British Journal of Nutrition (2022), 128, 1509–1517 doi:10.1017/S0007114521004359

Inhibited maturation of astrocytes caused by maternal *n*-3 PUFA intake deficiency hinders the development of brain glial cells in neonatal rats

Tatsuro Yamamoto[1]*†, Ayako Yamamoto[1], Hiroki Tanabe[1] and Naomichi Nishimura[1,2]†

[1]*Department of Nutritional Sciences, Faculty of Health and Welfare Science, Nayoro City University, Nayoro, Hokkaido 096-8641, Japan*

[2]*College of Agriculture, Academic Institute, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

(Submitted 9 April 2021 – Final revision received 13 October 2021 – Accepted 27 October 2021 – First published online 2 November 2021)

Abstract

The brain is rich in long-chain PUFA, which play an essential role in its development and functions. Here, we examined the impact of maternal *n*-3 PUFA intake deficiency during gestation and lactation on the development of glial cells in the pup's developing cerebral cortex. In addition, using myelination as indicator and the anti-myelin basic protein as measurement to establish the relationship between the number of glial fibrillary acidic protein (GFAP)-positive cells and the development of oligodendrocytes, we determined the myelination state of the somatosensory cortex at postnatal day 14. Rat dams were fed either a control (Cont) or an *n*-3 PUFA-deficient (Def) diet for 60 d (acclimatisation: 14 d; gestation: 21 d; and lactation: 21 d). Pups lactated from dams throughout the experiment. The distribution pattern of astrocytes in pups on postnatal day 7 was immunohistochemically analysed using GFAP and brain lipid binding protein (BLBP) as markers for mature astrocytes and astrocyte-specific radial glial cells, respectively. It was observed that, when compared with Cont pups, GFAP-positive cells decreased, BLBP-positive cells increased and myelinated structures were sparser in the somatosensory cortices of Def pups. In the open field test on postnatal day 21, behavioural parameters did not differ between groups. Our results indicated that inhibited maturation of astrocytes caused by maternal *n*-3 PUFA deficiency hindered the development of brain glial cells of neonatal rats; hence, maternal *n*-3 PUFA intake during the gestation and lactation periods may have been crucial for the brain cell composition of pups.

Key words: ***n*-3 PUFA: Astrocyte: Oligodendrocyte: Myelin sheath: Rats**

Cell membranes, in particular brain cells, are rich in PUFAs, which are the structural components of phospholipids that are mostly located in the sn-2 position[1,2]. An intake of a diet deficient in these fatty acids may lead to a decrease of DHA (22:6 (*n*-3)) and an increase in docosapentaenoic acid (22:5 (*n*-6))[3,4] in the brain, resulting in impaired visual functions and behaviour and learning changes in mature humans and rodents[5,6]. Previous studies showed that a maternal *n*-3 PUFA deficiency during pregnancy affects neuronal migration and laminar formation in the hippocampus and the cerebral cortex[7,8]. Those studies also showed that fatty acid binding protein 7 (also known as brain lipid binding protein, BLBP) assisted in the uptake of *n*-3 PUFA into cells and was abundant in neuronal epithelial cells at the early stage of brain development and essential for the maintenance of neuroepithelial cells. Those studies suggested that *n*-3 PUFA were key molecules in brain development[9]. Nonetheless, past work mostly focused on the relationship between *n*-3 PUFA and neuronal migration and differentiation, upon intake of *n*-3 PUFA. Therefore, to the best of our knowledge, little work has been conducted to elucidate the status of the development of glial cells when diets are deficient in *n*-3 PUFA.

Glial cells are 10-fold more abundantly distributed in the brain than neurons. Barres (2008) reported that glial cells aided in the migration, survival and maintenance of neurons, by adjusting neurotransmission and nourishing neurons[10]. Furthermore, glial cells have been reported to be capable of modulating the electrical activity of neurons and to perhaps directly participate in the information processing in the central nervous system[11]. In the mammalian central nervous system, whilst neurons are generated primarily during the embryonic period, glia are mostly formed after birth[12,13]. In addition, studies using mice showed that whilst in early development neural stem cells differentiated mainly to neurons, in late development, they did to astrocytes (a type of glia cell), which peaked in numbers immediately after birth[13]. Sakayori *et al.* showed that in mice fed a high *n*-6/*n*-3

Abbreviations: BLBP, brain lipid binding protein; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein.

* **Corresponding author:** Tatsuro Yamamoto, email tyama@nayoro.ac.jp

† These authors contributed equally to this work.

ratio diet, the timing of astrocyte and neuron production was disrupted, and the number of astrocytes produced during embryonic stages increased[14]. These findings seem to indicate that *n*-3 PUFA are important factors for the regulation of astrocyte production.

Previous studies reported the relationship between psychiatric disorders in infancy and nutritional factors[15,16]. Thus, investigating the role of *n*-3 PUFA in the brain may lead to further understanding of the mechanisms of psychiatric disorders in order to develop new nutritional treatments. The present study was designed to investigate whether postnatal glial development in rat pups was influenced by maternal dietary *n*-3 PUFA deficiency. To achieve this, we conducted a comparative histological analysis using anti-glial fibrillary acidic protein (GFAP), anti-myelin basic protein (MBP) and anti-BLBP antibodies on postnatal day 7 and 14. Anti-GFAP and anti-MBP were used to identify mature astrocytes and myelin sheaths, respectively. In addition, BLBP was used as a marker for cells that exhibited astrocyte-like properties in radial glial cells[17–19]. Furthermore, since it has been shown that the decrease in DHA in the brain and the disturbance of fatty acid balance caused by the intake of *n*-3 PUFA-deficient diet induce anxiety behaviour[14], behavioural changes in *n*-3 PUFA-deficient rat pups were monitored using an open field test. These studies revealed the importance of mother's intake of *n*-3 fatty acid, especially α-linolenic acid, for the brain development of pups.

This study is to clarify whether *n*-3 fatty acid, especially α-linolenic acid, consumed by mother rats is important for the brain development of their offspring, because DHA is abundantly synthesised from α-linolenic acid in the liver of mother rats.

Materials and methods

The present study was carried out with the approval of the Committee for Animal Care and Welfare of Nayoro City University. Animals were kept and cared for as per the Guidelines for the Care and Use of Laboratory Animals issued by Nayoro City University.

