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

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Abstract **Rats fed a semipurified diet supplemented with 3% (w/w) safflower oil [Saf, n-3 fatty acid deficient, high linoleic acid (18:2n-6)] through two generations exhibit decreased correct response ratios in a brightness-discrimination learning test compared with rats fed 3% perilla oil [Per, high α-linolenic acid (18:3n-3)]. This is associated with a decreased DHA (22:6n-3)-to-arachidonic acid (20:4n-6) ratio in brain lipids. In the first set of experiments, dietary oil was shifted from Saf to a mixture of 2.4% safflower oil plus 0.6% DHA after weaning (Saf-DHA), but all parameters measured in the learning test were essentially unchanged. Brain 22:6n-3 content of the Saf-DHA group reached that of the Per group but the levels of 20:4n-6 and docosatetraenoic acid (22:4n-6) did not decrease to those of the Per group at the start of the test. In the second set of experiments, dietary oil was shifted to a mixture of 0.6% safflower oil plus 1.2% oleic acid (OA) plus 1.2% DHA (Saf-OA-DHA group) with 18:2n-6 content comparable to that of the Per group. The Saf-OA-DHA group exhibited a learning performance similar to that of the Per group; brain 22:6n-3, 20:4n-6, and 22:4n-6 contents were also comparable to those of the Per group. These results indicate that the altered learning behavior associated with a long-term n-3 fatty acid deficiency is reversed by supplementing 22:6n-3 after weaning, when the levels of competing n-6 fatty acids in the diet and brain lipids are limited.**—Ikemoto, A., M. Ohishi, Y. Sato, N. Hata, Y. Misawa, Y. Fujii, and H. Okuyama. **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.*** **2001.** 42: **1655–1663.**

Supplementary key words arachidonic acid • brain • docosahexaenoic acid • learning ability • linoleic acid • α-linolenic acid

Long chain PUFA derived from n-6 or n-3 fatty acids are fundamental components of membrane lipids in the central nervous system (1–3). These PUFA, mainly arachidonic acid (20:4n-6) and DHA (22:6n-3), are derived from essential fatty acid precursors, linoleic (18:2n-6) and α-linolenic acids (18:3n-3), respectively, which must be supplied from exogenous sources in mammals.

Besides its role as structural components, 20:4n-6 is well characterized as a precursor of bioactive mediators, such as prostaglandins, which are involved in sleep regulation (4), febrile response (5), and pain perception (6), as well as in inflammatory responses. Free 20:4n-6 is known to activate protein kinases and ion channels, inhibit neurotransmitter uptake, and enhance synaptic transmission (7). 20:4n-6 and platelet-activating factor derived from 20:4n-6-containing phospholipids are suggested to function as retrograde messengers in long-term potentiation of synapses in the hippocampus CA1 region (8, 9) and to be involved in the migration of neurons in the cerebral cortex (10). Two 20:4n-6 derivatives, anandamide (11) and 2-arachidonoylglycerol (12, 13), have been identified and characterized as endogenous cannabinoid receptor (CB1) ligands that suppress long-term potentiation (14) and dopamine-induced facilitation of motor activity (15). However, the role played by these 20:4n-6 metabolites in learning behavior is still to be clarified.

The essential role of n-3 fatty acid in the presence of n-6 fatty acids in central nervous system functions has been established relatively recently as reviewed elsewhere (16). The decreased correct response ratio in the safflower oil (Saf, n-3 fatty acid deficient, high 18:2n-6)-fed rats compared with perilla oil (Per, high 18:3n-3)-fed rats in a brightness-discrimination learning test was associated with a marked reduction in brain 22:6n-3 content and a small but significant increase in 20:4n-6 content (17–19). The altered learning behavior has been correlated with reduced synaptic vesicle density in the hippocampus CA1 region (20) and some structural changes in the brain microsomal membrane surface (21). In a model system

Abbreviations: CRR, correct response ratio; DHA, 22:6n-3; OA, oleic acid; Per, perilla oil; Saf, safflower oil.

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with PC12 cells, 22:6n-3 was shown to enhance whereas 20:4n-6 was shown to suppress both phospholipid biosynthesis and neurite elongation induced by a nerve growth factor (22, 23). A significantly decreased nerve growth factor content was also noted in the hippocampus of the Saf group (24). However, the significance of these observations to the altered learning behavior is still to be defined in functional terms.

