Impact of dietary n-3 polyunsaturated fatty acids on cognition, motor skills and hippocampal neurogenesis in developing C57BL/6J mice


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Abstract

Maternal intake of omega-3 polyunsaturated fatty acids (n-3 PUFA) is critical during perinatal development of the brain. Docosahexaenoic acid (DHA) is the most abundant n-3 PUFA in the brain and influences neuronal membrane function and neuroprotection. The present study aims to assess the effect of dietary n-3 PUFA availability during the gestational and postnatal period on cognition, brain metabolism and neurohistology in C57BL/6J mice.

Female wild-type C57BL/6J mice at day 0 of gestation were randomly assigned to either an n-3 PUFA deficient diet (0.05% of total fatty acids) or an n-3 PUFA adequate diet (3.83% of total fatty acids) containing preformed DHA and its precursor α-linolenic acid. Male offspring remained on diet and performed cognitive tests during puberty and adulthood. In adulthood, animals underwent 31P magnetic resonance spectroscopy to assess brain energy metabolites. Thereafter, biochemical and immunohistochemical analyses were performed assessing inflammation, neurogenesis and synaptic plasticity.

Compared to the n-3 PUFA deficient group, pubertal n-3 PUFA adequate fed mice demonstrated increased motor coordination. Adult n-3 PUFA adequate fed mice exhibited increased exploratory behavior, sensorimotor integration and spatial memory, while neurogenesis in the hippocampus was decreased. Selected brain regions of n-3 PUFA adequate fed mice contained significantly lower levels of arachidonic acid and higher levels of DHA and dihomo-γ-linolenic acid.

Our data suggest that dietary n-3 PUFA can modify neural maturation and enhance brain functioning in healthy C57BL/6J mice. This indicates that availability of n-3 PUFA in infant diet during early development may have a significant impact on brain development.

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Keywords: Brain development; Animal models; Omega-3 fatty acid supplementation; Cognition; Neurohistology; Phosphorus magnetic resonance imaging

1. Introduction

Long-chain polyunsaturated fatty acids (LCPUFA) in the human diet are important for maintaining health. During evolution in some cultures, LCPUFA dietary intake has changed from a diet rich in omega-3 polyunsaturated fatty acids (n-3 PUFA) to a diet deficient in n-3 PUFA [1–3]. Within the western dietary pattern, n-6 PUFA, saturated fatty acid and trans fatty acids levels have gradually

Abbreviations: 31P MRS, 31P magnetic resonance spectroscopy; adq, adequate; ALA, α-linolenic acid; ARA, arachidonic acid; ATP, adenosine triphosphate; CD36, cluster of differentiation 36; DCX, doublecortin; def, deficient; DEPC, diethylpyrocarbonate; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IML, inner molecular layer; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acids; MCF-1, monocyte chemoattractant protein-1; MR, magnetic resonance; MWM, Morris water maze; n-3 PUFA, omega-3 polyunsaturated fatty acids; NPD1, neuroprotectin D1; OML, outer molecular layer; PBS, phosphate-buffered saline; PCr, phosphocreatine; PDE, phosphodiesterase; PF, paraformaldehyde; Pi, inorganic phosphate; PME, phosphomonoesters; PND, postnatal day; PSD95, postsynaptic density protein 95; qRT-PCR, quantitative real-time polymerase chain reaction; SIPB, synaptophysin immunoreactive presynaptic bouton; SL, stratum lucidum; SR, stratum radiatum; TNF-α, tumor necrosis factor-α.

☆ Grants, sponsors and funding sources: This work was financed by a research grant of Mead Johnson Pediatric Nutrition Institute.

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http://dx.doi.org/10.1016/j.jnutbio.2014.08.002
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increased, whereas n-3 PUFA levels have decreased [1]. This shift in fatty acid composition is also reflected in human breast milk [4–7].

LCPUFA play an important role in brain development, especially during the growth spurt in the last trimester of pregnancy and the early postnatal period up to 2 years of age [8–10]. Docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA; 20:4n-6) are the most abundant LCPUFA in the brain [10]. Placental fatty acids are dependent on the maternal supply. After birth, breast milk is the only source of essential fatty acids for breast-fed infants.

Deficiency studies in rodents have demonstrated the importance of n-3 PUFA such as DHA, eicosapentaenoic acid (EPA; 20:5n-3) and their precursor α-linolenic acid (ALA; 18:3n-3) for brain development and cognitive functioning [11–14]. Research has suggested that an n-3 PUFA deficiency may lead to a variety of neuronal and psychological abnormalities, such as attention-deficit hyperactivity disorder, depression, schizophrenia, autism and anxiety [11,12,15–19]. N-3 PUFA deficiency in rodents can result in hyperactivity, a feature underlying these neuropsychiatric disorders [14,20,21]. It has been proposed that n-3 PUFA deficiency may affect neurotransmission, especially within the dopaminergic and serotonergic systems, as a consequence of altered membrane fluidity and related receptor functions [11,19]. Furthermore, n-3 PUFA are important for the regulation of synaptic plasticity, as well as learning and memory by their involvement in regulating gene expression and retinoid signaling pathways [22–26].

A sufficient dietary intake of n-3 PUFA is necessary for maintaining a healthy LCPUFA status. This is due to the fact that both ALA and linoleic acid (LA; 18:2n-6, the precursor of n-6 PUFA) can compete for the same conversion enzymes, namely delta-5-desaturase and delta-6-desaturase [27,28]. A high LA intake interferes with the desaturation and elongation of ALA and thereby with the conversion of ALA via EPA to DHA [28]. This imbalance in the n-6/n-3 ratio also results in the production of the n-6 ARA, leading to the formation of more pro-inflammatory eicosanoids, while the n-3 EPA is the precursor of anti-inflammatory eicosanoids [3,27,29]. DHA can also interfere with neuroinflammation, as it is the precursor for resolvins and neuroprotectants, such as neuroprotectin D1 (NPD1) [29,30]. NPD1 induces signaling for homeostatic maintenance of cellular integrity and can inactivate pro-apoptotic and pro-inflammatory signaling [27]. Therefore, it has been proposed that a balanced n-6/n-3 ratio is critical for maintaining a healthy brain and immune status.

