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Effects of Dietary γ-Linolenic Acid and Prenatal Ethanol on Mouse Brain and Behavior

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WAINWRIGHT, P. E., Y.-S. HUANG, S. LÉVESQUE, L. MUTSAERS, D. McCUTCHEON, P. BALCAEN AND J. HAMMOND. *Effects of dietary γ-linolenic acid and prenatal ethanol on mouse brain and behavior.* PHARMACOL BIOCHEM BEHAV **53**(4) 843-852, 1996. – Pregnant B6D2F$_1$ mice were treated with ethanol (25% Kcal) from days 5-17 of gestation. The diet was supplemented with either 18 : 2n-6 [linoleic acid (LA)] or 18 : 3n-6 [γ-linolenic acid (GLA)] throughout the study. Ethanol reduced 20 and 22 carbon n-6 and n-3 fatty acids in the brains of adult offspring. Feeding of GLA, compared with LA, increased levels of 20 : 3n-6 and 22 : 4n-6, but reduced 22 : 6n-3, particularly in the offspring of dams administered ethanol during gestation; adult brain weight was also lower in this group. Ethanol reduced the number of viable litters and adult body weight, and GLA reduced birth weight. Neither prenatal ethanol nor GLA affected open-field activity in adult males, nor did either treatment have an effect on the duration of immobility in the forced swimming test. However, GLA did affect circadian activity by increasing running wheel activity during the dark cycle, and decreasing it slightly during the light cycle. These results do not support a beneficial effect of GLA in preventing the developmental effects of ethanol; we suggest caution in the administration of high doses of GLA with ethanol during pregnancy.

γ-Linolenic acid Essential fatty acids Brain phospholipid Prenatal ethanol Circadian wheel-running activity Forced swimming test Mice

POLYUNSATURATED fatty acids, particularly arachidonic acid [20 : 4n-6 (AA)] and docosahexaenoic acid [22 : 6n-3 (DHA)], are found in high concentrations in brain phospholipid, particularly in synaptosomal, microsomal, and mitochondrial fractions (7,34). As shown in Fig. 1, they are synthesized by animals from the 18-carbon fatty acids linoleic acid [18 : 2n-6 (LA)] and linolenic acid [18 : 3n-3 (LnA)] through a series of desaturation and elongation reactions (10). LA and LnA are called essential fatty acids because, unlike plants, animals lack the capacity to synthesize these 18-carbon precursors de novo, and are therefore dependent on dietary supply.

Polyunsaturated fatty acids (PUFA) have the potential to influence neural function through their effects on membrane properties, as well as through their role as precursors of the eicosanoids (i.e., prostaglandins and related compounds), which serve an important regulatory function (9). γ-LnA [18 : 3n-6 (GLA)] is the product of the δ6 desaturation of LA. It is further chain-elongated to form dihomo-γ-LnA [20 : 3n-6 (DGLA)], which is in turn desaturated to form AA (17). DGLA and AA can be metabolized through the cyclooxygenase pathway to the 1 and 2 series prostaglandins, respectively. Based on this, GLA has been proposed to be a compound of nutritional and medical importance, particularly in situations where δ6-desaturase activity may be compromised (22). In this article we address the effects of long-term dietary GLA supplementation on brain fatty acid composition and behavioral outcomes, including effects on mice that have been exposed to ethanol during gestation.

Ethanol has been shown to be among the factors which influence the activities of the desaturase enzymes (27). The observation that ethanol increases the ratio of LA to AA in various membrane fractions suggests that ethanol inhibits the activity of the δ6 desaturase, which is instrumental in the conversion of LA to GLA, as well as δ5 desaturase, which is involved in the conversion of DGLA to AA [reviewed in (30,33)]. It is thought that some of the pharmacologic effects

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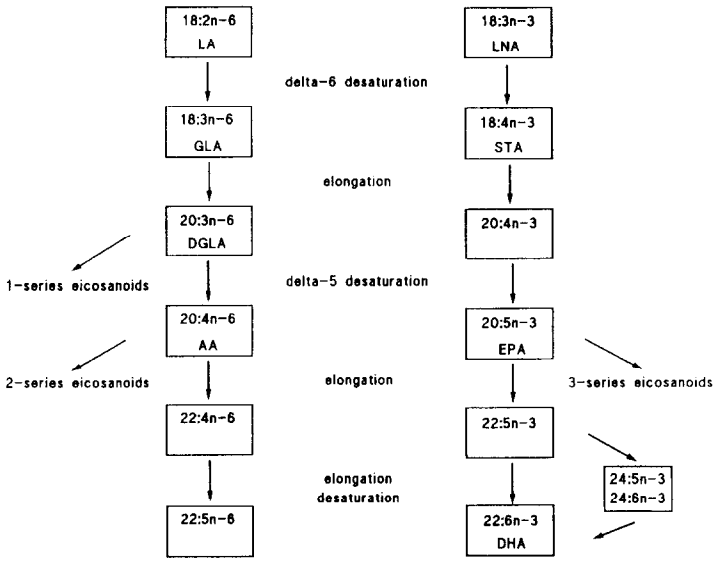

FIG. 1. Schematic representation of major pathways of n-3 and n-6 fatty acid (FA) biosynthesis. In FA nomenclature, the first number denotes the length of the carbon chain; the second number, following the colon, refers to the number of double bonds; and the third number, after n-, represents the number of carbons from the methyl end of the molecule to the first double bond. LA = linoleic acid; GLA = γ-LnA; DGLA = dihomo-γ-LnA; AA = arachidonic acid; LnA = linolenic acid; STA = stearidonic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.

of ethanol may be related to its effects on membrane fatty acid composition and prostaglandin (PG) production (5, 20,21,23). This is supported by studies which show that both GLA and PGE_1 potentiate some of the acute behavioral effects of ethanol and reduce the severity of withdrawal symptoms in alcohol-addicted animals (32,35). These effects can be prevented by the administration of inhibitors of PG synthesis, which suggests that they are mediated through PG production (14,15,31). Based on these observations, it has been suggested that PGE_1 may be implicated in alcoholism, and also possibly in affective disorders (22). This hypothesis states that although ethanol initially increases PGE_1 production, chronic use, through inhibition of δ6-desaturase activity, will prevent replenishment of DGLA stores and eventually result in a deficiency of DGLA and hence PGE_1. Thus, the physiologic and behavioral disturbances attendant to chronic alcoholism should be ameliorated by dietary supplementation of GLA (20).