Animals and diets

Twelve female and twelve male 8-week-old Sprague-Dawley rats were purchased from Japan SLC. The animals (approval number: 09–11) were housed in individual cages with screen bottoms made of stainless steel and kept in a room maintained at 23 ± 2°C, with 50–70 % relative humidity, and in a 12 h/12 h light–dark cycle (light period, 07.00–19.00). The animals had free access to food and water. Two different dietary formulations were prepared by altering the fatty acid composition of AIN-93 diet: adequate *n*-3 PUFA content (control, Cont) and deficient *n*-3 PUFA content (deficient, Def) diets (Tables 1 and 2). Female rats were equally divided (*n* 6) and fed these diets 2 weeks prior to mating and afterwards, throughout gestation. In contrast, all male rats were fed only Cont for 14 d. On day 14, one female rat from each group was mated overnight with one male and became pregnant. Pregnancy was determined by the presence of a vaginal plug. After mating, the male was removed from the cage, and the day was considered as embryological day 0·5. Some of the pups were used to analyse fatty acid composition and morphological changes in the brain, while the remaining pups were used for behavioural study.

Table 1. Nutrient composition of control (Cont) and *n*-3 PUFA-deficient (Def) diets of rat dams

	Cont	Def
	g/kg	
Casein	200	200
Maize starch	529·486	529·486
Sucrose	100	100
Cellulose	50	50
Mineral mix*	35	35
Vitamin mix†	10	10
L-cystine	3	3
Choline bitartrate	2·58	2·58
t-BHQ	0·014	0·014
Hydrogenated coconut oil	20	25
Soyabean oil	50	–
Sunflower oil	–	45

Cont, *n*-3 PUFA-adequate control; Def, *n*-3 PUFA-deficient; t-BHQ, tertiary butylhydroquinone.
* AIN-93G mineral mix.
† AIN-93 vitamin mix.

Table 2. Fatty acid composition of diets of rat dams

	Cont	Def
	%	
16:0 (palmitic acid)	0·63	0·28
18:0 (stearic acid)	0·18	0·12
18:1 (oleic acid)	0·81	1·35
18:2 (linoleic acid)	2·76	2·71
18:3 (α-linolenic acid)	0·64	0·01
20:4 (arachidonic acid)	0	0·01
n-6/*n*-3	4	272
18:2 *n*-6/18:3 *n*-3	4	271

Cont, *n*-3 PUFA-adequate control; Def, *n*-3 PUFA-deficient.

Tissue preparation

On postnatal day 7 and 14, two pups were randomly selected from each of the litters of the pregnant rats, anesthetised with Na pentobarbital (100 mg/kg body weight; intraperitoneal injection) and killed by transcardial perfusion with 4 % paraformaldehyde in 0·1 M phosphate buffer (pH 7·4). After perfusion, the brains of the pups were immediately removed from the skulls and each divided in half along the longitudinal fissure of the cerebrum. The left and right cerebral hemispheres were used for lipid analysis and the evaluation of the distribution pattern of glial cells in the cerebral cortex, respectively. The cerebral hemispheres chosen for lipid analysis were immediately frozen in liquid N_2 and stored at −80°C until further analysis. The other hemispheres chosen for immunohistochemical analysis were postfixed in 4 % paraformaldehyde in 0·1 M phosphate buffer (pH 7·4) for 1 h at 4°C. Afterwards, the brain samples were dehydrated in a series of alcohol solutions ranging from 70 to 100 %

ethanol, cleared in xylene and embedded in paraffin. The brain hemispheres were then coronally sectioned with a thickness of 4 μm, using a sliding microtome (Leica, SM-2000R). The sections were mounted on MAS-coated slide glasses (Matsunami).

Lipid analysis

Lipids were extracted from the cerebral hemispheric samples (*n* 6, each group) on postnatal day 7 and 14 as per the method of Folch *et al.* (1957)[20]. Next, fatty acids were converted to methyl ester derivatives and quantified by GC. The experiment was conducted following previously reported procedures[2,21]. A GC system (GC-17A, Shimadzu) equipped with a CP-Sil 88 column (0·25 mm × 50 m, film thickness 0·2 μm, Agilent Technologies) and a flame ionisation detector was used for the fatty acid methyl ester analysis. The composition of the fatty acids was calculated based on their gas chromatographic areas.

Evaluation of distribution pattern of glial cells in the cerebral cortices of suckling pups

To examine the distribution pattern of glial cells in the cerebral cortices of suckling pups, we carried out an immunohistochemical analysis on the glial cells of their cerebral cortices on postnatal day 7 and 14. Cerebral cortical sections were incubated with the following primary antibodies: mouse anti-GFAP (1:1000; Frontier Science, Inc.), rabbit anti-BLBP (1:1000; Sigma-Aldrich, Inc.) and anti-MBP (1:1; Nichirei Biosciences, Inc.). Prepared sections were deparaffinised in xylene, rehydrated in 100 % alcohol for 10 min three times and then in alcohol with the following descending grades: 95, 90, 80 and 70 %, for 5 min each. Next, the sections were incubated in 0·01 M citric acid buffer (pH 6) for 40 min at 98°C, to activate the antigens, and incubated overnight at 4°C with the primary antibodies. For the detection of the GFAP, the sections were incubated at 4°C for 2 h, using biotin-conjugated mouse or rabbit IgG (Vector Laboratories) as a secondary antibody, and reacted with diaminobenzidine. Similarly, for the detection of BLBP and MBP, the sections were incubated at 4°C for 2 h using Alexa Fluor 488-conjugated rabbit IgG (Invitrogen). Diaminobenzidine and fluorescent signals were detected using an upright fluorescence microscope (Axio imager, Zeiss). Three microscopic fields of view were measured per individual, and the same process was performed on the brain tissue of six animals in each group. The positive cells per field were evaluated by two external blinded assessors.

Open field test. To evaluate the spontaneous locomotor activity and anxiety-like behaviour, we carried out an open field test on postnatal day 21. Open field test was performed in the morning (09.00–12.00). Two male rats randomly selected from the same litter were used for the open field test, with a total of twelve rats in each group. Female rats were not used in the test due to expected sex effects[22]. The rats were transported to the behaviour testing room 30 min before the test to habituate to the conditions of the room. The open field arena (90 cm × 90 cm × 30 cm) made of the iron plate (Yui sheet metal industry, Inc.) was used. The field area was divided equally into thirty-six sections by drawing lines. A rat was placed gently in a corner region of the field. Its behaviour was recorded by a video camera from above. After a 5-min trial, the rat was returned to its home cage. Then, the open field arena was cleaned with alcohol and wiped with a Kimtowel (Nippon Paper Crecia Co.) to dry the field. The total number of moving compartments and time spent in the centre region were measured while observing the recorded images. Locomotor activity and anxiety-like behaviour were assessed by the total number of moving compartments and the time spent in the centre region of the open field, respectively.