Clinical studies have shown that (perinatal) LCPUFA supplementation may be beneficial for healthy neural development in both preterm and full-term infants [31–42]. Studies in preterm infants underline the significance of the timing of supplementation because these infants cannot fully benefit from the accumulation of LCPUFA starting in the last trimester of gestation. Studies in full-term infants show that both prenatal and postnatal interventions are able to improve cognition, corresponding with the findings in preterm infants that it is important to start supplementation in the last trimester of gestation [37,38,41,43–50]. However, in humans, the outcome of infant n-3 PUFA supplementation on long-term brain development appears to be subtle [38,40,45–57], whereas rodent studies have shown more pronounced beneficial effects of n-3 PUFA supplementation [11,22,51,58–61]. This may be explained by the fact that human supplementation studies are limited to noninvasive parameters and often encounter difficulties when studying a broad lifespan for long-term effects: these studies rarely exceed the infant age. Experiments with rodents offer the opportunity to overcome these limitations of human studies and enable us to study the effect of n-3 PUFA availability during gestation on brain development in more detail throughout life.

Therefore, the aim of this study was to obtain detailed insights into the mechanisms underlying the long-term beneficial effects of n-3 PUFA availability during gestation and throughout life in mice. A broad combination of parameters was determined to assess effects on behavior, brain structure and function. With behavioral and cognitive tests, changes in cortical and hippocampal functionality were studied. Cerebral metabolite status was measured to assess energy metabolism and neuronal membrane turnover, immunohistochemistry evaluated neurogenesis and synaptic plasticity, and quantitative real-time polymerase chain reaction (qRT-PCR) was used to study gene expression of inflammatory markers.

2. Methods and materials

2.1. Animals and diets

Mouse C57BL/6j dams (3–4 months old; Harlan Laboratories Inc., Horst, The Netherlands) were used for breeding and randomly assigned to either n-3 PUFA deficient (n-3 def; n=10) or n-3 PUFA adequate (n-3 adq; n=11) diet at the first day of gestation (GD 0). The isocaloric diets were based on AIN93M and only differed in n-3 PUFA composition (Table 1) [62–64]. As shown in Table 1, the n-3 def diet contained 2.55% ALA and 1.28% DHA of total fatty acids, while the n-3 def diet contained 0.05% ALA and 0.00% DHA (Research Diets Services, Woerden, The Netherlands). Diets are comparable to those used in other studies so that the results can be easily compared [14,63,64]. It was demonstrated that n-3 PUFA supplementation contributed to increased spatial learning and memory, although the mechanisms involved were not elucidated [14,63]. Offspring of the dams was maintained on the corresponding diet throughout the whole study. To normalize litter sizes, they were culled to 3 males and 3 females per dam. Six parallel groups of male offspring were used, which were either tested and sacrificed on postnatal day (PND) 30 (n-3 def=13, n-3 adq=12) or PND 60 (n-3 def=11, n-3 adq=12), including cognition and brain biochemistry parameters, or used for brain histology at PND 60 (n-3 def=7, n-3 adq=9). One male pup from each dam was represented in each parallel group for testing and histology (Fig. 1).

Starting from GD 0, the dams were housed individually in Phenotypers (Noldus, Wageningen, The Netherlands) and remained there during birth and until weaning of the offspring. The male offspring was housed in groups of 3. All mice were housed in the central animal facility with temperature controlled at 21°C, an artificial 12:12 h light:dark cycle (lights on at 7:00 a.m.), continuous music playing in the background.

### Table 1

<table>
<thead>
<tr>
<th>Composition of experimental diets</th>
<th>Ingredient</th>
<th>Amount (g/100 g diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n-3 def</td>
<td>n-3 adq</td>
</tr>
<tr>
<td>Protein:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alacid 710 (Vit free casein)</td>
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<td>20</td>
</tr>
<tr>
<td>Carbohydrate:</td>
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<td></td>
</tr>
<tr>
<td>Cornstarch</td>
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<td>15</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>10</td>
</tr>
<tr>
<td>Dextrose</td>
<td>19.9</td>
<td>19.9</td>
</tr>
<tr>
<td>Maltose-dextrin</td>
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<td>15</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>5</td>
</tr>
<tr>
<td>Salt-mineral mix</td>
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<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix</td>
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<td>1</td>
</tr>
<tr>
<td>l-Cystine</td>
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<td>0.3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
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<td>0.25</td>
</tr>
<tr>
<td>TBHQ</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Fat:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogenated coconut oil</td>
<td>8.1</td>
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<tr>
<td>Safflower oil</td>
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</tr>
<tr>
<td>Flaxseed oil</td>
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<td>0.48</td>
</tr>
<tr>
<td>DHASCO</td>
<td>–</td>
<td>0.3</td>
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<tr>
<td>Fatty acid composition (%):</td>
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<tr>
<td>Total saturated fatty acids</td>
<td>80.8</td>
<td>75.6</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>4</td>
<td>4.8</td>
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<tr>
<td>Saturated fatty acids</td>
<td>18:2n-6 (LA)</td>
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</tr>
<tr>
<td></td>
<td>18:3n-3 (ALA)</td>
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</tr>
<tr>
<td></td>
<td>20:2n-6 (ARA)</td>
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</tr>
<tr>
<td></td>
<td>22:6n-3 (DHA)</td>
<td>1.28</td>
</tr>
<tr>
<td>0-6/0-3</td>
<td>303</td>
<td>6.2</td>
</tr>
<tr>
<td>18:2n-6/18:3n-3</td>
<td>302</td>
<td></td>
</tr>
</tbody>
</table>

All diets were isocaloric and based on AIN93M [63]. The diets only differed in n-3 fatty acid composition [64,65]. The n-3 adequate diet contained 3.83% n-3 fatty acids (2.55% ALA and 1.28% DHA), while the n-3 deficient diet contained 0.05% n-3 fatty acids (0.05% ALA and 0.00% DHA).

The ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; DHASCO, docosahexaenoic acid single cell oil; LA, linoleic acid; n-3 adq, n-3 PUFA adequate diet; n-3 def, n-3 PUFA deficient diet; TBHQ, tertiary butylhydroquinone; Vit free, vitamin free.
during the light period and cage enrichment consisting of a plastic shelter and cotton nesting material. Food and water were available ad libitum. The experiments were performed according to Dutch federal regulations for animal protection and were ethically approved by the Veterinary Authority of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

Due to some technical problems during the experiments, not all mice could be included for statistical analysis for each parameter. For example, some mice were excluded from further analysis of phosphorus magnetic resonance spectroscopy, since the obtained spectra did not meet inclusion criteria.

2.2. Behavioral and cognitive tests

Dams and pups were monitored in Phenotyper cages for 24 h per day during PND 0–21 (birth until weaning). Combined total activity was measured as well as administration on opening of the eyes. Pups were weighed on PND 14, 21, 30 and 60. Behavioral and cognitive tests on the male offspring were performed starting at PND 30 (pubertal age) and PND 60 (young adult age) (Fig. 2). All tests were performed in the same order (open field, rotarod, prepulse inhibition) during the light cycle between 8:00 a.m. and 5:00 p.m. The Morris water maze (MWM) was only performed at young adult age, after animals had performed the behavioral tests.

2.2.1. Open field

The open field test was performed at PND 30 and 60 as a measure for locomotion and exploration. The mice were placed individually in a square open field (45 cm × 45 cm × 30 cm) with transparent Plexiglas walls. Their activity was recorded for 30 min. Locomotion was automatically registered with EthoVision XT8.5 (Noldus), while exploration was manually scored. The duration (s) of walking, wall leaning, rearing, sitting and grooming were scored and analyzed as previously described [65,66].

2.2.2. Rotarod

Motor coordination was studied using the rotarod at PND 30 and 60. The mice were placed on a rotating rod (3.18 cm in diameter; IITC Inc., Woodland Hills, CA, USA) and their ability to remain on the device was recorded as latency to fall (s). Trials were performed at both fixed speed and accelerated speed. All trials had a maximum duration of 300 s and the intertrial interval was 20 min. First, mice were accustomed to the task by placing them on a stationary drum for a minute followed by a test trial at 10 rpm. Next, the fixed speed trials were performed at 10, 15 and 20 rpm. Finally, two trials at accelerating speeds (4–40 rpm) followed.

2.2.3. Prepulse inhibition

The prepulse inhibition test was also performed at PND 30 and 60 to study sensorimotor integration as previously described by Streijger et al. [65]. Startle reactivity was measured in a startle response system, the SR-LAB (San Diego Instruments, San Diego, CA, USA). A nonrestrictive Plexiglas restrainer (4 cm diameter) on a Plexiglas platform was placed inside a ventilated and sound attenuated startle chamber. A high-frequency speaker in this chamber produced the various acoustic stimuli and the background noise (set at 70 dB). The whole-body startle response of the mouse produced vibrations of the platform, which were detected and transduced...
by a piezoelectric accelerometer mounted underneath the platform, which was connected to an automated system.

The testing session started with a 5-min habituation to the 70 dB background noise. Next, three blocks of startle pulses followed. The first block consisted of 5 pulses at 120 dB. Subsequently, the mice were presented with 5 blocks that all contained 2 startle pulse alone trials (120 dB), 4 prepulse trials in which 72, 74, 78 or 86 dB startle stimuli were followed by a 120 dB pulse and 1 no stimulus trial (70 dB background), in pseudorandomized order. The last block consisted of 5 startle pulse trials at 120 dB again. The prepulses had a duration of 20 ms, with a 100 ms interval between the onset of the prepulse and the onset of the pulse. The startle response was measured during 50 ms, starting at the onset of the pulse, with an amplitude read-out expressed in arbitrary units. The intertrial interval range was 10–20 s.

2.2.4. MVM

The effect of dietary n-3 PUFA availability on spatial learning and memory was tested using the MWM at PND 60. The young adult mice were placed at different starting positions in a circular pool (120 cm diameter) that was filled with water (21–22°C, made opaque by adding milk powder). The mice were trained to find a submerged platform (8 cm diameter) 1 cm below the water surface located in the northeast quadrant of the pool by using distant visual cues. The spatial cues were present on the four walls surrounding the pool at a distance of 0.5 m. During all trials, the observer was present in the room and always located at the same location (behind a curtain surrounding the setup).

Acquisition phase: mice performed 4 acquisition trials (maximal swimming time, 120 s; 30 s on platform; intertrial interval, 60 min) per day during 4 consecutive days. Starting positions were south, north, east and west. All trials were recorded and the latency to find the platform (s) was used as measure for spatial learning. Probe phase: all mice performed a single probe trial on day 5 (1 day after acquisition), in which the platform was removed from the pool. They were allowed to swim for 120 s and all trials were recorded and analyzed with EthoVision XT8.5 (Noldus). Time spent searching in the target quadrant and the number of platform crossings were used as a measure for spatial memory.

