Original Research

Neurochemical and behavioural impact of C18 fatty acids in male mice postweaning

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Abstract

Dietary components, particularly essential fatty acids, affect the expression and maintenance of normal physiological phenotypes. However, the influence of C18 fatty acids that are abundantly present in the normal diet is unclear. We focused on the behavioural and neurochemical effects of C18 fatty acids during postweaning development in male mice. An AIN-93G diet supplemented with 8% stearic acid (C18:0), 3% oleic acid (C18:1), 3% linoleic acid (C18:2) or 3% α-linolenic acid (C18:3) was provided from four weeks of age for eight weeks. At 12 weeks of age, novel exploratory behaviour and social interaction tests were carried out. One week after the last behavioural test, the brain of each mouse was removed. The frequency of social interactive behaviour was decreased by approximately 70% in the C18:0 group compared to the basal diet group, but there was no difference in cumulative time. The frequency of social interaction showed a positive correlation to cumulative time in mice fed with the experimental diets except for C18:0. Dietary C18 fatty acids following weaning had no impact on brain fatty acid composition except for the C18:3 diet. Furthermore, the neurochemical properties to be especially noted were that choline acetyltransferase activity was absolutely higher in C18:0 diet-fed mice than in the other groups, especially in the frontal cortex where it was 1.7-fold higher than in the basal diet-fed group. The present results reveal a significant possibility of neurochemical and behavioural effects of dietary fatty acids, and saturated fatty acids are of special importance during the postweaning period.

Keywords: Saturated fatty acid, postweaning period, choline acetyltransferase, monoamine oxidase, novel-exploratory behaviour, mice

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Introduction

For all animal species including humans, the diet is an indispensable resource to provide energy and essential nutrients. Especially, lipids are particularly important not only as a highly efficient source of energy but also as a major component of plasma membranes. The brain has the second greatest concentration of lipids, immediately after adipose tissue. Membrane lipids comprise 50–60% of the solid matter in the adult brain,[1] of which approximately 35% are in the form of long-chain polyunsaturated fatty acids (LCPUFAs), particularly arachidonic acid (C20:4 *n*-6) and docosahexaenoic acid (C22:6 *n*-3; DHA).[2,3] These LCPUFAs are derived from essential fatty acid precursors, linoleic acid (C18:2 *n*-6) and α-linolenic acid (C18:3 *n*-3). Unlike in the other tissues, the fatty acid composition of brain tissue is considered to be relatively constant during the postweaning period, not responding as readily to changes in dietary fat composition.[4,5] However, the difference in oils in maternal diets induced specific components of (*n*-6) and (*n*-3) fatty acids in fetal growth cone membrane,[6] and insufficient dietary (*n*-6) and (*n*-3) fatty acids during development altered brain C20:4(*n*-6) and C22:6(*n*-3) levels.[7,8] Furthermore, these maternal diet-mediated reductions of LCPUFAs in the postnatal brain were accompanied by several aspects of brain function, including brain weight,[8,9] decreased dopamine and serotonin levels,[10–12] habituation,[13] auditory-evoked brain responses and learning behaviour[8,14] when measured in postweaned animals. Consequently, the type of lipids in a diet, i.e. fatty acid composition, is considered to be extremely important for the expression and maintenance of normal physiological phenotypes, but the influence of C18 fatty acids, the most abundant of these groups, is unclear.

Genetic background is considered to govern the behavioural phenotypes determined in adulthood. However, a number of studies have indicated a critical role for

Table 1 Composition of the experimental diets based on AIN-93G

Ingredient	Basal	C18:0	C18:1	C18:2	C18:3
Milk casein	39.7486	39.7486	39.7486	39.7486	39.7486
β-Corn starch	20	20	20	20	20
α-Corn starch	13	13	13	13	13
Sucrose	10	10	10	10	10
Cellulose	5	5	5	5	5
AIN-93 mineral mixture	4	4	4	4	4
AIN-93 vitamin mixture	1	1	1	1	1
L-Cystine	0.3	0.3	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25	0.25	0.25
Butylhydroquinone	0.014	0.014	0.014	0.014	0.014
Safflower oil	2	2	2	2	2
Stearic acid	–	8	–	–	–
Oleic acid	–	–	2	–	–
Linoleic acid	–	–	–	2	–
Linolenic acid	–	–	–	–	2

Note: Dietary compositions are expressed as g% (w/w).

environmental conditions including nutritional factors in the development of the central nervous system and various behavioural processes. The postweaning period is fundamentally crucial for the establishment of social-behavioural phenotypes in animals that live in a group such as mice. Furthermore, nutritional composition in those periods has a major impact in the majority of animals. Behavioural indices of activation are closely associated with several neural systems including the dopaminergic, noradrenergic, serotonergic and cholinergic afferents systems.[15] In this study, we focused on catalytic enzymes for cholinergic and monoaminergic neurotransmission which strongly influence a variety of behavioural phenotypes including social behaviour as indices of neurochemical effects of C18 fatty acids during postweaning development in male mice.

