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Modulation of learning, pain thresholds, and thermoregulation in the rat by preparations of free purified α-linolenic and linoleic acids: Determination of the optimal ω3-to-ω6 ratio

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ABSTRACT **Ingested polyunsaturated fatty acids are postulated to lead to changes in central nervous system activity, presumably by altering the lipid composition of neuronal membranes. In support of this hypothesis, we and other investigators have previously demonstrated cognitive effects in rats fed oils that contain both α-linolenic acid (18:3ω3) and linoleic acid (18:2ω6), with the relative content of α-linolenic acid being seen as the critical variable. The present study in rats examined the effects of preparations containing different ratios of highly purified free α-linolenic acid to linoleic acid (about 25 mg/kg of body weight daily) on learning performance (Morris water tank), pain thresholds (heated plate), and thermoregulatory control of *d*-amphetamine-induced hypothermia during 4 weeks of treatment. Preparations with ω3-to-ω6 ratios ranging from 1:3.5 to 1:5 (specifically a ratio of 1:4) produced significant favorable effects on all of these variables. Although the specific mode of action remains to be elucidated, these results suggest that such preparations of free fatty acids should be evaluated in the treatment of memory disorders and pain conditions.**

Previous evidence suggests that certain biological constituents, when administered in pure form or ingested in food, can function as drugs. They may induce changes in the chemical composition of structures in the brain and consequently modify brain activity in experimental animals (1, 2). For example, it has been hypothesized that the mode of action of tryptophan, tyrosine, and choline involves their role as precursors for brain neurotransmitters (1). The ratio between the level of tryptophan and large neutral amino acids (the total of tyrosine, phenylalanine, leucine, isoleucine, and valine) in the plasma is reported to be a critical determinant of brain tryptophan bioavailability (1). Changes in the levels of these amino acids in the central nervous system are postulated to induce changes in the functional activity of brain neurotransmitters and consequently in behavior.

However, there are several observations regarding other types of food components that cannot be satisfactorily accounted for by such an explanation—e.g., the cognitive effects of soybean oil and the regional decrease in the level of cholesterol after learning (3, 4). We have also previously proposed (2, 4–6) that diet-induced changes in the lipid composition of neuronal membranes may mediate the observed changes in learning and behavior. It should be emphasized that this hypothesis, the neuronal membrane functional modification hypothesis (2), does not contradict but supplements the neurotransmitter precursor hypothesis (1).

Several researchers have examined the effects of various oils in the diet on brain development, brain biochemistry, and behavior (7–11). Most of the studies showed that oils containing either α-linolenic acid (18:3ω3) (such as soybean oil and perilla oil) or docosahexanoate (22:6ω3) had beneficial effects on various types of learning (7–11). These studies are consistent with our own observations that rats fed a soybean source lipid diet exhibited a significantly improved capacity in an environmentally cued testing paradigm (4–6). In addition, they exhibited a higher pain threshold and were protected from *d*-amphetamine-induced hypothermia when exposed to an ambient temperature of 4°C. In contrast, rats fed a lard or sunflower source diet did not differ from rats fed a control (Chow) diet. None of the diets induced changes in the level of motor activity (4–6).

The initial hypothesis that attempted to explain these results focused on the amount of polyunsaturated fatty acids (PUFAs) in soybean oil. However, sunflower oil, which contains a higher level of PUFAs than soybean oil, failed to produce the positive effects of soybean oil (5, 6). Since soybean oil contains a considerably higher level of α-linolenic acid (8–9%) than sunflower oil (about 0.4%), we postulated that the relative quantity (ratio) of α-linolenic acid to linoleic acid (18:2ω6), rather than the absolute quantities of the fatty acids, was the critical factor for brain bioavailability and the central nervous system-mediated effects.

