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Dietary omega-3 deficiency exacerbates inflammation and reveals spatial memory deficits in mice exposed to lipopolysaccharide during gestation

V.F. Labrousse^{1,2*}, Q. Leyrolle^{1,2,3*}, C. Amadiou^{1,2}, A. Aubert^{1,2}, A. Sere^{1,2}, E. Coutureau⁴, S. Grégoire⁵, L. Bretillon⁵, V. Pallet, P^{1,2}, Gressens^{3,6}, C. Joffre^{1,2}, A. Nadjar^{1,2£}, S. Layé^{1,2£}

¹INRA, Nutrition et Neurobiologie Intégrée, UMR 1286, 33076 Bordeaux, France

²Univ. Bordeaux, Nutrition et Neurobiologie Intégrée, UMR 1286, 33076 Bordeaux, France

³PROTECT, INSERM, Université Paris Diderot, Sorbonne Paris Cité, F-75019 Paris, France

⁴Centre National de la Recherche Scientifique, Institut de Neurosciences Cognitives et Intégratives d'Aquitaine, Unité Mixte de Recherche 5287, 33076 Bordeaux, France, Université de Bordeaux, Institut de Neurosciences Cognitives et Intégratives d'Aquitaine, 33076 Bordeaux, France.

⁵Centre des Sciences du Goût et de l'Alimentation, AgroSup Dijon, CNRS, INRA, Université Bourgogne Franche-Comté, Dijon, France.

⁶Centre for the Developing Brain, Department of Division of Imaging Sciences and Biomedical Engineering, King's College London, King's Health Partners, St. Thomas' Hospital, London, SE1 7EH, United Kingdom

*Co-first authors

£ Co-senior and corresponding authors

sophie.laye@inra.fr; agnes.nadjar@u-bordeaux.fr

Key words: omega-3; PUFA; MIA; LPS; microglia; cytokines; memory

Abstract

Maternal immune activation (MIA) is a common environmental insult on the developing brain and represents a risk factor for neurodevelopmental disorders. Animal models of *in utero* inflammation further revealed a causal link between maternal inflammatory activation during pregnancy and behavioural impairment relevant to neurodevelopmental disorders in the offspring. Accumulating evidence point out that proinflammatory cytokines produced both in the maternal and fetal compartments are responsible for social, cognitive and emotional behavioral deficits in the offspring.

Polyunsaturated fatty acids (PUFAs) are essential fatty acids with potent immunomodulatory activities. PUFAs and their bioactive derivatives can promote or inhibit many aspects of the immune and inflammatory response. PUFAs of the n-3 series ('n-3 PUFAs', also known as omega-3) exhibit anti-inflammatory/pro-resolution properties and promote immune functions, while PUFAs of the n-6 series ('n-6 PUFAs' or omega-6) favor pro-inflammatory responses. The present study aimed at providing insight into the effects of n-3 PUFAs on the consequences of MIA on brain development. We hypothesized that a reduction in n-3 PUFAs exacerbates both maternal and fetal inflammatory responses to MIA and later-life defects in memory in the offspring.

Based on a lipopolysaccharide (LPS) model of MIA (LPS injection at embryonic day 17), we showed that n-3 PUFA deficiency 1) alters fatty acid composition of the fetal and adult offspring brain; 2) exacerbates maternal and fetal inflammatory processes with no significant alteration of microglia phenotype, and 3) induces spatial memory deficits in the adult offspring. We also showed a strong negative correlation between brain content in n-3 PUFA and cytokine production in MIA-exposed fetuses. Overall, our study is the first to address the deleterious effects of n-3 PUFA deficiency on brain lipid composition, inflammation and

memory performances in MIA-exposed animals and indicates that it should be considered as a potent environmental risk factor for the apparition of neurodevelopmental disorders.

Introduction

Maternal immune activation (MIA), occurring in a context of bacterial or viral infection, is a common environmental insult on the developing brain and represents a risk factor for neurodevelopmental disorders such as schizophrenia, autism or cerebral palsy (Bilbo et al., 2018; Estes and McAllister, 2016; Fleiss and Gressens, 2012; Hagberg et al., 2015; Knuesel et al., 2014; Madore et al., 2016; Patterson, 2011, 2009; Van Steenwinckel et al., 2014). MIA has recently been associated to altered connectivity in the prefrontal cortex, temporo-parietal junction, and basal ganglia of neonates and toddlers, further linking prenatal inflammation to psychiatric risk in humans (Spann et al., 2018).

Animal models of *in utero* inflammation (triggered by viral polyinosinic:polycytidylic [poly(I:C)] or bacterial [lipopolysaccharide, LPS] mimics) further revealed a causal link between maternal inflammatory activation during pregnancy, disorganized brain cytoarchitecture and behavioural impairment relevant to neurodevelopmental disorders in the offspring (Bilbo and Schwarz, 2012; Choi et al., 2016; Deverman and Patterson, 2009; Estes and McAllister, 2016; Fernández de Cossío et al., 2017; Hui et al., 2018; Shin Yim et al., 2017). Accumulating evidence point out that proinflammatory cytokines produced both in the maternal and fetal compartments, are responsible for social, cognitive and emotional behavioral deficits in the offspring (Ashdown et al., 2006; Bilbo et al., 2018; Cai et al., 2000; Estes and McAllister, 2015; Golan et al., 2005; Hao et al., 2010; Liverman et al., 2006; Urakubo et al., 2001). Interleukin-1beta (IL-1 β), IL-6 and Tumor Necrosis Factor alpha (TNF α) disrupt brain structures connectivity involved in social, emotional and memory processes (Baharnoori et al., 2009; Cai et al., 2000; Fatemi et al., 2009; Giovanoli et al., 2015; Golan et al., 2005; Graciarena et al., 2010; Lowe et al., 2008; Samuelsson et al., 2006). More recently, IL-17 has been shown to mediate maternal poly-IC-induced social behavior impairment and abnormal cortical development in offspring (Choi et al., 2016; Shin Yim et

al., 2017). A particular role has been attributed to maternal and fetal IL-6 in offspring cognitive disorders in humans (Spann et al., 2018). Indeed, prenatal exposure to IL-6 recapitulates the deficits in hippocampal synaptic transmission and spatial learning that are classically observed in adult offspring in a context of MIA (Patterson, 2009; Samuelsson et al., 2006). These deficits can be reversed by treating the mothers with an anti-IL-6 antibody (Mouihate and Mehdawi, 2016). Hence, the production of IL-6 in the pregnant mother and in the fetal brain is critical to MIA-induced cognitive impairment in the offspring.

Polyunsaturated fatty acids (PUFAs) are essential fatty acids with potent immunomodulatory activities (Calder 2005). PUFAs and their bioactive derivatives can promote or inhibit many aspects of the immune and inflammatory response. Notably, PUFAs of the n-3 series ('n-3 PUFAs', also known as omega-3) exhibit anti-inflammatory/pro-resolutive properties and promote immune functions, while PUFAs of the n-6 series ('n-6 PUFAs' or omega-6) favor pro-inflammatory responses (Calder, 2006, 2001; Layé et al., 2018; Orr et al., 2013b). As vertebrates lack the necessary enzymes for *de novo* synthesis of n-6 and n-3 PUFAs, these fatty acids have to be provided by the diet (Bazinet and Layé, 2014). When increased by dietary or genetic approaches, we and others showed that n-3 PUFAs down-regulate the production of proinflammatory cytokines both at the periphery and in the brain, while n-6 PUFAs promote their synthesis and release (Delpech et al., 2015b, 2015a; Fourrier et al., 2017; Hopperton et al., 2017, 2016; Labrousse et al., 2012; Madore et al., 2014; Mingam et al., 2008; Orr et al., 2013a). The central anti-inflammatory effects of n-3 PUFAs are mainly mediated by docosahexaenoic acid (DHA, 22:6 n-3), the main long chain (LC) n-3 PUFA accumulating in the brain (Bazinet and Layé, 2014; Layé et al., 2018; Orr et al., 2013b). DHA targets microglia, the brain resident innate immune cells to dampen the production and action of the proinflammatory cytokines IL-6, IL-1 β and TNF α (De Smedt-Peyrusse et al., 2008; Fourrier et al., 2017; Inoue et al., 2017; Mancera et al., 2017; Nadjar et al., 2016; Tremblay et

al., 2016). Conversely, low level of DHA in the brain enhances the production of proinflammatory cytokines and affects microglia phenotype and function, with a polarization of these cells to a pro-inflammatory phenotype (Delpech et al., 2015b; Madore et al., 2014; McNamara et al., 2010; Nadjar et al., 2016).