2.3. 31P MRS

At the end of the behavioral testing, the young adult mice underwent phosphorus magnetic resonance spectroscopy (31P MRS) to quantify energy metabolites and phospholipid content in the brain. MR measurements were performed on a shielded 7 T/300 mm horizontal-bore MR magnet interfaced to a clinical console (ClinScan, Bruker Biospin, Ettlingen, Germany). A homemade probe was designed to fit the mouse head consisting of a ring-shaped 31P coil (121.7 MHz) for localized 31P MRS and a surface H1 coil (300.4 MHz) for MR imaging and localized field shielding. The coils were positioned with a perpendicular field orientation to avoid coupling. Mice were placed in a stereotactic holder to prevent unwanted movement during scanning. They were anesthetized using 2% isoflurane (Abbott, Cham, Switzerland) in a 2:1:0.5:2:1:2 N2O mixture and body temperature was maintained at about 37°C with a heated airflow and monitored with a rectal optical temperature probe. Respiration of the animal was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc., Stony Brook, NY, USA). MR images in the coronal, transversal and longitudinal orientation were acquired to visualize the anatomy and morphology of the mouse brain.

Contour was enhanced. For synaptophysin, only the SIPBs between 0.1–2 μm were included in the analysis [68, 69]. The cortex of the right hemispheres of the brains collected at PND 30 and 60 were analyzed by DANTEC (Williston, VT, USA). Quantified hippocampus regions were based on the mouse brain atlas of Franklin & Paxinos (third edition, 2008).

2.4. Quantification of DCX

At PND 30, 40 and 60 the mice were decapitated and whole brains were collected and snap frozen in liquid nitrogen. The brains were stored at −80°C until analysis. The left cerebral hemispheres and erythrocytes collected from blood were processed to analyze fatty acid composition at (early) adult age. Brain tissue was divided into four regions; brain stem, cortex, subcortical region and cerebellum. Samples were kept at −80°C until analysis. Preparation and analysis of fatty acid methyl esters (FAME) was performed with a one-step homogenization/methylation procedure as described previously [70]. Briefly, about 50 mg of tissue was treated simultaneously with an aqueous solution and an organic solution that extract, separate and methylate fatty acids. FAME were quantified with a S800 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a BPX70 fused silica column (25 μm × 0.25 mm i.d.; 25 μm film; SGE Inc., Austin, TX, USA). FAME structures were positively identified by covalent-adduct chemical ionization tandem mass spectrometry on a Saturn 2000 ion trap mass spectrometer (Varian, Inc., Walnut Creek, CA, USA). An equal-weight FAME mixture was used daily to measure response factors that were applied to the day’s analyses. The concentration of the fatty acids is expressed as micrograms of fatty acid per milligram of wet tissue weight.

2.5. Biochemistry

2.5.1. Fatty acid analysis

The mice that were tested on behavior on PND 30 and those that were tested on behavior and underwent 31P MRS on PND 60 were transcardially perfused with 0.1 M PBS. Subsequently, mice were decapitated and whole brains were collected and snap frozen in liquid nitrogen. The brains were stored at −80°C until analysis. The left cerebral hemispheres and erythrocytes collected from blood were processed to analyze fatty acid composition at (early) adult age. Brain tissue was divided into four regions; brain stem, cortex, subcortical region and cerebellum. Samples were kept at −80°C until analysis. Preparation and analysis of fatty acid methyl esters (FAME) was performed with a one-step homogenization/methylation procedure as described previously [70]. Briefly, about 50 mg of tissue was treated simultaneously with an aqueous solution and an organic solution that extract, separate and methylate fatty acids. FAME were quantified with a S800 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a BPX70 fused silica column (25 μm × 0.25 mm i.d.; 25 μm film; SGE Inc., Austin, TX, USA). FAME structures were positively identified by covalent-adduct chemical ionization tandem mass spectrometry on a Saturn 2000 ion trap mass spectrometer (Varian, Inc., Walnut Creek, CA, USA). An equal-weight FAME mixture was used daily to measure response factors that were applied to the day’s analyses. The concentration of the fatty acids is expressed as micrograms of fatty acid per milligram of wet tissue weight.

2.5.2. qRT-PCR

The cortex of the right hemispheres of the brains collected at PND 30 and 60 were analyzed for inflammatory markers and synaptic plasticity with qRT-PCR. The subcortical area of the right hemispheres collected at PND 30 and 60 was analyzed for neurogenesis with qRT-PCR. Brain tissue was analyzed for interleukin-1β (IL-1β),...
interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), cluster of differentiation 36 (CD36), monocyte chemotactant protein-1 (MCP-1), synaptophysin and DCX.

Brain tissue was collected in 1 mL cold Trizol (Invitrogen, Paisley, UK) and homogenized using a dispersing machine (ultra-Turrax; IKA Werke GmbH & Co. KG, Staufen, Germany). After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in 25 μL RNA-free diethylpyrocarbonate (DEPC)-treated water. The RNA concentration of the tissue was measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, DE, USA). First-strand cDNA synthesis was performed using 1 μg RNA dissolved in a 10-μL solution containing RNA-free DEPC-treated water, 2 μL of 5× Script reaction mix and 0.5 μL iScript reverse transcriptase (Script cDNA synthesis kit; Bio-Rad Laboratories B.V., Hercules, CA, USA) at 25°C for 5 min, at 42°C for 30 min and at 85°C for 5 min and cooled down to 4°C. The cDNA samples were stored at −80°C.

qRT-PCR was performed in a total volume of 10 μL buffer solution containing 2 μL of template cDNA (diluted 1:10), 5 μL of 2× Power SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA), 2.92 μL RNA-free DEPC-treated water and 0.04 μL of each primer (100 μM). Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), IL-1α, IL-6, TNF-α, CD36, MCP-1, synaptophysin and DCX were designed using Vector Primer Express software (Applied Biosystems) (Table 2). The optimal temperature cycling protocol was determined to be 95°C for 10 min followed by 40 reaction cycles at 90°C for 15 s and at 60°C for 1 min, using a StepOnePlus real-time PCR system (Applied Biosystems). Subsequently, the melt curve temperature protocol was determined to be 95°C for 15 s, 60°C for 20 s and 90°C for 15 s with a slope of 0.6°C. Absolute quantities were determined using standard curves, and the validity of the results was confirmed by including appropriate negative controls. The quantity of cDNA was calculated for each sample with StepOne Software version 2.2.2. To evaluate differences, relative gene expression ratios were calculated according to the comparative C(T) method (also referred to as the 2(-ΔΔCT) method) [71]. Relative Ct values were calculated by subtracting the Ct value of the housekeeping gene GAPDH from the Ct values of IL-1α, IL-6, CD36, MCP-1, TNF-α, synaptophysin and DCX. For each primer, two independent qRT-PCR runs were performed, and the means of their relative values were used for statistical analysis.