The use of alcohol by women of childbearing age is of particular concern because of the threat it poses to the health of the unborn child (2). Dietary deficiencies of essential fatty acids during development have been associated with various adverse outcomes, including severe growth retardation and behavioral anomalies [reviewed in (25,38)]. Chronic administration of ethanol increases urinary PG output and amplifies indices of essential fatty acid deficiency (4). This then leads to the question of whether some of the deleterious effects of ethanol consumption during pregnancy, specifically those on growth and activity, might be mediated through essential fatty acid deficiency, specifically of the long-chain n-6 fatty acids. If so, the administration of GLA, by circumventing the effects of ethanol on δ6-desaturase activity, may improve fatty acid status and thereby improve developmental outcomes.

There is also the question of whether, independent of the effects of prenatal ethanol exposure, long-term dietary administration of GLA is reflected in changes in brain fatty acid composition and associated behaviors. Sleep disturbances, which are characteristic of depressive disorders, are relieved by the use of tricyclic antidepressants (16), and a recent study suggests that unsaturated free fatty acids may play a role in regulating antidepressant binding sites in rat brain (36). There is also an accumulating body of evidence that PGs are involved in the regulation of sleep-wake activity, with PGE_1 having an awaking effect [reviewed in (19)]. We were therefore particularly interested in obtaining measures of circadian activity in relation to dietary GLA supplementation, as well as indications of effects on a standard screening test of antidepressant activity.

The present study addressed the effects of prenatal ethanol exposure and chronic dietary supplementation with GLA on reproduction and offspring development, as well as the behavior of the adult male pups. The specific questions were as follows:

1) Will the long-term supplementation of the maternal diet with GLA counteract the effects of prenatal ethanol exposure on growth and development?
2) What are the effects of prenatal ethanol exposure and chronic dietary supplementation of GLA on adult brain fatty acid composition?
3) What are the effects of prenatal ethanol exposure and long-term dietary GLA supplementation on a) open-field activ-

ity and b) circadian activity as measured in a running wheel?

4) Does long-term dietary supplementation of GLA have effects similar to that of the tricyclic depressants in decreasing the time spent immobile in a forced swimming test?

METHODS

Subjects

Parents were B6D2F$_1$ hybrid mice purchased from Charles River Breeding Laboratories (St. Constant, P.Q., Canada) at 4 weeks of age. They were maintained under a reversed 12 L : 12 D schedule at 22 ± 1°C, with tapwater and lab chow (Purina 5001, protein, 23.0%; fat 4.5%; Bioserv, Frenchtown, NJ) available ad lib. The animals were group housed in standard opaque plastic cages containing a Beta-Chip hardwood bedding and toilet tissue for nesting material. Breeding commenced when the mice were 11 weeks old.

Experimental Design

The experiment was conducted in four groups ordered in a 2 × 2 factorial design, with the addition of a fifth untreated lab chow control group. The factors were dietary oil composition, 18 : 2n-6 (LA) vs. 18 : 3n-6 (GLA) and ethanol [25% ethanol-derived calories (EDC)] vs. maltose dextrin (substituted isocalorically for the ethanol). The dietary treatment was maintained throughout the experiment, whereas the ethanol was fed from days 5–17 of gestation. During the ethanol treatment, the four groups other than that fed chow were fed an average daily amount of liquid diet per gram body weight to ensure equivalence of caloric intake. This amount had been calculated on each day of pregnancy from previous studies using this diet (40,42). It should be noted that comparison of the so-called "normal" chow control group with the safflower-treated control group allows one to assess possible nutritional effects associated with the feeding of liquid diets and oil-supplemented chow.

TABLE 1

SELECTED FATTY ACID COMPOSITION OF DIETARY OILS*

	Experimental Oil (GLA)	Control Oil (LA)
Oils (proportion)		
Safflower	10	20
GLA 70	10	0
ALA 60	2	2
α-Tocopherol acetate	4 IU/g oil	4 IU/g oil
Fatty acids (%)		
14 : 0	0.08 ± 0.05	0.05 ± 0.09
16 : 0	3.26 ± 0.19	6.38 ± 0.05
16 : 1w7	0.41 ± 0.07	0.09 ± 0.03
18 : 0	1.17 ± 0.08	2.26 ± 0.01
18 : 1w9	9.07 ± 0.23	13.21 ± 0.05
18 : 2w6	42.93 ± 1.49	70.44 ± 0.61
18 : 3w6	34.51 ± 1.82	0.00
18 : 3w3	6.65 ± 0.33	6.39 ± 0.08
Other	0.82 ± 0.04	0.28 ± 0.06
20 : 2w6	0.23 ± 0.00	0.05 ± 0.04
20 : 3w6	0.00	0.00
20 : 4w6	0.12 ± 0.01	0.21 ± 0.04
20 : 5w3	0.05 ± 0.00	0.06 ± 0.05
22 : 4w6	0.00	0.11 ± 0.02
22 : 5w6	0.08 ± 0.01	0.14 ± 0.01
22 : 5w3	0.00	0.00
22 : 6w3	0.00	0.00
totaln-6	77.89	70.90
total n-3	6.65	6.39

*Fatty acids are expressed as wt% of total fatty acids. Values represent means ± SD based on three replications.

Diets

The dietary oils were constituted from a mixture of safflower oil and free fatty acid concentrates (ALA 60 and GLA 70) manufactured and supplied by Callanish Laboratories (UK). The proportion of each of these oils in the mixtures and the representative fatty acid composition is shown in Table 1.