Most neuronal cells cease dividing and proliferating after weaning in the rat; therefore, the nutritional status during gestation and lactation is considered crucial for the development of brain and neural functions. In fact, a decreased learning ability in a maze test, which was induced by feeding a fat-free diet deficient in both the n-6 and n-3 fatty acids, was not restored by supplementing these fatty acids after parturition (25, 26). These observations led to the conclusion that proper intake of essential fatty acids during the last week of gestation is critical. Consistently in the rat, the major brain growth spurt is reported to occur postnatally; 22:6n-3 and 20:4n-6 increase rapidly during the period of rapid brain cell division between birth and 20 days of age (27). In contrast, we have observed that a shift from the Saf diet to the Per diet after weaning restores both the brain 22:6n-3 content and brightness-discrimination learning behavior in the rat. This indicate that n-3 deficiency during gestation and lactation in the presence of n-6 fatty acids does not bring about irreversible damage to brain (28). Therefore, we expected that 22:6n-3 supplementation after weaning will restore learning behavior when the brain 22:6n-3 level is restored. Here, we report that the level of n-6 fatty acids in the brain is another critical factor for the reversibility of n-3 fatty acid deficiency-induced alterations in brightness-discrimination learning behavior in the rat.

MATERIALS AND METHODS

Materials

DHA ethyl ester (95.8% DHA and 4.2% eicosapentaenoate) was a product of Harima Chemicals (Tsukuba, Japan). Oleic acid (OA) ethyl ester was purchased from Wako Pure Chemical Industries (Osaka, Japan). Heptadecanoic acid was purchased from Funakoshi (Tokyo, Japan). The semipurified diet (Clea Japan, Tokyo) contained by weight 48.0% corn starch, 25.1% milk casein, 8.2% cellulose, 5.1% sucrose, 2.0% Okanol (a mixture of carbohydrates), 6.1% mineral mixture, 1.4% vitamin mixture, 0.4% DL-methionine, 0.6% choline chloride, and 3% oil. Vegetable oils (Saf and Per) were purchased from a local market. The fatty acid compositions of the experimental diets are shown in **Table 1**. Saf-DHA was prepared by mixing Saf (80% by weight) and DHA ethyl ester (20%). Saf (20%), OA ethyl ester (40%), and DHA ethyl ester (40%) were mixed to make Saf-OA-DHA, in which the 18:2n-6 content was adjusted to that of Per.

TABLE 1. Fatty acid composition of the diet

Fatty Acid	Saf	Saf-DHA	Saf-OA-DHA	Per
16:0	6.6	5.3	1.3	5.4
18:0	2.3	1.8	0.5	1.4
18:1	15.8	12.6	43.2	15.1
18:2 n-6	73.9	59.1	14.8	14.8
18:3 n-3	0.8	0.7	0.2	63.0
20:0	0.3	0.2	0.1	0.1
20:1	0.2	0.2	Trace	0.2
20:5 n-3	ND	0.8	1.7	ND
22:6 n-3	ND	19.1	38.3	ND
n-6/n-3	90.24	2.86	0.37	0.23

Values represent percentages of total fatty acids (w/w). Saf, Safflower oil; Saf-DHA, a mixture of 80% safflower oil and 20% DHA ethyl ester; Saf-OA-DHA, a mixture of 20% safflower oil, 40% DHA ethyl ester, and 40% oleic acid ethyl ester; Per, perilla oil; ND, not detected.

Animals

All animals used in the experiments were maintained under controlled lighting (24-h cycle with lights on from 7:00 to 19:00 h) at 23 ± 2°C and 50 ± 10% humidity. Female Donryu rats (F_0) (a conventional specific pathogen-free strain from SLC, Shizuoka, Japan) at 4 weeks of age were fed the Saf diet (n-3 fatty acid deficient, n = 28 and 24 in the first and second experiments, respectively) or the Per diet (n-3 fatty acid sufficient, n = 20 and 16 in the first and second experiments, respectively) for 7 weeks. The rats (F_0) were mated at 11 weeks of age and the numbers of litters (F_1) were 308 (Saf) and 199 (Per) in the first experiment and 299 (Saf) and 212 (Per) in the second experiment. The offspring (F_1) were randomized at 1 week of age and the litter size was adjusted to 11 per dam. At 3 weeks of age, the male pups (F_1) from the Saf diet group were weaned and divided randomly into two groups. One group was fed the same Saf diet and the other group was fed a Saf-DHA diet or a Saf-OA-DHA diet in the first and second experiments, respectively. The male pups (F_1) from the Per diet group were fed the same Per diet. The brain fatty acid composition was analyzed at the indicated age, using 4 male rats (F_1) in each group. The brightness-discrimination learning test was started at 11 weeks of age. The 12 male rats (F_1) in each group were used in the test.

Brightness-discrimination learning test

The conditions of the test have been described previously (17–19). Briefly, food consumption by the rats was controlled to decrease their body weight to 85% of the normal body weight in 1 week. This level was maintained throughout the experiments. Rats were trained to press a lever to obtain diet pellets. When the total time to obtain 40 pellets was less than 10 min, we judged that the rat had completed the shaping process. All the rats completed this process within 5 days. Then, the brightness-discrimination learning test was started.