Materials and methods

Animals and diets

All experiments were performed in accordance with the Guidelines for Animal Experiments, College of Bioresource Sciences, Nihon University and Nihon University's Animal Care and Use Committees. Primiparous mice of the dd strain (closed colony-derived non-inbred strain; albino) obtained from our own breeding colony were mated with male dd mice, housed in individual cages at $23 \pm 2^{\circ}$C under a 14-h light:10-h dark (lights on at 06:00) photoperiod until the behavioural analysis. They were allowed free access to food (Labo MR Standard; Nosan Corporation, Yokohama, Japan) and water. Three days after delivery, litter size was, in principle, adjusted to six pups, and the body weight of the mothers and litters, and food and water intake were measured once daily (09:00) to estimate the lactating performance. Each litter was weaned at 21 days of age, and male mice were housed three/cage with the control diet (Labo MR Stock; Nosan Corporation) for one week. At four weeks of age, mice were supplied with the respective diets described below in place of the control diets, and further housed for eight weeks.

A defatted, powderized AIN-93G diet (Research Diets, New Brunswick, NJ) was supplemented with 2% (w/w) safflower oil (MP Biomedicals, Solon, OH) as the basal diet, and 8% stearic acid (Wako Pure Chemical Industries, Osaka, Japan; C18:0), 3% oleic acid (Wako Pure Chemical; C18:1), 3% linoleic acid (Wako Pure Chemical; C18:2) or 3% α-linolenic acid (MP Biomedicals; C18:3, Table 1) was further added. The absorbability of long-chain saturated fatty acids is relatively lower compared with unsaturated fatty acid in diets. In previous research using fat-free powdered diet, the ratio of fatty acid contents was adjusted with added natural oil, 2–3%, and 2–3% of free unsaturated fatty acids and 6–8% in the case of saturated fatty acids (SAFAs). In this study, the amounts of the additive respective fatty acids added were based on Clarke et al.,[16] especially considering the low absorbability of C18:0. Distilled water was added to create a paste. Thereafter, each diet was lyophilized at -30°C, under vacuum less than 0.15 Torr, for 24–48 h. Immediately after the lyophilization, diets were vacuum-packed and stored at -20°C.

Determination of novel-social exploratory behaviour

When 12 weeks of age, the mice were moved from the breeding room to a behaviour-analysing room, maintained with a 14-h light:10-h dark (lights off at 12:00) photoperiod, to habituate to the environment. After one week of habituation, the behavioural test was carried out between 13:00 and 16:00 under dim illumination. A 60×45 cm open arena made of dark brown opaque acrylic plates with a wall height of 40 cm was used. A cylindrical tube with a diameter of 11.4 cm and a height of 20.5 cm, the lower part made of stainless steel with many small holes and the upper part made of a transparent acrylic plate, was placed at the centre of the arena. Each male mouse was placed alone on one side

of the arena and allowed to habituate to the test environment for 3 min. A social partner, a castrated dd mouse aged five to seven weeks from a breeding colony in our laboratory, was then placed in the cylindrical tube. Novel exploratory behaviour and social interactions were recorded with a digital video camera for 5 min. The behavioural activities analysed were as follows. Latency to start social behaviour: time until the first active approach towards the social partner. Social interaction: frequency and cumulative time of following and sniffing the partner during a 5-min test. These behavioural parameters are considered to be social partner-directed activities. Exploratory rearing behaviour: the frequency of rearing with hind legs. Self-grooming: frequency of bouts of self-grooming of the nasal region by the forelegs. These behavioural parameters are considered to be non-partner-directed activities. After the recording, the mice were returned to their home cages, and the test arena was immediately swept and cleaned with 35% isopropanol. Individual behavioural tests were conducted six times a day at 15–20-min intervals. One week after the last behavioural test, the whole brain of each mouse was removed, weighed and stored at −80°C.

Analysis of brain fatty acid composition

One cerebral hemisphere obtained from each mouse was lyophilized, and 15 mg of minced tissue and 250 mg of Glass beads (acid-washed, 150–212 μm; Sigma–Aldrich, St. Louis, MO) were collected in a test tube, weighed and crushed with a glass bar. The extraction of lipids and preparation of fatty acids methyl esters were carried out with a Fatty Acid Methylation Kit (Nakalai Tesque, Kyoto, Japan). Crude fatty acid methyl ester was infused into Bond Elut SI (cartridge silica gel packed column; Agilent Technologies, Santa Clara, CA), and purified using a Fatty Acid Methyl Ester Purification Kit (Nakalai Tesque). Finally, the purified samples were eluted from the column with 5 mL of a *n*-hexane-diethyl ether mixture (4:1, v/v; Wako Pure Chemical) and dissolved in 100 volumes of acetone (Wako Pure Chemical), and the solvent was evaporated away.

Fatty acid composition was analysed by gas chromatography (7890A GC System; Agilent Technologies) with an automatic liquid sampler (7683B; Agilent Technologies). Separation was carried out on a ULBON HR-SS-10, 50 m × 0.25 mm capillary column (FFS type; Shinwa Chemical Industries, Kyoto, Japan). The carrier-gas flow (He) rate was 0.8 mL/min. The temperature of the column oven was programmed in the isothermal mode to increase from 150 to 210°C at a rate of 3°C/min. The injector and detector temperatures were both maintained at 250°C. Fatty acids were identified by comparison with standard mixtures including margaric acid (Nu-Chek Prep, Elysian, MN; C17:0) as an internal standard. Areas were calculated with GC ChemStation (Agilent Technologies), and fatty acid concentrations were represented as a percentage of the total fatty acid content.