Whereas the importance of linoleic acid for normal health as well as for brain development and the maintenance of normal brain function had already been demonstrated (12), the biological effects of α-linolenic acid [which is also traditionally classified as an essential fatty acid (EFA)] are only recently becoming clarified. The selective enrichment of elongated ω3 fatty acids in the retina and the brain and their relative resistance to depletion has puzzled many investigators. Experiments with ^{14}C-labeled fatty acids have shown a preferential brain uptake of α-linolenic over linoleic acid (13). Early studies also suggested that α-linolenic acid may have a biochemical function distinct from that of linoleic acid, because decreases in 5′-mononucleotidase activity in the brain, caused by lipid deprivation, could be normalized only by linolenic acid supplementation (14). Although there were until now only a few clinical reports of α-linolenic acid deficiency (15–17), experiments in monkeys and rats have shown visual and learning impairment after consumption of diets deficient in ω3 fatty acids (18–20). These studies prompted a recent surge of interest in the role of α-linolenic acid and its derivatives in brain development, brain and retinal function, and maintenance of normal well-being (21–33).

The aims of this study were to test the hypothesis that the ratio of α-linolenic to linoleic acid is a key factor in mediating the beneficial effects of PUFAs and to identify the optimal ratio of these free fatty acids. To avoid changes in the percentage of fatty acid in commercially available oils and to exclude the confounding effects of other fatty acids or lipids, the test materials were prepared from highly purified free

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Abbreviations: PUFA, polyunsaturated fatty acid; EFA, essential fatty acid.

Table 1. Nutritional factors

Group	Food intake, kcal	Weight gain, g
A	2565 ± 39	237 ± 4.7
B	2575 ± 80	230 ± 7.0
C	2545 ± 75	235 ± 2.8
D	2534 ± 68	237 ± 4.6
E	2543 ± 72	239 ± 6.1
F	2562 ± 57	235 ± 3.3
G	2586 ± 48	238 ± 3.9
H	2533 ± 61	234 ± 5.5

The values given are the means ± SEM from nine rats per group. The unsaturated fatty acid treatments had no effect on the amount of food intake (kcal) or on the rate of body weight gain. The data were calculated at the end of treatment. Group A, 0.9% NaCl; groups B–H, α-linolenic acid-to-linoleic acid ratios of 1:3, 1:3.5, 1:4, 1:4.5, 1:5, 1:5.5, and 1:6, respectively.

linoleic and linolenic acids. We tested the effects of mixtures with various ratios on learning, motor activity, pain threshold, and thermoregulation in adult male rats.

MATERIALS AND METHODS

Test Material. α-Linolenic (0.92 g/cm^3) and linoleic (0.90 g/cm^3) free fatty acids, both ≈99% pure (as evaluated by capillary gas chromatography), were purchased from Sigma (L2367 and L1376). The test substances were stored at 4°C in the dark. A stock solution (1 ml) containing the two fatty acids (0.25 ml), mineral oil (0.73 ml), and α-tocopherol (0.02 ml) was prepared every 3 days. Seven different stock solutions with different ratios of the two fatty acids (see *Experimental Design* below) were used in the experiment.

Animals. Male Long Evans hooded rats [1 month of age and ≈100 g (range, 90–110 g)] were purchased from local breeders. They were housed individually in hanging stainless steel, wire-mesh cages in a well-ventilated room that was air-conditioned by means of a system designed to maintain the room temperature at an average of 22°C and a relative humidity of about 45%. The room was illuminated by a fluorescent light that simulates the spectrum of the sun (Vita-Lite; Dura-Test, Clifton, NJ) to permit an artificial 24-hr cycle of 12 hr of light (from 6 a.m. to 6 p.m.) daily. Tap water and Israeli Chow diet were available ad libitum.