In the original schedule, either a bright light (100 lx) or a dim light (3 lx) was shown on a screen randomly but with equal frequency; the lever-pressing responses under the bright light (R^+) were reinforced with diet pellets whereas no pellet was given on lever pressing under the dim light (R^-). The correct response ratio (CRR), an index of learning ability, was calculated as follows: CRR (%) = $100 \times [R^+/(R^+ + R^-)]$. One session consisted of 20 times each of the positive and negative stimuli, and the session was run every day for 20 or 25 days (1 session/day).

After the original schedule, we carried out the reverse schedule, in which the stimuli were reversed; the lever-pressing responses (R^+) under the dim light were reinforced with diet pellets whereas no pellet was given on lever pressing under the bright light (R^-). In the reverse schedule, the session was run every day for 20 or 25 days (1 session/day).

Lipid analysis

The brains of F_1 rats at the indicated weeks of age were removed, frozen, and stored at −80°C until analysis. Total lipids

were extracted with chloroform-methanol according to the method of Bligh and Dyer (29). Fatty acids were converted to their methyl esters by treatment with 5% HCl in methanol and were quantified by capillary column gas-liquid chromatography (Shimazu, Kyoto, Japan), using heptadecanoic acid as an internal standard as described previously (17).

Statistical analysis

The data from the brightness-discrimination learning test were compared by two-way ANOVA with repeated measures (diet × session). A difference was considered significant at $P < 0.05$. Fatty acid compositions at each week of age were compared by one-way ANOVA followed by a Bonferroni multiple comparison test. A difference was considered significant at $P < 0.01$.

RESULTS

Effect of a shift from Saf diet to Saf-DHA diet on brain fatty acid composition

The weight gain, litter size, brain weight, and total fatty acid content in the brain were not significantly different among the dietary groups (data not shown). The time course of the changes in n-3 PUFA levels in the brain is shown in **Fig. 1**. At 3 weeks of age, just after weaning, n-3 fatty acid deficiency in the Saf group led to a marked decrease in the 22:6n-3 level to one-fourth as compared with the Per group. In the Saf-DHA group, the 22:6n-3 level increased rapidly from 3 to 6 weeks of age and reached the level of the Per group at 9 weeks of age. In the Per group the DHA precursor 22:5n-3 was detectable (about 0.4–1.3%) but was negligible in the other two groups. Neither 18:3n-3 nor eicosapentaenoic acid (20:5n-3) was detected in any of the groups (data not shown).

The time course of the changes in n-6 PUFA levels in the brain is shown in **Fig. 2**. In the Saf group a marked decrease in the 22:6n-3 level was compensated for by increases in 20:4n-6, docosatetraenoic acid (22:4n-6), and docosapentaenoic acid (22:5n-6). In the Per group, 22:5n-6 was not detected throughout the period whereas it accounted for 9.5% of the total at 3 weeks of age in the Saf group. In the Saf-DHA group, the 22:5n-6 level decreased rapidly almost to the level of the Per group at 9 weeks of age, and then reached the level of the Per group at 18 weeks of age. However, the levels of 20:4n-6 and 22:4n-6 in the Saf-DHA group were between those of the Saf and Per groups throughout the period. The levels of 18:2n-6 and eicosatrienoic acid (20:3n-6) in the Per group were low (0.6–1.4% and 0.4–1.2%, respectively) but were higher than those in the Saf group, and their levels were restored in the Saf-DHA group at 9 weeks of age (data not shown). The n-6/n-3 ratios in the brain fatty acids were 4.51 ± 0.22, 1.05 ± 0.03, and 0.71 ± 0.03 at 9 weeks of age, and 3.23 ± 0.41, 1.03 ± 0.03, and 0.85 ± 0.02 at 18 weeks of age in the Saf, Saf-DHA, and Per groups, respectively. These values in the Saf and Saf-DHA groups were significantly higher than those of the Per group ($P < 0.01$).

Saturated fatty acids accounted for 45–49% of the total fatty acids, and age-related changes were not significant in all the dietary groups (data not shown). Monounsaturated fatty acids accounted for 19–29% of total fatty acids, and their levels in the Saf group were slightly but significantly lower than those in the Per group (23.7% vs. 27.6% at 9 weeks of age). In the Saf-DHA group, their levels reached those of the Per group at 9 weeks of age (data not shown).