The maternal dietary status in n-3 PUFAs is crucial for the offspring, as the embryo cannot produce its own DHA and therefore entirely depends on maternal supply (Gibson et al., 1996). DHA is transferred from the mother to the offspring during gestation (blood supply) and lactation (milk) (Hanebutt et al., 2008; Innis, 2005; Lewis et al., 2018). Preterm infants or infants from mothers with dietary deficit in n-3 PUFAs have limited n-3 PUFA stores in the body and brain (Larque et al., 2002; Makrides et al., 1994). This might enhance the immune response and aggravate the cognitive deficits associated with prenatal infection.

The present study aimed at providing insight into the effects of n-3 PUFA dietary intake during pregnancy and lactation on the consequences of MIA on brain development. We hypothesized that a reduction in maternal dietary n-3 PUFA exacerbates both maternal and fetal inflammatory responses to MIA and later-life defects in hippocampal connectivity and related memory behavior in the offspring. To this end, we evaluated developmental changes in offspring brain fatty acid composition as well as proinflammatory cytokine production in response to MIA (LPS at embryonic day 17) in a well-established dietary model of n-3 PUFA deficiency (Lafourcade et al., 2011; Madore et al., 2014; Mingam et al., 2008; Moranis et al., 2012). Long-term memory and hippocampal integrity were evaluated in the adult offspring. Our results revealed that maternal n-3 PUFA deficiency worsens the effects of prenatal LPS on memory performances and brain pro-inflammatory cytokines production in the offspring. We also showed that IL-6 production is strongly anti-correlated to brain DHA levels, emphasizing the crucial role of this fatty acid in the development of later life cognitive abilities.

MATERIALS AND METHODS

Animals

Animal husbandry and experimental procedures were in accordance with the EU Directive 2010/63/EU for animal experiments and approved by the national ethical committee for care and use of animals (approval ID A13169). Every effort was made to minimize suffering and the number of animals used. All experiments were made on C57BL6/J males and females (Charles River, Arbresle, France). Mice were maintained under standard housing conditions on corn cob litter in a temperature ($23\pm 1^{\circ}\text{C}$) and humidity (40-50%) controlled animal room with a 12h light/dark cycle (07h-19h) and *ad libitum* access to food and water.

Diet and treatment

N-6 and n-3 LC PUFAs can be biosynthesized from their dietary precursors, respectively linoleic acid (18:2n-6 or LA) and α -linolenic acid (18:3n-3 or ALA) (Lands et al., 1990). Female C57BL6/J mice were fed with isocaloric diets containing 5% fat with a high (n-3 deficient diet) or low LA/ALA ratio (n-3 balanced diet) across gestation and lactation to modulate n-3/n-6 PUFAs in the offspring (Delpech et al., 2015b; Madore et al., 2014; Mingam et al., 2008; Moranis et al., 2012). **When studied at adulthood, the male offspring was kept under the same diet as their dams after weaning, except for data presented in Figure 1B (reversal experiment in Y-maze) for which offspring were fed with n-3 PUFA balanced diet until weaning and then exposed to n-3 PUFA deficient diet until behavioral assessment.**

At G17 (17 days after mating), MIA was triggered by the intraperitoneal (i.p.) administration of LPS (*E. Coli* 0127:B8, Sigma Inc, St. Louis, MO, USA; $0.12\text{ }\mu\text{g/g mouse}/100\text{ }\mu\text{l}$). The

administration of the corresponding volume of saline solution (NaCl 0.9%, "Saline") was used as a control (Golan et al., 2005; Roumier et al., 2008).

We generated 4 cohorts of mice in total. Cohort 1 was used to assess memory performances in adults (57 males from 15 dams). In cohort 2, we quantified fatty acids levels and cytokine production in fetuses and dams (30 dams were used, fetuses from the same litter were pooled for $n=1$). Cohort 3 was used for FACS analysis (22 dams were used, fetuses from the same litter were pooled for $n=1$). Cohort 4 was used to assess cFos expression and measure fatty acid composition of adult livers and brainstems (40 males from 18 dams were used).

Assessment of memory performances

Memory tests always took place in the morning (between 8:00AM and 11:00AM). 3 months old mice were first handled for 5 min every day for two weeks before initiation of the experiments. All experiments were performed in a room adjacent to the vivarium with light intensity at 78 Lux.

Spatial recognition. The hippocampus-dependent spontaneous spatial recognition in the Y-maze was used in a two-trial procedure as previously described (Delpech et al., 2015b; Labrousse et al., 2009; Moranis et al., 2012). A Y-shaped maze was used, where each arm was 34 cm long, 8 cm wide and 14 cm high. The maze floor was covered with sawdust which was mixed between trials in order to remove olfactory cues. Extra-maze visual cues were placed around the testing room and kept constant during all tests. During the first trial (trial 1), one arm of the Y-maze was closed and mice were allowed to visit the two other arms for 5 min. After a 30-min inter-trial interval (ITI), mice were placed back in the starting arm with free access to all three arms for 5 min (trial 2). We quantified the time animals spent exploring the novel and familiar arms during the first 3 minutes of trial 2 (Labrousse et al., 2012).

Novel object recognition. This task was used as a hippocampus-independent task (Dere et al., 2007) and performed as previously described (Labrousse et al., 2009). Briefly, mice were acclimatized to a 40x40 cm cage made of white-coated plywood with 16 cm-high walls, 15 min per day for a week before training. As before, the maze floor was covered with sawdust which was mixed between trials in order to remove olfactory cues. During a first 10-min training period, two identical objects with a particular shape and color were presented to mice. After a 30-min ITI, one of the familiar objects was replaced by a novel object with a different shape and color, to test for memory retention. During the 5 min of testing, exploration of an object was defined as pointing the nose towards the object and/or touching the object with the nose.

Immunohistochemistry

cFos protein expression was assessed in the CA1 and dentate gyrus (DG) of the hippocampus of adult mice 60 min after the completion of the Y-maze task (trial 2), i.e. 90 min after acquisition (Labrousse et al., 2009). A decrease of this marker in the hippocampus is known to predict dampened neuronal activation and is associated with spatial memory impairments (Labrousse et al., 2012). Animals were deeply anesthetized with pentobarbital, intracardially perfused with Phosphate Buffer Saline (PBS, pH 7.4) for 5 min, followed by 10-min perfusion with 4% paraformaldehyde (PFA). Brains were removed, post-fixed in 4% PFA for 4h at 4°C, cryoprotected in 30% sucrose for 24h, quickly frozen in liquid azote, and stored at -80°C before sectioning. Free-floating 30µm coronal sections were collected throughout the hippocampus, using a cryostat. cFos immunohistochemistry was then performed as previously described (Labrousse et al., 2009). Briefly, rabbit polyclonal antiserum raised against cFos (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:1000 in Tris Buffer Saline (TBS) containing 0.3% Triton X-100, 2% donkey serum, 1% Bovine Serum Albumin (BSA), and

sections were incubated overnight at room temperature (RT), before incubation for 2 h with biotinylated donkey anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech Europe, Freiburg, Germany). Slices were then incubated for 2 h with avidin-biotin peroxidase complex (1:1000; Vectastain ABC kit, Vector laboratories, Burlingame, CA) and staining was revealed using diaminobenzidine and the nickel-enhanced glucose oxidase method (Shu et al., 1988). Sections were then mounted onto gelatine-coated slides. The procedure also included negative controls with omission of the primary antibody, which did not show any immunoreactivity (data not shown).

Brain sections were examined under a light microscope with a 10X objective (Nikon Eclipse E 400) and images were captured by a high-resolution digital Nikon DXM 1200 camera (Nikon Corporation, Champigny-sur-Marne, France). Camera aperture, magnification, light power, and exposure time were fixed for all images. ACT-1 software (Nikon Corporation, Champigny-sur-Marne, France)-generated images were stored. Image editing software (Adobe Photoshop, Adobe Systems, San Jose, CA) was used to adjust size, brightness, and contrast (same settings for all images). Quantification of cFos-immunoreactive cells was performed with NIH-imaging software Scion Image (Frederick, MD). The number of cFos positive cells was quantified from all consecutive sections containing the structure of interest, across both hemispheres.