Experimental Design. Seventy-two rats (12 each month for 6 months) were randomized to one of eight treatment groups (each with 9 rats): group A, saline (0.9% NaCl); groups B–H, α-linolenic acid-to-linoleic acid ratios of 1:3 (B), 1:3.5 (C), 1:4 (D), 1:4.5 (E), 1:5 (F), 1:5.5 (G), and 1:6 (H). An equal volume (1 ml) of placebo (0.9% NaCl) or test material (2.25 mg per rat with an initial average weight of 100 g, thus about 25 mg/kg) was injected intraperitoneally daily on days 1–28 in a double-blind fashion. The bolus consisted of 2.25 mg of free fatty acids (0.01 ml of the stock solution; see *Test Material* above) mixed with mineral oil to make 1 ml.

Observations. Testing was carried out immediately before the start of administration and was repeated at the end of weeks 1, 2, 3, and 4. The animals were weighed at the start and then again at the end of week 4. Baseline measurements in the learning apparatus were obtained in eight trials daily on 3 consecutive days (days −3, −2, and −1) immediately prior to the start of daily injections for 4 weeks. At the end of each week of treatment, testing was repeated 8 times daily on 3 consecutive days. Week 1 tests correspond to study days 7, 8, and 9; week 2 tests correspond to study days 14, 15, and 16; week 3 tests correspond to study days 21, 22, and 23; and week 4 tests correspond to study days 28, 29, and 30. All tests were administered between 10 a.m. and 2 p.m. using the same equipment, test instruments, and personnel. All testing was performed by an experimenter who was unaware of the diets fed to the individual subjects. Daily food intake was measured and converted into kcal. The order of the additional testing was as follows: on the first day of the 3-day testing periods, motor activity was measured (day 28 only), whereas pain threshold was measured on the second day (days −2, 8, 15, 22, and 29), and thermoregulation as well as retention of old learning was tested on the third day (days −1, 9, 16, 23, and 30).

The Learning Apparatus. The Morris water tank (see ref. 34 for complete review of the learning model), a circular tank (110 cm in diameter), was filled with water (to the level of 40 cm), which was made opaque by the addition of powdered milk, so that rats swimming in the tank were unable to see an escape platform (7.5 cm in diameter) submerged 2 cm below water level. Each animal was released facing the wall in one of four predetermined starting points each separated by 90° around the inner perimeter. While the rat was in the tank, it was able to observe the contents of the room. Special care was given to keep things in the room in the same location. The rat could navigate in the tank only by external cues. Each rat was tested 8 times per day in the tank. The order of the starting points was determined by random selection. To prevent possible effects of a magnetic field, each rat was allowed 120 sec to find the platform, with an interval of 20 sec between trials. The maximum duration of the test for each rat

Table 2. Number of trials to reach criterion (10 sec)

	Number of trials					
	Days pretreatment	Days after start of treatment				
Group	−3, −2, −1	7, 8, 9	14, 15, 16	21, 22, 23	28, 29, 30	*P*
A	19.6 ± 3.3	19.0 ± 3.7	20.3 ± 2.5	18.5 ± 2.9	19.1 ± 2.7	NS
B	20.1 ± 4.1	18.0 ± 4.0	19.9 ± 4.5	17.1 ± 4.0	17.0 ± 3.2	NS
C	17.1 ± 3.3	12.5 ± 2.1*	10.7 ± 4.1*	5.6 ± 2.5*	5.6 ± 2.5*	0.01
D	18.5 ± 2.0	9.3 ± 2.6*	7.1 ± 2.9*	6.1 ± 2.5*	6.1 ± 2.5*	0.001
E	19.1 ± 2.3	14.2 ± 3.7*	12.8 ± 3.9*	9.0 ± 3.4*	9.0 ± 3.4*	0.01
F	19.5 ± 3.5	16.1 ± 2.6	11.2 ± 1.1*	7.9 ± 1.0*	7.9 ± 1.0*	0.01
G	19.7 ± 3.8	18.1 ± 3.3	18.4 ± 2.9	18.6 ± 2.6	18.6 ± 2.6	NS
H	21.0 ± 4.0	20.0 ± 3.0	19.6 ± 3.1	19.1 ± 3.0	19.1 ± 3.0	NS
P	NS	0.01	0.01	0.01	0.01	

Groups (nine rats per group) are as identified in the legend to Table 1. Observations were made before start of treatment and at the end of weeks 1–4. Values represent the means ± SEM of eight tests daily on 3 consecutive days. Unsaturated fatty acid treatment with ω3-to-ω6 ratios of 1:3.5–1:5 (groups C–F) had a significant effect on learning. NS, not statistically significant.