Determination of choline acetyltransferase and acetylcholinesterase activities

The frontal region of the cerebral cortex (FC), the frontal edge of the cerebral cortex divided coronally into three equal parts and the hippocampus were dissected from the frozen whole brain. The dissected tissues were homogenized with a Teflon homogenizer in 5 mL of 25 mM sodium phosphate buffer (pH 7.4) per gram of wet weight and centrifuged at 15,000 *g* for 60 min at 4°C. The supernatant was obtained as an enzyme solution. The protein concentration was determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

The determination of choline acetyltransferase (ChAT) activity was modified from the methods of Kaneda and Nagatsu.[17] A volume of 100 μL of enzyme solution diluted to 3 mg protein/mL was mixed with 100 μL of substrate solution containing 1 mM choline chloride, 0.4 mM acetyl-CoA, 0.2 mM eserine sulphate, 0.3 mM sodium chloride and 20 mM EDTA-2Na in 0.1 M sodium phosphate buffer (pH 7.4), and incubated at 37°C for 60 min. The enzyme solution boiled at 95°C for 5 min was used as the negative control. The reaction was stopped with 50 μL of 1 M perchloric acid in an ice bath. After 10 min, 6 μL of 1 mM isopropyl homocholine (IPHC) was added as an internal standard, and the mixture was centrifuged at 1600*g* for 10 min at 4°C. Finally, the supernatant was filtered with a 0.45-μm filter unit to clean the sample.

The determination of acetylcholinesterase (AChE) activity was modified from methods of Kaneda *et al*.[18] A volume of 100 μL of enzyme solution diluted to 0.1 mg protein/ml was mixed with 98 μL of 0.306 M sodium chloride in 0.153 M sodium phosphate buffer (pH 7.0), 2 μL of 3 M magnesium chloride and 40 μL of 6 mM ACh, and incubated at 37°C for 30 min. For the negative control, the enzyme solution was boiled at 95°C for 5 min. The reaction was stopped with 80 μL of 5% methaphosphoric acid in an ice bath. After 10 min, 40 μL of 1 mM IPHC was added as an internal standard, and then the solution was treated as described above.

The acetylcholine (ACh) synthesized by ChAT and choline degraded from ACh by AChE were measured using an HPLC system (HP-1100; Hewlett Packard, Mount View, CA) with a postcolumn enzymatic reaction. A volume of 10 μL of sample for ACh detection and 5 μL for choline detection were directly injected on a reverse-phase column (Eicompak AC-GEL, 4.6 × 150 mm; Eicom, Kyoto, Japan) where ACh and choline were separated before entering into an enzyme reacting column (Eicom AC-Emzympak; Eicom) containing immobilized acetylcholine esterase and choline oxidase, which converted the ACh to choline, and finally to hydrogen peroxide. The mobile phase consisted of a 100 mM phosphate-buffered solution (pH 8.5) containing 0.8 mM sodium 1-decanesulfonate (Sigma–Aldrich) and 1.68 mM tetramethylammonium chloride (Sigma–Aldrich). The flow rate was 0.6 mL/min and the column oven was set at 33°C. The hydrogen peroxide was detected using HPLC with the postcolumn derivatization system referring to the methods of Wang and Glaze.[19] Two reagent delivery pumps (LC-20AD; Shimadzu Corporation, Kyoto, Japan), equipped with an enzyme reacting column, were used to pump the

Table 2 Body and brain weights, and the ratio of brain/body weight in the C18 diet-fed mice

	Basal	C18:0	C18:1	C18:2	C18:3
Body (g)	43.41 ± 1.12	40.14 ± 1.56	42.00 ± 1.73	40.16 ± 0.82	41.46 ± 0.70
Brain (g)	0.412 ± 0.006	0.406 ± 0.009	0.429 ± 0.008	0.408 ± 0.009	0.406 ± 0.007
Brain/body ratio	0.0095 ± 0.0002	0.0102 ± 0.0004	0.0103 ± 0.0005	0.0102 ± 0.0004	0.0098 ± 0.0002

Note: Each diet was administered to mice for eight weeks from four to 12 weeks of age. Results are expressed as the mean ± SEM, $n = 6$ per group.

fluorescence reagent consisting of 32 units/L chloroperoxidase (Sigma–Aldrich) and 7.9 mM 4-hydroxyphenyl-acetic acid (Merck Chemicals, Darmstadt, Germany) in 0.05 M phosphate buffer (pH 8.5) at 0.2 mL/min (first reaction) and for ionization using a 0.2 M sodium hydroxide (Wako Pure Chemical) solution at 0.1 mL/min (second reaction) into two mixing tees. The elute from the enzyme column met the fluorescence reagent in the first tee mixer and then passed through a reactor coil made of 0.3-mm I.D. teflon tubing. After a reaction time of 1 min, the flow entered the other tee mixer and met sodium hydroxide. The retention time during the second reaction was approximately 0.8 min. These reactions were carried out in a column oven (Shimadzu Corporation) at 80°C. Fluorescence intensity was measured with excitation and emission wavelengths at 250 nm and 400 nm, respectively (HP-1100, FLD G1321A; Hewlett Packard). The procedure used for the determination of choline and ACh was that described by Yamamuro *et al*.[20]

Determination of monoamine oxidase activities

Monoamine oxidase activities (MAO)-A and -B were analysed using an Amplex Red Monoamine Oxidase Kit (Life Technologies, Carlsbad, CA). Protein (enzyme) solutions extracted from the regions described above were used for the determination of both enzymatic activities. For MAO-A, 100 μL of enzyme solution diluted to 1 mg protein/mL was mixed with 0.2 μL of the MAO-A-specific inhibitor clorgyline (0.5 mM) and pre-incubated for 30 min at room temperature. After the preincubation, 100 μL of benzylamine substrate solution was mixed on a 96-well micro plate, and then incubated for 60 min. For the determination of MAO-B, pargyline as a MAO-B-specific inhibitor and tyramine as a reaction substrate were used instead of clorgyline and benzylamine. Following a 60-min incubation, the reaction product, resorufin, was measured using a Synergy 2 Multi-Mode Plate Reader (Bio Tek Instruments, Winooski, VT) with excitation and emission wavelengths at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm.