Quantitative Real-Time PCR

Two μ g of total RNA were reverse transcribed with Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Cergy-Pontoise, France). Quantitative PCR was then performed using the Applied Biosystems Assay-on-Demand Gene Expression Products protocol as previously described (Madore et al., 2013; Rey et al., 2016). Briefly, TNF α , IL-1 β , IL-6, IL-10, IL-6 receptor (gp130), CD11b and housekeeping gene (β 2-microglobulin) cDNA were

amplified by PCR by using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ). Fluorescence was determined using an AB 7500 Real Time PCR system (Applied Biosystems, Foster city, CA) and final quantification was performed using the comparative threshold (Ct) method (Livak and Schmittgen, 2001). For each experimental sample, the difference between target and housekeeping gene Ct values (ΔCt) was used to normalize for difference in the amount of total nucleic acid added to each reaction and the efficiency of the RT step. Values were then expressed relative to the mean ΔCt value obtained for the balanced-saline group (calibrator ΔCt) by subtracting ΔCt for each experimental sample from calibrator ΔCt ($=\Delta\Delta\text{Ct}$). The amount of target gene (linear value) normalized to the housekeeping gene and relative to the calibrator was determined by $2^{-\Delta\Delta\text{Ct}}$ (relative fold change). Data are presented as mean relative fold change $\pm$ SEM.

Cytokine assays

Maternal blood samples and placenta were collected in EDTA-coated vials and centrifuged for 15min at 13000 rpm at 4°C. Proteins were extracted using a Bioplex extraction kit (Bio-rad Laboratories, Beverly, MA). All samples were aliquoted and stored at -80°C until use. IL-6 measurement in placenta extracts was performed by ELISA according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Cytokines were measured in the maternal blood by using a LINCOplex multiplex kit (IL-1 β , IL-6, TNF α , IL-10; Millipore).

Analysis of Fatty Acid Contents

Lipids were extracted from fetal brains, placenta, adult offspring's liver and brainstem as previously described (Delpech et al., 2015b, 2015b; Joffre et al., 2016; Labrousse et al., 2012; Lafourcade et al., 2011). Briefly, fatty acid methyl esters were analyzed on a Hewlett-Packard 5890 series II gas chromatograph equipped with a split/splitless injector, a flame ionization

detector (Palo Alto, CA, USA), and a CPSil88-silica capillary column (100m × 0.25mm i.d., film thickness 0.20µm, Varian, Les Ulis, France). Injector and detector were maintained at 250°C and 280°C, respectively. Hydrogen was used as a carrier gas (inlet pressure 210 kPa). The oven temperature was fixed at 60°C for 1min, increased to 85°C at a rate of 3°C/min and then to 190°C at a rate of 20°C/min and left at this temperature for 65min. Fatty acid methyl esters were identified by comparing with commercial standards.

Isolation of microglia and FACS analyses

Microglia were isolated from E17 brain homogenates as previously described (Delpech et al., 2015a; Madore et al., 2013). Briefly, brains were rinsed in PBS and meninges were removed. Brains were homogenized in Hanks' Balanced Salt Solution (HBSS), pH 7.4 passing through a 70 µm nylon cell strainer. Homogenates were centrifuged at 600g for 6 min. Supernatants were removed and cell pellets were re-suspended in 70% isotonic Percoll (GE-Healthcare, Aulnay sous Bois, France). A discontinuous Percoll density gradient was set up as follows: 70%, 50%, 35% and 0% isotonic Percoll. Gradients were centrifuged at 2000g for 20 min. Microglia cells were collected at the interphase between the 70% and 50% Percoll layers (Frank et al., 2006; Nair et al., 2007). Cells were washed and counted with a hemacytometer. Microglial cells were re-suspended in Phosphate Buffer Saline solution (PBS)/0.1% Bovine Serum Albumin (BSA) to perform flow cytometry analysis. Microglial preparations were incubated with anti-CD16/CD32 antibody (eBiosciences, Paris, France) to block Fc receptors for 10 min on ice. Cells were washed and then incubated for 45 min with the appropriate conjugated antibodies: anti-CD11b-APC, anti-CD45-PerCP Cy5.5, anti-MHC-II-FITC, anti-CD86-FITC, anti-CD130-APC, anti-TLR4-APC (eBiosciences) and anti-CD36-PE, anti-CD206-FITC, anti-CD126-PE (Biolegend, Saint Quentin Yvelines, France) antibodies. Cells were washed and then suspended in PBS/BSA 0.1% for analysis. Non-specific binding was

assessed by using non-specific, isotype-matched antibodies. Antigen expression was determined using a Becton–Dickinson LSRFortessa™ multicolor cytometer. Ten thousand events were recorded for each sample and isotype matched-conjugate. Data were analyzed using FlowJo software and gating for each antibody was determined based on non-specific binding of appropriate negative isotype stained controls.

Statistical analyses

All data are expressed as means $\pm$ SEM. Normality and homoscedasticity of data was assessed by Shapiro-Wilk test and Brown-Forsythe test respectively. Then results were analysed using two-way analysis of variance (ANOVA) (diet x prenatal treatment) using group as between factor when applicable or Kruskal-Wallis test. Significant effects were further analysed either with Bonferroni *post-hoc* test when interaction was statistically positive or with Mann-Whitney *U*-test. Specific comparison between novel and familiar arms was assessed with paired t-tests. To analyse recognition index in the novel object recognition task, we performed one sample t-tests (comparison to chance level). For all results, statistical significance was set at $p < 0.05$.

We used principal component analysis (PCA) to identify cytokines and fatty acids patterns in E17 fetuses 3h after Saline or LPS injection. The PCA is a dimension-reduction approach that summarizes data into principal components (PC) that maximize the variance of the data considered. These PCs were uncorrelated linear combinations of the initial variables, which can be interpreted as “patterns”. PCA generates factor loadings that reflect the correlation of each variable with the PC and a principal component score for each individual that corresponds to a linear combination of the cytokines and fatty acids that load heavily within each pattern. A higher component score indicated a higher adherence to that pattern. To facilitate the interpretation of our patterns we have calculated the correlations between each

variable and the PCs. We kept only the variables that had a correlation coefficient significantly different from 0. The number of components/patterns was selected according to their eigenvalues, each component with an eigenvalue higher than 1 was retained (Kaiser criteria). Statistical analyses were performed using R version 3.3.0 (FactoMineR).

Results

Effect of dietary manipulations and MIA on fatty acid composition in the fetus and adult offspring.

Both dietary n-3 PUFAs and MIA alter fatty acid composition in the fetal brain.

Fatty acids levels were measured in the fetal brain (Table 1) 3 h after MIA. ANOVA analyses revealed a significant effect of the diet. In n-3 deficient mice, n-6 species (total n-6 PUFAs and docosapentaenoic acid - DPA) and the n-6/n-3 PUFA ratio were increased while n-3 PUFAs species (total n-3 PUFA and docosahexaenoic acid - DHA) were decreased (Two-way ANOVA, Diet effect, DPA n-6: $p=0.007$, DHA: $p=0.03$, Total n-6: $p=0.008$, Total n-3: $p<0.001$, n-6/n-3 ratio: $p=0.003$). MIA also decreased brain levels in n-3 PUFAs, especially DHA (Two-way ANOVA, Treatment effect, DHA: $p=0.002$, Total n-3: $p<0.001$, n-6/n-3 ratio: $p=0.008$, Total PUFAs: $p=0.035$). There was no significant interaction between diet and treatment (statistics in Supplementary Table 1).

We also analysed lipid composition of the placenta. In this tissue, n-3 PUFA deficiency decreased total PUFA levels ($p=0.04$) and increased n-6/n-3 ratio ($p<0.001$). In addition, we observed an increase in n-6 species (Two-way ANOVA, Diet effect, Total n-6: $p=0.007$, DPA n-6: $p<0.001$, ALA: $p<0.001$) and a decrease in n-3 PUFAs (Two-way ANOVA, Diet effect, Total n-3: $p<0.001$, EPA: $p<0.001$, DHA: $p<0.001$) in the placenta of n-3 PUFA deficient

mice. However, no significant effect of MIA and no interaction between diet and treatment were revealed (statistics in Supplementary Table 2).

These data suggest that 1) maternal dietary manipulation affected brain PUFA composition (both n-3 and n-6 species) very early in development; 2) MIA specifically decreased DHA levels in the fetal brain without altering other lipids; 3) the placenta and fetal brain were differentially affected by the diet and MIA.

Both dietary n-3 PUFAs and MIA alter fatty acid composition in the adult offspring.

