardian; all minors provided assent. Participants had no current or past history of a Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, axis I psychiatric disorder as assessed by structured diagnostic interview (Kaufman et al., 1997; First et al., 2001). Other exclusion criteria included: (1) intellectual disability, (2) learning disability, (3) MRI contraindications, (4) pregnancy, and

(5) major medical illness. Mean full scale IQ was measured using the Wechsler Abbreviated Scale of Intelligence. Handedness was determined using the Edinburgh Handedness Inventory (Oldfield, 1971). Subjects were administered an omega-3 dietary intake questionnaire (self-report by patient, or parent or guardian for minors) to estimate dietary DHA and EPA intake (McNamara et al., 2013). This study was approved by the Institutional Review Board of the North Shore—Long Island Jewish Health System. All experiments on the subjects were conducted in accordance with the Declaration of Helsinki.

DTI acquisition

MRI exams were conducted at North Shore University Hospital, Manhasset, NY, on a 3T GE scanner (GE Signa HDx; General Electric, Milwaukee, WI, USA). DTI data were acquired using single shot echo-planar imaging, and double spin echo to decrease distortions due to eddy currents, with the following parameters: repetition time = 14000 ms, echo time = minimum, matrix = 128×128 , field of view = 240 mm, slice thickness = 2.5 mm, and 51 contiguous axial slices aligned to the anterior and posterior commissures. A total of 36 DTI volumes were obtained for each subject that included 31 volumes with diffusion gradients applied along 31 non-collinear directions ($b = 1000 \text{ s/mm}^2$) and 5 volumes without diffusion weighting.

DTI processing and analysis

All scans were reviewed by a radiologist to ensure that no gross abnormalities were evident. All images were visually inspected for gross artifacts. DTI data were processed and analyzed using the FMRIB Software Library (FSL; www.fmrib.ox.ac.uk/fsl/ – last accessed 15-08-2016). Head motion and eddy current induced distortions were corrected through affine registration of the diffusion-weighted images to the first B0 image. The gradient directions were corrected according to the rotation parameters of this linear correction. Next, non-brain tissue was removed using the Brain Extraction Tool in FSL. The DTIFIT tool was then used to fit a diffusion tensor model to the raw diffusion data at each voxel, fitting the model with weighted least squares.

DTI tractography

Nine major cerebral WM tracts were selected for our analysis: two interhemispheric tracts, i.e. splenium and genu of the corpus callosum (CC, which connects the bilateral occipital lobes as well as parietal and temporal cortices and the bilateral frontal lobes, respectively); two projection tracts, i.e. corticospinal tract (CST, which connects the motor cortex to the spinal cord) and anterior thalamic radiation (ATR, which connects thalamic nuclei to the frontal lobe); five association tracts, i.e. inferior longitudinal fasciculus (ILF, which connects the anterior temporal lobe to the occipital lobe), inferior fronto-occipital fasciculus (IFOF, which connects the frontal lobe to the occipital lobe as well as parietal and temporal cortices), superior longitudinal fasciculus (SLF, which connects the frontal lobe to the parietal and temporal lobes), cingulum (which projects from the frontal lobe to the temporal lobe beneath the cingulate gyrus), and uncinate fasciculus (which connects the frontal lobe to the anterior temporal pole). The probable trajectories of these tracts were traced as follows. Within-voxel probability density functions of the principal diffusion direction were estimated using Markov Chain Monte Carlo sampling in FSL's BEDPOSTX tool (Behrens

et al., 2003). A spatial probability density function was then estimated across voxels based on these local probability density functions using FSL's PROB-TRACKX tool (Behrens et al., 2003), in which 5000 samples were taken for each input voxel with a .2 curvature threshold, .5-mm step length, and 2000 steps per sample. For each tract, seed masks, waypoints, termination and exclusion masks were defined on the MNI152 T1 1-mm template (see (Peters et al., 2014a) and appendix B below for more details). Masks were normalized to each subject's diffusion space using FSL's Linear Registration Tool (Jenkinson and Smith, 2001), applying the affine parameters obtained by co-registering the first b0 volume to the MNI152 T1 1-mm template using trilinear interpolation. The resulting tracts were thresholded at a normalized probability value, which had been determined iteratively by visual inspection in an independent test sample and then confirmed in a larger sample to produce successful tracings in each individual (Peters et al., 2014a) (see Fig. 1). Mean FA, RD and AD of each tract were then extracted for analysis. FA was chosen as the primary DTI measure, and RD and AD as secondary measures to further explore the microstructural features underlying FA findings.