*The *P* value in the last column indicates the *P* value relative to the pretreatment value for that group. The *P* value in the bottom row indicates the *P* value relative to the control (saline; group A) for those days.

Table 3. Time in the "wrong" location

	Time in the wrong location					
	Day pretreatment	Day after start of treatment				
Group	−1	9	16	23	30	*P*
A	22.9 ± 3	24.3 ± 4	19.0 ± 3	22.3 ± 4	25.1 ± 4	NS
B	18.5 ± 3	19.4 ± 4	20.6 ± 6	20.6 ± 4	20.1 ± 5	NS
C	20.3 ± 4	30.9 ± 2*	35.3 ± 4*	39.2 ± 4*	49.4 ± 3*	0.001
D	19.5 ± 3	24.1 ± 3	29.3 ± 4*	36.6 ± 4*	39.1 ± 4*	0.01
E	20.8 ± 4	25.1 ± 4	30.1 ± 3*	33.1 ± 4*	36.1 ± 5*	0.01
F	19.4 ± 3	22.1 ± 3	29.1 ± 5*	30.1 ± 5*	32.2 ± 5*	0.01
G	22.8 ± 4	19.4 ± 3	19.0 ± 3	19.6 ± 4	18.1 ± 4	NS
H	19.1 ± 5	18.7 ± 5	19.9 ± 4	21.1 ± 3	19.6 ± 5	NS
P	NS	0.01	0.001	0.001	0.001	

Groups (nine rats per group) are as identified in the legend to Table 1. The means ± SEM of the first two trials are shown. Unsaturated fatty acid treatment with ω3-to-ω6 ratios of 1:3.5–1:5 (groups C–F) had a significant effect on retention of old learning. NS, not statistically significant.
*The *P* value in the last column indicates the *P* value relative to the pretreatment value for that group. The *P* value in the bottom row indicates the *P* value relative to the control (saline; group A) for that day.

was 16 min, and three rats were tested each hour. The rats were tested on 3 consecutive days. During this period, the platform was in the same location in the tank. For each of the 24 trials (eight trials × three days), the latency to reach the platform was recorded. A cutoff criterion, defined as the first successful trial with a maximum latency of 10 sec without any increase in latency on a later trial, was used to calculate an index of learning ability (rate of learning) for each diet group.

After the completion of the eighth trial on day 3, the platform was removed and placed in a different location in the tank. The time that the rats spend in the "old" (wrong) position for two trials was used to calculate the resistance to extinction (retention of old learning). To our knowledge, this is the first time that this method has been used in a Morris water tank.

The Level of Motor Activity. This endpoint was assessed in an open field apparatus by recording the number of horizontal movements (infrared photobeam crossings) and rearing movements (determined from videotapes) made during the 15-min sessions. The apparatus was very similar to the one previously described by Coscina and Yehuda (4).

Pain Threshold. A plate, 60 × 60 cm, was held at 58 ± 0.2°C by a thermostatic bath (Hakka, Karlswke, Germany). The animal was placed on the plate. The latency (to the nearest 0.1 sec) to lick the paw was recorded (35).

Thermoregulation. As previously described (36), the basal colonic temperature of each rat was measured (Yellow Springs Instruments telethermometer, model 43TA), after which the rat was injected intraperitoneally with *d*-amphetamine (15.0 mg/kg) and placed immediately into a 4°C cold room for 1 hr. The temperature was recorded again after 60 min in the cold room.