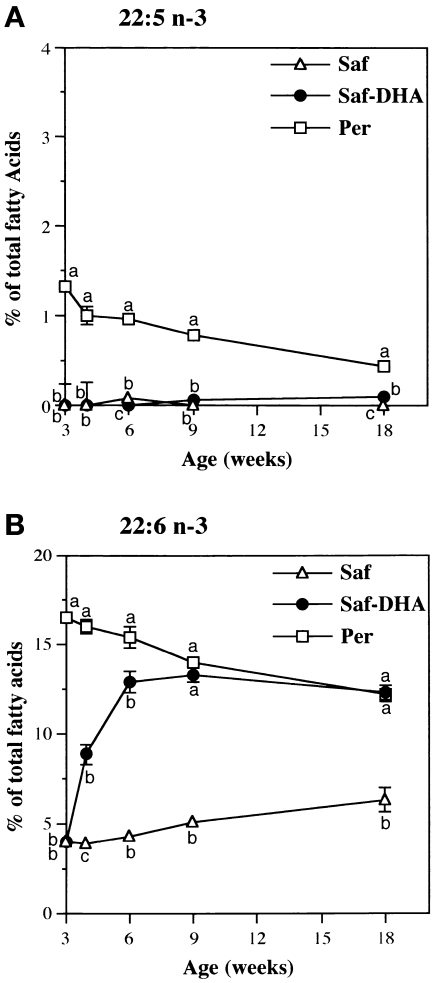

Fig. 1. Effect of a shift of Saf diet to Saf-DHA diet after weaning on brain n-3 PUFA level. Fatty acid compositions of total lipids extracted from the brain of F_1 rats fed a semipurified diet supplemented with Saf, Saf-DHA, or Per were analyzed. Values represent means ± SD for four rats. Values at each week of age with different letters are significantly different ($P < 0.01$) in one-way ANOVA followed by Bonferroni's test. In some cases error bars are included within the symbols. A: 22:5n-3. B: 22:6n-3.

Effect of a shift of Saf diet to Saf-DHA diet on learning behavior

The brightness-discrimination learning test (the original and the reverse schedule) was performed from 11 to 18 weeks of age. In the original schedule (**Fig. 3**), the number of R^+ increased as the session progressed similarly among the three dietary groups. In contrast, the number of R^- increased during the first several sessions and thereafter reached plateau levels and then decreased in all the groups. However, the Saf group took significantly longer times before the number of R^- began to decrease. The number of R^- in the Per group was signifi-

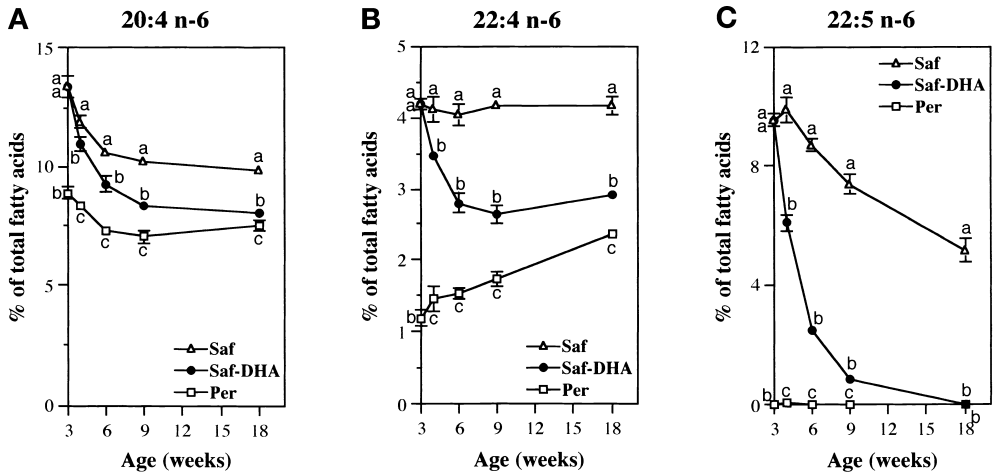

Fig. 2. Effect of a shift of Saf diet to Saf-DHA diet after weaning on brain n-6 PUFA level. A: 20:4n-6. B: 22:4n-6. C: 22:5n-6. See legend to Fig. 1.

cantly less than in the Saf group. As a result, the CRR was significantly lower in the Saf group than in the Per group. These are typical of the results we observed. When the Saf diet was shifted to the Saf-DHA diet after weaning, all parameters of the test were similar to those of the Saf group and the learning performance was not restored to the level of the Per group.

In the reverse schedule, the number of R^+ increased as the sessions progressed similarly among the three groups as in the original schedule (**Fig. 4**). The number of R^- decreased rapidly and then reached a low plateau level in the Per group. However, in the Saf and Saf-DHA groups, the number of R^- was significantly higher and the CRR was significantly lower than in the Per group. Thus, a long-term n-3 fatty acid deficiency led to decreased learning ability (CRR) regardless of the conditions of the light stimuli (original and reverse schedule). The Saf-DHA diet did not restore the learning ability to the level of the Per group even though the brain 22:6n-3 level was restored.