The control oil (LA) contained n-6 fatty acids (LA, 18 : 2n-6) from safflower oil and n-3 fatty acids (α-LnA, 18 : 3n-3) from ALA 60; the experimental oil (GLA) contained long-chain n-6 (GLA, 18 : 3n-6) from GLA 70 in addition to LA and LnA. The n-3 : n-6 ratio in both oils was 0.09. A concern with the use of long-chain fatty acids is their oxidative potential; for this reason, 4 IU of the antioxidant α-tocopherol acetate was added to each gram of the prepared oil mixture. Oils and prepared diets were stored in the refrigerator under argon, and fresh diet was fed daily. We added 4 g of the oil mixture to 100 g of chow, resulting in 21% of the total calories being derived from fat; 3% of these were derived from GLA in the treated group. The fatty acid composition of the mixed chow is shown in Table 2.

During the ethanol treatment period, the animals were provided with liquid diets formulated for mice (Ref. 2187; Bio-Serv) as their sole source of food and fluid. In these diets, 20% of the calories were provided by protein (casein), 25% by carbohydrate (maltose-dextrin), 20% by fat (the experimental oils), and a further 25% by ethanol or, in the case of the control, maltose-dextrin. GLA provided 7% of the calories in the treated group during this period.

Breeding Protocol

At 3 weeks before mating, the females were assigned randomly to three groups: the experimental group was fed chow supplemented with oil containing GLA (GLA group), the control group was fed chow supplemented with the control oil (SAFF group), and the third group was given chow with no oil supplementation (LC group). Animals were mated daily starting at the beginning of the dark phase, and were checked for the presence of copulatory plugs 7 h later. The day on which a vaginal plug was detected was considered to be day 0 of gestation, and all subsequent days refer to days postconception. On day 0, pregnant dams within each dietary oil group were assigned randomly to either ethanol treatment (ETOH) or the maltose-dextrin control (CON) condition. The animals were weighed on days 0 and 5, and every 2 days thereafter, and the amount of food was adjusted accordingly. All groups were fed the CON diet until day 5. The ETOH was implemented gradually, with animals receiving 6.25% EDC on day 5, 12.5% EDC on day 6, 25% EDC on days 7–16, and 12.5% on day 17. On day 18 the animals were returned to chow (supplemented with the appropriate oil) and water ad lib. Birth occurred on day 19 or 20, and all dams and litters were weighed on day 21. At this time, litters were culled to six (four males and two females where possible). On day 32 all pups in a litter were assessed for eye-opening on a scale from 0.0–1.0

TABLE 2
SELECTED FATTY ACID COMPOSITION OF CHOW DIETS*

Fatty Acids (%)	Chow with LA†	Chow with GLA†	Lab Chow‡
14 : 0	0.63 ± 0.19	0.86 ± 0.02	1.39 ± 0.02
16 : 0	12.47 ± 0.20	10.78 ± 0.12	18.06 ± 0.11
16 : 1w7	1.10 ± 0.15	1.48 ± 0.09	2.59
18 : 0	5.40 ± 0.53	4.42 ± 0.04	7.51 ± 0.07
18 : 1w9	19.17 ± 0.50	18.35 ± 0.11	28.34 ± 0.22
18 : 2w6	42.75 ± 0.39	36.19 ± 0.15	31.72 ± 0.25
18 : 3w6	0.20 ± 0.28	16.06 ± 0.15	0.25 ± 0.00
18 : 3w3	5.23 ± 0.37	4.69 ± 0.02	3.29 ± 0.02
Other	1.52 ± 0.52	0.90 ± 0.06	0.96
20 : 2w6	1.34 ± 0.08	0.23 ± 0.05	0.15 ± 0.01
20 : 3w6	0.00	0.00	0.05 ± 0.07
20 : 4w6	1.17 ± 0.11	0.32 ± 0.04	0.47 ± 0.03
20 : 5w3	0.44 ± 0.38	0.79 ± 0.02	1.35 ± 0.02
22 : 4w6	0.00	0.00	0.25 ± 0.03
22 : 5w6	0.00	0.00	0.00
22 : 5w3	0.00	0.03 ± 0.06	0.20 ± 0.02
22 : 6w3	0.85 ± 0.11	0.66 ± 0.01	1.47 ± 0.04

*Fatty acids are expressed as wt% of total fatty acids. Values represent means ± SD based on three replications.
†Oils added to Purina 5001: 4-100 g.
‡Purina 5001 contains 4.5% fat.

(available from the author on request). All assessment was done independently of knowledge of the treatment group. Animals were weaned on day 43 postconception (about 23 days after birth), and ear-notched for identification. One male from each litter was assigned randomly to the open-field/wheel-running condition or to the forced swimming test. Males weaned from the same litter were housed three to a cage, which contained a plastic "plumber's elbow" to prevent fighting, and were maintained on their respective diets for the duration of the study. The use of males for the behavioral tests was dictated by our previous work in which prenatal ethanol resulted in hyperactivity in weanling males (41), and also by the fact that males were the standard used in the development of the forced swimming test (28).

Determination of Blood Alcohol Concentration (BAC)

At 1430 h on day 15, 100 μl of blood was withdrawn from the tip of the tail into a heparinized capillary tube and centrifuged; the plasma was frozen for subsequent determination of BAC. All control animals underwent the same procedure, and a few samples were kept as baseline controls. BAC were determined using the ultraviolet NAD/NADH determination method described in Sigma technical bulletin 332-UV (St. Louis, MO), with standards and reagents obtained from Sigma. All assays were run in duplicate and compared against a standard curve.

Open-Field Activity

When they were 7 weeks old (from birth), males were tested for activity in the open field. The apparatus used was an enclosure measuring 120 × 120 × 30 cm. The floor was painted white and divided by black lines into 15-cm squares (64 total). The animal was placed in the field and its behavior observed for 3 min. Computer-assisted measures (Videomex V, Columbus Instruments, OH) were obtained for the number of internal and external squares entered, the total distance travelled, and the time spent ambulatory, resting, or engaged in stereotypic behavior. Vertical activity in the form of rearing was recorded manually by trained observers and was defined as the animal standing on two hind legs with its nose in the air and two front paws off the ground. The reliability of this measure, determined by interobserver correlation, was 0.93. The apparatus was wiped down with 70% ethanol between each testing session.