Statistics. Group comparisons were made using ANOVA (one-way repeated measures) with subsequent contrast *t* tests.

RESULTS

Effect of Food Intake and Body Weight. As shown in Table 1, treatment with the test material had no effect on food intake or body weight in any of the groups.

Effect on Learning. Tables 2 and 3 and Fig. 1 present the results of the learning experiments. At the end of weeks 1–4, groups C, D, and E (i.e., ratios of 1:3.5, 1:4, and 1:4.5) showed a significant reduction in the number of trials necessary to reach the criterion (10 sec) as compared to baseline—i.e., before start of treatment (Table 2 and Fig. 1). This was also observed for group F (ratio 1:5) after week 2.

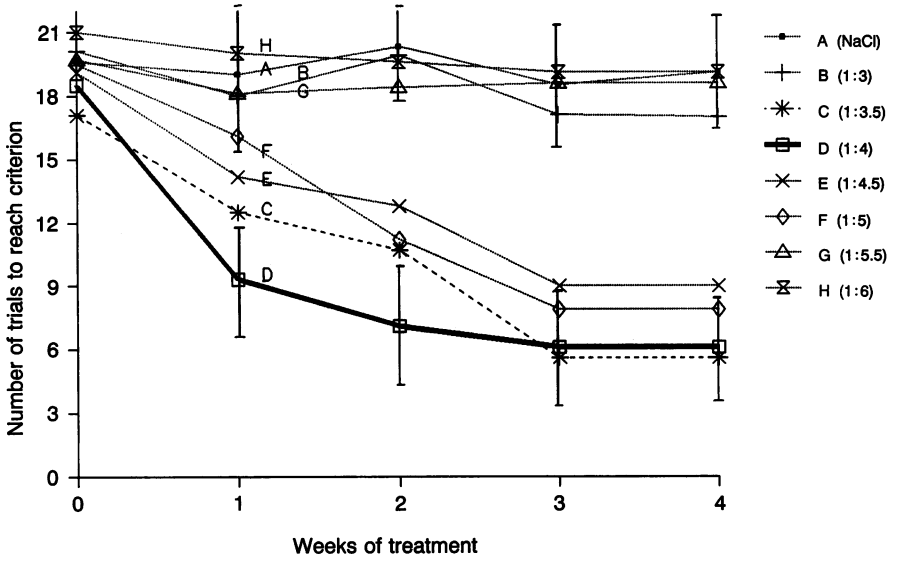

FIG. 1. Improvement of learning (Morris water tank testing during 4 weeks of treatment at 25 mg per kg per day) in the rat by α-linolenic/linoleic acid mixtures with ω3-to-ω6 ratios of 1:3.5–1:5 (groups C–F). The values plotted are the means; error bars indicate the SEM.

ANOVA shows that the ratio 1:4 (group D) differs statistically at repeated measures at the level of $P < 0.001$, indicating that this ratio may be most effective in improving performance in a cognitive task. At the end of weeks 1–4, group C (ratio 1:3.5) and at the end of weeks 2–4, groups D, E, and F (ratios of 1:4, 1:4.5, and 1:5) also showed significant effects on the retention of old learning as determined by the duration of time spent in the wrong (old) platform location (Table 3).

Effect on Motor Activity. At the end of weeks 1–4, none of the treatment groups showed any significant effects on horizontal or vertical movement, as determined by counts of infrared photo beam crossings and the frequency of rearings (data not shown).

Effect on Pain Threshold. At the end of weeks 1–4, groups D and E (ratios of 1:4 and 1:4.5) and at the end of weeks 2–4 and 3–4, groups C and F (ratios of 1:3.5 and 1:5), respectively, also showed a significant effect on pain threshold (analgesia) as determined by the latency for the rat to lick the paw after being placed on a hot plate (Table 4).

Effect on Thermoregulation. At the end of weeks 1–4, groups C–F (ratios of 1:3.5–1:5) showed significant protection from *d*-amphetamine-induced hypothermia, as determined by colonic temperature measurements before and after placement in a cold room (4°C) for 1 hr (data not shown).