Statistic analysis

The significance of differences between dietary groups was tested by one-way ANOVA with Duncan's new multiple range test, and the significance of a correlation between variables was evaluated using a simple correlation coefficient. The criterion for significance was $P < 0.05$ in all cases.

Results

Body and brain weight, and brain fatty acid compositions

To check for malnutrition or overnutrition caused by the experimental diets, other mice were fed with the control diet (Labo MR Stock; Nosan Corporation) from three to 12 weeks of age. The body weight of the control diet-fed mice (40.66 ± 0.90 g, $n = 8$) tended to be lower slightly than that of the basal diet-fed mice ($t = -1.937$, $P = 0.0766$). The four C18-fatty acid diets had no effect on body and brain weights, and the ratio of brain/body weight of the basal diet-fed mice (Table 2).

The fatty acid profiles of the five diet groups are given in Table 3. Nine kinds of fatty acids were detected in the brain: two SAFAs, palmitic acid (C16:0) and stearic acid (C18:0); three monounsaturated fatty acids (MUFAs), oleic acid (C18:1 *n*-9), vaccenic acid (C18:1 *n*-7) and gadoleic acid (C20:1 *n*-9) and four polyunsaturated fatty acids (PUFAs), linoleic acid (C18:2 *n*-6), arachidonic acid (C20:4 *n*-6), adrenic acid (C22:4 *n*-6) and DHA (C22:6 *n*-3). α-linolenic acid was not detected in any of the dietary groups. The ratio of SAFA in the brain of mice fed with the basal diet tended to be higher than that in the other groups. Ratios of brain DHA, PUFA and (*n*-3)/(*n*-6) in the C18:3 diet-fed mice were significantly higher than those in the other groups.

Novel-social exploratory behaviour

Table 4 summarizes the behavioural parameters of the groups. The C18 fatty acid diets had no effect on the latency to start social behaviour, rearing frequency or bouts of self-grooming. The frequency of social interactive behaviour was decreased approximately 70% in the C18:0 group compared to the basal diet-fed group ($F_{(4,85)} = 4.454$, $P = 0.0026$; Figure 1B) and significant differences were seen from the other groups, whereas there was no difference in cumulative time ($F_{(4,85)} = 1.537$, $P = 0.1989$; Figure 1A). The frequency of social interactive behaviour showed a significant positive correlation to cumulative time in the basal ($P = 0.0045$), C18:1 ($P = 0.0001$), C18:2 ($P = 0.0059$) and C18:3 ($P = 0.0045$) groups, but not in the C18:0 group ($R^2 = 0.005$, $P = 0.7652$; Figure 2).

ChAT and AChE activities in the FC and hippocampus

The ChAT activity in the FC was increased by approximately 1.7-fold in the C18:0 group compared to the basal diet-fed group, but no significant difference was seen among the other groups ($F_{(4,35)} = 12.488$, $P = 0.0001$; Figure 3A). In the hippocampus, the C18:0 and C18:3

Table 3 Brain fatty acid compositions in the C18 diet-fed mice

	Dietary groups				
Fatty acids	**Basal**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
C16:0	24.1 ± 0.64	20.6 ± 0.14	20.5 ± 0.17	21.9 ± 0.51	21.8 ± 0.17
C18:0	21.7 ± 0.59	19.9 ± 0.11	19.7 ± 0.08	20.5 ± 0.38	19.9 ± 0.14
C18:1 (*n*-9)	16.2 ± 0.51	15.0 ± 0.07	15.1 ± 0.15	14.9 ± 0.26	15.6 ± 0.20
C18:1 (*n*-7)	5.0 ± 0.16	4.1 ± 0.08	3.9 ± 0.02	4.2 ± 0.12	4.3 ± 0.06
C18:2 (*n*-6)	0.3 ± 0.01	0.3 ± 0.03	0.4 ± 0.03	0.6 ± 0.04	0.5 ± 0.02
C18:3 (*n*-3)	N.D.	N.D.	N.D.	N.D.	N.D.
C20:1 (*n*-9)	1.7 ± 0.09	1.7 ± 0.05	1.8 ± 0.06	1.5 ± 0.07	1.4 ± 0.08
C20:4 (*n*-6)	9.1 ± 0.59	9.6 ± 0.03	9.8 ± 0.07	9.5 ± 0.15	9.1 ± 0.10
C22:4 (*n*-6)	2.5 ± 0.17	3.2 ± 0.07	3.3 ± 0.01	3.1 ± 0.09	2.4 ± 0.03
C22:6 (*n*-3)	11.7 ± 1.30 a	14.0 ± 0.39 a	14.6 ± 0.19 a	12.7 ± 0.51 a	18.1 ± 0.11 b
SAFA	45.8 ± 1.22	40.5 ± 0.18	40.3 ± 0.23	42.4 ± 0.89	41.8 ± 0.30
MOFA	22.9 ± 0.74	20.8 ± 0.17	20.7 ± 0.21	20.7 ± 0.34	21.3 ± 0.32
PUFA	23.5 ± 1.99 a	27.2 ± 0.40 a	28.0 ± 0.28 a	25.8 ± 0.76 a	30.1 ± 0.19 b
(*n*-3)/(*n*-6)	0.97 ± 0.06 a	1.06 ± 0.03 a	1.08 ± 0.01 a	0.96 ± 0.02 a	1.51 ± 0.02 b