Wheel Running

Running wheels with a diameter of 40.2 cm were installed into standard mouse cages. These were equipped with a magnet and counterbalance to record wheel revolutions. The magnetic switch was activated with each cycle of the wheel; this was recorded through connection to an analogue counter. The distance run was calculated as Distance (m) = Number of Revolutions × 0.402. The males were introduced into these individual cages at 9 weeks of age (from birth) and allowed 1 week to acclimatise to the apparatus. Activity was then measured twice daily for 4 consecutive days at the beginning of the dark and light cycles.

Forced Swimming Test

The procedure used was an adaptation of that described previously for mice (29). The primary use of this test is to screen for clinically effective antidepressants, which reduce the amount of time that the animals spend immobile during the test (i.e., not swimming or attempting to escape). The question we asked was whether a dietary intervention, alone or in combination with a tricyclic antidepressant, desmethylimipramine (DMI) (Sigma Biochemicals) might have a similar outcome. Because this test had not been validated previously on the $B6D2F_2$ mouse population, a standard dose-response curve using DMI was run on a separate group of animals fed lab chow. The apparatus consisted of two clear Plexiglas cylinders (diam. 10 cm × height 30 cm). These were placed in a clear plastic mouse cage containing 10 cm water at a temperature of 22 ± 1°C and were separated by an opaque plastic panel. The depth of water was that which prevented the animals from reaching the bottom with their tails and thereby using them for support. The animals were tested in pairs by being placed in the cylinders for 6 min and their behavior recorded using a Quasar VM701-K camcorder. The water was replaced between tests. Using video editing equipment, the tapes were scored subsequently for the time spent immobile during the last 4 min of testing. This was defined as the animal's making only those movements necessary to keep itself afloat. All scoring was done with the observer blind to the treatment condition of the animal. The interobserver reliability on this measure, determined by having two independent observers score a random subset of 10 tapes, was 0.97. To obtain the standard curve, 10 animals were assigned randomly to each of five DMI dosage conditions: 0, 3.75, 7.5, 15, and 30 mg/kg. The drug was dissolved in physiologic saline and administered intraperitoneally 1 h before testing, using an injection volume of 0.02 ml/g body wt. Animals in the treatment groups were tested twice, first with saline, then a week later with 7.5 mg/kg DMI. We followed this method, rather than counterbalancing the treatments, because we were most interested in the effects of the GLA treatment alone and did not want to risk the possibility of long-term carry-over effects from the drug. Moreover, there is evidence that the magnitude of learned immobility in rats is not altered by repeated testing (18).

Brain Lipids

At the conclusion of the wheel-running test, the animals were killed with an overdose of halothane; their brains were removed and weighed, and then frozen at −80°C for subsequent biochemical analysis. The brain lipids were extracted with chloroform/methanol (1 : 1, v/v) containing 0.02% BHT (w/v) using a modified Bligh and Dyer method (6). The total lipids were then fractionated into different lipid fractions by thin-layer chromatography (TLC) using g-silica gel plates (Analtech GF) and a solvent system, chloroform/methanol/acetic acid/water (50 : 30 : 2, by vol.). The fatty acids in the different phospholipid classes, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were methylated with 14% boron trifluoride in methanol and analyzed on a gas chromatograph (8420; Perkin Elmer, Norwalk, CT) equipped with a flame ionization detector and a 15 m × 0.32 mm (ID) capillary column (Supelco Wax 10, Bellefonte, PA). The temperature program for the GLC run consisted of 2 min at 160°C, followed by a 2°C/min increase to 190°C. After 10 min at 190°C, the temperature was increased 5°C/min to 220°C, followed by 2 min at 220°C. Identification of fatty acids was made via comparison of retention times with authentic standards.

Statistical Analyses

The maternal and pup growth data were analyzed using the general linear model (GLM) provided by the Statistical Analysis System (SAS) to perform analysis of variance (ANOVA), with the treatment effects analyzed through the use of preplanned comparisons, as outlined in Table 3. The standard dose-response curve on the Porsolt test was described using regression analysis. The α level was set at 0.05. The litter mean score was used as the unit of analysis for the data on pup growth and development (1), and the behavioral tests and brain fatty acid determinations were conducted on only one adult male from each litter. Table 3 shows the number of dams that were assigned to each group at conception and the numbers that eventually provided live litters for the study.

RESULTS

Growth and Development

Table 3 shows the data on reproductive outcome and pup development. There were fewer viable litters in the ethanol-exposed dams [χ^2 (1) = 7.1, p = 0.008]. BAC were not significantly different between the two oil-treated groups (mean ± SEM, mg %: GLA/ETOH 277 ± 25, SAFF/ETOH 216 ± 20). GLA reduced pup weight at birth [$F(1, 44) = 6.23$, $p < 0.05$], and birth weight was also lower in SAFF/CON than in LC [$F(1, 44) = 54.07$, $p < 0.0001$]. These weight differences were no longer apparent at day 32 post-conception or weaning. As predicted, ethanol retarded eye opening [$F(1, 45) = 3.73$, $p < 0.03$ (one-tailed)]. Adult males exposed prenatally to ethanol were lighter [$F(1, 40) = 8.96$, $p < 0.005$], and those exposed to both GLA and ethanol had lighter brains [Interaction $F(1, 41) = 4.14$, $p < 0.05$].

Brain Fatty Acid Composition

As expected, 20 : 4n-6 and 22 : 6n-3 were the predominant PUFA in brain phospholipid. The treatment effects on selected fatty acids in brain PC and PE fractions, expressed as weight percent of total methyl esters, are shown in Tables 4 and 5, respectively.