Analysis of erythrocyte fatty acid concentrations

Whole blood was collected into EDTA-coated BD Vacutainer tubes and centrifuged for 20 min (1900×g, at 4 °C). Plasma and the platelet rich interface were removed, and the erythrocytes were washed three times with 0.9% saline and then stored at –80 °C until analysis. Fatty acids in erythrocytes were analyzed by capillary gas chromatography as their methyl esters, as described previously (Peters et al., 2009). Fatty acid concentrations were expressed as nmol/μmol Hb. All samples were processed in a blinded manner.

Analysis of *in*PLA₂ activity

Plasma *in*PLA₂ activity was measured using a continuous kinetic fluorometric assay. We used the commercially available fluorescent substrate PED6 (Cat. No. D23739 In-Vitrogen, Carlsbad, CA, USA). PED6 incorporates a BODIPY® FL dye-labeled sn-2 acyl chain and a dinitrophenyl quencher group. Cleavage of the dye-labeled acyl chain by *in*PLA₂ eliminates the intramolecular quenching effect of the dinitrophenyl group, resulting in a corresponding increase in fluorescence (Hendrickson et al., 1999). Thus, the measured fluorescence intensity kinetics are directly linked to *in*PLA₂ activity. The fluorescent reaction product has its maximum absorption at 505 nm, and its maximum emission at 515 nm. Fluorescence was measured using a microplate reader (FLUOstar Omega, BMG LABTECH GmbH, Offenburg, Germany), equipped to pipette and to dispense reagents automatically. We used a filter combination of Ex 485 nm/Em 520 nm.

To provide the quantitation of intracellular PLA₂, all measurements were conducted in a calcium-depleted environment, established by adding ethylene glycol tetra acetic acid (EGTA) to the reactions. For calibration we used a standard dilution series of bee venom PLA₂ (SIGMA 29279-1MG). The wells of a 96-well microplate were filled with HEPES buffer, then 5 μl of plasma or the respective standard solution was added. After recording baseline values, 5 μl of PED6 solution (dissolved in dimethyl sulfoxide to obtain a 200 mM stock solution) was added via the reagent dispenser. The total measurement time was 70 s per well. For calculating *in*PLA₂ activities, the ascent of the curve and time interval after

adding PED6 (slope/min) and calibration curves were used. The resulting enzyme activity was normalized to the total protein concentration of the respective plasma sample. This yields specific activity in (pmol/min)/mg protein.

Statistical analysis

We first composed global WM tract measures by averaging each DTI value across all nine WM tracts, because a previous study indicated a global effect rather than a tract-specific effect of LC-PUFAs on white matter microstructure (Peters et al., 2014b). Specifically, we had observed a global effect of FADS haplotype (which is strongly associated with LC-PUFA concentrations in blood) on brain white matter microstructure from childhood into adulthood (Peters et al., 2014b), using the same tractography procedure as the present study. Similarly, and in accordance with prior analyses (Peters et al., 2009, 2013), individual concentrations of polyunsaturated and monosaturated fatty acids (i.e., omega-3 [C18:3n3, C20:5n3 (=eicosapentaenoic acid, EPA), C22:5n3 (=docosapentaenoic acid, DPA), C22:6n3 (=docosahexaenoic acid, DHA)], omega-5 [C14:1n5], omega-6 [C18:2n6, C18:3n6, C20:2n6, C20:3n6, C20:4n6 (=arachidonic acid, AA), C22:4n6, C22:5n6], omega-7 [C16:1n7, C18:1n7], and omega-9 [C16:1n9, C18:1n9, C20:3n9, C24:1n9 (=nervonic acid, NA)]) were added to compose a global measure of total long-chain unsaturated fatty acid (LC-UFA) concentration. Significant associations with the global measures were followed by in-depth analyses of individual WM tracts and individual LC-UFAs of interest (i.e. AA, DPA, EPA, DHA, and NA). Exploratory analyses were also performed for associations among the individual WM tracts and individual LC-UFAs that were not identified due to the global measure thresholds. DTI measures can have non-normal distributions, therefore, we tested whether our DTI data met the assumption of a normal distribution for parametric testing using the Kolmogorov–Smirnov test.

Linear and nonlinear associations were tested with Pearson's or partial correlations and quadratic regression, respectively, in the Statistical Package for the Social Sciences, version 11.5.1 (IBM, Armonk, New York; <http://www.spss.com> – last accessed 15-08-2016). The predictor variables for quadratic regressions were first demeaned to reduce collinearity effects. *p*-Values of <0.05, two-tailed, were considered statistically significant. Bonferroni correction was applied to exploratory associations (i.e. not surviving the global measure thresholds): total LC-UFA concentration or *inPLA*₂ activity with individual WM tract values (significance threshold $p = 0.050/9$ WM tracts = 0.006); individual LC-UFA concentrations with average WM tract values (significance threshold $p = 0.050/5$ LC-UFAs = 0.010); individual LC-UFAs with individual WM tracts (significance threshold $p = 0.050/(5 * 9) = 0.001$).