Statistical analysis

To determine the adequate sample size to identify significant differences in the concentrations of DHA in the brains of the pups, a Student's *t* test power analysis was carried out using the G × Power statistical package version 3.1.9.6. The sample size was then calculated considering an α probability of 0·05 with a power of 0·80. The effect size was estimated using the results from a preliminary study conducted at these premises (unpublished results). From the power analysis, it was determined that the required sample size per group was six dams. Values obtained from the body weight, the food consumption data and the fatty acid profiles in the brains were expressed as means ± SEM. Data were analysed by the Student's *t* test using SAS JMP software (version 10.0, SAS Institute). The total area of cells and fibres which expressed GFAP and MBP per unit of area, and the number of BLBP-positive cells in the primary somatosensory cortices were quantified using ImageJ software (version 1.52, NIH). Eighteen different slices from each diet group were counted and observed. Mean values were significant if $P < 0{\cdot}05$.

Results

DHA concentrations in the brains of n-3 PUFA-deficient pups

No differences were observed in food intake (Fig. 1(a)) or body weight gain between non-pregnant and pregnant females within the two dietary groups (Fig. 1(b)). Similarly, no differences were detected on postnatal day 0, 7 and 14 between the body weight gain of pups from the Cont and the Def groups (Fig. 1(c)).

To investigate whether the *n*-3 PUFA intake deficiency in dams affected the brain fatty acid profiles, the fatty acid composition of the brain hemispheres of pups was analysed on postnatal day 7 and 14. As observed on postnatal day 7, maternal *n*-3 PUFA intake deficiency did not exert a significant effect on the levels of palmitic acid (16:0) and stearic acid (18:0), which are SFA, or oleic acid (18:1, *n*-9) and arachidonic acid (20:4, *n*-6), which are unsaturated fatty acids (Fig. 2(a)). However, on the same day, the concentrations of DHA (22:6, *n*-3) in the brains were significantly lower in pups from dams fed the Def diet than in those of pups whose dams were fed the Cont diet (Fig. 2(a)). On postnatal day 14, similar differences were also observed in the brains (Fig. 2(b)).

Effect of n-3 PUFA deficiency on the postnatal development of astrocytes

To compare the postnatal development of astrocytes on postnatal day 7 in pups, an immunohistochemical analysis using

British Journal of Nutrition

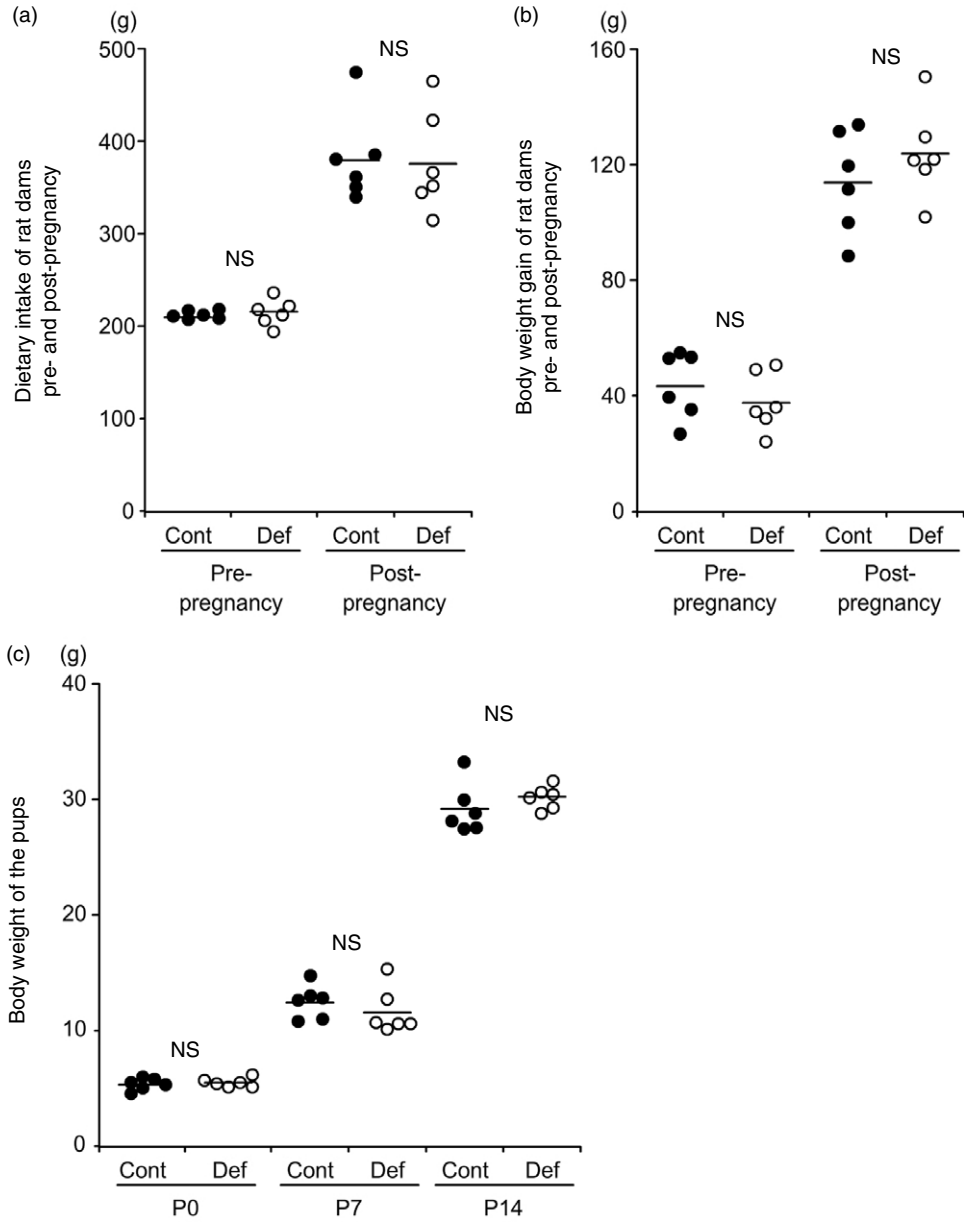

Fig. 1. Food intake and body weight gains 2 weeks pre-pregnancy and 3 weeks post-pregnancy of dams, and body weights of pups on postnatal day 0, 7 and 14 in the Cont and the Def groups. (a) Food intake of dams. (b) Body weight gains of dams. (c) Body weights of pups on postnatal day 0, 7 and 14. In the graph, closed circles indicate Cont individuals and open circles indicate Def individuals. As the data between the two groups are equally distributed, the bars in the graph show the mean values. Cont, *n*-3 PUFA-adequate control; Def, *n*-3 PUFA-deficient.