2.6. Statistical analyses

Data are expressed as mean±S.E.M. and were analyzed with SPSS for Windows 20.0 software (SPSS Inc., Chicago, IL, USA). The repeated measures analysis of variance was used for the open field parameters (with the repeated measure: 10 min intervals), the first and last block of pulse-alone trials in the prepulse inhibition (with the repeated measure: time) and the acquisition phase of the MWM (with the repeated measure: time) and the acquisition phase of the MWM (with the repeated measure: repeated measure: time) and the acquisition phase of the MWM (with the repeated measure: repeated measure: time).

Table 2

qRT-PCR primers

<table>
<thead>
<tr>
<th>Primers (mouse)</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5'-TCTGAGACACATGTTAGTTGCACT-3'</td>
<td>5'-GTTACAGAGTATGATGCTCAG-3'</td>
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<tr>
<td>IL-1α</td>
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<td>CD36</td>
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<tr>
<td>MCP-1</td>
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<tr>
<td>Synaptophysin</td>
<td>5'-TCTGGGATCCACTGGCCTTG-3'</td>
<td>5'-TCTGGGATCCACTGGCCTTG-3'</td>
</tr>
<tr>
<td>DCX</td>
<td>5'-AGGGCTGTCGAGGACCTG-3'</td>
<td>5'-GGCGCTGTCGAGGACCTG-3'</td>
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</tbody>
</table>

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; MCP-1, monocyte chemotactant protein-1; qRT-PCR, quantitative real-time polymerase chain reaction; TNF-α, tumor necrosis factor α.

CD36, cluster of differentiation 36; DCX, doublecortin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; MCP-1, monocyte chemotactant protein-1; qRT-PCR, quantitative real-time polymerase chain reaction; TNF-α, tumor necrosis factor α.

Fig. 3. Locomotion in the open field. Activity (total walking distance) was measured over a 30-min period. Values represent mean±S.E.M., n=8–10 per diet, *P≤0.05. (a) n-3 adq diet increased time spent walking on PND 60 compared to PND 30. Furthermore, n-3 adq increased time spent walking compared to n-3 def on PND 60. (b) n-3 adq diet decreased time spent grooming on PND 60 compared to PND 30. Additionally, n-3 adq decreased time spent grooming compared to n-3 def on PND 60. (c) n-3 adq diet increased time spent leaning on PND 60 compared to PND 30. n-3 adq, n-3 PUFAs adequate diet; n-3 def, n-3 PUFAs deficient diet; PND, postnatal day.
3. Results

3.1. Cognitive tests

There were no differences between the two dietary groups in general developmental parameters such as opening of the eyes or activity of the litter in the Phenotypers (data not shown). Activity of the dam with litter increased over time for both diet groups (data not shown). Dietary intervention had no effects on body weight of the two groups throughout the study (data not shown).

3.1.1. Open field

The open field was used to assess locomotion (total walking distance) and exploration (walking, wall leaning, rearing, sitting) and grooming for 30 min.

In the open field, no effects of diets could be detected on locomotion at both PND 30 and 60. However, the young adult mice fed n-3 adq diet spent more time walking (P<.01) and less time grooming (P=.027) than pubertal mice (Fig. 3). This effect was not found in the n-3 def diet.

Furthermore, we observed no dietary effects in exploratory behavior in pubertal mice in the total 30 min. However, young adult mice on the n-3 adq diet spent more time walking (P=.031) and less time grooming (P=.027) than pubertal mice fed the n-3 adq diet (Fig. 4a and b). Moreover, they showed more time wall leaning (P=.033) and walking (P=.024) but less time grooming (P=.027) than their n-3 def fed littermates across the 30 min of the test (Fig. 4a–c). Subsequently, we observed that the n-3 adq fed young adult mice spent more time walking than their n-3 def fed littermates (P=.024; Fig. 4a). Lastly, the n-3 adq fed mice demonstrated less time grooming (P=.027) than their n-3 def fed littermates over 30 min (Fig. 4b).

3.1.2. Rotarod

The rotarod is designed to assess motor coordination. Mice performed a motor task on a rotating rod at fixed speeds or acceleration and the latency to fall was recorded.

No dietary effect was observed at fixed speeds (10, 15 and 20 rpm) in both pubertal and young adult mice (data not shown). However, n-3 adq fed pubertal mice demonstrated a higher latency to fall (P=.001) compared to n-3 def fed pubertal mice at accelerated speed (4–40 rpm) (Fig. 5). Young adult mice showed no diet effect at accelerated speed (data not shown). However, the n-3 adq fed mice on PND 60 display a lower latency to fall than n-3 def fed mice on PND 30 (P=.01; Fig. 5). Comparing performances in all groups, the n-3 adq fed mice at pubertal age performed best among all groups.

3.1.3. Prepulse inhibition

The prepulse inhibition test studies sensorimotor integration by measuring the startle response in mice that are administered acoustic pulses with or without a preceding softer prepulse.