Because sex differences in white matter maturation have been described (Simmonds et al., 2014), we explored sex effects on significant results by re-analyzing them separately for males and females.

RESULTS

Thirty healthy individuals (63% male; 60% Caucasian) between the ages of 9 and 20 years (14.8 ± 2.9) were included. Mean full scale IQ was 109 ± 11 . For handedness, median laterality quotient was 0.8 (-0.7 to 1) (Table 1).

Tractography of the right SLF failed in one subject, therefore this subject was not included in the analyses of the global WM tract measures. Inspection of the distributions of LC-UFA concentrations revealed one subject with values of ≥ 3 SD from the mean for total LC-UFAs and AA. Analyses were performed without these outliers.

All Kolmogorov–Smirnov tests were nonsignificant for FA, RD and AD of each white matter tract ($p(\text{all}) > 0.300$), indicating that the distributions were statistically normal.

Age did not show significant associations with total LC-UFA concentration, any of the individual LC-UFA concentrations, or *in*PLA₂ activity ($p(\text{all}) > 0.050$), but did show significant linear correlations with FA of the cingulum and IFOF ($p(\text{all}) < 0.050$). Therefore, analyses with WM tracts were conducted adjusting for age.

Associations between *in*PLA₂ activity and WM tract microstructure

Plasma *in*PLA₂ activity did not show linear correlations with average WM tract FA, RD or AD ($p(\text{all}) > 0.100$). Plasma *in*PLA₂ activity did not show significant linear correlations with individual WM tract FA, RD or AD values ($p(\text{all}) > 0.050$, Bonferroni corrected).

Plasma *in*PLA₂ activity showed a significant U-curved quadratic association with average WM tract RD ($\beta = 0.413$, $p = 0.036$) (Fig. 2A). Plasma *in*PLA₂ activity also showed a trend-level quadratic association with average WM tract FA following an inverted U-curve ($\beta = 0.342$, $p = 0.076$) (Fig. 2B). Plasma *in*PLA₂ activity did not show significant quadratic association with average WM tract AD ($\beta = 0.223$, $p = 0.302$).

Among the individual WM tracts, plasma *in*PLA₂ activity showed significant quadratic association with RD of the genu of CC ($\beta = 0.682$, $p < 0.001$), but not RD of the other WM tracts ($p(\text{all}) > 0.100$). Plasma *in*PLA₂ activity showed significant quadratic association with FA of the genu of CC ($\beta = -0.631$, $p = 0.001$), but not FA of the other WM tracts ($p(\text{all}) > 0.100$). Plasma *in*PLA₂ activity did not show significant association with AD of the individual WM tracts ($p(\text{all}) > 0.100$, except for the genu of CC $p = 0.072$).

Associations between erythrocyte LC-UFA concentrations and WM tract microstructure

Total LC-UFA concentration did not show significant linear correlation with average WM tract FA ($r = -0.071$, $p = 0.724$).

Among the individual WM tracts, total LC-UFA concentration did not show significant linear correlation with individual WM tract FA values ($p(\text{all}) > 0.100$, Bonferroni corrected). Among the individual LC-UFAs, none of the concentrations showed significant linear correlation with average WM tract FA ($p(\text{all}) > 0.100$, Bonferroni corrected). Further exploratory analyses among *each* of the five LC-UFAs and nine FA values of the individual WM tracts, showed no significant correlations ($p(\text{all}) > 0.050$, Bonferroni corrected).

Analyses were repeated for WM tract RD and AD. Total LC-UFA concentration did not show significant linear correlation with average WM tract RD ($r = 0.068$, $p = 0.668$) or AD ($r = 0.124$, $p = 0.538$).

Among the individual WM tracts, total LC-UFA concentration did not show significant linear correlation with RD or AD of the nine WM tracts ($p(\text{all}) > 0.100$, Bonferroni corrected). Among the individual LC-UFAs, none of the concentrations showed significant linear correlation with average WM tract RD or AD ($p(\text{all}) > 0.100$, Bonferroni corrected). Further exploratory analyses among each of the five LC-UFAs and nine individual WM tracts, showed a significant positive linear correlation between DHA concentration and AD of the splenium of CC ($r = 0.626$, $p < 0.050$, Bonferroni corrected) and a nonsignificant positive linear correlation between DHA and RD of the splenium of CC ($r = 0.477$, $p > 0.100$, Bonferroni corrected). Correlations between DHA and the other eight WM tracts and between the other four LC-UFAs and nine WM tracts were nonsignificant ($p(\text{all}) > 0.050$, Bonferroni corrected).