Effect of a shift of Saf diet to Saf-OA-DHA diet on brain fatty acid composition

We considered two possibilities: *i*) n-3 fatty acid deficiency during gestation and lactation causes irreversible learning impairments, and *ii*) n-6 fatty acids such as 20:4n-6 and 22:4n-6 are significantly associated with learning impairments. In the next set of experiments, we examined a mixture of Saf, OA, and DHA (Saf-OA-DHA), in which the

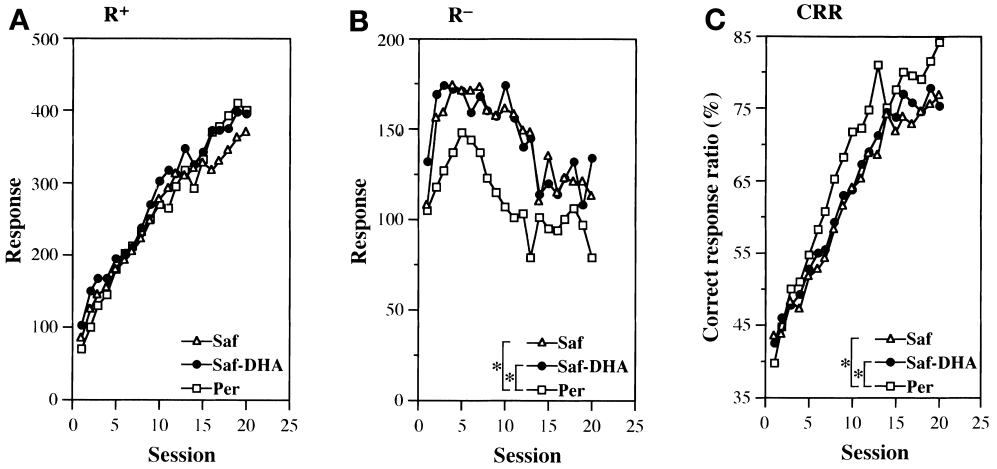

Fig. 3. Effect of a shift of Saf diet to Saf-DHA diet after weaning on learning behavior in a brightness-discrimination learning test (original schedule). The original schedule of the brightness-discrimination learning test in F_1 rats fed a semipurified diet supplemented with Saf, Saf-DHA, or Per was followed, as described in text. The lever-pressing responses under bright light (A: R^+) were reinforced with diet pellets whereas no pellet was given on lever pressing under dim light (B: R^-). The correct response ratio (C: CRR) was defined as $100 \times [R^+/(R^+ + R^-)]$. The asterisk indicates a statistically significant difference between the indicated dietary groups ($P < 0.05$ evaluated by two-way ANOVA using diet and session as sources of variations).

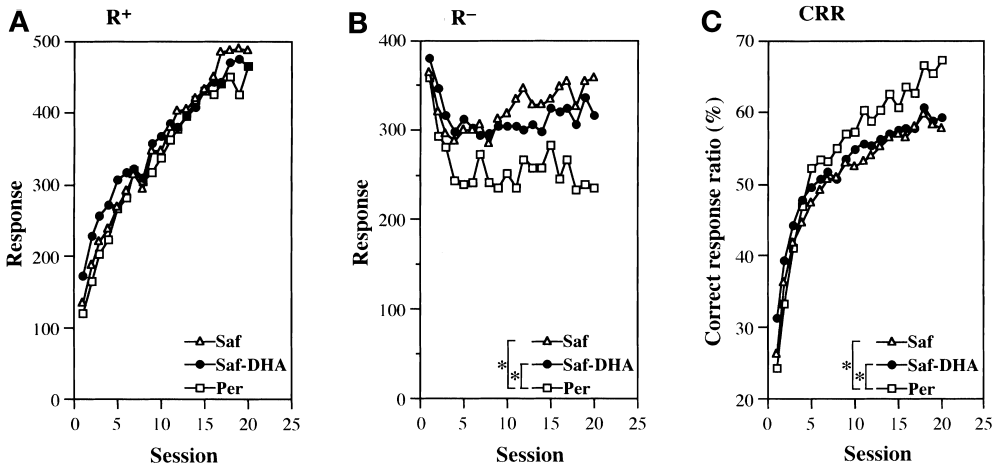

Fig. 4. Effect of a shift of Saf diet to Saf-DHA diet after weaning on learning behavior in a brightness-discrimination learning test (reverse schedule). The reverse schedule of the brightness-discrimination learning test in F_1 rats fed a semipurified diet supplemented with Saf, Saf-DHA, or Per was followed, as described in text. See legend to Fig. 3. The lever-pressing responses under the dim (A: R^+) were reinforced with diet pellets while no pellet was given on lever-pressing under the bright light (B: R^-). The correct response ratio (C: CRR) was defined as $100 \times R^+/(R^+ + R^-)$.