an anti-GFAP antibody was conducted. It was observed that GFAP-positive cells and their projections were distributed from the superficial to the deep layers of the somatosensory cortices in all pups, and these cells were especially abundant in the superficial glial limitans and the deep layers (Fig. 3(a)–(d)). The density of GFAP-positive areas in the deep layers of the somatosensory cortices was significantly lower in pups from the Def group when compared with pups from the Cont group (Fig. 3(e)). These reductions in GFAP-expressing cells and projections were also observed in the primary somatosensory cortices of pups from the Def group on postnatal day 14 (online Supplemental Fig. 1).

Brain lipid binding protein-expressing cells in n-3 PUFA-deficient pups

On postnatal day 7 and 14, GFAP-positive areas were significantly lower in the Def pups than in the Cont pups. We theorised that these results were probably associated with the thickness of the astrocyte projections as well as the number of astrocyte cells. Therefore, the number of positive cells of radial glial cells, which are the progenitor cells of astrocytes, in the two groups was compared, using BLBP as an astrocyte-specific radial glial cell marker[17–19]. On postnatal day 7, BLBP-expressing cells were observed in all layers of the primary somatosensory cortices of both groups (Fig. 4(a) and (b)). Nonetheless, the number of

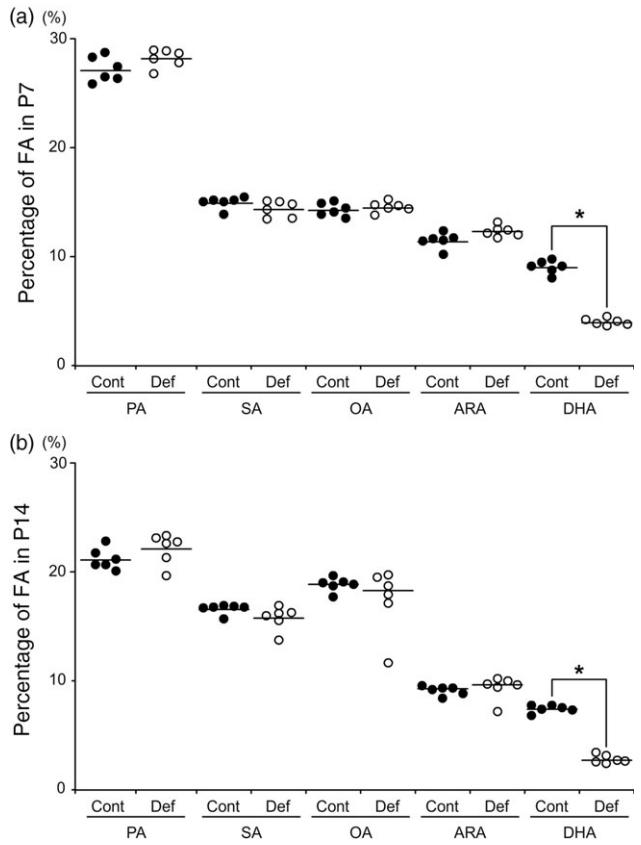

Fig. 2. Fatty acid composition of the cerebral hemisphere of Cont (closed circles) and Def pups (open circles). (a) Postnatal day 7 (P7). (b) Postnatal day 14 (P14). Asterisks indicate significant differences ($P < 0{\cdot}01$). In the graph, closed circles indicate Cont individuals and open circles indicate Def individuals. The OA and ARA data in B are unequally distributed, and therefore the bars show the median. For the other data, the bars in A and B show the mean values because they are equally distributed. Cont, *n*-3 PUFA-adequate control; Def, *n*-3 PUFA-deficient, PA, palmitic acid; SA, stearic acid; OA, oleic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid.