Quadratic regressions between total or individual LC- UFA concentrations and average or individual WM tract FA/RD/AD values, respectively, were not significant ($p(\text{all}) > 0.050$, Bonferroni corrected), with the exception of a significant U-curved association between total LC- UFA concentration and AD of the ILF ($\beta = 0.592$, $p < 0.050$, Bonferroni corrected).

Sex differences

The quadratic association between *inPLA*₂ activity and average WM tract RD was significant for males ($\beta = 0.654$, $p = 0.018$), but not for females ($\beta = 0.035$, $p = 0.899$). Among the individual WM tracts, the quadratic association between *inPLA*₂ activity and RD of the genu of CC was significant for males ($\beta = 0.793$, $p = 0.002$), but not for females ($\beta = 0.388$, $p = 0.277$). The quadratic association between *inPLA*₂ activity and FA of the genu was significant for males ($\beta = -0.779$, $p = 0.004$), and trend-level significant for females ($\beta = -0.576$, $p = 0.061$).

The positive linear correlation between DHA concentration and AD of the splenium of CC was significant for males ($r = 0.725$, $p < 0.001$), but not for females ($r = 0.539$, $p = 0.108$). The quadratic association between total LC-UFA concentration and AD of the ILF was significant for males ($\beta = 0.709$, $p = 0.002$), but not for females ($\beta = 0.080$, $p = 0.876$).

Associations between *inPLA*₂ activity and LC-UFA concentrations

Plasma *inPLA*₂ activity did not show significant linear or quadratic association with total LC-UFA concentration ($p > 0.100$). There were no significant linear or quadratic associations between *inPLA*₂ activity and AA, EPA, DPA, DHA or NA concentrations ($p(\text{all}) > 0.100$).

Associations between dietary DHA and EPA intake and LC-UFA concentrations

Estimated dietary EPA intake correlated significantly with erythrocyte EPA concentration ($r = 0.389$, $p = 0.037$) and DHA concentration ($r = 0.401$, $p = 0.031$). Estimated dietary DHA

intake correlated significantly with erythrocyte EPA concentration ($r = 0.401$, $p = 0.031$) and DHA concentration ($r = 0.386$, $p = 0.038$).

DISCUSSION

In the present study, we observed significant curvilinear associations between plasma *in*PLA₂ activity and brain WM tract microstructure in healthy youth. Specifically, U-curved associations with WM tract RD were paralleled by inverted U-curved associations with WM tract FA. This pattern may suggest that both lower and higher *in*PLA₂ activity is associated with lower degree of WM myelination, while intermediate *in*PLA₂ activity is associated with the highest degree of WM myelination (Song et al., 2002). These associations between *in*PLA₂ activity and DTI measures of WM tract myelination are, to our knowledge, novel observations. It is relevant to note here that PLA₂ activity in serum and CSF are closely correlated (Smesny et al., 2008; Talib et al., 2013). These curvilinear relationships support the notion that there might be an optimum of physiological *in*PLA₂ activity and membrane LC-UFA turnover associated with healthy WM myelination (Smesny et al., 2014), while excessive *in*PLA₂ activity may reduce WM myelination. This is in line with findings of increased *in*PLA₂ activity being associated with structural brain WM abnormalities in patients with schizophrenia including first-episode patients (Smesny et al., 2010).

Overall, we found little association between erythrocyte membrane LC-UFA concentrations and brain WM tract microstructure. This suggests that the observed associations between *in*PLA₂ activity and WM microstructure do not reflect a direct link to erythrocyte membrane LC-UFA biostatus, but may be mediated by individual LC-UFAs that occur at the sn-2 position of membrane phospholipids and are enriched in phospholipids of myelin. This is supported by our exploratory analysis of individual LC-UFAs indicating a positive linear association between DHA concentration and WM tract AD (and to a lesser extent RD) of the splenium of CC. Because higher overall diffusivity may suggest lower degree of myelination (Song et al., 2002), this positive correlation between erythrocyte DHA and WM tract diffusivity may suggest that mobilization of DHA from the sn-2 position of membrane phospholipids by *in*PLA₂ is necessary for healthy WM myelination. We also observed a significant U-curved association between total LC-UFA concentration and AD of the ILF, which requires replication in an independent sample.