Fatty acid composition was then analysed in the brainstem of adult offspring. Almost all fatty acids species were modified by the diet (Two-way ANOVA, Diet effect, saturated fatty acid SFA: $p=0.003$, monounsaturated fatty acid MUFA: $p<0.001$, LA: $p<0.001$, dihomo- γ -linolenic acid DGLA: $p<0.001$, arachidonic acid AA: $p<0.001$, docosatetraenoic acid DTA n-6: $p<0.001$, DPA n-6: $p<0.001$, Total n-6: $p<0.001$, eicosapentaenoic acid EPA: $p<0.001$, DPA n-3: $p<0.001$, DHA: $p<0.001$, Total n-3: $p<0.001$, n-6/n-3 ratio: $p<0.001$, Total PUFAs: $p<0.001$). Moreover, DHA and total n-3 PUFAs were also significantly increased by MIA, though differences were very slight (Two-way ANOVA, Treatment effect, DHA: $p=0.05$, Total n-3 PUFAs: $p=0.05$) (Table 3, statistics in Supplementary Table 3). This shows that while MIA significantly decreased DHA levels by 29-39% in fetal brain, a slight 4% increase in DHA concentrations was observed in the brainstem of adult offspring. The dietary manipulation, however, strongly affected brain lipid composition in the long term (for all classes of fatty acids studies, i.e. PUFAs, MUFAs and SFAs).

Fatty acid composition was also significantly altered by the diet in the liver of adult offspring (Two-way ANOVA, Diet effect, AA: $p<0.001$, DTA n-6: $p<0.001$, DPA n-6: $p<0.001$, Total n-6: $p<0.001$, ALA: $p<0.001$, EPA: $p<0.001$, DHA: $p<0.001$, Total n-3: $p<0.001$, n-6/n-3 ratio: $p<0.001$). MIA significantly decreased SFA ($p=0.011$) and N-3 PUFAs (ALA: $p=0.013$,

EPA: $p=0.018$, DHA: $p=0.004$, Total n-3: $p=0.004$). As revealed by the interaction between diet and treatment, a significant decrease of n-3 PUFAs levels was measured in n-3 balanced fed mice that were exposed to MIA compared to n-3 PUFA balanced mice (Bonferonni *post-hoc* tests, ALA: $t(11)=4.322^{**}$, EPA: $t(11)=4.11^{*}$, DHA: $t(11)=5.76^{***}$ and total n-3 PUFAs: $t(11)=5.70^{***}$) (Table 4, statistics Supplementary Table 4).

Altogether, lipid analyses revealed 1) a strong effect of the dietary manipulation on fetal brain lipid composition as early as at E17; 2) a long-term effect of the diet on brain and body lipid composition; 3) a strong effect of MIA on DHA levels, 3 h after LPS treatment, while the impact is much lighter at adulthood; 4) a differential impact of diet and MIA on brain and peripheral organs at adulthood.

Effect of n-3 PUFA deficiency on memory performances in MIA-exposed animals.

N-3 PUFA deficiency reveals spatial memory deficits in MIA-exposed adult mice.

Both MIA and n-3 PUFA deficiency have been shown to influence memory performances (Golan et al., 2005; Janssen et al., 2015). We thus evaluated spatial memory in adult offspring using a Y-Maze task (Labrousse et al., 2009). While balanced-LPS mice spent significantly more time exploring the novel arm, MIA-exposed mice fed with n-3 deficient diet (Deficient-LPS) failed to discriminate the novel arm from the familiar one (Figure 1A) (Paired t-tests; Deficient-LPS: $t(12)=0.069$, $p=0.94$; Balanced-LPS: $t(6)=2.655$, $p=0.037$). Both deficient-saline and balanced-saline groups spent significantly more time in the novel arm than in the familiar one (Paired t-tests; Deficient-saline: $t(7)=3.206$, $p=0.014$; Balanced-saline: $t(6)=4.892$, $p=0.002$).

The perinatal period is a critical window to reveal the deleterious effects of n-3 PUFA deficiency in MIA exposed animals.

We further examined whether the deleterious effects of n-3 PUFA deficiency on spatial memory abilities mainly relied on their brain accretion during development or both on brain accretion during development and turnover at adulthood. Animals were thus fed with n-3 PUFA balanced diet until weaning and then exposed them to n-3 PUFA deficient diet until behavioral assessment. All animals spent more time in the novel arm than in the familiar one (Figure 1B) (Paired t-tests; Balanced-saline: $t(10) = 13.16$, $p < 0.001$; Balanced-LPS: $t(8) = 4.04$, $p = 0.0037$; Balanced/Deficient-saline: $n = 14$, $t(13) = 5.557$, $p < 0.001$; Balanced/Deficient-LPS: $n = 8$, $t(7) = 2.956$, $p = 0.02$).

N-3 PUFA deficiency does not affect recognition memory in MIA-exposed adult mice.

We then assessed recognition memory using Novel Object Recognition (NOR). LPS-treated mice failed to discriminate the novel object regardless of the diet while saline-treated animals spent significantly more time exploring the new object than the familiar one (Figure 1C) (One-Sample t-tests; Deficient-saline: $t(8) = 6.099$, $p = 0.0003$; Deficient-LPS: $t(13) = 0.1115$, $p = 0.91$; Balanced-saline: $t(7) = 5.95$, $p = 0.0006$; Balanced-LPS: $t(6) = 0.5949$, $p = 0.5926$).

Dietary n-3 PUFAs deficiency reveals MIA-induced decrease in cFos expression in the dentate gyrus (DG).

We then investigated cFos expression, as a marker of neuronal activation, 60 min after trial 2 of the Y-Maze task (Figure 2). In the CA1 region of the hippocampus, the number of cFos-positive cells was significantly decreased in MIA-exposed mice as compared to saline-treated animals, in both diet groups. However, this decrease was greater in n-3 deficient mice treated with LPS when compared to n-3 balanced animals exposed to MIA. The number of cFos-

positive cells was not significantly modified by MIA in the DG of n-3 PUFAs balanced fed mice, n-3 PUFA deficiency revealed MIA-induced cFos expression decrease (CA1: Kruskal-Wallis test: $K(4,24)=17.84$, $p=0.0005$, Mann-Whitney comparisons: Deficient Saline vs Deficient LPS $p=0.002$; Balanced Saline vs Balanced LPS $p=0.002$; Deficient LPS vs Balanced LPS $p=0.03$. DG: Kruskal-Wallis test: $K(4,24)=11.85$, $p=0.008$, Mann-Whitney comparisons: Deficient Saline vs Deficient LPS $p=0.009$; Balanced Saline vs Balanced LPS $p=0.30$; Deficient LPS vs Balanced LPS $p=0.009$).

N-3 PUFA deficiency exacerbates MIA-induced cytokine production in both maternal and fetal compartments.

Numerous studies have shown that the effects of MIA on the developing brain are mediated mostly by the indirect production of pro-inflammatory cytokines such as IL-6, in both maternal and fetal compartments (Ashdown et al., 2006; Bilbo and Schwarz, 2012; Mouihate and Mehdawi, 2016). We thus assessed the impact of n-3 PUFAs on maternal cytokine expression in response to MIA using bioplex assay. The levels of pro-inflammatory cytokines IL-1 β , IL-6, TNF α , and anti-inflammatory cytokine IL-10 were measured in maternal blood samples 3 h after MIA (Figure 3A). Prenatal exposure to LPS significantly increased the protein expression of IL-6, TNF α , IL-1 β and IL-10. Moreover, n-3 PUFA deficiency exacerbated MIA-induced IL-6 and TNF α production in maternal blood (Figure 3A) (Mann-Whitney comparisons: IL-6: Deficient Saline vs Deficient LPS $p<0.001$; Balanced Saline vs Balanced LPS $p<0.001$; Deficient LPS vs Balanced LPS $p<0.001$; IL-1 β : Deficient Saline vs Deficient LPS $p=0.005$; TNF- α : Deficient Saline vs Deficient LPS $p<0.001$; Balanced Saline vs Balanced LPS $p=0.002$; Deficient LPS vs Balanced LPS $p=0.002$; IL-10: Deficient Saline vs Deficient LPS $p<0.001$; Balanced Saline vs Balanced LPS $p<0.001$).

In the placenta, MIA-induced IL-6 production was also exacerbated in n-3 PUFAs deficient mice (Mann-Whitney comparisons: Deficient Saline vs Deficient LPS $p<0.001$; Balanced Saline vs Balanced LPS $p<0.001$; Deficient LPS vs Balanced LPS $p<0.001$).