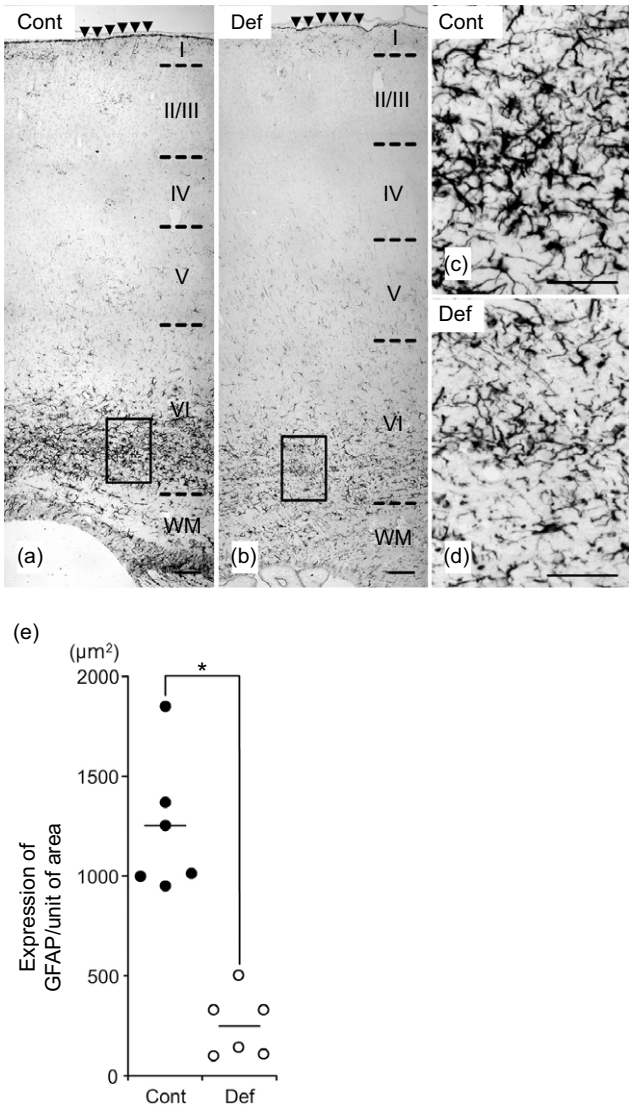

Fig. 3. Distribution of glial fibrillary acidic protein (GFAP)-positive astrocytes in the somatosensory cortices of Cont and Def pups on postnatal day 7. I-VI, cortical layers I-VI. GFAP-positive cells and their projections were distributed from the superficial to the deep layers of the somatosensory cortices of Cont (a) and (c) and Def pups (b) and (d). The arrowheads in (a) and (b) indicate the glial limitans on the surface of the pia matter. (c) and (d) are enlarged images of the areas in the squares in a and b, respectively. The GFAP-positive areas in the deep layers of the somatosensory cortices were measured using ImageJ software (e). In the graph, closed circles indicate Cont individuals and open circles indicate Def individuals. As the data between the two groups are unequally distributed, the bars in the graph show the median. Asterisk indicates different from the Cont group ($^*P < 0{\cdot}05$). Scale bars = 50 μm in a and b and 25 μm in c and d, respectively. Cont, *n*-3 PUFA-adequate control; Def, *n*-3 PUFA-deficient, WM, white matter.

BLBP-expressing cells per unit of area was significantly greater in Def pups than in Cont pups (Fig. 4C).

Myelination in n-3-deficient pups on postnatal day 14

As astrocyte maturation has been reported to be a factor promoting myelination by oligodendrocytes, the development of myelination in the somatosensory cortices was immunohistochemically compared on postnatal day 14 using an anti-MBP antibody. Neat myelin sheaths were observed in the white matter and cortical regions of the primary somatosensory cortices of Cont pups, whereas these myelin sheaths were found more sparsely in the same regions in Def pups (Fig. 5(a)–(d)). The density of MBP-positive areas in the white matter of the somatosensory cortices was significantly lower in pups from the Def group compared with pups from the Cont group (Fig. 5(e)).

Open field test in n-3-deficient pups on postnatal day 21

To evaluate behavioural changes in *n*-3 PUFA-deficient pups resulted from decreased astrocyte distribution and myelination in the brain, we performed the open field test on postnatal day 21 in both groups. The total number of moving compartments (Fig. 6(a)), assessing locomotor activity, and the time spent in the centre region (Fig. 6(b)), assessing anxiety-like behaviour, did not differ significantly between the two groups.

Discussion

In the present study, we examined whether maternal *n*-3 PUFA intake deficiency affected the development of astrocytes and

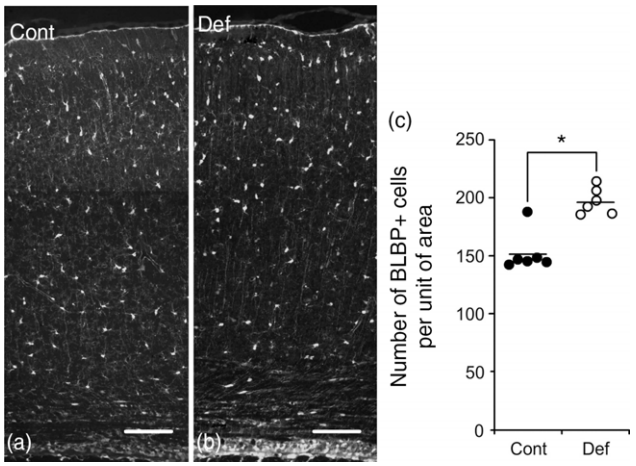

Fig. 4. Changes in the number of BLBP-expressing cells in the somatosensory cortices of Cont and Def pups on postnatal day 7. (a) and (b) Distribution of BLBP-expressing cells. (c) The number of expressing cells. In the graph, closed circles indicate Cont individuals and open circles indicate Def individuals. As the data between the two groups are equally distributed, the bars in the graph show the mean values. All scale bars = 100 μm in (a) and (b). Cont, *n*-3 PUFA-adequate control; Def, *n*-3 PUFA-deficient.

oligodendrocytes in the cerebral cortices of rat pups. The body weights of dams during pregnancy were not different regardless of whether *n*-3 PUFA were adequately supplied or not (Fig. 1(a) and (b)), as reported by other investigators[7]. The present study also demonstrated that body weights of pups during lactation (e.g. postnatal days 0, 7 and 14) were similar between Cont and Def pups (Fig. 1(c)). Although in general, the presence or absence of maternal dietary nutrients affects the growth of embryos and neonates, in the case of *n*-3 PUFA intake deficiency, there was not apparent effect on growth. Our results were consistent with those from previous reports[7,23].

The present study demonstrated that the DHA levels in the cerebral hemispheres of Def pups significantly decreased when compared with those of Cont pups. The results may have been more pronounced when diets with a higher *n*-6/*n*-3 ratio were used, which would have promoted *n*-3 deficiency. DHA uptake by brain cells is pivotal during both fetal and neonatal periods, when brain development and formation are promoted. Also, during these periods, the rate of DHA accumulation in the brain is faster than after adulthood is reached[24–26]. Therefore, it is necessary to adequately supply DHA to fetuses and neonates via the maternal placenta and milk because of their low ability to synthesise DHA on their own[27,28]. In the present study, dams in the Def group were fed a diet containing extremely low levels of *n*-3 PUFA, especially α-linolenic acid, which is used in the liver as a substrate for DHA synthesis[29]. Lactating mothers with low intakes of *n*-3 PUFA produce milk with remarkably high *n*-6/*n*-3 ratios[30]. Thus, although in the present work the fatty acid profiles of milk from dams were not measured, it can be reasonably assumed that for the Def pups to be *n*-3-deficient as it was observed on postnatal day 7 and 14, milk from the dams must have been consistently deficient in *n*-3 PUFA throughout the experiment. Whilst the enzymes for DHA biosynthesis have a high affinity for *n*-3 PUFA, when there is an *n*-3 PUFA deficiency, they can also promote the elongation and unsaturation of *n*-6