The relative lack of correlations between erythrocyte LC-UFA concentrations and DTI indices of WM tract myelination may have several explanations. First, lack of correlation may arise from simultaneous age-related changes in LC-UFA levels and WM myelination in adolescence (Carver et al., 2001; Jakobik et al., 2009). Both DHA levels and DTI indices of myelination are found to increase during this age period (Carver et al., 2001; Jakobik et al., 2009). However, in our sample, no correlations were observed between age and LC-UFA concentrations, and analyses were adjusted for age. Second, it is feasible that indicators of LC-UFA biosynthesis better reflect cerebral LC-UFA biostatus, compared to erythrocyte LC-UFA composition. This notion is supported by our prior DTI study demonstrating that haplotype of the FADS gene cluster, which regulates LC-PUFA biosynthesis, is associated with brain WM development in adolescence (Peters et al., 2014b). A prior postmortem study indicated that FADS haplotype affected PUFA composition of prefrontal cortex, but likely

not through local synthesis, because haplotype affected cortical PUFA levels but not cortical FADS gene expression or transcript quantity (Freemantle et al., 2012). The authors concluded that LC-PUFA composition of the brain more likely depends on uptake from peripheral circulation due to liver synthesis levels (controlled by the FADS gene cluster) (Freemantle et al., 2012). This concurs with studies that showed that brain membrane fatty acids are replaced on a daily basis from plasma (Chen et al., 2008; Rapoport, 2008). Thus, biological measures that reflect peripheral LC-PUFA biosynthesis and membrane LC-PUFA incorporation and turnover may be most informative during healthy development, compared to erythrocyte membrane concentration. This is consistent with postmortem observations that erythrocyte and cerebral cortex LC- PUFA concentrations seem to be poorly correlated during adolescence (Carver et al., 2001). In addition to this, dietary intake of preformed LC-PUFAs (i.e., EPA, DHA, AA), which bypasses FADS, may also affect cerebral LC- PUFA biostatus.

We found some indications for sex differences. The relationships between *in*PLA₂ activity, LC-UFA concentrations and WM microstructure were significant for males, but not for females, which suggests that LC- UFA homeostasis is implicated in WM development in boys only. Such a sex-specific effect may be explained by an interaction of LC-UFA homeostasis with sex hormones. Indeed, sex hormones have been implicated in healthy WM development. For instance, one study found that healthy WM maturation paralleled pubertal changes and differed between males and females (Asato et al., 2010); another study found significant relationships between bioavailable testosterone and WM volume in healthy male adolescents, but not female adolescents (Perrin et al., 2008). Taken together, these findings could suggest that LC-UFA homeostasis and sex hormones act in concert to drive WM development in male adolescence. The sex effects in the present study, however, must be considered tentative due to our small sample of females.

Our findings in healthy adolescents contrast with findings in first-episode schizophrenia patients, in whom we found positive correlations between erythrocyte LC- UFA concentrations and DTI indices of WM myelination (Peters et al., 2009, 2013). This contrast may be due to specific pathogenic mechanisms in schizophrenia underlying relationships between peripheral UFAs and WM microstructure. In schizophrenia, LC-PUFA deficiencies are well-established (Hoen et al., 2012) and generalized LC-PUFA depletion may lead to concurrent low erythrocyte LC-PUFA as well as low cerebral LC-PUFA concentrations and consequently reduced WM myelination. Alternatively, inflammation and oxidative stress have been implicated in the pathophysiology of schizophrenia and both these factors may lead to concurrently reduced erythrocyte and cerebral membrane LC-PUFA levels as well as reduced WM integrity (Khan et al., 2002; Arvindakshan et al., 2003). Nevertheless, the observed association between DHA and AD of the splenium of CC in our data may suggest that DHA deficiencies cause white matter abnormalities in schizophrenia (Peters and Karlsgodt, 2015) by disrupting cleavage of membrane-bound DHA by *in*PLA₂, and thereby compromising the physiological process of myelin membrane remodeling and repair by *in*PLA₂. Such an effect could have therapeutic implications, e.g. treatment of white matter abnormalities in schizophrenia through DHA or EPA supplementation. This notion is corroborated by DTI data demonstrating that fish oil (including DHA and EPA) supplementation increases brain white matter FA in depressed patients while decreasing

symptoms (Chhetry et al., 2016). In addition to the above, effects of medication and substance use may play a role in the contrasting results between patients and healthy youth.