18 : 2n-6. The levels of 18 : 2n-6 were greater in SAFF/CON than in LC, PC [$F(1, 41) = 23.83$, $p < 0.0001$], or PE

TABLE 3

REPRODUCTIVE OUTCOME AND PUP DEVELOPMENT OF B6D2F$_1$ MOUSE DAMS TREATED WITH ETHANOL DURING GESTATION AND LONG-TERM DIETARY SUPPLEMENTATION OF GLA*

	1: GLA/ETOH	2: GLA/CON	3: SAFF/ETOH	4: SAFF/CON	5: Lab Chow	Effects ($p < 0.05$)†
Dams‡						
Day 0	17	15	16	17	15	
Day 21	6	10	8	14	11	
Dam weight (g)						
Day 0	21.6 ± 0.3	22.4 ± 0.5	21.8 ± 0.4	21.6 ± 0.2	21.6 ± 0.3	
Day 17	29.2 ± 0.7	29.7 ± 1.0	31.4 ± 1.4	30.0 ± 0.8	36.8 ± 0.7	NUT
Litter size (day 21)	6.5 ± 0.6	7.6 ± 0.0.6	8.0 ± 1.7	8.0 ± 0.5	8.5 ± 0.4	
Pup weight (g)§						
(Birth) day 21	1.2 ± 0.04	1.3 ± 0.04	1.4 ± 0.06	1.4 ± 0.03	1.8 ± 0.5	GLA NUT
Day 32	6.3 ± 0.4	6.7 ± 0.3	6.7 ± 0.2	7.0 ± 0.2	6.8 ± 0.2	
(Weaning) day 43	9.4 ± 0.7	10.1 ± 0.3	9.3 ± 0.3	10.2 ± 0.2	10.9 ± 0.3	
Males (11 weeks)	18.6 ± 0.8	21.8 ± 0.7	20.3 ± 1.0	21.3 ± 0.4	22.6 ± 0.5	ETOH
Pup brain weight (11 weeks)	0.363 ± 0.009	0.387 ± 0.004	0.385 ± 0.007	0.386 ± 0.004	0.398 ± 0.004	ETOH INT
Pup eye opening (day 32)	0.24 ± 0.008	0.34 ± 0.005	0.26 ± 0.006	0.34 ± 0.03	0.42 ± 0.004	ETOH‖

*Values represent means ± SEM.

†ETOH = ethanol effect (groups 1 + 3 vs. 2 + 4); GLA = GLA effect (groups 1 + 2 vs. 3 + 4); INT = interaction (groups 1 + 4 vs. 2 + 3); NUT = nutritional effect (group 4 vs. 5).

‡Days refer to days postconception.

‖ETOH effect on eye opening significant at $p < 0.06$.

§Days refer to days postconception.

TABLE 4

SELECTED FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE FRACTION OF ADULT BRAIN PHOSPHOLIPIDS, EXPRESSED AS WT% OF TOTAL FATTY ACIDS*

Fatty Acid	GLA/ETOH (n = 6)	GLA/CON (n = 10)	SAFF/ETOH (n = 8)	SAFF/CON (n = 12)	Lab Chow (n = 10)	Effects ($p < 0.05$)
C16 : 0	49.99 ± 1.35	42.61 ± 1.60	46.13 ± 1.12	45.19 ± 0.67	46.45 ± 1.98	ETOH, INT
C18 : 0	10.29 ± 0.39	10.95 ± 0.67	11.32 ± 0.54	10.83 ± 0.44	10.28 ± 0.63	
C18 : 1n-9	18.07 ± 0.85	20.44 ± 0.57	19.64 ± 0.13	19.89 ± 0.34	19.59 ± 0.55	ETOH, INT
C18 : 1n-7	5.87 ± 0.16	6.26 ± 0.16	6.08 ± 0.10	6.08 ± 0.11	6.09 ± 0.09	
C18 : 2n-6	0.51 ± 0.17	0.84 ± 0.02	1.10 ± 0.05	1.20 ± 0.04	0.81 ± 0.05	ETOH, GLA, NUT
C20 : 3n-6	0.39 ± 0.13	0.70 ± 0.03	0.37 ± 0.03	0.43 ± 0.02	0.38 ± 0.08	ETOH, GLA
C20 : 4n-6	3.48 ± 0.57	5.39 ± 0.49	4.08 ± 0.49	4.75 ± 0.36	3.77 ± 0.49	ETOH
C22 : 4n-6	0.17 ± 0.07	0.47 ± 0.06	0.34 ± 0.06	0.45 ± 0.04	0.29 ± 0.09	ETOH
C22 : 5n-6	2.54 ± 0.85	1.48 ± 0.17	1.03 ± 0.10	1.63 ± 0.30	2.62 ± 0.47	INT
C22 : 6n-3	2.55 ± 0.38	4.01 ± 0.44	3.55 ± 0.67	3.92 ± 0.46	3.41 ± 0.51	

*Values represent means ± SEM. ETOH = ethanol effect (GLA/ETOH + SAFF/ETOH vs. GLA/CON + SAFF/CON); GLA = GLA effect (GLA/ETOH + GLA/CON vs. SAFF/ETOH + SAFF/CON); INT = interaction (GLA/ETOH + SAFF/CON vs. GLA/CON + SAFF/ETOH); NUT = nutritional effect (SAFF/CON vs. LC).

[$F(1, 41) = 30.51, p < 0.0001$]. They were reduced by GLA feeding in both PC [$F(1, 41) = 54.56, p < 0.0001$] and PE [$F(1, 41) = 72.86, p < 0.0001$] and were also reduced by ETOH in PC [$F(1, 41) = 10.92, p < 0.01$].

20 : 3n-6. The levels of 20 : 3n-6 were increased by GLA feeding in both PC [$F(1, 41) = 6.08, p < 0.05$] and PE [$F(1, 41) = 429.69, p < 0.0001$]. They were reduced by ETOH in PC [$F(1, 41) = 9.13, p < 0.01$], particularly in the GLA/ETOH group [Interaction $F(1, 41) = 3.95, p = 0.054$].