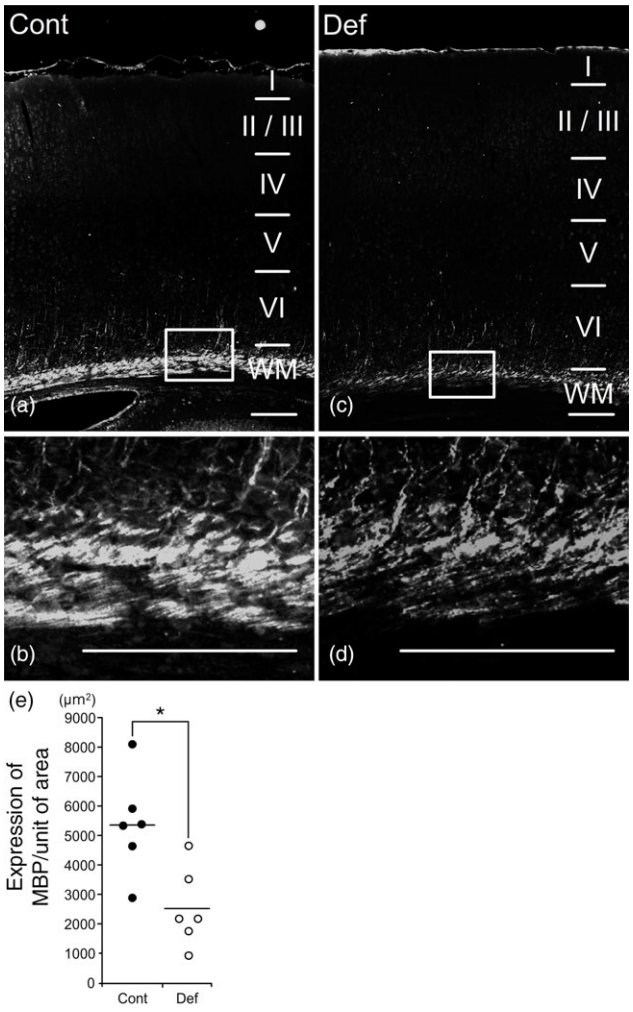

Fig. 5. Changes in the distribution of MBP-positive fibres in the somatosensory cortices in Cont and Def pups on postnatal day 14. (a) and (b), Cont pups. (c) and (d), Def pups. I-VI, cortical layers I-VI. Images in (b) and (d) are the areas in the rectangles in (a) and (c), respectively. The MBP-positive areas in the white matter of the somatosensory cortices were measured using ImageJ software (e). In the graph, closed circles indicate Cont individuals and open circles indicate Def individuals. As the data between the two groups are equally distributed, the bars in the graph show the mean values. Scale bars = 100 μm in (a) and (c) and 50 μm in (b) and (d). Cont, *n*-3 PUFA-adequate control; Def, *n*-3 PUFA-deficient; WM, white matter.

PUFA. Thus, it is not surprising that the content of *n*-6 PUFA such as docosapentaenoic acid (22:5, *n*-6) proportionately increases in the brain of *n*-3 PUFA-deficient rats as the concentration of DHA decreases[31]. In the present study, similar changes were also observed. Our results seemed to suggest that a deficiency of *n*-3 PUFA in maternal milk had a crucial effect on the fatty acid composition of cells in the central nervous system of pups, especially on the proportion of DHA. Such changes in the fatty acid profiles of brain would necessarily affect brain functions at later stages because they change the fluidity of cell membranes.

In the present study, on postnatal day 7 and 14, a decrease in GFAP-positive areas was observed in the somatosensory cortices of Def pups (Fig. 3 and online Supplemental Fig. 1). One of the possible reasons for this may be that *n*-3 PUFA are essential molecules for astrocyte maturation. In addition, the astrocyte

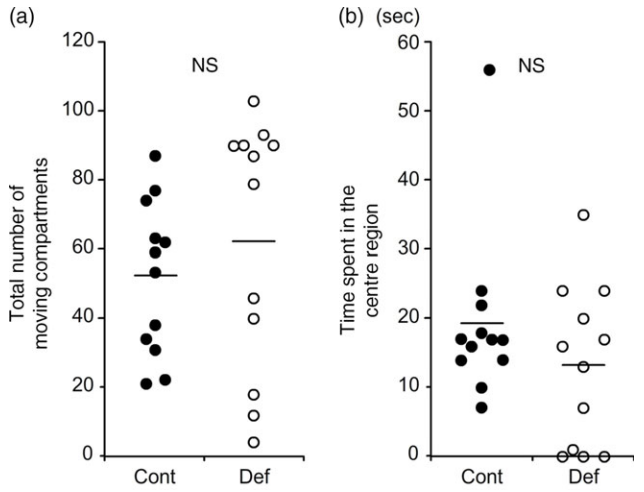

Fig. 6. Open field test for Cont and Def pups on postnatal day 21. The total number of moving compartments (a), assessing locomotor activity, and the time spent in the centre region (b), assessing anxiety-like behaviour. In the graph, closed circles indicate Cont individuals and open circles indicate Def individuals. As the data between the two groups are equally distributed, the bars in the graph show the mean values.

projections were thinner in brain samples from Def pups than in those from Cont pups (Fig. 3(c) and 3(d)). Moreover, astrocytes in Def pups seemed to be more immature when compared with those of Cont pups. We believed that the DHA level necessary for the proper maturation of astrocytes was insufficient for the Def group. In this regard, in a recent study that generated induced pluripotent stem cell-derived neural stem cell lines from patients with major depressive disorder, Yu *et al.* reported that *n*-3 PUFA promoted the differentiation of neural stem cells into astrocytes[32].

The fact that areas of GFAP-positive cells decreased in Def pups seemed to be unrelated to the decreased number of astrocytes in cerebral cortices. For example, Sakayori *et al.* demonstrated an increase in astrocyte production during the embryonic stage of mice consuming a high-*n*-6/*n*-3-deficient diet[14]. Furthermore, astrocyte production peaks immediately after birth in normal development[13]. Therefore, in the present work, it is believed that the number of astrocytes did not change by postnatal day 7. However, it is important to consider that the timing of astrocyte production in the cerebral cortex differed between Cont and Def pups, which may have been indicated by the decrease in GFAP-positive cells in Def pups. Furthermore, we found that the number of cells expressing BLBP, which represents the cellular lineage of postnatal astrocytes[17–19], was higher in the Def group than in the Cont group (Fig. 4), suggesting that *n*-3 PUFA intake deficiency, probably that of DHA, had an inhibitory effect on the differentiation of radial glial cells into astrocytes during postnatal brain development. Due to the lack of brain samples in this study, we were unable to quantify the expression levels of the proteins, which will be clarified in the future.