At both ages, no dietary effects could be detected (data not shown). However, an age effect was found in the n-3 def diet; mice fed the n-3 def diet at PND 60 demonstrated a stronger prepulse inhibition than at PND 30, which was not the case in n-3 adq fed mice. A habituation effect to the stimuli was observed in pubertal mice (P=.001; Fig. 6a) and in the young adult mice (P=.004; Fig. 6b). However, at young adult age, this habituation effect was stronger in the n-3 adq fed mice than in the n-3 def fed mice. Both diet groups showed a stronger habituation at PND 60 compared to PND 30 (P=.009).

3.1.4. MWM

In the MWM task, spatial learning is tested in the acquisition phase where the mice have to find a hidden platform. Spatial memory is assessed in a subsequent trial where the platform is removed from the pool.

The MWM was only performed at PND 60. In the acquisition phase, both diet groups showed a learning effect (P=.002), but there was no significant difference between the two diet groups (P>.05; Fig. 7a). In the probe phase, we focused on the first 30 s of the trial. The n-3 adq fed mice spent more time in the target quadrant than the n-3 def fed mice (P=.007; Fig. 7b) and also showed more platform crossings (P=.029;
Fig. 7c) over the area where the platform used to be located. The n-3 def fed mice performed below 25% chance level ($P=0.004$) while the n-3 adq fed mice performed at 25% chance level.

3.2. 31P MRS

After performing the MWM, the mice underwent 31P MRS to measure the content of the main phosphorylated metabolites in the brain at PND 60. The metabolite levels (PCr, $\gamma$ and $\alpha$-ATP, Pi, PME and PDE) showed no differences between the diets for all metabolites in the young adult mice (data not shown).

3.3. Immunohistochemistry

Immature neurons were visualized in young adult mice with a polyclonal antibody against DCX. DCX-positive cells were counted in the subgranular zone of the hippocampus as a measure for..
4.5 μn

Erythrocytes demonstrated lower levels of ARA (fed animals) as compared to n-3 def fed mice (Table 3). On PND 30, n-3 adq diet increased expression of synaptophysin compared to n-3 def. On PND 60, n-3 adq diet decreased synaptophysin expression compared to PND 30. n-3 adq, n-3 PUFA adequate diet; n-3 def, n-3 PUFA deficient diet; NS, not significant.

Values represent mean±S.E.M., n=9-10 per diet, *P≤.01; Table 3). On PND 30, n-3 adq diet increased expression of synaptophysin compared to n-3 def. On PND 60, n-3 adq diet decreased synaptophysin expression compared to PND 30. n-3 adq, n-3 PUFA adequate diet; n-3 def, n-3 PUFA deficient diet; NS, not significant.

Table 3
Fatty acid analysis of erythrocytes and brain tissue at (young) adult age

<table>
<thead>
<tr>
<th></th>
<th>n-3 def %</th>
<th>n-3 adq %</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td></td>
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</tr>
<tr>
<td>ARA</td>
<td>12.41±1.31</td>
<td>9.13±0.94</td>
<td>NS</td>
</tr>
<tr>
<td>DGLA</td>
<td>1.24±0.14</td>
<td>1.35±0.07</td>
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<td>DHA</td>
<td>&lt;0.001±0.00</td>
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<tr>
<td>DPA</td>
<td>1.55±0.28</td>
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<td>≤.01</td>
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<tr>
<td>Brain stem</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARA</td>
<td>7.07±0.17</td>
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<tr>
<td>DGLA</td>
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<tr>
<td>DHA</td>
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<tr>
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<tr>
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<tr>
<td>DHA</td>
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<tr>
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<tr>
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<td>DPA</td>
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<td>Cerebellum</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>≤.01</td>
</tr>
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</table>

Values represent mean±S.E.M., and P≤.01 indicates that means were not equal. ARA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid (osbond acid); n-3 adq, n-3 PUFA adequate diet; n-3 def, n-3 PUFA deficient diet; NS, not significant.

4. Discussion

The present study aimed to investigate the influence of dietary n-3 PUFA availability during the development of C57BL/6J mice on behavior, cognition, cerebral metabolism and brain plasticity using behavioral and cognitive tests, 31P MRS, immunohistochemistry and biochemical approaches. An overview of the main results is shown in Table 4. Overall, significant effects on motor coordination, exploratory behavior, sensorimotor integration, spatial memory and neurogenesis were observed. These findings indicate that dietary intake of both ALA and preformed DHA during early development has a significant impact on neural and brain development as well as on cognition and behavior.