Our study has some limitations. We did not directly measure LC-UFA composition and myelin content of the brain WM. Phosphorus magnetic resonance spectroscopy could provide more direct insight into phospholipid metabolism in the brain WM (Richardson et al., 2001; Yao et al., 2002). DTI may be combined with different MRI modalities to more specifically measure WM myelin content, e.g. quantitative T₁ imaging (Stuber et al., 2014). Another limitation is the relatively small sample, which may not be representative of all adolescents.

CONCLUSION

Our findings provide further insight into the role of membrane lipid metabolism in healthy brain WM development during late childhood and adolescence. Our data suggest that there are optimal physiological *inPLA*₂ activity levels associated with healthy WM myelination during these age periods. Myelination may be mediated by cleavage of docosahexaenoic acid from membrane phospholipids by *inPLA*₂. In light of the evidence for elevated *inPLA*₂ activity in schizophrenia, these findings have implications for our understanding of the role of LC-PUFA homeostasis in myelin-related neurodevelopmental disorders.

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R.K. McNamara co-designed the study, advised on the fatty acid analysis and interpreted the results, and co-wrote the manuscript. P.R. Szeszko co-managed the imaging data-collection and – analysis, and co-wrote the manuscript. S. Smesny managed and supervised the PLA₂ analysis, interpreted the results and co-wrote the manuscript. T. Ikuta co-managed and co-analyzed the imaging data. P. DeRosse co-designed the study, co-wrote the protocol and advised on the statistical analysis. F.M. Vaz managed and supervised the fatty acid analysis. B. Milleit, U.C. Hipler, C. Wiegand, and J. Hesse developed the *inPLA*₂ assay and performed the PLA₂ analyses. G. Paul Amminger advised on interpretation of the PLA₂ and fatty acid results. A.K. Malhotra co-designed the study, co-wrote the protocol, and co-managed the overall execution of the study. B.D. Peters designed the study and co-wrote the protocol, supervised the data-collection and - processing, managed and performed the literature searches and analyses, and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

APPENDIX A

Classification of intracellular calcium-independent PLA₂ (*inPLA*₂)

The superfamily of phospholipase A₂ enzymes is expanding rapidly. A first conclusive classification of PLA₂s was proposed by Dennis et al. (Dennis, 1994). Adding several more

recently described PLA₂s, Balsinde et al. (Balsinde et al., 1999) put forward an updated classification based on the structural comparison of PLA₂ enzymes. For clinical research purposes two dyadic classifications are still popular: cytosolic versus secretory PLA₂s and calcium-dependent versus calcium-independent PLA₂s. Secretory PLA₂s (sPLA₂s) require calcium for their activities at millimolar levels, cytosolic PLA₂s (cPLA₂s) are sensitive to micromolar levels of calcium and “independent” PLA₂s (iPLA₂s) are completely independent of calcium for their activities (reviewed by Taketo and Masahiro, 2002). Integrating the moleculargenetical and the dyadic classifications the here investigated *in*PLA₂ activity includes group IVC, VIA and B characterized by high molecular weight. Group IV and VI indicate cytosolic and calcium-independent subgroups while A, B and C relate to the diverse isoenzymes in each of these subgroups.

Analysis of *in*PLA₂-activity

In this study plasma *in*PLA₂ activity was measured using a continuous kinetic fluorometric assay, as described in the Experimental Procedures. The *in*PLA₂ activity investigated here includes the activity of different PLA₂ isoenzymes: cytosolic PLA₂ (cPLA₂, requiring micromolar doses of calcium), and intracellular calcium-independent PLA₂ (iPLA₂, completely calcium-independent), both summarized as intracellular PLA₂ (*in*PLA₂).

In order to measure intracellular calcium-independent PLA₂ (*in*PLA₂) exclusively, all measurements were conducted in a calcium-depleted environment, as described in the Experimental Procedures.

*in*PLA₂ activation pathways

Some of the known *in*PLA₂ activation pathways are: (i) receptors activated by cytokines (e.g., TNF α , IL 1 α) (Bickford et al., 2012), growth factors, and hormones (Brenner, 1982); (ii) via G-protein (guanosine triphosphate binding proteins) coupled processes related to dopaminergic, muscarinic, NMDA- and AMPA receptors (Gilman, 1987; Clapham and Neer, 1997); (iii) via free PUFA availability, and (iv) via protein kinase C α - and C ϵ -mediated phosphorylation of the enzyme (Sugita et al., 2010; Zhao et al., 2012). In the case of membrane damage, the activity of cPLA₂ and iPLA₂ (as *in*PLA₂ sum activity) in turn is increased by interaction with extracellular secretory non-pancreatic, and calcium-dependent PLA₂ isoenzymes (Garcia and Kim, 1997; Balsinde et al., 1998; Kuwata et al., 2000; Kurrasch-Orbaugh et al., 2003).