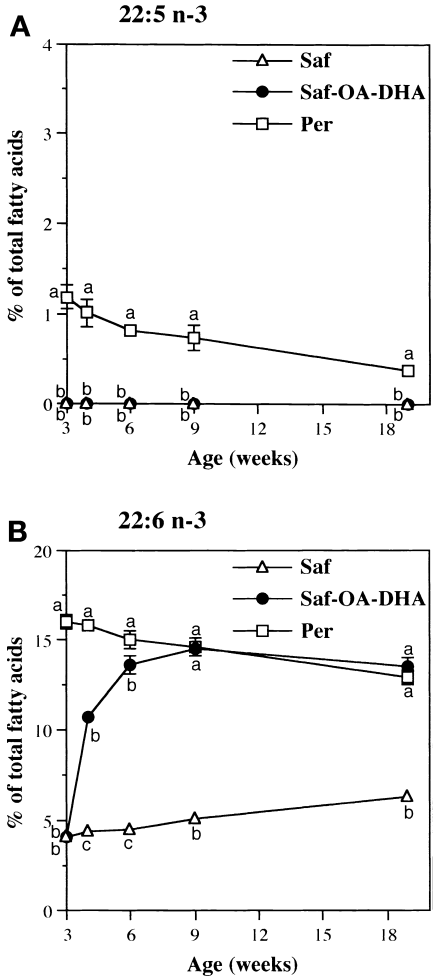

Fig. 5. Effect of a shift of Saf diet to Saf-OA-DHA diet after weaning on brain n-3 PUFA level. Fatty acid composition of the Saf-OA-DHA diet is shown in Table 1. A: 22:5n-3. B: 22:6n-3. See legend to Fig. 1 for details.

22:6n-3 level was doubled compared with the Saf-DHA mixture, and the 18:2n-6 level was adjusted to that of the Per diet by supplementing with oleic acid (18:1n-9).

As in the first set of experiments, the weight gain, litter size, brain weight, and total fatty acid content in the brain were not significantly different among the three dietary groups (data not shown). In the Saf-OA-DHA group, the 22:6n-3 level increased rapidly from 3 to 6 weeks of age, reaching the Per group level completely at 9 weeks of age (**Fig. 5B**). In the Per group, 22:5n-3 was detected at low levels (about 0.4–1.3%) but its level was negligible in the Saf and Saf-OA-DHA groups (Fig. 5A). Thus, the changes in n-3 PUFA in the Saf-OA-DHA group were similar to those observed in the Saf-DHA group (Fig. 1).

The changes in n-6 PUFA in the brain lipids are shown in **Fig. 6**. In contrast to the results with the Saf-DHA diet (Fig. 2), the Saf-OA-DHA diet decreased the 20:4n-6 and 22:4n-6 levels to the levels of the Per group almost completely by 9 weeks of age. At 19 weeks of age, those levels were even lower than those in the Per group. The levels of other fatty acids such as 18:2n-6 and 20:3n-6, saturated and monounsaturated fatty acids in the Saf-OA-DHA group, were similar to that in the Saf-DHA group (data not shown). The n-6/n-3 ratios in the brain fatty acids were 4.49 ± 0.06, 0.77 ± 0.02, and 0.73 ± 0.02 at 9 weeks of age, and 3.16 ± 0.15, 0.74 ± 0.02, and 0.85 ± 0.02 at 19 weeks of age in the Saf, Saf-OA-DHA, and Per groups, respectively. The values in the Saf group were significantly higher than in the Saf-OA-DHA and Per groups, but the differences between the two dietary groups were not significant.

Effect of a shift of Saf diet to Saf-OA-DHA diet on learning ability

In the original schedule, the Saf-OA-DHA group exhibited fewer positive responses (R^+) compared with the Per

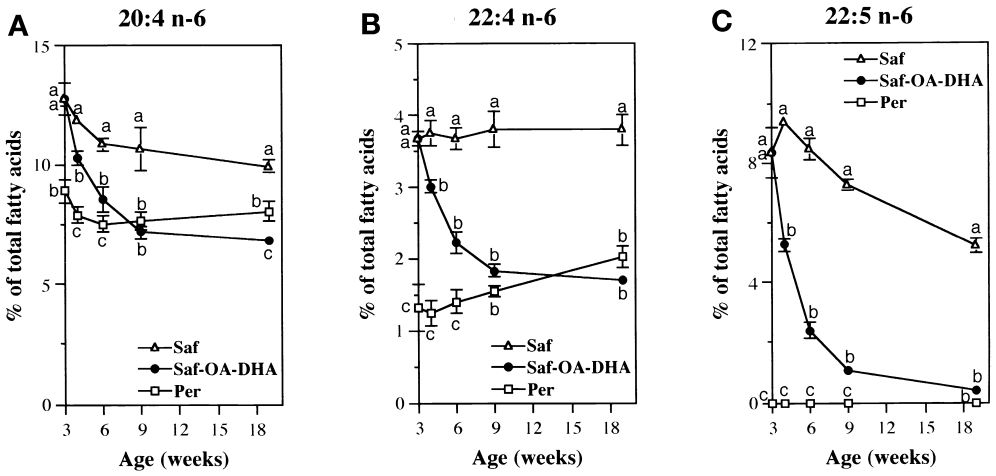

Fig. 6. Effect of a shift of Saf diet to Saf-OA-DHA diet after weaning on brain n-6 PUFA level. A: 20:4n-6. B: 22:4n-6. C: 22:5n-6. See legend to Fig. 1 for details.