Note: Fatty acid compositions are expressed as mg/100 g. Results are expressed as the mean ± SEM, $n = 6$ per group. Different lowercase letters indicate a significant difference between diets ($P < 0.05$).
SAFA: saturated fatty acids; MOFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Table 4 Behavioural data in the C18 diet-fed mice

	No. of mice	Latency (sec)	Rearing activity (frequency)	Self-grooming (frequency)
Basal	18	7.9 ± 1.4	38.4 ± 4.3	1.4 ± 0.4
C18:0	19	7.7 ± 1.0	32.8 ± 3.2	0.7 ± 0.3
C18:1	20	7.9 ± 0.9	40.7 ± 4.2	1.2 ± 0.3
C18:2	17	7.3 ± 1.2	35.7 ± 3.6	0.9 ± 0.3
C18:3	16	8.1 ± 1.4	45.3 ± 4.2	0.8 ± 0.3

Note: Each diet was administered to mice for eight weeks from four to 12 weeks of age. Results are expressed as the mean ± SEM.

groups had higher levels than the basal diet-fed group. The C18:2 group, conversely, was 64% of the basal group ($F_{(4,35)} = 32.927$, $P = 0.0001$; Figure 3B).

Except in the C18:3 group, AChE activity did not exhibit greater variation than ChAT activity. AChE activities in the FC and hippocampus of C18:3 diet-fed mice were increased significantly when compared with the basal diet-fed group, but the increases were only 27% and 31%, respectively (FC; $F_{(4,34)} = 3.099$, $P = 0.0281$; Figure 4A, hippocampus; $F_{(4,35)} = 8.553$, $P = 0.0001$; Figure 4B).

MAO activities in the FC and hippocampus

The MAO-A activity in the FC was 34% higher in the C18:2 diet-fed mice than in the basal group, but no difference was seen among the other groups ($F_{(4,35)} = 9.264$, $P = 0.0001$; Figure 5A). In the hippocampus, MAO-A activity was 23% lower in the C18:0 group than the basal diet group. There was no significant difference among the other groups ($F_{(4,35)} = 10.265$, $P = 0.0001$; Figure 5B).

In comparison with the basal diet group, MAO-B activities in the FC of C18:0 and C18:1 diet-fed mice was 22% and 21% lower, respectively ($F_{(4,35)} = 8.195$, $P = 0.0001$; Figure 6A). The activities in the hippocampus of the C18:0 and C18:1 groups were also lower, 37% and 23%, respectively ($F_{(4,35)} = 29.314$, $P = 0.0001$; Figure 6B).

Discussion

In many mammalian species, the greater region of the brain is fundamentally immature at birth and undergoes developmental changes in composition and structure in the postnatal period. During the developmental period, from the embryonic stage to early infancy, amounts of LCPUFAs in the brain increase rapidly, suggesting that these fatty acids play an essential role in the acquisition of inherent brain function.[21,22] The fatty acid composition of the brain is thought to be governed primarily by levels of fatty acids in maternal milk, or the maternal diet, until weaning. In general, the maternal diet has little effect on the distribution of lipid classes of milk fat, but PUFA levels are strongly dependent on the types of fatty acids in the maternal diet. Plasma free fatty acids and lipoproteins are an essential source of fatty acids in the brain, and dietary fatty acids are likely reflected in plasma fatty acid profiles.[23] Fatty acids, including arachidonic acid and DHA, injected into plasma are incorporated rapidly into the brain.[24–26] However, the fatty acid composition of the brain postweaning is considered relatively constant, not responding as readily to changes in dietary fat composition.[4,5] In this study, dietary C18 fatty acids following weaning had no significant impact on brain fatty acid composition except

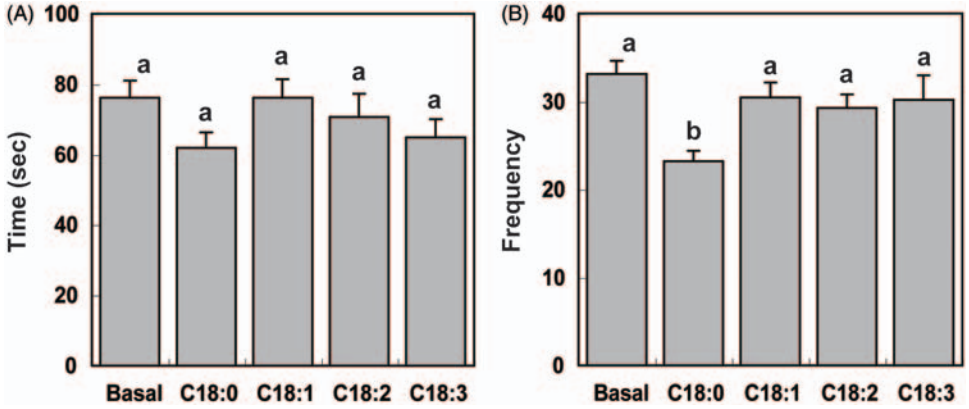

Figure 1 Cumulative time (A) and frequency (B) of social interaction with a novel-social partner during a 5-min novel-social exploratory behaviour test in the basal ($n = 18$), C18:0 ($n = 19$), C18:1 ($n = 20$), C18:2 ($n = 17$), and C18:3 ($n = 16$) diet-fed mice during postweaning. Values are means ± SEMs. Different small letters indicate a significant difference between the groups ($P < 0.05$)