Finally, fetal brain cytokines were measured by RTqPCR (Figure 3C). MIA significantly increased mRNA expression of IL-6 and this was exacerbated in n-3 PUFA deficient mice. The expression of other pro-inflammatory markers such as IL-1 β , TNF- α , IL-6 receptor and CD11b was not significantly affected by the diet or MIA (Mann-Whitney comparisons: IL-6, Deficient Saline vs Deficient LPS $p=0.002$; Balanced Saline vs Balanced LPS $p=0.004$; Deficient LPS vs Balanced LPS $p=0.009$).

Neither n-3 PUFA deficiency nor MIA modulate microglia phenotype.

We already showed that n-3 PUFA deficiency modulates microglia phenotype, exacerbating their reactivity to LPS (Delpech et al., 2015a). To assess the phenotype of microglia, we measured the surface expression of various receptors on isolated microglia from fetuses, 3 h after LPS or saline injection. Flow cytometry data showed that microglial phenotype was not altered neither by the MIA nor by the diet in pup brain at E17 (Figure 4A). Because the expression of IL-6 mRNA was significantly increased in the fetal brain 3 h post-LPS, we also measured the expression of IL-6 receptor (CD126/CD130 subunits) protein at the surface of microglia and could not find any difference between groups (Figure 4B).

Specific association between n-3 LC-PUFA and pro-inflammatory cytokines.

We further assessed correlations between fatty acid profile and cytokine expression within the fetal brain. For each brain, we quantified both cytokine mRNAs and fatty acids, enabling us to seek patterns of expression of these factors within the population using principal component analysis (PCA). PCA showed a marked separation of all variables according to 3 main

patterns (Kaiser criteria) (Figure 5A). The table represents all variables that allow defining each pattern. Values correspond to the correlation coefficient between each variable and the pattern they are part and give the weight of each factor in defining the pattern. Figure 5B is a schematic representation of the correlation between variables within each pattern. The length of each arrows is proportional to the weight of the corresponding factor in defining the pattern. Two arrows with opposite directions means that variables are anti-correlated. The first pattern ("pattern 1", defined by 9 variables among all variables measured) explained 49.28% of the total variance within the population and revealed an anti-correlation between IL-6, n-6/n-3 ratio, AA/DHA ratio on one hand and DHA, n-3 PUFAs, DHA/DPA on the other hand (Figure 5B). Pattern 2 explained 25.22% of the total variance within the population. It revealed a positive score for AA, DTA, DPA and n-6 PUFAs suggesting that fetuses can be classified according to their n-6 PUFAs levels as well. The third component ("Pattern 3") explained 15.93% of the total variance within the population. It revealed a positive score for pro-inflammatory cytokines and a negative score for EPA.

Overall, PCA reported negative relationship between long-chain n-3 PUFAs levels and pro-inflammatory cytokines expression within the fetal brain, DHA levels being more negatively correlated to IL-6 expression while EPA content was negatively correlated to TNF α and IL-1 β production.

Discussion

Only few studies have assessed the impact of n-3 PUFAs on the behavioral and molecular consequences of MIA (Delattre et al., 2017; Li et al., 2015; Weiser et al., 2015). These reports showed the protective effect of DHA supplementation on cognitive, emotional and social behaviors in animals treated with LPS (Delattre et al., 2017) or polyI:C (Li et al., 2015; Weiser et al., 2015) during prenatal period. Yet, this study is the first to address the

deleterious effects of n-3 PUFA deficiency on brain lipid composition, inflammation, microglial phenotype and memory performances in the offspring. We here demonstrated that maternal dietary n-3 PUFA deficiency 1) alters fatty acid composition of the fetal and adult offspring brain; 2) exacerbates maternal and fetal inflammatory processes with no significant alteration of microglia phenotype, and 3) induces spatial memory deficits in the adult offspring. We also showed a strong negative correlation between brain content in LC n-3 PUFA and cytokine production in MIA-exposed fetuses.

Both n-3 PUFAs and MIA alter fatty acid composition in the brain of the offspring.

The developing brain expresses the enzymatic machinery to convert precursors of PUFAs into long chains such as DHA and AA (Green and Yavin, 1996). PUFAs start accumulating in rodents very early during fetal life, yet it accelerates dramatically around gestational day 17 (Clandinin, 1999; Cunnane and Chen, 1992; Green and Yavin, 1996; Kuipers et al., 2012; Sinclair and Crawford, 1972). We measured fetal brain fatty acid composition as early as at E17 and showed for the first time that maternal dietary exposure to n-3 PUFA deficient diet from E0 to E17 was sufficient to significantly affect offspring's brain lipid composition. Notably, these effects were still visible in adult offspring, suggesting that no compensatory mechanisms were put in place later on.

MIA altered brain fatty acid composition in fetus brain as soon as 3 hours post-LPS injection, in both dietary groups, while no modification was seen in the placenta. DHA concentration was significantly decreased in MIA-exposed mice. As a plausible explanation, PUFAs can be unesterified from the membrane and converted into bioactive mediators (Orr et al., 2013a; Serhan, 2017). EPA and DHA derivatives display potent anti-inflammatory and pro-resolutive activities (Layé et al., 2018; Serhan, 2017). Resolvin D1 and E1 derived respectively from DHA and EPA exert potent anti-inflammatory properties pushing microglia toward a pro-

resolution phenotype (Bisicchia et al., 2018; Harrison et al., 2015; Kantarci et al., 2018; Li et al., 2014; Rey et al., 2016; Xu et al., 2013; Zhu et al., 2016). MIA-induced fetal brain inflammation is likely to promote cleavage of DHA out of plasma membranes and conversion into lipid mediators as part of the local inflammatory response (Calder, 2017). Hence, this would lead to decrease in total DHA content in offspring's brain, as observed here.

N-3 PUFA deficiency induces memory deficits in MIA-exposed adult offspring.

We showed that systemic maternal inflammation produces long-term behavioral deficits in n-3 PUFA deficient adult offspring. Animals could not differentiate the novel arm from the familiar one in the Y-maze task, as a surrogate of deficits in spatial memory. This suggests that low brain n-3 PUFA content is correlated to poor cognitive abilities and that it may represent a risk factor for neurodevelopmental disorders (Madore et al., 2016). This also echoes many studies indicating that hippocampal n-3 PUFA levels modulate cognitive performances (Joffre et al., 2014). We and others have previously shown that increased brain content of DPA n-6, together with reduced brain DHA content, is associated with behavioral impairment in adult rodents fed with diet deprived of n-3 PUFAs (Lafourcade et al., 2011; Larrieu et al., 2012, 2014; Lim et al., 2005; Mingam et al., 2008).

Surprisingly, we also found that when fed a n-3 balanced diet, LPS-treated animals were able to discriminate the novel arm from the familiar one, suggesting that MIA did not impact their spatial memory. This is in contradiction with previous reports showing behavioral deficits in mice exposed to LPS at E17, such as memory impairment in the Morris water maze task (Golan et al., 2005), anxiety and depressive-like symptoms (in the elevated plus maze and forced swimming test, (Enayati et al., 2012)) or amphetamine-induced hyper-locomotion (Zager et al., 2012). Moreover, other studies using different MIA models (intraperitoneal injection of polyI:C, mimicking viral infection, or different time of LPS injection) showed

protective effects of n-3 PUFAs on social interaction and dopaminergic system function (Delattre et al., 2017; Fortunato et al., 2017). In all these studies, mice were fed with standard chow, while we fed control mice with n-3 PUFA balanced diet. We previously showed that fatty acid composition is different between standard chow (A04) and n-3 PUFA balanced diet (Joffre et al., 2016). Specifically, the n-6/n-3 PUFA ratio is higher in the standard diet as compared to the n-3 PUFA balanced diet, leading to differences in brain fatty acid composition, which might explain the discrepancies in behavioral outcome (Joffre et al., 2016). Moreover, dose/source of LPS and mouse strains vary in all these studies, which might also account for differential effects on behavior (Enayati et al., 2012; Golan et al., 2005; Zager et al., 2012). Enayati et al., for instance, had a closer look to the dose effect of prenatal LPS on anxiety and mood disorders and showed that the behavioral alterations were highly dependent on the dose of endotoxin injected to the mother (ranging from 50 to 500 mg/kg LPS; (Enayati et al., 2012)).