DISCUSSION

The results of this study show that administration of α-linolenic acid and linoleic acid preparations with ratios of the fatty acids ranging from 1:3.5 to 1.5 has a significant effect on learning (Table 2 and Fig. 1), which cannot be explained by changes either in food intake or weight gain (Table 1) or in motor activity (data not shown). Furthermore, as all animals were about 1 month of age, there was no difference in the stage of development between treatment and control groups that could impact on the results. Formulations with a ratio of 1:4 have been selected for further experimental and clinical evaluation. The effect of these specific PUFA preparations on pain threshold (Table 4) and thermoregulation (data not shown) after 1–4 weeks of administration suggests that the activity is mediated via central sites, since *d*-amphetamine-induced hypothermia is regulated by dopaminergic neurons in the brain (36).

Linoleic and α-linolenic acids and their elongated and desaturated derivatives are polyunsaturated EFAs, which presently are recognized to have several important biological functions. Approximately 20% of the dry weight of the brain consists of EFAs, which are incorporated into phospholipids that are critically important for the structural integrity of neuronal membranes, membrane fluidity (28), and membrane-related functions such as receptor, enzyme, and ion channel kinetics, as well as eicosanoid functions (37–39).

The ω3 and ω6 fatty acid families are closely interrelated with positive and negative feedback regulation of desaturating conversion enzymes (40). Although alteration of the fatty acid composition of brain lipids by varying levels of ingested EFAs was demonstrated almost three decades ago (41), only recently has research focused on determination of the optimal ω3-to-ω6 ratio in adult diet and in infant or parenteral formulas. Whereas Neuringer *et al.* (18) considered a ratio in the range 1:4–1:10 to be prudent, several recent reports (42–45) support our observation that a 1:4 ratio may be optimal. A recent North Atlantic Treaty Organization conference on essential fatty acids recommended a ratio of 1:4 (42). Analysis of the fatty acid composition of human milk from Canadian Eskimos eating a traditional diet showed a ω3-to-ω6 ratio of 1:4 (43). Wainwright *et al.* (44) found evidence in the developing mouse that maximal incorporation of ω3 fatty acids in the phosphatidylethanolamine fraction of the brain membrane occurred with a ratio of 1:4. Clark *et al.* (45) found evidence that maximal ω3 incorporation into erythrocyte membranes (a postulated marker for brain membranes) in human infants occurred after use of formulas with a ratio of 1:4. It is possible that such a ratio may optimize enzyme kinetics within the ω3 and ω6 fatty acid families to allow maximal conversion to the elongated and desaturated species. Thus, it is likely that at least some of the beneficial effects of certain PUFA formulations are related to optimal incorporation of ω3 fatty acids into the brain membranes, without causing a concomitant inhibition of ω6 conversions (and subsequent depletion of ω6 derivatives) in the membrane due to negative feedback regulation. This concept is also in agreement with a recent report that expressed caution in the use of ω3 supplements in infant formulas without additional ω6 supplementation (46).

A fundamental question is how can a specific ratio of EFA in administered preparations be biologically meaningful. The basic diet (Israeli Chow) according to specifications by the manufacturer and our own biochemical analysis contains about 0.15 mg of α-linolenic acid per kg of diet and about 35 mg of linoleic acid per kg of diet (i.e., a ratio of 1:233; total fat about 5.1%). This could indicate that the feeding of a diet low in α-linolenic acid may have caused a relative EFA deficiency and cognitive dysfunction at baseline in all groups as well as during the total study period in the control groups. If so, the observation that supplemental α-linolenic acid and linoleic