Once activated, intracellular PLA₂ isoenzymes move to the inner phospholipid layer of the membrane (in the CNS containing mainly PUFAs bound to phosphatidylethanolamine) and neutralize oxidatively damaged PUFAs at the sn-2 position of the glycerophospholipids, generating lysophospholipids, free damaged PUFAs and reactive oxygen species. Thus, PLA₂ isoenzymes act within a complex interrelated system that is highly sensitive to calcium. Different cytosolic PLA₂ isoenzymes interact to potentiate membrane repair/remodeling or breakdown processes (Balsinde et al., 1999; Law et al., 2006).

APPENDIX B

Placement of seed masks, way-points, termination and exclusion masks for probabilistic tractography

Genu of Corpus Callosum

A seed mask of the midsagittal section of the genu was derived from the ICBM-DTI-81 WM atlas, provided in FSL. An exclusion mask was drawn posterior to the seed mask, excluding fibers tracing posterior to the seed. The resulting tracts of each subject were thresholded at a normalized probability value of 0.005.

Splenium of Corpus Callosum

A seed mask of the midsagittal section of the splenium was derived from the ICBM-DTI-81 WM atlas, provided in FSL. An exclusion mask was placed anterior to the seed mask, excluding fibers tracing anterior to the seed. The resulting tracts of each subject were thresholded at a normalized probability value of 0.005.

Corticospinal Tract

A seed mask of precentral gyrus WM was derived from the Harvard Oxford subcortical atlas, provided in FSL, and a second seed mask was manually drawn in the pons. The resulting tracts of each subject were thresholded at a normalized probability value of 0.01.

Anterior Thalamic Radiation

A seed mask of the thalamus was derived from the HarvardOxford subcortical atlas, provided in FSL, and then manually edited according to the MNI152 T1 brain to exclude the medial and lateral geniculate nuclei. A way-point of prefrontal WM was derived from the HarvardOxford atlas, provided in FSL, which was also used as a termination mask (i.e. fibers were terminated when they reached prefrontal WM). A second way-point was manually drawn in the anterior limb of the internal capsule (ALIC), on 3 coronal slices in the anterior section of the ALIC. An exclusion mask of occipital, temporal, parietal, and sensory-motor (including supplementary motor) gray matter (GM) was derived from the HarvardOxford cortical atlas, provided in FSL. This exclusion mask was manually expanded to exclude fibers tracing into the brainstem or the contra-lateral hemisphere. The resulting tracts of each subject were thresholded at a normalized probability value of 0.005.

Inferior Longitudinal Fasciculus (ILF)

Occipital and temporal seed masks were derived from the HarvardOxford atlas, provided in FSL, based on the trajectory of the ILF in the Johns Hopkins University (JHU) tractography atlas, provided in FSL. The occipital seed mask comprised the occipital fusiform WM, occipital pole WM, temporal occipital fusiform WM, cuneus WM, inferior lateral occipital WM, lingual WM, and intracalcarine WM. The temporal seed mask comprised the temporal pole WM, anterior temporal fusiform WM, and anterior middle temporal WM. An exclusion mask was drawn anterior to the temporal pole. The resulting tracts of each subject were thresholded at a normalized probability value of 0.01.

Inferior Fronto-Occipital Fasciculus (IFOF)

Frontal and occipital seed masks were derived from the HarvardOxford atlas, provided in FSL, based on the trajectory of the IFOF in the JHU tractography atlas, provided in FSL. The frontal seed comprised the frontal pole WM, inferior frontal gyrus (pars triangularis) WM, medial frontal WM, and orbito-frontal WM; the superior section of the frontal pole was removed as it is not included in the main trajectory of the IFOF. The occipital seed mask comprised the inferior division of the lateral occipital WM, supracalcarine WM, fusiform occipital WM, and lingual WM. A way-point was manually drawn in the anterior section of the temporal stem, including the external capsule and WM medial to the insular cortex, according to the trajectory of the IFOF in the JHU tractography atlas, provided in FSL. An exclusion mask was drawn in each contra-lateral hemisphere. The GM of the seed regions was also defined as a termination mask. The resulting tracts of each subject were thresholded at a normalized probability value of 0.01.