Overall, our data show for the first time that n-3 PUFA deficiency is a risk factor for the development of cognitive impairment in the context of MIA. This is in line with clinical data showing that n-3 PUFAs deficiency increases the risk of developing ADHD, autism or schizophrenia (Al-Farsi et al., 2013; Brigandi et al., 2015; Crippa et al., 2016; Hoen et al., 2013; Jory, 2015; Kirby et al., 2010; Mostafa et al., 2015; Parletta et al., 2016; Pawelczyk et al., 2015; Solberg et al., 2016; van der Kemp et al., 2012; Vancassel et al., 2001; Wiest et al., 2009). Conversely, interventional studies supplemented mothers and/or infants with n-3 PUFAs over the perinatal period with mixed results (Amminger et al., 2015, 2007; Bent et al., 2011; Cooper et al., 2016; Dubnov-Raz et al., 2014; Gillies et al., 2012; Gould et al., 2014; Huss et al., 2010; Jiao et al., 2014; Mankad et al., 2015; McGorry et al., 2017; Meguid et al., 2008; Ooi et al., 2015; Politi et al., 2008). Hence, we need more preclinical studies to better

characterized the type of n-3 PUFAs required (precursors vs EPA alone or in combination with DHA), duration and time-window of the supplementation.

Structure-dependent effects of n-3 PUFA deficiency on MIA-exposed mice.

In addition to the Y-maze task, we also tested cognitive abilities of the offspring in the novel object recognition task. The spatial memory test (Y-maze) is considered as hippocampus-dependent (Dellu et al., 2000) whereas the novel object recognition test is cortex-dependent and hippocampus-independent (Dere et al., 2007). Our data showed that n-3 PUFA deficiency revealed memory deficits in the Y-maze while it did not in the novel object recognition task, as n-3 PUFA balanced mice were already impaired. These results suggest a specificity of action of n-3 PUFAs on fetal hippocampus. Our group and others previously showed that PUFA accretion is region-dependent in adults (Carrie et al., 2000; Joffre et al., 2016). We found that the hippocampus was specifically enriched in PUFAs when compared to cortical regions (Joffre et al., 2016). This might explain why our dietary manipulations more greatly affect hippocampus-dependent behavior. More studies are required to understand why hippocampus is more sensitive to dietary n-3 PUFA intake and what are the cellular and molecular consequences in that region.

N-3 PUFA deficiency exacerbates maternal and fetal cytokine production but does not affect microglia phenotype.

We showed that n-3 PUFA deficiency exacerbates MIA-induced pro-inflammatory cytokine, more specifically IL-6, production in maternal plasma, placenta and fetal brain. Our data are in accordance with an extensive literature showing increased levels of cytokines (including IL-6) in embryonic serum and brain after MIA (Ashdown et al., 2006; Beloosesky et al., 2006; Fidel et al., 1994; Gayle et al., 2004; Gilmore et al., 2005; Meyer, 2006; Paintlia et al.,

2004; Urakubo et al., 2001). **Clinical studies showed that giving n-3 PUFA could alleviate perinatal inflammatory processes associated with various pathologies such as ASD, ADHD or perinatal depression (Chang et al., 2018a, 2018b; Madore et al., 2016; Su et al., 2008).** Yet, our study is the first to report the aggravating action of maternal n-3 PUFA deficiency on inflammation, specifically on IL-6 release in both maternal and fetal compartment. Many studies already showed the anti-inflammatory action of n-3 PUFAs both *in vitro* on microglial cell cultures and *in vivo* on various pathological models but none ever addressed the (for extensive review, (Layé et al., 2018)). However, we are the first to demonstrate the exacerbating action of n-3 PUFA deficiency on inflammation in a model of MIA. This complete previous studies from our group showing that mice developed under a n-3 PUFA deficient diet express higher levels of IL-1 β and IL-6 mRNA in the hippocampus at post-natal day 21 (Madore et al., 2014). Of note, we previously demonstrated that feeding animals with n-3 PUFA deficient diet starting at weaning was not sufficient to increase cytokine production in the hippocampus of adult control animals while it only affected their ability to mount an inflammatory response after acute intraperitoneal LPS injection (Delpech et al., 2015b). This highlights the perinatal period as a critical window for n-3 PUFA deficiency to increase basal inflammatory processes and suggests that the developmental PUFA accretion phase is decisive for later cellular and behavioral activities while n-3 PUFA deficiency later in life has less drastic consequences. We here confirmed this assumption by feeding animals with n-3 PUFA deficient diet only after weaning and could not show any deleterious effect of the diet on cognitive abilities in the Y-maze task. This emphasizes the need for nutritional recommendations for pregnant and lactating women (Huffman et al., 2011; Kim et al., 2017).

Since n-3 PUFAs can regulate microglial function (for review, (Layé et al., 2018; Nadjar et al., 2016)), we also assessed microglial phenotype in all experimental groups. Indeed, these cells are highly versatile and can acquire diverse phenotypes, characterized by distinctive surface receptors expression profile, and associated with various functions including inflammatory or anti-inflammatory cytokine production (Garden and Möller, 2006; Madore et al., 2014). We could not find any effect neither of diet nor of treatment on microglial phenotype, suggesting that n-3 PUFA-mediated anti-inflammatory effects are not due to alteration of microglial status. This confirms our previous results showing that microglial phenotype is not affected 2 hours after peripheral injection of LPS to adult mice (Madore et al., 2013). Yet, one cannot exclude that fetal microglia differently responded to inflammation in our model. More studies are needed to further characterize the combined impact of n-3 PUFAs and maternal immune activation on microglial function, especially on its role on neuronal development and maturation ((Paolicelli et al., 2011; Schafer et al., 2012; Tremblay et al., 2010; Zhan et al., 2014) for review, (Bilimoria and Stevens, 2015; Frost and Schafer, 2016; Mosser et al., 2017)).

PCA analysis revealed a strong negative correlation between DHA content and IL-6 expression in the fetal brain, while EPA levels were inversely correlated to TNF α and IL-1 β expression. Hence, the increase in IL-6 expression is likely to be due to decreased DHA concentration in the fetal brain. Even though more studies are required to better understand the molecular link between DHA levels and IL-6 expression, our observations confirmed a previous report in which we found that a single *in vivo* administration of a highly stable form of DHA specifically reduced LPS-induced IL-6 mRNA synthesis in the hippocampus but not in the hypothalamus. This was correlated to the downregulation of the IL-6 receptor subunit gp130 and an inhibition of IL-6-induced nuclear translocation of STAT3 *in vitro* (Fourrier et

al., 2017). This corroborates our observations that 1) DHA levels and IL-6 production are anti-correlated; 2) that DHA affects brain function in a region-specific manner. Moreover, recent clinical data from a randomized control trial revealed a negative correlation between DHA and IL-6 blood levels in mothers supplemented with n-3 PUFAs during pregnancy and lactation (Rodriguez-Santana et al., 2017).

N-3 PUFA deficiency exacerbates cytokine production as a potential mechanism supporting memory deficits

Proinflammatory cytokines, such as IL-1 β , TNF α and IL-6, have been directly linked to modulation of memory abilities in various animals models (Delpech et al., 2015b; Yirmiya and Goshen, 2011). Moreover, IL-6 is considered as a key player in MIA-induced behavioral abnormalities, corroborating our observations (Dammann and Leviton, 1997; Gilmore et al., 2004; Samuelsson et al., 2006; Smith et al., 2007). Samuelsson and colleagues reported that prenatal exposure to IL-6 induces neuronal loss in the hilus, leading to hippocampal cognitive impairment in adulthood (Samuelsson et al., 2006). As an alternative explanation, we cannot exclude that n-3 PUFA deficiency directly affects neuronal development and maturation. Previous reports showed that DHA regulates neurogenesis, synaptogenesis, myelinogenesis and neuronal outgrowth (for extensive review, see (Joffre et al., 2014; Luchtman and Song, 2013)). For instance, application of DHA on neuronal cultures increases the number of neurons with more mature morphology (Calderon and Kim, 2004) and increases the number of synapses (Cao et al., 2009; Kim et al., 2011). In vivo, n-3 PUFA supplementation increases the number of neurons in the cortex and hippocampus (Coti Bertrand et al., 2006), favors neurite growth (Calderon and Kim, 2004; Cao et al., 2009), promotes long term synaptic plasticity (Cao et al., 2009) and increases myelin-specific protein expression (Salvati et al., 2008). We thus need to further decipher the relative weight of pro-inflammatory processes

and direct action of lipids on neurons in the deleterious effects of n-3 PUFA deficiency on cognition.

Overall, our study is the first to address the deleterious effects of n-3 PUFA deficiency on brain lipid composition, inflammation and memory performances in MIA-exposed animals and indicates that it should be considered as a potent environmental risk factor for the apparition of neurodevelopmental disorders.