group (**Fig. 7**). The number of R^- decreased in the order Saf group > Per group > Saf-OA-DHA group. The CRR was significantly lower in the Saf group than in the Per and Saf-OA-DHA groups.

The recovery of learning impairment was observed more clearly in the reverse schedule (**Fig. 8**). The R^+, R^-, and the CRR of the Saf-OA-DHA group were essentially the same as those of the Per group, which were different from those of the Saf group. Thus the n-3 fatty acid deficiency during gestation and lactation induced learning impairment that was reversed by shifting to the Saf-OA-DHA diet after weaning (Figs. 7 and 8) but not by shifting to the Saf-DHA diet (Figs. 3 and 4).

DISCUSSION

The inferior learning capacity of rats associated with n-3 fatty acid deficiency was first reported on the basis of a simple Y-maze test (30). However, this result was not reproduced with a more complex X-maze test (31). Harman et al. (32) reached an apparently opposite conclusion by using a different maze test. In a radial maze with eight arms, we were unable to establish any statistically significant difference in the learning ability between rats fed the Saf diet and those fed the Per diet through two generations (A. Ikemoto, M. Ohishi, Y. Fujii, and H. Okuyama, unpublished observations). Thus, the conclusions from maze tests are inconsistent. However, it is now clear from our results obtained with a computer-programmed brightness-discrimination learning apparatus (17–19, 28) and from those of the other studies (33–38) that n-3 fatty acid deficiency causes impaired learning performance in the rat.

A 22:6n-3-rich fish oil diet was as effective as a Per diet in the learning test (H. Okuyama et al., Japan Patent Publication, 1990-153629, 1990), and the learning ability in the brightness-discrimination learning test as well as the

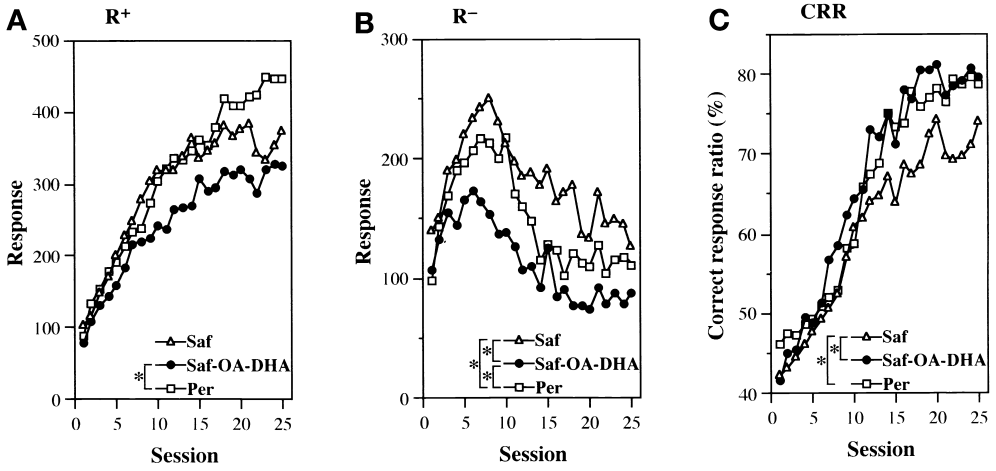

Fig. 7. Effect of a shift of Saf diet to Saf-OA-DHA diet after weaning on learning ability (original schedule). The original schedule of the brightness-discrimination learning test in F_1 rats fed a semipurified diet supplemented with Saf, Saf-OA-DHA, or Per was followed, as described in text. A: R^+. B: R^-. C: CRR. See legend to Fig. 3 for details.