In the present study, on postnatal day 14, myelination of oligodendrocytes in Def pups was sparser than in Cont pups (Fig. 5). Yu *et al.* reported that *n*-3 PUFA promoted the production of neurotrophic factors from astrocytes[32]. One of the major trophic factors produced by astrocytes is a brain-derived neurotrophic factor, which stimulates the production of oligodendrocytes and the synthesis of myelin proteins, when the brain is exposed to a demyelination-causing injury[33,34]. Those studies, along with the results from the present study, suggest that the sparse myelination observed in Def pups may have been due to a lack of neurotrophic factors (e.g. brain-derived neurotrophic factor), resulting from astrocyte immaturity. It is recommended that further investigation on the extent to which myelination progresses long term in *n*-3 PUFA-deficient brains be conducted to determine whether sparse myelination is associated or not with a delayed maturation of oligodendrocytes.

Previous animal studies have shown that decreased *n*-3 PUFA in the brain increases several anxiety-related behaviours[35,36]. To evaluate the potential behavioural effects of inhibited maturation of astrocytes and the development of glial cells in *n*-3 PUFA-deficient pups on postnatal day 7 and 14, we carried out an open field test on postnatal day 21. Because it is difficult to conduct behavioural tests on pre-weaned pups (at postnatal day 7 and 14), we conducted the test on pups immediately after weaning (at postnatal day 21), but no significant difference was observed in behavioural parameters between the two groups (Fig. 6). This may be because the development of the brain structure of *n*-3 PUFA-deficient pups caught up with that of *n*-3-adequate pups at postnatal day 21. Unfortunately, we were not able to examine the brain structure of these rats in this study. Further investigation of not only the arrangement of glial cells at postnatal day 21 but also the layered structure of the cerebral cortex and the distribution of interneurons would provide further important insight into the effects of *n*-3 PUFA deficiency from fetus to infancy. *n*-3 PUFA deficiency has been also reported to be related to learning[37,38] and fear memory[39], and thus, it is necessary to examine the effects using a brightness discrimination learning test and a passive avoidance test in our model in the future.

In summary, our results showed that maternal *n*-3 PUFA intake deficiency, which inhibited maturation of astrocytes, hindered the development of brain glial cells of neonatal rats. Since previous reports showed that nutritional factors were related to the onset of some psychiatric disorders and neuropathy during the neonatal period[15,16], our findings may represent an important advance in our understanding of the initial mechanisms of these psychiatric diseases and could help lead to the development of nutritional treatments to be given during the perinatal period. Finally, although we conducted immunohistochemical analysis of astrocytes and the myelin sheaths of oligodendrocytes to elucidate the effect of *n*-3 PUFA deficiency on the brain structure, the underlying mechanisms by which maternal *n*-3 PUFA intake deficiency impaired the development of the brain structure in pups remained unclear. Further investigation is recommended to establish the association between the impaired development of the brain structure and abnormal behaviours due to maternal *n*-3 PUFA intake deficiency. Finally, although we showed that maternal *n*-3 deficiency could affect the development of glial cells in the rat pup brain in this study, the *n*-3-deficient diet used in this study is very extreme, with an *n*-6/*n*-3 ratio of 270. It should be emphasised that the ratio in the human diet is between 4 and 20[40–42], and the changes in brain

structure observed in this study in rat pups born and suckled from *n*-3-deficient dams do not directly apply to humans.

Acknowledgements

The authors thank editors at BioScience Proofreaders (Tokyo, Japan) for their assistance in the manuscript editing. In addition, the authors thank Miss. Ayuko Moriuchi, Miss. Chisato Kon and Miss. Maya Kato for technical assistants of the morphological analysis, and Toshio Terashima, Emeritus Professor of Division of Structural Medicine and Anatomy, Kobe University Graduate School of Medicine, for valuable comments regarding the manuscript.

The present study was supported in part by JSPS KAKENHI (Grant Numbers JP19700519 and JP21700759) from Japan Society for the Promotion of Science, Japan.

T. Y. and N. N. contributed to the conceptualisation, investigation, formal analysis, project administration and writing (original draft); A. Y and H. T. contributed to the investigation, formal analysis and writing (review and editing); T. Y. contributed to the funding acquisition.

The authors have no conflict of interest associated with the present study.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114521004359