Regarding behavioral and cognitive outcomes, n-3 PUFA availability had no effects on locomotion, although total walking distance and activity of the pups increased as they reached weaning age (PND 21). This observation is in agreement with other studies [60,72-74]. Significantly more exploratory behavior, reflected by more time spent walking and wall leaning, was found in the open field in the n-3 adequate fed mice at adult age, but not at pubertal age. Carrié et al. demonstrated increased locomotion and an increase in the exploratory parameter rearing in n-3 PUFA supplemented adult female mice compared to control mice [59]. However, Coluccia et al. observed no difference in rearing between n-3 PUFA supplemented and control rats at both pubertal age and adult age [60]. In yet another study, rearing and horizontal movement were decreased in n-3 PUFA deprived rats [73]. These studies define exploratory parameters purely as rearing or sometimes vertical movement when drawing their conclusions on exploration. In the current study, no dietary effect in the time spent rearing could be observed, but indeed, an increase in time spent on wall leaning in animals with an adequate n-3 PUFA intake was demonstrated, which is also a vertical movement. Additionally, n-3 PUFA adequate fed young adult mice also displayed more walking, which is considered as an exploratory horizontal movement [73]. Furthermore, grooming has been suggested as a measure for anxiety because mice are known to display grooming under stressful conditions [75]. We observed less grooming in the adequate n-3 PUFA group at adult age, but not in puberty, while the n-3 adq fed mice displayed a decrease in synaptophysin mRNA expression on PND 60 compared to PND 30 (P=.001). No diet effects were detected in DCX mRNA expression of the subcortical region at either PND 30 or 60 (data not shown).
PUFA deficient mice did not display age differences. This indicates that n-3 PUFA supplementation may decrease anxiety-related behavior; however, no specific tests of anxiety were included in the current study. In the prepulse inhibition test of the acoustic startle reflex, a habituation effect to the 120 dB pulses was found in pubertal and young adult mice. At young adult age, this habituation effect was significantly stronger in animals with adequate n-3 PUFA intake. This reflects improved habituation to startle stimuli and therefore our data point to an improvement of cortical development by adequate n-3 PUFA intake [76]. Fedorova et al. also measured prepulse inhibition in 8-week-old mice on n-3 PUFA deficient and n-3 PUFA supplemented diets [11]. In that study, the n-3 PUFA supplemented mice showed a stronger inhibition to the acoustic pulses than mice on the deficient diet. Additionally, a habituation process was observed in supplemented, but not in deficient animals, which is in line with the results of this present study. In this experiment, we observed a stronger habituation process in our n-3 PUFA adequate mice. In a schizophrenia rat model, LCPUFA have been shown to be able to ameliorate the impaired prepulse inhibition [61]. Schizophrenia is a condition that mostly develops during early adulthood and has been associated with LCPUFA deficiency [14,20,20,21]. These data suggest that LCPUFA supplementation supports dopaminergic neurotransmission processes, by improving membrane fluidity. More testing at time points later in life could provide more information on the full potency of LCPUFA. It has been reported that high levels of n-3 PUFA were associated with a negative effect on neural development by prolonging auditory brain stem conduction times and a delay in auditory startle reflex in rodents [77,78]. These studies show the importance of dosage but indirectly also stress the relevance of the n-6/n-3 ratio, which deserves further attention.

In the current study, n-3 PUFA adequate mice displayed a higher latency to fall in the accelerating rotarod test at pubertal age, but not at young adult age. A higher latency reflects improved performance, since the animals were able to stay longer on the rotarod. The n-3 PUFA deficient mice did not demonstrate differences between puberty and adulthood. The increased rotarod latency in n-3 PUFA adq mice suggests that the n-3 PUFA adequate diet was associated with an accelerated development of motor coordination. In contrast to our results, Collucia et al. showed that n-3 PUFA supplementation in both juvenile and adult rats improved performance in the rotarod at fixed but not accelerating speeds [60]. During development, Purkinje cells are innervated by multiple climbing fibers. Around PND 21, elimination of synapses occurs, leading to mono-innervation of Purkinje cells [79]. Bearzatto et al. compared three common wild-type mouse strains and suggested a delay in the elimination of multiple climbing fibers innervating the Purkinje cells in adult C57BL/6J mice [79]. Our findings have led us to postulate that the possible delay in elimination of multiple climbing fibers in C57BL/6J mice as described by Bearzatto et al. might be shortened by adequate dietary n-3 PUFA availability. As a result, the plateau in the development of motor coordination may be reached already before adulthood.

The results of the MWM test indicate that spatial memory at PND 60 was improved in n-3 PUFA adq animals, but spatial learning remained unaffected. Several studies have stated the probable relation of learning and memory functions with brain fatty acid status [12,14]. Moriguchi et al. showed that n-3 PUFA deficiency in second- and third-generation deprived adult rats caused an impairment in performing the MWM task [80]. The second-generation n-3 adequate fed rats showed improved spatial learning and memory as compared to the deficient rats, and the effect was even stronger in the third generation. However, Carrié et al. found no effects on spatial learning or memory in adult female mice demonstrated in both an n-3 PUFA deficiency model and an n-3 PUFA supplementation study [59,72]. A study performed by Kavraal et al. underlined the importance of n-3 PUFA availability starting early on at gestation in rats [81]. In that study, both dams and offspring were supplemented with n-3 PUFA, and offspring was also supplemented with n-3 PUFA from gestation and throughout life. However, in contrast to our results, an increase in spatial learning was demonstrated, whereas there was no significant effect on spatial memory. The limitation of that study was that the supplemented diet was compared to a standard rat chow, which contained 1.9% ALA, preventing complete n-3 PUFA depletion [81]. These studies show the importance of choosing the right starting point for supplementation and composition of diets.

In the present study, 31P MRS demonstrated no effects of n-3 PUFA availability on phosphorylated energy and lipid metabolites. As neurogenesis was decreased in n-3 PUFA adequate animals, a change in the PME levels reflecting membrane formation was expected. A clinical 31P MRS study in epilepsy patients showed that n-3 PUFA supplementation reduced membrane phospholipid breakdown, whereas it improved brain energy metabolism [82]. However, a postmortem observational study of schizophrenia patients revealed no changes in phospholipid metabolism measured by 31P MRS [83]. Studies using 1H MRS have further elucidated the effect of n-3 PUFA supplementation on cerebral metabolite status [13,84]. McNamara et al. demonstrated a decrease of myo-inositol in the prefrontal cortex of n-3 PUFA deficient adult rats [13]. It is hypothesized that this reduction may be caused by impaired astrocyte maturation and deficits in osmotic regulation. Another study using 1H MRS revealed
that ethyl-EPA has the ability to protect the human brain from early psychosis by increasing glutathione levels in the temporal lobes and increasing glutamate/glutamine levels in the left hippocampus [85]. These studies indicate that 1H MRS may provide a more sensitive method to detect effects on cerebral metabolite status that would help to understand the changes in behavioral and cognitive tests in the present experimental setup, such as improvement of spatial memory in the MWM and neuronal functionality as reflected by decreased neurogenesis. Furthermore, we were only able to measure whole brain metabolite status with our 31P MRS method. Possibly, measurements distinguishing between different brain regions such as hippocampus and cortex would be more sensitive to detect metabolite changes.