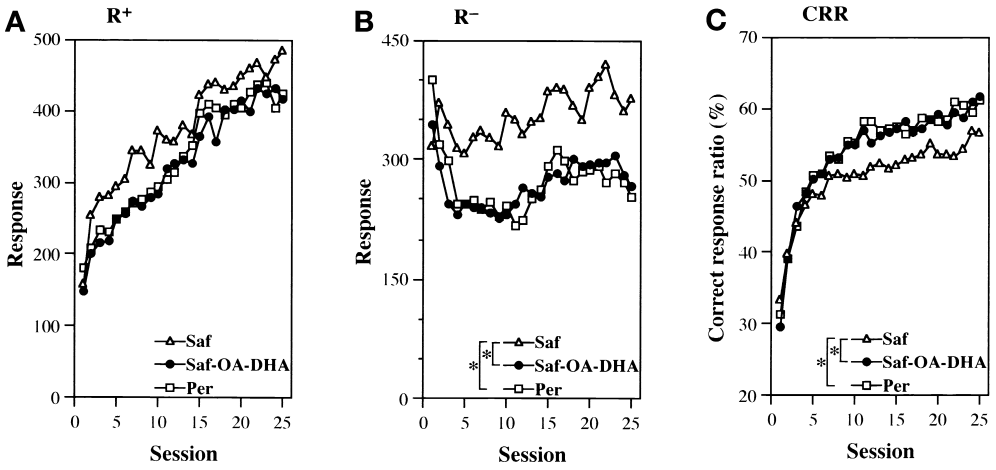

Fig. 8. Effect of a shift of Saf diet to Saf-OA-DHA diet after weaning on learning ability (reverse schedule). The reverse schedule of the brightness-discrimination learning test in F_1 rats fed a semipurified diet supplemented with Saf, Saf-OA-DHA, or Per was followed, as described in text. A: R^+. B: R^-. C: CRR. See legend to Fig. 4 for details.

brain 22:6n-3 level were restored to the level of the Per group when the Saf diet was shifted to the Per diet after weaning (28). Therefore, we concluded that n-3 fatty acid is essential for the maintenance of learning performance and that n-3 deficiency in the presence of n-6 fatty acid during gestation and lactation does not lead to irreversible damage to the brain (28). The data presented here support our earlier interpretation. Moriguchi, Griener, and Salem (38) have reported that learning and cognitive behavior is related to brain 22:6n-3 status, which, in turn, is influenced not only by the weaning diet but also by the milk received from the mother during lactation. However, the Saf-DHA diet did not restore the learning ability but the Saf-OA-DHA diet did (Figs. 3, 4, 7, and 8). The brain 22:6n-3 levels of the two dietary groups reached that of the Per group at 9 weeks of age before the learning test but the recovery of 22:6n-3 tended to exhibit a slightly steeper slope with the Saf-OA-DHA diet than in the Saf-DHA group (Figs. 1B and 5B). Reversal of 22:6n-3 deficiency has been investigated in several tissues of the rat (39), suggesting that transport-related processes may limit the rate of 22:6n-3 repletion in the brain. It is possible, then, that the difference in the recovery rate of brain 22:6n-3 affects learning performance. However, the lower learning performance of the Saf-DHA group was observed throughout the original and reverse schedule (for 2 months after complete recovery of the brain 22:6n-3 level), which indicates that the difference in the recovery rates of 22:6n-3 is not the major factor influencing the reversibility of the learning performance under the conditions examined. The n-6/n-3 ratio of the Saf-DHA group was higher than in the Per group before and after the learning test but that of the Saf-OA-DHA group decreased to that of the Per group at 9 weeks of age. A difference in brain fatty acid status of both dietary groups was found when examining n-6 fatty acid levels (Fig. 2A and B and Fig. 6A and B), indicating that not only n-3 fatty acids but also n-6 fatty acids (20:4n-6 and 22:4n-6) are involved in learning performance and its reversibility. The 20:4n-6 metabolites may be involved in learning behavior because indomethacin affected both the numbers of R^+ and R^- in the rat (C. Fujiwara, S. Yuasa, S. Watanabe, T. Kobayashi, and H. Okuyama, unpublished observations).

As to the mechanism by which n-3 fatty acid deficiency induces altered behavior, altered monoaminergic neurotransmission (40, 41), enhanced brain protein synthesis (42), decreased brain phospholipid synthesis (43), decreased pineal melatonin release (44), and altered 12-hydroxyeicosatetraenoic acid and melatonin levels (45) have been proposed, although enhanced protein synthesis has not been confirmed in our case (46). Salem and Niebylski (47) suggested that a specific molecular species of 22:6n-3-containing phospholipid is critical for optimal neural function. Our data suggest that n-6 fatty acids (20:4n-6 and 22:4n-6) may also affect phospholipid bilayer properties and interactions of phospholipids and proteins. Here, we have provided evidence that increased n-6 PUFA (20:4n-6 and 22:4n-6) level in the brain under n-3 fatty acid deficiency is another critical factor affecting the reversibility of learning performance in the brightness-discrimination learning test. Because n-6 and n-3 fatty acids are competitive at many enzymatic steps, it is important to limit the intake of n-6 fatty acids when n-3 fatty acids are supplemented in order to prevent alterations in learning behavior (16).

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