20 : 4n-6. ETOH decreased 20 : 4n-6 slightly in PC [$F(1, 41) = 6.83, p < 0.05$] but not in PE.

22 : 4n-6. In PE, the levels of 22 : 4n-6 in SAFF/CONT were higher than in LC group [$F(1, 41) = 14.90, p < 0.001$], with a similar trend in PC ($0.05 < p < 0.06$). ETOH decreased 22 : 4n-6 in both PC [$F(1, 41) = 9.25, p < 0.01$] and PE [$F(1, 41) = 13.71, p < 001$]. GLA increased 22 : 4n-6 slightly in PE [$F(1, 41) = 7.02, p < 0.05$].

22 : 5n-6. In PC, the levels of 22 : 5n-6 were increased in GLA/ETOH, but decreased in SAFF/ETOH [Interaction $F(1, 41) = 4.19, p < 0.05$]. ETOH decreased 22 : 5n-6 slightly in PE [$F(1, 41) = 5.42, p < 0.05$].

22 : 6n-3. In PE the levels of 22 : 6n-3 were lower in SAFF/CON than in LC [$F(1, 41) = 9.11, p < 0.01$]. GLA reduced levels of 22 : 6n-3 in PE [$F(1, 41) = 14.38, p < 0.0005$], particularly in the GLA/ETOH group [Interaction $F(1, 41) = 4.60, p < 0.05$]. Although the trend of the data in GLA/ETOH was similar in PC, this was not significant.

The effects on the saturated and monounsaturated effects can be summarized as follows: In both PC and PE, levels of 16 : 0 were increased by prenatal ethanol, particularly when combined with GLA; levels of 18 : 0 were slightly decreased by ethanol in PE. Levels of 18 : 1n-9 and 18 : 1n-7 were decreased by ethanol in PC and increased in PE, with these effects being pronounced in the GLA groups.

Behavior

The behaviors measured in the open field failed to show any significant differences (data not shown). However, there

TABLE 5

SELECTED FATTY ACID COMPOSITION OF PHOSPHATIDYLETHANOLAMINE FRACTION OF BRAIN GLYCEROPHOSPHOLIPIDS, EXPRESSED AS WT% OF TOTAL FATTY ACIDS*

Fatty Acid	GLA/ETOH (n = 6)	GLA/CON (n = 10)	SAFF/ETOH (n = 8)	SAFF/CON (n = 12)	Lab Chow (n = 10)	Effects ($p < 0.05$)
C16 : 0	11.35 ± 1.96	7.13 ± 0.28	7.66 ± 0.22	7.11 ± 0.06	6.66 ± 0.12	ETOH, GLA, INT
C18 : 0	16.67 ± 0.70	17.30 ± 0.30	15.93 ± 0.46	17.69 ± 0.44	16.44 ± 0.54	ETOH
C18 : 1n-9	12.45 ± 0.50	11.17 ± 0.24	11.65 ± 0.21	11.58 ± 0.15	11.96 ± 0.13	ETOH, INT
C18 : 1n-7	2.99 ± 0.23	2.50 ± 0.06	2.69 ± 0.05	2.64 ± 0.03	2.69 ± 0.05	ETOH, INT
C18 : 2n-6	0.76 ± 0.02	0.66 ± 0.02	1.13 ± 0.06	1.07 ± 0.03	0.76 ± 0.06	GLA, NUT
C20 : 3n-6	1.24 ± 0.05	1.27 ± 0.02	0.80 ± 0.01	0.81 ± 0.01	0.81 ± 0.02	GLA
C20 : 4n-6	13.18 ± 0.15	13.76 ± 0.12	13.49 ± 0.31	13.00 ± 0.28	12.92 ± 0.30	
C22 : 4n-6	4.35 ± 0.32	4.98 ± 0.10	4.21 ± 0.05	4.48 ± 0.07	3.90 ± 0.05	ETOH, GLA, NUT
C22 : 5n-6	0.39 ± 0.02	0.41 ± 0.02	0.34 ± 0.01	0.42 ± 0.03	0.38 ± 0.01	ETOH
C22 : 6n-3	23.00 ± 0.70	25.10 ± 0.40	26.76 ± 0.77	26.15 ± 0.55	28.52 ± 0.64	GLA, INT, NUT

*Values represent means ± SEM. ETOH = ethanol effect (GLA/ETOH + SAFF/ETOH vs. GLA/CON + SAFF/CON); GLA = GLA effect (GLA/ETOH + GLA/CON vs. SAFF/ETOH + SAFF/CON); INT = interaction (GLA/ETOH + SAFF/CON vs. GLA/CON + SAFF/ETOH); NUT = nutritional effect (SAFF/CON vs. LC).

was a trend ($p < 0.08$) toward increased stereotypy in the GLA-treated groups [mean (s) ± SEM; GLA/ETOH 52.5 ± 6.2; GLA/CON 44.6 ± 3.0; SAFF/ETOH 40.9 ± 4.7; SAFF/CON 39.3 ± 4.3; LC 42.5 ± 4.7]. The 4 days of running-wheel activity scores were averaged separately for the dark and light cycle (Fig. 2).

Repeated-measures ANOVA indicated a main effect of Period [$F(1, 41) = 221.58$, $p < 0.001$], with animals, as expected, being much more active during their dark cycle. There was also a Group × Period interaction [$F(4, 41) = 2.86$, $p < 0.04$]. A further simple effects analysis showed that GLA were more active than SAFF during the dark cycle [$F(1, 41) = 9.79$, $p < 0.005$] and slightly less active during the light cycle [$F(1, 41) = 4.70$, $p < 0.05$]. Despite the apparent trend in the data for the ETOH animals to be more active, and particularly for the SAFF/ETOH to be more active than the GLA/ETOH in the light cycle, in neither case was there a significant main effect of ETOH, nor a GLA × ETOH interaction.