Superior Longitudinal Fasciculus (SLF)

A seed mask was manually drawn in the frontal part of the SLF (just anterior to the precentral gyrus) based on the trajectory of the SLF in the JHU tractography atlas, provided in FSL. A way-point was manually drawn in the frontal section of the SLF just posterior to the seed mask, and a second way-point comprising WM of the middle temporal gyrus (MTG) was derived from the HarvardOxford atlas, provided in FSL. A termination mask was drawn anterior to the seed mask and inferior to the MTG WM, and combined with MTG GM as derived from the HarvardOxford atlas, provided in FSL. The resulting tracts of each subject were thresholded at a normalized probability value of 0.01.

Cingulum

A seed mask of the anterior cingulum was manually drawn on 3 coronal slices just anterior to the genu of CC. A second seed mask was manually drawn on three coronal slices just posterior to the splenium of the CC. An exclusion mask was drawn anterior to the cingulate cortex, inferior to the level of the hippocampus and in each contra-lateral hemisphere. The resulting tracts of each subject were thresholded at a normalized probability value of 0.05.

Uncinate Fasciculus (UF)

Frontal and temporal seed masks were derived from the HarvardOxford atlas, provided in FSL, based on the trajectory of the UF in the JHU tractography atlas, provided in FSL. The frontal seed mask comprised the frontal pole WM, frontal medial WM, subcallosal WM, and orbito-frontal WM; only WM below the level of the anterior cingulate cortex was included. The temporal seed mask comprised the temporal pole WM. An exclusion mask was drawn posterior to the planum polare. The resulting tracts of each subject were thresholded at a normalized probability value of 0.05.

Abbreviations

AD	axial diffusivity
CC	corpus callosum

DTI	diffusion tensor imaging
FA	fractional anisotropy
FADS	fatty acid desaturase
IFOF	inferior fronto-occipital fasciculus
ILF	inferior longitudinal fasciculus
LC-PUFAs	long-chain polyunsaturated fatty acids
RD	radial diffusivity
SLF	superior longitudinal fasciculus
WM	white matter

Phospholipase A₂

enzymes that catalyze the cleavage of fatty acids from the second carbon group of glycerol; this particular phospholipase specifically recognizes the sn-2 acyl bond of phospholipids

Unsaturated fatty acid (UFA)

lipid in which the constituent hydrocarbon chain possesses one or more ('poly') carbon-carbon double bonds; "unsaturated" refers to the fact that the molecules contain less than the maximum amount of hydrogen

Diffusion tensor imaging

magnetic resonance imaging technique that enables the measurement of restricted diffusion of water in tissue, which can be used to trace neural tracts and measures the diffusion properties along these tracts

Fractional anisotropy (FA)

a scalar value between zero and one that describes the degree of anisotropy of the diffusion of water molecules (by Brownian motion) along the neural tracts; a value of zero means that diffusion is isotropic, i.e. it is unrestricted (or equally restricted) in all directions; a value of one means that diffusion occurs only along one axis and is fully restricted along all other directions

Axial diffusivity (AD)

the diffusivity of the water molecules along the principal axis of the ellipsoid shape that is created by the water diffusion in an anisotropic medium, such as brain white matter

Radial diffusivity (RD)

the diffusivities of the water molecules in the two minor axes of the ellipsoid shape that is created by the water diffusion in an anisotropic medium

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Fig. 1. Representative examples of the nine studied white matter tracts. Top panel: red = superior longitudinal fasciculus; green = anterior thalamic radiation; yellow = inferior fronto-occipital fasciculus; blue = inferior longitudinal fasciculus. Bottom panel: red = cingulum; green = uncinate fasciculus; yellow = genu (left) and splenium (right) of corpus callosum; blue = corticospinal tract. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Quadratic relationships between plasma phospholipase A₂ activity and average white matter tract radial diffusivity (panel A) or fractional anisotropy (panel B) in healthy youth ($n = 29$).

Table 1Subject characteristics ($n = 30$)

Age, mean $\pm$ SD	14.8 $\pm$ 2.9
Sex, %	
Male	63
Race, %	
Caucasian	60
IQ, mean $\pm$ S.D. ^a	109 $\pm$ 11
Handedness, median (range) ^b	0.8 (−0.7 to 1)
SES, mean $\pm$ S.D. ^c	2.04 $\pm$ 0.79

^aIntelligence quotient was measured using the Wechsler Abbreviated Scale of Intelligence.

^bLaterality quotient was determined using the Edinburgh Handedness Inventory; quotient of 1 indicates complete right-handedness, quotient of −1 indicates complete left-handedness.

^cSES = Social Economic Status, as determined using the Hollingshead Two Factor Index of Social Position; for minors, this was based on the head of household. SES was scored as I, II, III, IV, or V, where a lower score indicates higher SES ($n = 25$, data missing for five subjects).