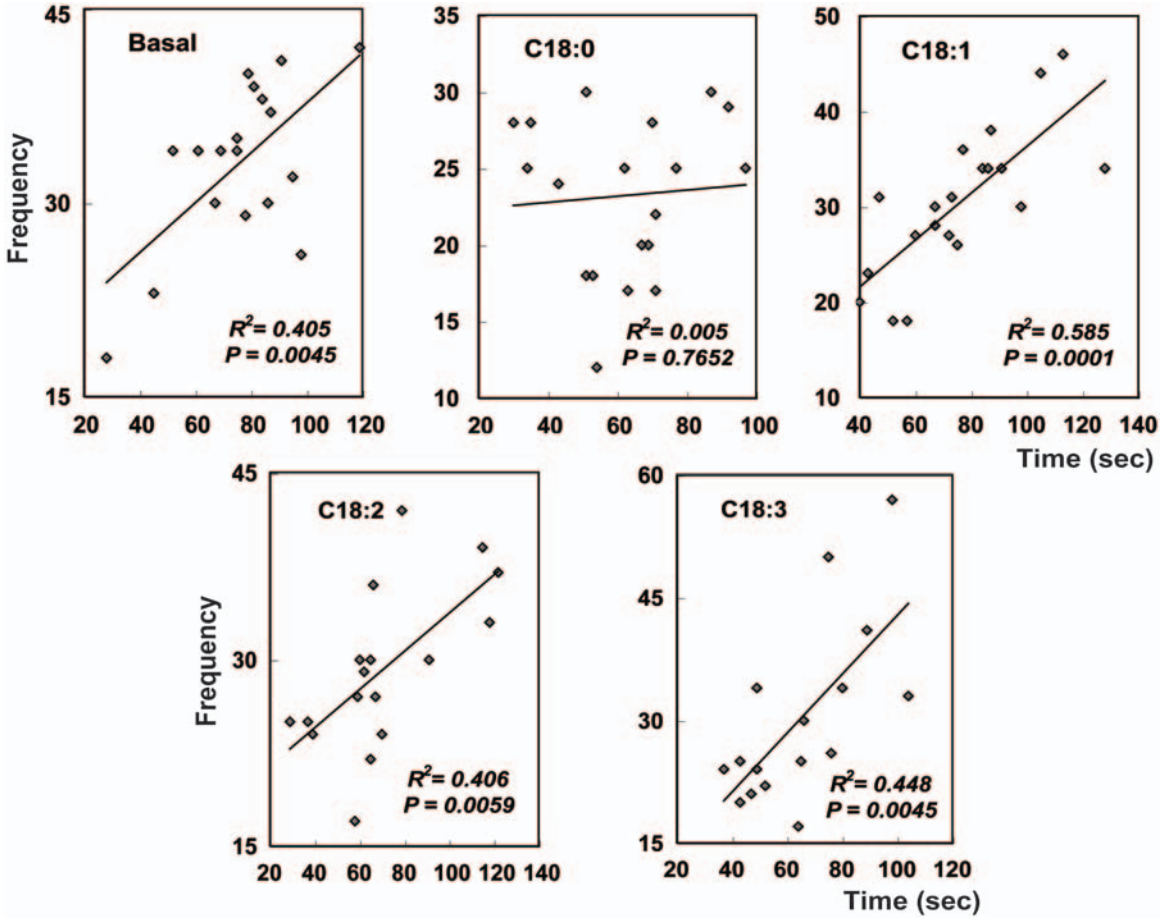

Figure 2 Correlations between cumulative time and frequency of social interaction with a novel-social partner during a 5-min novel-social exploratory behaviour test in the basal ($n = 18$), C18:0 ($n = 19$), C18:1 ($n = 20$), C18:2 ($n = 17$) and C18:3 ($n = 16$) diet-fed mice during postweaning

for the C18:3 diet, which increased the DHA, PUFA and (n-3)/(n-6) ratios, coinciding with findings described earlier. Furthermore, C18:3 n-3 was not detected in the brain even in the C18:3 diet-fed mice, similarly to previous studies.[27–29] The various changes in neurochemical properties and behavioural traits by C18 fatty acids in diet confirmed in this study are not likely dependent on the alteration of membrane fluidity due to brain fatty acid components.

It has become evident that dietary fatty acids, mainly PUFAs, act on the central nervous system. Experimental studies have shown behavioural and neurochemical abnormalities in animals fed diets unbalanced in fatty