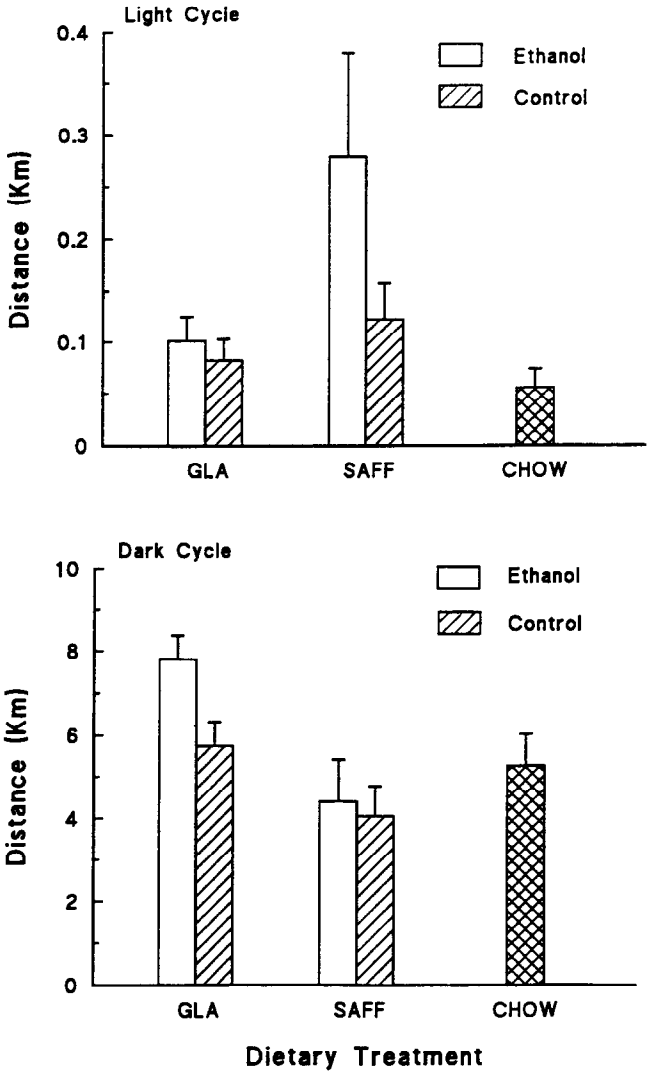

FIG. 2. Circadian running-wheel activity of adult B6D2F$_2$ male mice treated with ethanol during gestation and fed diets supplemented with γ-LnA. The chow group represents a reference group fed standard laboratory chow. GLA increased activity in the dark (active) phase and decreased activity in the light (inactive) phase. ($p < 0.05$. Note the change of scale between light and dark).

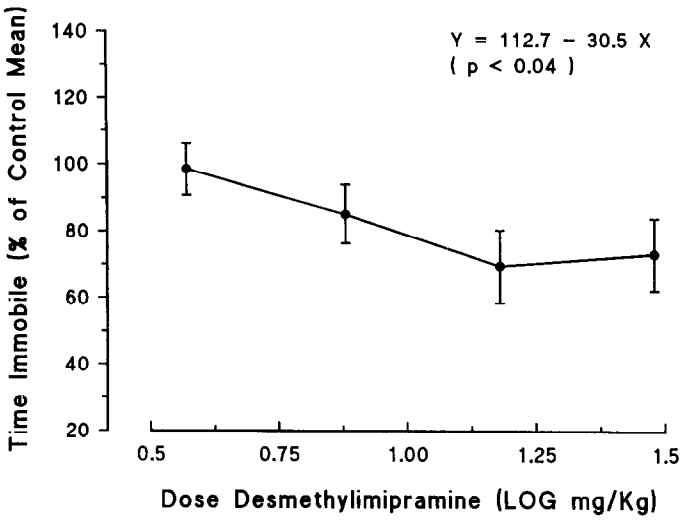

FIG. 3. Standard dose-response curve of adult male B6D2F$_2$ male mice treated with desmethylimipramine (DMI) showing decreases in the time spent immobile during the last 4 min of a 6-min test in the forced swimming task ($p < 0.05$).

Forced Swimming Test

As seen in Fig. 3, there was a significant dose-response relationship between DMI and immobility scores in this population ($p < 0.04$), with the animals showing a decrease in immobility up to 15 mg/kg, which then appeared to plateau between 15 and 30 mg/kg. This confirms data obtained in other studies using CD1 mice (27). In contrast, as seen in Fig. 4, there were no significant effects of GLA or ETOH on this measure, either alone or combined with 7.5 mg/kg DMI.

DISCUSSION

The intent of this study was to investigate the effects in mice of chronic dietary GLA supplementation on reproduction, growth and development, brain fatty acid composition, and measures of behavioral activity. It also sought to determine how such effects might interact with those of prenatal ethanol exposure.

We showed previously that administration of unsaturated fatty acids reduced the number of successful pregnancies in mice exposed to ethanol (41). In this previous work, pregnant mice were treated with ethanol and GLA only during gestation, and the pups were tested at weaning. GLA was administered via SC injection in doses of 20, 120, and 200 mg/kg, and AA in a dose of 200 mg/kg. There were significantly fewer successful pregnancies in the fatty acid-treated groups. Ethanol retarded behavioral development in both sexes and increased locomotion in the open field in males but, as in the present study, GLA had no preventive effect. Although the trend of the data in the present study was in a similar direction, the effect of GLA on reproductive outcome was not significant; however, it did reduce birth weight. Ethanol, on the other hand, reduced the number of viable litters and decreased adult weight. Adult brain weight was reduced by the