Fig 1: Dietary n-3 PUFA deficiency reveals spatial memory deficits in MIA-exposed adult mice. **A.** Graphical representation of the time spent (in second) in the novel or the familiar arm after a 30-min inter-trial interval (Y-maze task). N-3 PUFA deficiency reveals spatial memory deficits in MIA-exposed adult offspring (Balanced-Saline n=7; Balanced-LPS n=7; Deficient-Saline n=8; Deficient-LPS n=13). **B.** N-3 PUFA deficiency during the perinatal period is necessary to induce spatial memory deficits in the Y-maze task in MIA-exposed adult offspring (Balanced-Saline n=7; Balanced-LPS n=7; Balanced/Deficient-Saline n=14; Balanced/Deficient-LPS n=8). **C.** Graphical representation of the time spent (in second) interacting with the novel object after a 30-min inter-trial interval in the novel object recognition task. N-3 PUFA deficiency does not worsen MIA-induced memory deficits in this test (Balanced-Saline n=8; Balanced-LPS n=7; Deficient-Saline n=9; Deficient-LPS group n=14).

Fig 2: Dietary n-3 PUFAs deficiency reveals MIA-induced decrease in cFos expression in the dentate gyrus (DG). **A.** Quantification of cFos immunoreactivity in the CA1 region of the hippocampus. MIA-exposed animals showed a significant decrease in cFos immunoreactivity in both diet groups, yet, n-3 PUFA deficient mice displayed a greater inhibition. MIA-induced decrease in neuronal activation in the CA1 region of the hippocampus (n=6/group). **B.** Quantification of cFos immunoreactivity in the DG region of the hippocampus. N-3 PUFA deficiency induces MIA-mediated reduction in neuronal activation in the DG region of the hippocampus (n=6/group).

Fig 3: N-3 PUFA deficiency exacerbates MIA-induced cytokine production in both maternal and fetal compartments. Quantification of cytokine protein expression in maternal plasma (n=6-11 per group) (**A**) and in placenta (n=6-9 per group) (**B**) 3 hours after LPS

injection at G17. **C.** Quantification of cytokine mRNA expression in fetal brain 3 hours after LPS injection at E17 (n=6 per group). N-3 PUFA deficiency enhances MIA-induced cytokine production in all tissues studied.

Fig 4: Neither n-3 PUFA deficiency nor MIA modulate microglia phenotype 3 hours after LPS injection at E17. A. FACS analysis of some microglial surface markers expression level, 3 hours after LPS injection. Fetal brain microglial cells were gated using CD11b^{high} and CD45^{low} (n=3-7/group). **B.** FACS analysis of IL-6 receptor subunits expression level on microglia, 3 hours after LPS injection. Fetal brain microglial cells were gated using CD11b^{high} and CD45^{low} (n=3-7/group).

Fig 5: Specific association between n-3 LC-PUFA and pro-inflammatory cytokines. A. Separation of all variables according to 3 main patterns (Kaiser criteria). The table represents all variables that allow defining each pattern. Values correspond to the correlation coefficient between each variable and the pattern they are part and give the weight of each factor in defining the pattern. **B.** Schematic representation of the correlation between variables within each pattern. The length of each arrows is proportional to the weight of the corresponding factor in defining the pattern. Two arrows with opposite directions means that variables are anti-correlated. The first three principal axes (patterns 1 to 3) explained 90.4 % of the variance. Each color represent a group of variables: blue: n-3 PUFAs, orange: n-6 PUFAs and n-6/n-3 ratio, green: classes of fatty acids, red: pro-inflammatory cytokines.

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Figure 1

Figure 2

Figure 3

A Maternal Plasma

B Placenta

C Fetal Brain

Figure 4

Figure 5

A Correlation between variables and patterns

	Pattern 1	Pattern 2	Pattern 3
DHA	-0,973***		
EPA			-0,667*
DHA/DPA	-0,894***		
Total n-3 PUFAs	-0,977***		
AA		0,935***	
DTA		0,968***	
DPA		0,813***	
Total n-6 PUFAs		0,659*	
n-6/n-3	0,973***		
AA/DHA	0,957***		
Total SFA	-0,695*		
Total MUFA	0,830***		
Total PUFA	-0,736*		
IL-1 β			0,679*
IL-6	0,791**		
TNF α			0,821***

Cytokines-lipids pattern interpretation is based on the strongest correlation within each pattern. Non-significant associations were excluded to simplify the table.

A high pattern 1 score is interpreted as high concentration in IL-6 and low in n-3 PUFAs. A high pattern 2 score is interpreted as high concentration in n-6 PUFAs and a high pattern 3 score is interpreted as high concentration in IL-1 β and TNF α and low in EPA.

AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, Docosapentaenoic acid; DTA, docosatetraenoic acid; EPA, Eicosapentaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. ***p<0.001; ** p<0.01; *p<0.05.

B PCA analysis - Variables factor map

C

Table 1: Total fatty acid composition of fetal brain

Fatty acids	n-3 balanced		n-3 deficient		Statistical effects		
	Saline	LPS	Saline	LPS	LPS	Diet	LPS x Diet
Saturated (SFA)	45.2±1.46	44.6±0.38	43.9±0.60	43.31±1.12	NS	<0.05	NS
Monounsaturated (MUFA)	26.4±1.55	28.3±1.15	27.1±1.2	29.4±2.93	NS	NS	NS
Polyunsaturated (PUFA)	22.7±0.79	21.1±1.14	23.1±1.16	21.1±2.18	<0.05	NS	NS
18:2 n-6 (LA)	2.2±0.42	2.8±0.54	2.26±0.12	2.7±0.63	NS	NS	NS
18:3 n-6 (GLA)	0.05±0.01	0.06±0.01	0.06±0.01	0.09±0.05	NS	NS	NS
20:2 n-6 (EDA)	0.11±0.02	0.10±0.01	0.16±0.08	0.16±0.06	NS	<0.05	NS
20:3 n-6 (DGLA)	0.33±0.05	0.34±0.04	0.37±0.07	0.42±0.13	NS	NS	NS
20:4 n-6 (AA)	10.0±0.46	10.0±0.93	11.0±0.47	10.2±1.03	NS	NS	NS
22:4 n-6 (DTA)	2.2±0.18	2.1±0.31	2.6±0.28	2.2±0.27	NS	NS	NS
22:5 n-6 (DPA)	0.8±0.13	1.4±0.86	2.3±0.29	2.0±0.76	NS	<0.01	NS
Total n-6	15.7±0.90	16.9±1.63	18.7±0.82	17.8±1.26	NS	<0.01	NS
18:3 n-3 (ALA)	0.04±0.01	0.04±0.02	0.03±0.01	0.05±0.04	NS	NS	NS
20:5 n-3 (EPA)	0.08±0.02	0.05±0.04	0.05±0.03	0.05±0.04	NS	NS	NS
22:5 n-3 (DPA)	0.23±0.03	0.14±0.10	0.08±0.01	0.12±0.07	NS	<0.05	NS
22:6 n-3 (DHA)	6.7±1.10	4.1±0.67	4.2±0.37	3.0±1.07	<0.01	<0.01	NS
Total n-3	7.0±1.05	4.4±0.78	4.5±0.45	3.3±0.93	<0.001	<0.001	NS
n-6/n-3	2.3±0.39	4.0±1.14	4.1±0.47	5.8±1.33	<0.01	<0.001	NS

n = 3-4 in all groups.

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LA, Linoleic acid; GLA, gamma-linolenic acid; DGLA, di-homo-gamma-linolenic acid; EDA, eicosadienoic acid; AA, arachidonic acid; DTA, docosatetraenoic acid; DPA, Docosapentaenoic acid (n-6 and n-3); ALA, α -linolenic acid; EPA, Eicosapentaenoic acid; DHA, docosahexaenoic acid.