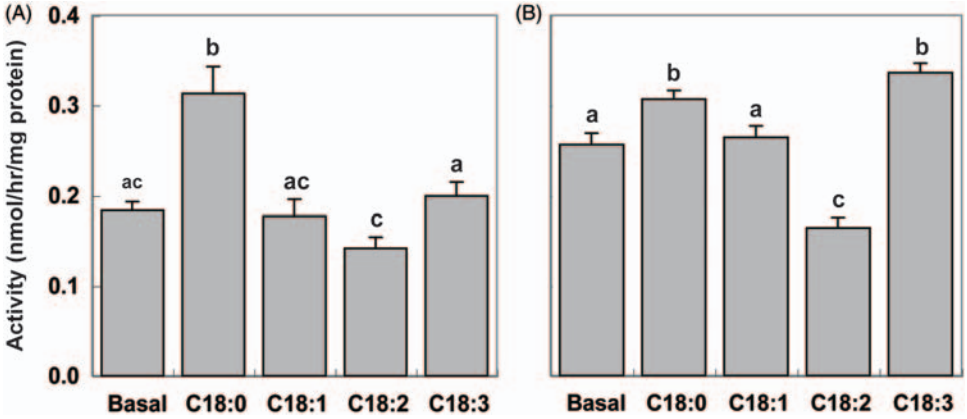

Figure 3 ChAT activity in the frontal cortex (A) and hippocampus (B) of mice fed the basal ($n = 8$), C18:0 ($n = 8$), C18:1 ($n = 8$), C18:2 ($n = 8$), and C18:3 ($n = 8$) diets during postweaning. Values are means ± SEMs. Different small letters indicate a significant difference between the groups ($P < 0.05$)

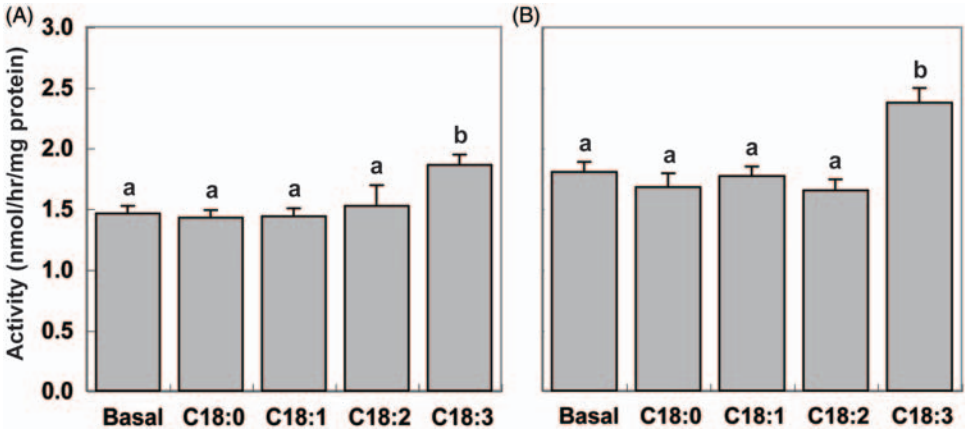

Figure 4 AChE activity in the region of frontal cortex (A) and hippocampus (B) of mice fed the basal ($n = 8$), C18:0 ($n = 8$), C18:1 ($n = 8$), C18:2 ($n = 8$), and C18:3 ($n = 7$) experimental diets during postweaning. Values are means ± SEMs. Different small letters indicate a significant difference between the groups ($P < 0.05$)

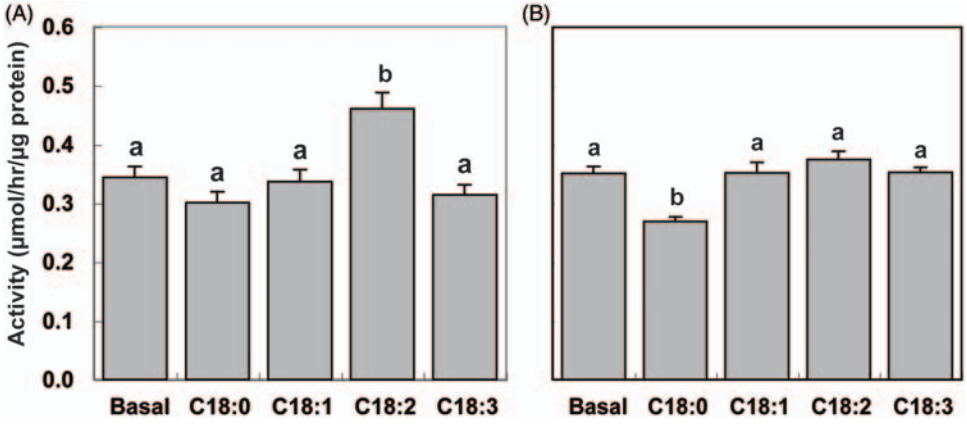

Figure 5 MAO-A activity in the region of frontal cortex (A) and hippocampus (B) of mice fed the basal ($n = 8$), C18:0 ($n = 8$), C18:1 ($n = 8$), C18:2 ($n = 8$), and C18:3 ($n = 7$) diets during postweaning. Values are means ± SEMs. Different small letters indicate a significant difference between the groups ($P < 0.05$)

acid components. A deficiency of *n*-3 PUFAs, e.g. α-linolenic acid, induces impaired behavioural responses involving learning ability and motivational processes.[30–33] These behavioural responses might be mediated through the monoaminergic system because monoamines are known to influence attention, motivation and emotion.[34] Although two forms of MAO, MAO-A and -B, catalyse the oxidative deamination of a number of biogenic amines