The qRT-PCR data on neurogenesis revealed no diet effect on DCX mRNA expression in the subcortical area (containing hippocampus and subcortical structures). (This may have led to a weakening of possible effects in DCX mRNA expression.) Another explanation may be that n-3 PUFA already exert their effect before puberty. Strikingly, immunohistochemical staining for DCX demonstrated decreased hippocampal neurogenesis in n-3 PUFA adequate fed young adult mice. To our knowledge, this is the first study to show diminished neurogenesis in young adult mice due to altered n-3 PUFA availability during development. Contrarily, several studies have illustrated an increase of neurogenesis after LCPUFA supplementation [58,61,86]. Niculescu et al. assessed hippocampal neurogenesis of n-3 PUFA supplemented mice at the end of the lactation period (PND 19) and detected an increase in cell proliferation [58]. In another study, dietary LCPUFA supplementation led to increased neurogenesis in juvenile rats [61]. Dietary DHA has been suggested to promote neurogenesis and synaptogenesis during embryonic development [22]. It should be noted that these studies were performed at an earlier age than the immunohistochemical staining in our experiment. Additionally, n-3 PUFA supplementation counteracted the reduction of hippocampal neurogenesis in an immune deprived mouse model [86]. Combined, these data suggest that n-3 PUFA availability may promote earlier neurogenesis, possibly already before pubertal age.

In the current study, immunohistochemical staining for synaptophysin and PSD95 showed no effect on presynaptic and postsynaptic density, respectively, indicating that synaptogenesis remained unaffected in adulthood. However, our results obtained by qRT-PCR indicate that synaptic plasticity may be increased at pubertal age in the cortex, while it remains unaffected at adult age. Several studies demonstrated an increased expression of presynaptic and postsynaptic proteins after n-3 PUFA supplementation, hinting at increased synaptogenesis either early on in life or during aging [22,51,86]. Our data on synaptophysin expression led us to hypothesize that synaptogenesis is stimulated at adolescent age on an n-3 PUFA adequate diet and reaches a plateau before adult age. We postulate that neurogenesis may already be accelerated before puberty, as our data show unaffected DCX mRNA expression during puberty and adulthood. Our immunohistochemistry data on hippocampal neurogenesis provide evidence that n-3 PUFA induce a plateau in neurogenesis before adulthood. In line with these results, the n-3 PUFA adequate animals showed enhanced performance on the rotarod in puberty, but not in adulthood. At young adult age, n-3 PUFA adequate fed mice still demonstrated improved spatial memory in the MWM. Additional gene expression analysis (for example, cAMP response element binding protein that is involved in long-term potentiation or peroxisome proliferator-activated receptor and retinoid X receptor that is involved in regulating synaptic plasticity and in learning and memory) may further support our findings.

In the present study, only DHA levels in erythrocyte membrane were affected by the dietary intervention, while all brain regions tested displayed decreased levels of ARA and increased levels of DHA and DGLA as well. This decrease in ARA might be explained by the competition of n-3 and n-6 PUFA for the conversion enzymes delta-5-desaturase and delta-6-desaturase, which favor the conversion of n-3 PUFA [27,28]. Another explanation could be a competition in tissue uptake due to the administration of preformed DHA [87]. These changes in n-3 and n-6 PUFA are in the same range as observed in previous studies in rats [14,80]. The results acquired in the current study underline the importance of brain fatty acid status during development. Besides, the expression of inflammatory markers remained unaffected by n-3 PUFA availability during development. N-3 PUFA supplementation has been shown to decrease neuroinflammation in immune deprived and aged mice [86,88]. It is not likely that the young, healthy C57Bl/6j mice used in our experiments are prone to inflammation at this stage in life, which is supported by the fact that n-3 PUFA availability did not affect inflammatory markers.

In summary, the current study indicates that adequate dietary n-3 PUFA levels early in life improve neural development in the cerebellar cortex during puberty, as reflected by improved motor coordination. Additionally at adult age, we observed cortical and hippocampal changes with increased habituation in the prepulse inhibition, increased exploratory behavior and decreased anxiety in the open field, increased spatial memory in the MWM, a decrease in neurogenesis and unaltered synaptogenesis in immunohistochemistry. qRT-PCR revealed that n-3 PUFA increased synaptogenesis in the cortex at pubertal age but did not change neurogenesis in the subcortical area. The overall level of cerebral phosphorylated metabolites as measured by in vivo 31P MRS was not altered by n-3 PUFA. Expression of inflammatory markers also remained unaffected in both puberty and young adulthood. Finally, we demonstrated that dietary modifications led to increased n-3 PUFA and decreased n-6 PUFA in cerebral fatty acid status, as well as increased DHA in erythrocyte membranes.

We postulate that perinatally administered dietary n-3 PUFA have the potential to improve cortical and hippocampal development and enhance cognitive functioning. While the present study focused on dietary n-3 PUFA availability in a healthy mouse model, it already yields a range of beneficial effects in brain functioning. Overall, our findings indicate that ensuring adequate dietary n-3 PUFA levels starting early in life may support optimal neural development in healthy full-term infants. Furthermore, our results implicate that it would be interesting to study the effect of a sufficient n-3 PUFA intake in disorders caused by an n-3 PUFA deficiency more extensively.

Acknowledgements

The authors would like to thank Professor Thomas Brenna for the fatty acid analysis of brain and erythrocytes. We would like to acknowledge Janneke Mulders and the Central Animal Laboratory of the Radboud university medical center for taking excellent care of our mice. Furthermore, we would like to thank Jos Dederen, Inge SMEETS, Annelies van Nuland, Maarten van Dijk, Sarita Dam and Elina Samani for their laboratory work.

This work was sponsored by Mead Johnson Pediatric Nutrition Institute.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2014.08.002.

References