References

1. Svennerholm L & Stallberg-Stenhagen S (1968) Changes in the fatty acid composition of cerebrosides and sulfatides of human nervous tissue with age. *J Lipid Res* **9**, 215–225.
2. Innis SM & de La Presa Owens S (2001) Dietary fatty acid composition in pregnancy alters neurite membrane fatty acids and dopamine in newborn rat brain. *J Nutr* **131**, 118–122.
3. Bourre JM, Durand G, Pascal G, *et al.* (1989) Brain cell and tissue recovery in rats made deficient in *n*-3 fatty acids by alteration of dietary fat. *J Nutr* **119**, 15–22.
4. Moriguchi N, Isokawa S, Ando A, *et al.* (2004) Experience with the use of salazosulfapyridine for intractable diarrhea after hematopoietic stem cell transplantation. *Int J Hematol* **80**, 463–466.
5. Heird WC & Lapillonne A (2005) The role of essential fatty acids in development. *Annu Rev Nutr* **25**, 549–571.
6. Bazan NG (2006) The onset of brain injury and neurodegeneration triggers the synthesis of docosanoid neuroprotective signaling. *Cell Mol Neurobiol* **26**, 901–913.
7. Coti Bertrand P, O'Kusky JR & Innis SM (2006) Maternal dietary (*n*-3) fatty acid deficiency alters neurogenesis in the embryonic rat brain. *J Nutr* **136**, 1570–1575.
8. Yavin E, Himovichi E & Eilam R (2009) Delayed cell migration in the developing rat brain following maternal *n*-3 α linolenic acid dietary deficiency. *Neurosci* **162**, 1011–1022.
9. Arai Y, Funatsu N, Numayama-Tsuruta K, *et al.* (2005) Role of Fabp7, a downstream gene of Pax6, in the maintenance of neuroepithelial cells during early embryonic development of the rat cortex. *J Neurosci* **25**, 9752–9761.
10. Barres BA (2008) The mystery and magic of glia: a perspective on their roles in health and disease. *Neuron* **60**, 430–440.
11. Newman EA & Zahs KR (1998) Modulation of neuronal activity by glial cells in the retina. *J Neurosci* **18**, 4022–4028.
12. Jacobson M (1991) *Developmental Neurobiology*, 3rd ed. New York: Plenum Press.
13. Qian X, Shen Q, Goderie SK, *et al.* (2000) Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* **28**, 69–80.
14. Sakayori N, Kikkawa T, Tokuda H, *et al.* (2016) Maternal dietary imbalance between *n*-6 and *n*-3 polyunsaturated fatty acids impairs neocortical development via epoxy metabolites. *Stem Cells* **34**, 470–482.
15. Carlson ES, Tkac I, Magid R, *et al.* (2009) Iron is essential for neuron development and memory function in mouse hippocampus. *J Nutr* **139**, 672–679.
16. Maekawa M, Takashima N, Matsumata M, *et al.* (2009) Arachidonic acid drives postnatal neurogenesis and elicits a beneficial effect on prepulse inhibition, a biological trait of psychiatric illnesses. *PLoS ONE* **4**, e5085.
17. Barry D & McDermott K (2005) Differentiation of radial glia from radial precursor cells and transformation into astrocytes in the developing rat spinal cord. *Glia* **50**, 187–197.
18. Schmid RS, Yokota Y & Anton ES (2006) Generation and characterization of brain lipid-binding protein promoter-based transgenic mouse models for the study of radial glia. *Glia* **53**, 345–351.
19. White RE, McTigue DM & Jakeman LB (2010) Regional heterogeneity in astrocyte responses following contusive spinal cord injury in mice. *J Comp Neurol* **518**, 1370–1390.
20. Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497–509.
21. Elias SL & Innis SM (2001) Infant plasma trans, n-6, and n-3 fatty acids and conjugated linoleic acids are related to maternal plasma fatty acids, length of gestation, and birth weight and length. *Am J Clin Nutr* **73**, 807–814.
22. Knight P, Chellian R, Wilson R, *et al.* (2021) Sex differences in the elevated plus-maze test and large open field test in adult Wistar rats. *Pharmacol Biochem Behav* **204**, 173168.
23. Pongrac JL, Slack PJ & Innis SM (2007) Dietary polyunsaturated fat that is low in (*n*-3) and high in (*n*-6) fatty acids alters the SNARE protein complex and nitrosylation in rat hippocampus. *J Nutr* **137**, 1852–1856.
24. Kuhn DC & Crawford M (1986) Placental essential fatty acid transport and prostaglandin synthesis. *Prog Lipid Res* **25**, 345–353.
25. Ruyle M, Connor WE, Anderson GJ, *et al.* (1990) Placental transfer of essential fatty acids in humans: venous-arterial difference for docosahexaenoic acid in fetal umbilical erythrocytes. *Proc Natl Acad Sci USA* **87**, 7902–7906.
26. Haggarty P, Page K, Abramovich DR, *et al.* (1997) Long-chain polyunsaturated fatty acid transport across the perfused human placenta. *Placenta* **18**, 635–642.
27. Carlson SE, Werkman SH, Peeples JM, *et al.* (1993) Arachidonic acid status correlates with first year growth in preterm infants. *Proc Natl Acad Sci USA* **90**, 1073–1077.
28. Weiler H, Fitzpatrick-Wong S, Schellenberg J, *et al.* (2005) Maternal and cord blood long-chain polyunsaturated fatty acids are predictive of bone mass at birth in healthy term-born infants. *Pediatr Res* **58**, 1254–1258.
29. Rapoport SI & Igarashi M (2009) Can the rat liver maintain normal brain DHA metabolism in the absence of dietary DHA? *Prostaglandins Leukot Essent Fatty Acids* **81**, 119–123.
30. Moriguchi T, Greiner RS & Salem N (2000) Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. *J Neurochem* **75**, 2563–2573.

31. Schiefermeier M & Yavin E (2002) *n*-3 Deficient and docosahexaenoic acid-enriched diets during critical periods of the developing prenatal rat brain. *J Lipid Res* **43**, 124–131.
32. Yu JZ, Wang J, Sheridan SD, *et al.* (2020) n-3 polyunsaturated fatty acids promote astrocyte differentiation and neurotrophin production independent of cAMP in patient-derived neural stem cells. *Mol Psychiatry* **435**, 417.
33. Fulmer CG, VonDran MW, Stillman AA, *et al.* (2014) Astrocyte-derived BDNF supports myelin protein synthesis after cuprizone-induced demyelination. *J Neurosci* **34**, 8186–8196.
34. Miyamoto N, Maki T, Shindo A, *et al.* (2015) Astrocytes promote oligodendrogenesis after white matter damage via brain-derived neurotrophic factor. *J Neurosci* **35**, 14002–14008.
35. Fedorova I & Salem N (2006) *n*-3 fatty acids and rodent behavior. *Prostaglandins Leukot Essent Fatty Acid* **75**, 271–289.
36. Lafourcade M, Larrieu T, Mato S, *et al.* (2011) Nutritional *n*-3 deficiency abolishes endocannabinoid-mediated neuronal functions. *Nat Neurosci* **14**, 345–350.
37. Bourre JM, Francois M, Youyou A, *et al.* (1989) The effects of dietary α-linolenic acid on the composition of nerve membranes, enzymatic activity, amplitude of electrophysiological parameters, resistance to poisons and performance of learning tasks in rats. *J Nutr* **119**, 1880–1892.
38. Ikemoto A, Ohishi M, Sato Y, *et al.* (2001) Reversibility of *n*-3 fatty acid deficiency-induced alterations of learning behavior in the rat: level of *n*-6 fatty acids as another critical factor. *J Lipid Res* **42**, 1655–1663.
39. Yamada D, Takeo J, Koppensteiner P, *et al.* (2014) Modulation of fear memory by dietary polyunsaturated fatty acids via cannabinoid receptors. *Neuropsychopharmacol* **39**, 1852–1860.
40. Sugano M (1996) Characteristics of fats in Japanese diets and current recommendations. *Lipids* **31**, S283–S286.
41. Lassek WD & Gaulin SJ (2011) Sex differences in the relationship of dietary Fatty acids to cognitive measures in american children. *Front Evol Neurosci* **3**, 5.
42. Simopoulos AP (2001) *n*-3 fatty acids and human health: defining strategies for public policy. *Lipids* **36**, S83–S89.

British Journal of Nutrition