Table 2: Total fatty acid composition of placenta

Fatty acids	n-3 balanced		n-3 deficient		Statistical effects		
	Saline	LPS	Saline	LPS	LPS	Diet	LPS x diet
SFA	40.0±1.20	40.6±1.13	40.7±2.48	40.9±2.92	NS	NS	NS
MUFA	16.9±1.25	18.7±0.53	19.5±4.21	19.8±2.19	NS	NS	NS
PUFA	37.05±0.77	35.96±1.11	35.6±3.13	33.4±2.32	NS	<0.05	NS
18:2 n-6 (LA)	6.4±0.37	6.0±0.16	5.8±0.16	6.5±0.92	NS	NS	NS
18:3 n-6 (GLA)	0.11±0.01	0.10±0.02	0.11±0.03	0.10±0.01	NS	NS	NS
20:2 n-6 (EDA)	0.48±0.05	0.43±0.10	0.37±0.08	0.51±0.14	NS	NS	NS
20:3 n-6 (DGLA)	1.36±0.06	1.23±0.06	1.14±0.09	1.13±0.14	NS	<0.01	NS
20:4 n-6 (AA)	16.2±0.76	15.5±1.21	17.1±1.81	15.2±1.25	NS	NS	NS
22:4 n-6 (DTA)	2.3±0.12	2.4±0.42	2.6±0.49	2.6±0.44	NS	NS	NS
22:5 n-6 (DPA)	0.9±0.12	1.0±0.12	3.6±0.21	3.0±0.67	NS	<0.001	NS
Total n-6	27.7±0.47	27.0±1.48	30.8±2.33	29.1±1.55	NS	<0.01	NS
18:3 n-3 (ALA)	0.12±0.02	0.14±0.03	0.03±0.03	0.04±0.01	NS	<0.001	NS
20:5 n-3 (EPA)	0.12±0.02	0.14±0.03	0.04±0.02	0.07±0.03	NS	<0.001	NS
22:5 n-3 (DPA)	0.64±0.04	0.61±0.08	0.16±0.03	0.20±0.05	NS	<0.001	NS
22:6 n-3 (DHA)	8.3±0.33	8.0±0.33	4.61±0.76	4.1±0.74	NS	<0.001	NS
Total n-3	9.4±0.32	9.0±0.42	4.83±0.81	4.4±0.79	NS	<0.001	NS
n-6/n-3	3.0±0.06	3.0±0.30	5.6±1.72	6.8±1.00	NS	<0.001	NS

n = 3-4 in all groups.

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LA, Linoleic acid; GLA, gamma-linolenic acid; DGLA, di-homo-gamma-linolenic acid; EDA, eicosadienoic acid; AA, arachidonic acid; DTA, docosatetraenoic acid; DPA, Docosapentaenoic acid (n-6 and n-3); ALA, α -linolenic acid; EPA, Eicosapentaenoic acid; DHA, docosahexaenoic acid.

Table 3: Total fatty acid composition of adult brainstem

Fatty acids	n-3 balanced		n-3 deficient		Statistical effects		
	Saline	LPS	Saline	LPS	LPS	Diet	LPS x Diet
SFA	36.9±0.40	38.0±0.30	38.5±0.30	38.8±1.0	<0.05	<0.01	NS
MUFA	28.7±0.52 ^b	27.4±0.52 ^b	25.8±0.11 ^a	26.1±0.88 ^a	NS	<0.001	<0.05
PUFA	24.0±0.45	24.9±0.66	26.2±0.63	26.0±0.61	NS	<0.001	NS
18:2 n-6 (LA)	0.45±0.01	0.4±0.03	0.38±0.03	0.4±0.02	NS	<0.001	NS
20:2 n-6 (EDA)	0.15±0.01	0.14±0.01	0.14±0.01	0.14±0.02	NS	NS	NS
20:3 n-6 (DGLA)	0.44±0.02	0.43±0.02	0.27±0.01	0.27±0.01	NS	<0.001	NS
20:4 n-6 (AA)	6.9±0.21	7.3±0.23	8.68±0.22	8.6±0.53	NS	<0.001	NS
22:4 n-6 (DTA)	2.1±0.03	2.1±0.07	2.93±0.04	2.9±0.02	NS	<0.001	NS
22:5 n-6 (DPA)	0.2±0.03	0.2±0.03	4.35±0.72	3.7±0.34	NS	<0.001	NS
Total n-6	10.2±0.25	10.6±0.32	16.8±0.75	16.1±0.78	NS	<0.001	NS
20:5 n-3 (EPA)	0.05±0.01	0.05±0	0.01±0	0.01±0	NS	<0.001	NS
22:5 n-3 (DPA)	0.17±0	0.17±0.01	0.04±0.01	0.04±0.01	NS	<0.001	NS
22:6 n-3 (DHA)	12.15±0.30	12.67±0.3	8.13±0.72	8.48±0.19	<0.05	<0.001	NS
Total n-3	12.37±0.30	12.89±0.3	8.18±0.72	8.53±0.19	<0.05	<0.001	NS
n-6/n-3	0.83±0.00	0.8±0.01	2.0±0.30	1.9±0.13	NS	<0.001	NS

n = 3-4 in all groups.

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LA, Linoleic acid; GLA, gamma-linolenic acid; DGLA, di-homo-gamma-linolenic acid; EDA, eicosadienoic acid; AA, arachidonic acid; DTA, docosatetraenoic acid; DPA, Docosapentaenoic acid (n-6 and n-3); ALA, α -linolenic acid; EPA, Eicosapentaenoic acid; DHA, docosahexaenoic acid. 18:3 n-6 (GLA, gamma-linolenic acid) and 18:3 n-3 (ALA, α -linolenic acid): non-detectable.

Table 4: Total fatty acid composition of adult liver

Fatty acids	n-3 balanced		n-3 deficient		Statistical effects		
	Saline	LPS	Saline	LPS	LPS	Diet	LPS x Diet
SFA	31.5±1.03	30.8±0.87	32.9±1.05	29.6±2.01	<0.05	NS	NS
MUFA	33.9±1.14	37.6±2.69	35.1±1.90	36.0±3.88	NS	NS	NS
PUFA	34.3±1.12	31.2±1.85	31.7±2.89	34.0±2.19	NS	NS	<0.05
18:2 n-6 (LA)	10.2±0.61	8.7±0.98	9.4±1.39	9.8±0.82	NS	NS	NS
18:3 n-6 (GLA)	0.20±0.05 ^a	0.12±0.01 ^b	0.17±0.03 ^a	0.21±0.02 ^a	<0.01	NS	NS
20:2 n-6 (EDA)	0.12±0.01	0.14±0.01	0.13±0.08	0.16±0.01	NS	NS	NS
20:3 n-6 (DGLA)	1.63±0.21	1.78±0.06	1.65±0.2	1.99±0.06	<0.01	NS	NS
20:4 n-6 (AA)	11.2±0.47	10.8±0.42	14.5±1.18	15.5±1.55	NS	<0.001	NS
22:4 n-6 (DTA)	0.2±0.01	0.2±0.02	0.4±0.04	0.4±0.06	NS	<0.001	NS
22:5 n-6 (DPA)	0.2±0.01	0.34±0.06	3.5±0.03	3.6±0.58	NS	<0.001	NS
Total n-6	23.8±0.84	22.1±1.32	29.8±3.04	31.8±2.23	NS	<0.001	NS
18:3 n-3 (ALA)	0.46±0.05 ^b	0.3±0.08 ^c	0.03±0.01 ^a	0.03±0.01 ^a	<0.05	<0.001	<0.05
20:5 n-3 (EPA)	0.63±0.08 ^b	0.43±0.1 ^c	0.03±0.01 ^a	0.03±0.01 ^a	<0.05	<0.001	<0.05
22:5 n-3 (DPA)	0.38±0.04 ^b	0.28±0.06 ^c	0.04±0.01 ^a	0.05±0.01 ^a	<0.05	<0.001	<0.05
22:6 n-3 (DHA)	8.4±0.35 ^b	7.0±0.51 ^c	0.9±0.11 ^a	1.0±0.22 ^a	<0.01	<0.001	<0.01
Total n-3	9.8±0.44 ^b	8.0±0.73 ^c	1.1±0.12 ^a	1.1±0.21 ^a	<0.01	<0.001	<0.01
n-6/n-3	2.4±0.09	2.8±0.09	28.7±3.94	28.4±3.10	NS	<0.001	NS

n = 3-4 in all groups. Differences in Bonferroni *post-hoc* comparisons were represented with letters ^{a,b}.

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LA, Linoleic acid; GLA, gamma-linolenic acid; DGLA, di-homo-gamma-linolenic acid; EDA, eicosadienoic acid; AA, arachidonic acid; DTA, docosatetraenoic acid; DPA, Docosapentaenoic acid (n-6 and n-3); ALA, α -linolenic acid; EPA, Eicosapentaenoic acid; DHA, docosahexaenoic acid.

Highlights

- Maternal omega-3 deficiency has deleterious effects on pups' brain development
- Maternal omega-3 deficiency alters fatty acid composition of the fetal and adult offspring brain
- Maternal omega-3 deficiency exacerbates maternal and fetal inflammation
- Maternal omega-3 deficiency induces spatial memory deficits in the adult offspring
- There is a strong negative correlation between brain content in omega-3 and cytokines production