Table 4. Pain threshold

	Latency to lick the paw after being placed on a hot plate, sec					
	Day pretreatment	Day after start of treatment				
Group	−2	8	15	22	29	*P*
A	7.9 ± 0.9	7.8 ± 0.8	8.0 ± 0.6	7.9 ± 0.9	8.1 ± 0.9	NS
B	8.0 ± 0.8	7.9 ± 0.7	8.0 ± 0.9	8.1 ± 0.7	7.8 ± 0.7	NS
C	7.8 ± 0.6	11.9 ± 0.7	13.9 ± 0.7*	16.5 ± 0.6*	20.1 ± 1.1*	0.01
D	8.1 ± 0.8	12.1 ± 0.6*	14.5 ± 0.6*	18.2 ± 0.7*	21.1 ± 0.9*	0.01
E	7.8 ± 0.6	9.0 ± 0.9*	9.0 ± 0.8*	14.1 ± 0.7*	17.4 ± 0.7*	0.01
F	8.1 ± 0.9	9.9 ± 0.9	11.5 ± 0.7	14.1 ± 0.7*	16.3 ± 0.7*	0.01
G	7.6 ± 0.7	8.0 ± 0.3	8.8 ± 0.8	8.0 ± 0.8	8.1 ± 0.9	NS
H	8.0 ± 0.9	8.0 ± 0.4	8.5 ± 0.5	8.3 ± 0.7	8.3 ± 0.7	NS
P	NS	0.05	0.01	0.01	0.01	

Groups (nine rats per group) are as identified in the legend to Table 1. Values given are the mean ± SEM. Unsaturated fatty acid treatments with ratios of 1:3.5–1:5 (groups C–F) caused analgesia in rats that were placed on a heated plate (58°C). NS, not statistically significant.

*The *P* value in the last column indicates the *P* value relative to the pretreatment value for that group. The *P* value in the bottom row indicates the *P* value relative to the control (saline; group A) for that day.

acid in a specific range of ratios may improve cognitive function becomes even more intriguing. This would suggest that such treatment may have potential therapeutic value in Alzheimer disease, where autopsy studies have shown reduced content of certain EFAs in affected brain regions (47).

We are presently unable to offer a definitive explanation for the effects of certain PUFA formulations on memory function, pain thresholds, and thermoregulation. Previous data provides evidence that PUFAs in the diet can decrease plasma levels of cholesterol and that membrane cholesterol levels (which are correlated with plasma levels) are inversely related to membrane fluidity (37, 38). Thus, it seems reasonable to postulate that the beneficial effects may be directly related to effects of the administrated EFAs on the composition and fluidity of neural membranes in the central nervous system. Another biologically important function of α-linolenic acid may be to provide acetate for the *de novo* synthesis of palmitic and other long-chain fatty acids, which are essential for membrane integrity (27). Furthermore, since elongated and desaturated EFAs are enriched in the brain and their conversion mechanisms have been reported to be competent in rats as well as in humans, even at an advanced age (48), it is likely that some of the beneficial effects of the administered EFAs are mediated by such longer chain derivatives.

We have previously demonstrated that treatment of rats with soybean oil (49) or certain peptides (50) also provides protection from *d*-amphetamine-induced hypothermia when the animals are placed in a cold room. Our theory is that *d*-amphetamine-induced hypothermia is mediated by the dopaminergic system in the striatum (51). It is possible that certain formulations of PUFAs affect the dopaminergic system, most likely the D_2 receptors.

In summary, our results show that treatment of rats for 2–4 weeks with preparations of α-linolenic acid in combination with linoleic acid in ratios ranging from 1:3.5 to 1:5 had a significant effect on the rate of learning, retention of old learning, pain thresholds (analgesia), and prevention of the *d*-amphetamine-induced hypothermic response to reduced ambient temperature. A clearer understanding of the mode of action of certain formulations of PUFAs will assist in the further evaluation in animal models as well as in human memory disorders, such as Alzheimer disease, and other degenerative disorders, where free radical formation, oxidation, deficiency of PUFAs (47, 52, 53) or degeneration of brain membrane phospholipids (54) have been implicated.

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