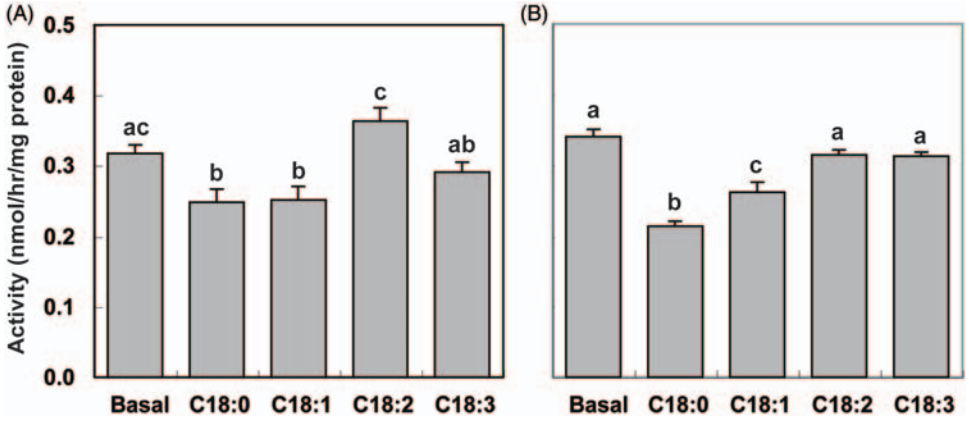

Figure 6 MAO-B activity in the frontal cortex (A) and hippocampus (B) of mice fed the basal ($n = 8$), C18:0 ($n = 8$), C18:1 ($n = 8$), C18:2 ($n = 8$), and C18:3 ($n = 7$) diets during postweaning. Values are means ± SEMs. Different small letters indicate a significant difference between the groups ($P < 0.05$)

in the brain and peripheral tissue, they show distinct substrate selectivity and inhibitor sensitivity. MAO-A is a catalytic enzyme for the neurotransmitter serotonin (5-HT), norepinephrine (NE) and dopamine (DA), and its activity is inhibited by clorgyline, whereas MAO-B has higher affinity for phenylethylamine and benzylamine, and its activity is inhibited by deprenyl.[35,36] Both MAO-A-deficient and MAO-B-deficient mice showed enhanced reactivity to stress in the forced-swim test.[37,38] Male MAO-A-deficient mice exhibited a marked increase in brain 5-HT and NE levels, and a modest increase in DA levels, and manifested enhanced aggression against intruder mice under standardized conditions.[37,39] Furthermore, a role for dietary PUFAs in monoaminergic neurotransmission focusing on the alteration of MAO activity was also pointed out.[40–43] In this study, the MAO-A activity in the frontal cortex was significantly higher in mice fed with the C18:2 diet than in the other groups, though no change in behavioural parameters was found, indicating that the increase of MAO-A in the frontal cortex is not concerned with behavioural traits investigated here. The MAO-B activity also fluctuated by C18 fatty acids in diet, but the variations were only a modest. Besides the cholinergic pathways which are also important for cognitive function, other research has focused on the influence of an *n*-3 PUFA-deprived diet on the central cholinergic neurotransmission system. The results indicated that the basal release of ACh in the hippocampus was higher in *n*-3 PUFA-deficient rats than in the control diet-fed groups and accompanied by a remarkable loss of DHA in the frontal cortex and hippocampus, but no change in AChE activity and the vesicular ACh transporter.[44] In addition, a dietary supplement of DHA also increased basal and/or evoked cholinergic neurotransmission,[45–47] and inhibited the hydrolysys of ACh.[48] In particular, AChE activity is reflected by changes in the biophysical properties of membranes,[49] and the present finding that dietary C18:3 during the postweaning period enhanced rates of brain PUFA and AChE activity largely coincided with the previous results.

Unlike in the developing brain, in the mature brain, the intake of saturated fatty acids rather than essential fatty acids may be important. Significant associations between dietary or supplemental saturated fatty acids and behavioural and neurochemical traits have been observed.[50–54] In many behavioural situations, increases in the frequency of novel-exploratory behaviour in an open-field, social partner recognition and so on, result in an increase in performance time. A strong positive correlation between frequency and time for social-exploratory performance in PUFA-supplemented and basal diet groups was also observed in this study, except for the C18:0 diet. The C18:0 diet significantly reduced the total number of behavioural bouts and had little effect on cumulative time for performance, indicating that a diet containing a relatively high ratio of saturated fatty acids, including stearic acid, during the postweaning period changes generic behavioural traits. Furthermore, the dispersion of C18:0 group was notably small when compared with the other groups, e.g. versus basal group, cumulative time: $F(1,35) = 1.5366$; frequency: $F(1,35) = 26.9898$. These results also indicated that C18:0 diet has an apparent effect on variability of social interaction bouts, especially lack of higher social interaction bouts.

ChAT activity in the brain was higher in the C18:0 diet-fed mice than basal diet-fed mice, especially in the frontal cortex where it was 1.7-fold higher. ChAT is an enzyme that catalyses the biosynthesis of the neurotransmitter ACh from acetyl-coenzyme A and choline, and is widely recognized as a specific marker for cholinergic neurons and their functions. Although it has been reported that a lack or excess of AChE activity in the brain alters behavioural reactions,[55–58] readily reflected in behavioural traits, little was known, until now, about the fundamental relationship between ChAT and behaviour, and/or between the intake of saturated fatty acids and neurotransmitter synthesis. The present results, however, indicate the potential for significant neurochemical and behavioural effects of dietary saturated fatty acids.

Author contributions: This study was a collaboration of all the authors. YYamamuro defined the research topic, designed the investigation, performed some of the laboratory experiments, analysed data, interpreted the results and

wrote the paper. YYamaguchi co-designed the experiments, performed the behavioural analysis and determined the enzymatic activities. SA and FT prepared the experimental diets and brain samples for biochemical analyses, and determined brain fatty acid compositions. All authors contributed to, and have read and approved the manuscript.

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