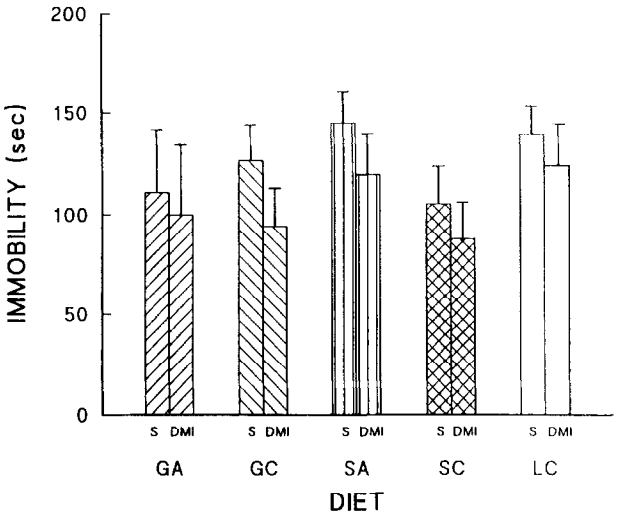

FIG. 4. Time spent immobile during the last 4 min of a 6-min forced swimming test in adult B6D2F$_2$ male mice treated with ethanol during gestation and fed diets supplemented with γ-LnA. Animals were tested twice, once after saline injection (S) and once after desmethylimipramine (DMI), 7.5 mg/kg. The chow group represents a reference group fed standard laboratory chow. GA = GLA/ETOH; GC = GLA/CON; SA = SAFF/ETOH; SC = SAFF/CON; LC = lab chow.

joint administration of GLA and ethanol. Thus, there is little evidence that GLA offsets the effects of ethanol; on the contrary, the combined effects might be viewed as deleterious in terms of reproductive outcome.

The effects on brain fatty acid composition are consistent with our previous findings on B6D2F$_2$ mice treated with the same regimen of ethanol prenatally (40). As seen previously, ethanol decreased 20- and 22-carbon fatty acids, and increased levels of 16 : 0; similar results have been reported recently in developing rats (11). What is noteworthy about the present findings is that they were seen in adult animals long after treatment had ceased; in our previous study they were seen 12 days after birth. This implies enduring effects in brain fatty acid composition of ethanol-induced changes during sensitive developmental periods. Despite their statistical significance, however, the magnitude of these findings is small, and their functional significance remains to be determined.

GLA increased levels of DGLA, particularly in the PC fraction (56%), but had no effects on AA. This differs from our previous findings in which, in the presence of high levels of fish oil containing long-chain n-3 fatty acids, GLA increased levels of AA (39,24); this may be related to differences in the n-3 : n-6 ratio in the two studies (0.5 and 0.25 previously, 0.09 currently). GLA and safflower oil decreased DHA (22 : 6n-3), particularly in the PC fraction. The GLA effect predominated in the group receiving ethanol, and GLA also increased levels of 22 : 5n-6 in this group. This replicates the commonly observed finding of an inverse reciprocity between levels of 22 : 6n-3 and 22 : 5n-6 in the brain (37). Thus, the combination of GLA and ethanol resulted in a group which might be described as slightly deficient in DHA (23% vs. 28% in lab chow control). This is important, given the role of DHA in retinal [reviewed in (28)] and also possibly brain function [reviewed in (38)].

The effects on activity are interesting. No differences were seen in the response to the open field. This differs from our previous findings showing that weanling males that had been exposed to ethanol prenatally were more active in the open field. This may be because our previous findings were with weanling animals and the animals in the present study were adult. A recent study in rats (12) using a treatment regimen of ethanol and evening primrose oil (100 mg/kg evening primrose oil, consisting of approximately 10% GLA, provided as a dietary supplement), administered 3 weeks before mating and throughout the study, showed that evening primrose oil increased locomotor activity particularly in the daytime, and decreased the latency to find the platform in the Morris maze, thereby offsetting the depressant effects of ethanol consumption. The statistical analysis of these data does not include the effects of litter, which may have contributed to inflation of power (1); and the continuous administration of ethanol does not allow separation of acute effects from those of prenatal exposure. Also, there does not appear to be a control oil which would allow the definitive attribution of the findings to the GLA content of evening primrose oil.

In the present study, ethanol was administered during gestation only, whereas GLA treatment continued throughout and was compared with a safflower oil control. Although GLA had no effect on open-field activity, it did affect circadian wheel-running activity. Activity was increased by GLA in the most active phase, the dark cycle, and slightly decreased by GLA in the light cycle. The GLA-induced decrease in activity during the light cycle appeared to be larger in the ETOH group, relative to the high activity of the SAFF/ETOH group, suggesting a protective effect. However, this was not supported statistically as a significant interaction, which may be related to the high variability in the SAFF/ETOH group, as well as the relatively small number of animals. Generally, the effects of the centrally administered PGs on overt behavior are sedatory (8,13), which is paradoxical in light of their effects on electroencephalographic measures of wakefulness (19). This suggests that different mechanisms with different sensitivities may be operative. Given that there was no behavioral sedation observed in the open field, these differences in circadian activity are consistent with the findings of "awaking" effects of PGE in rodents (26).

The response of the standard animals to the antidepressant DMI on the Porsolt test confirms the reliability of the test in this population of mice. However, neither prenatal ethanol nor GLA affected the duration of immobility, either alone or in response to 7.5 mg/kg of DMI. This does not support the hypothesis that GLA has typical antidepressant activity. Interestingly, in a study on male rats chronically exposed to ethanol, and their offspring, ethanol consumption did not affect the fathers' performance on this test, but their 3-mo-old offspring did show decreased immobility (3).

One point is especially important when interpreting the present results. A consistent theme in the literature relating PGs to physiologic and behavioral outcomes is that of bell-shaped dose-response curves. The level of GLA supplementation used in this study was intentionally high, to obtain maximal effects on incorporation into tissue phospholipid. Further work which addresses dose-response effects on circadian activity might prove interesting in this regard.

In summary, prenatal ethanol exposure had long-term effects in decreasing 20- and 22-carbon fatty acids in brain membrane phospholipid; the effects on 22 : 6n-3 were exacerbated when ethanol was administered with GLA. Chronic dietary GLA supplementation resulted in a relative increase in long-chain n-6 fatty acids in brain phospholipid and affected circadian wheel-running activity. These results do not support the hypothesis that GLA offsets the developmental effects of pre-

natal ethanol exposure; rather, they indicate caution in the use of high levels of GLA with ethanol during gestation.

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