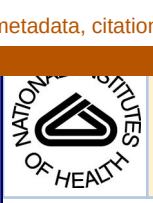

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Maternal α-linolenic acid availability during gestation and lactation alters the postnatal hippocampal development in the mouse offspring

Mihai D. Niculescu[a,b,*], **Daniel S. Lupu**[a], and **Corneliu N. Craciunescu**[b]

[a]UNC Nutrition Research Institute, Kannapolis, NC 28081, United States

[b]Department of Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States

Abstract

The availability of ω-3 polyunsaturated fatty acids is essential for perinatal brain development. While the roles of docosahexaenoic acid (the most abundant ω-3 species) were extensively described, less is known about the role of α-linolenic acid (ALA), which is the initial molecular species undergoing elongation and desaturation within the ω-3 pathways. This study describes the association between maternal ALA availability during gestation and lactation, and alterations in hippocampal development (dentate gyrus) in the mouse male offspring, at the end of lactation (postnatal day 19, P19). Postnatal ALA supplementation increased cell proliferation (36% more proliferating cells compared to a control group) and early neuronal differentiation, while postnatal ALA deficiency increased cellular apoptosis within the dentate gyrus of suckling pups (61% more apoptotic cells compared to a control group). However, maternal ALA deficiency during gestation prevented the increased neurogenesis induced by postnatal supplementation. Fatty acid analysis revealed that ALA supplementation increased the concentration of the ω-3 species in the maternal liver and serum, but not in the brain of the offspring, excepting for ALA itself. Interestingly, ALA supplementation also increased the concentration of dihomo γ-linolenic acid (a ω-6 species) in the P19 brains, but not in maternal livers or serum. In conclusion, postnatal ALA supplementation enhances neurogenesis in the dentate gyrus of the offspring at postnatal day 19, but its beneficial effects are offset by maternal ALA deficiency during gestation. These results suggest that ALA is required in both fetal and postnatal stages of brain development.

Keywords

Flaxseed oil; Linolenic acid; Brain development; Hippocampus; Dentate gyrus

1. Introduction

Omega-3 fatty acids (FA) play an essential role in brain development and cognition during fetal development and after birth (Innis, 2007a, 2009). The most abundant omega-3 FA is

*Corresponding author at: 500 Laureate Way, Kannapolis, NC 28081, United States. Tel.: +1 704 250 5029; fax: +1 704 250 5001. Mihai_Niculescu@unc.edu (M.D. Niculescu).

docosahexaenoic acid (DHA), the final product of a desaturation and elongation cascade that, in mammalians, starts with the α-linolenic acid (ALA, Fig. 3) (Innis, 2009). While extensive studies clearly defined the role of DHA and its metabolic precursors in neurodevelopment and neuroprotection (Niemoller and Bazan, 2010), considerably less is known about the roles of ALA in brain development. Although most studies suggested that ALA's beneficial effects are associated to its role as a DHA precursor (Niemoller and Bazan, 2010), a limited number of *in vitro* and *in vivo* studies also indicated that ALA may act as a signaling molecule, activating a variety of pathways, including IGF signal transduction (Seti et al., 2009), syntaxin-dependent neurite outgrowth (Darios and Davletov, 2006), and mitogen-activated protein kinase pathways (Ren and Chung, 2007).

During gestation and early post-natal life, the neonate's brain is experiencing the so called brain growth spurt, a substantial acceleration in growth, cellular proliferation, and neuronal and glial differentiation (Hoffman et al., 2009). Supplementation of the lactating mothers with ω-3 polyunsaturated fatty acids (PUFAs) was beneficial for increasing children's cognitive abilities like the progression of language acquisition, and visual acuity (Innis et al., 2001; Jensen et al., 2005).

Less is known about the relationship between postnatal brain development and the ALA maternal intakes during gestation and lactation. Gestational ALA deficiency reduces hippocampal neurogenesis (Coti Bertrand et al., 2006). Rats exposed to ALA deficiency *in utero*, and supplemented with ALA after birth, maintained lower levels of DHA than in controls (Li et al., 2006).

The aim of this study was to determine whether (1) maternal ALA availability during lactation alters hippocampal neurogenesis in the offspring at the end of lactation period and (2) gestational ALA availability interacts with this outcome.

2. Materials and methods

All reagents were purchased from Sigma–Aldrich (St. Louis, MO) if not otherwise specified.

2.1. Animals, tissue collection, and dietary exposure

All animal procedures were approved by the UNC Institutional Animal Care and Use Committee. Mouse C57BL/6J females (10 weeks old, Jackson Lab., Bar Harbor, ME) were maintained in a climate-controlled environment and exposed to a 12 h light/dark cycle daily, housed four per cage, and offered water and food *ad libitum*. The animals were randomly assigned into two initial feeding groups for 30 days prior, and during gestation. One feeding group (ALA control, C, $n = 12$) was given a defined control diet (AIN-93G, DYETS, Bethlehem, PA) containing soybean oil as fat source, while a second group (ALA deficient, D, $n = 12$) was fed an AIG-93G modified diet with corn oil as the only source of fatty acids (DYETS). Beginning with day 15, estrus cycle was induced and synchronized among the females from same cage by using dirty male bedding containing male urine (Whitten effect). After 30 days, the females were bred overnight with males that were maintained at all times on the C diet. The following morning, males were separated from females. Pregnant females were identified by either the presence of the vaginal plug (gestation day E0), or by palpation

beginning with gestation day E11. Five days before the expected birth date, each female was isolated in a different cage. One day prior to delivery date, the two groups were randomly split each into two subgroups, respectively. Six animals from each group remained on the same diet during the birth process and during the entire lactation period (C–C and D–D, respectively). The other six animals from each group were switched to a custom AIN-93G diet (ALA supplemented, S) containing flaxseed oil as the only source of fatty acids (C–S and D–S, respectively). The fatty acid composition of each diet is indicated in Table 1. All pregnant mice were allowed to give birth and to feed their litter until postnatal day 19 (P19, considering date of birth as day 0). Before termination, the body weight of all lactating mice and their litters was measured. On day P19 the pups and mothers were anesthetized with a single intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight, Henry Schein Inc., Melville, NY). The mice were kept on a heating pad to maintain body temperature. The pups were decapitated, the skulls were opened and the brains were either immersed in 4% formaldehyde for 24 h or snap-frozen in liquid nitrogen and stored at −80 °C. Maternal livers were extracted, snap frozen in liquid nitrogen and stored at −80 °C. Maternal blood was collected with a 5 ml syringe directly from the heart and then left to coagulate for serum separation. The second day fixed brains were stored for further paraffin embedding in 0.1 mol/l phosphate buffer, pH 7.4. One lactating female from the D–S group cannibalized its pups and was excluded from the study. Therefore, in the C–S group, only $n = 5$ adult females with their litters were included for further analysis. In this study male pups were used for subsequent determinations.

2.2. Immunohistochemistry

Fixed male brains were paraffin-embedded and 5 μm coronal serial sections were cut and applied on glass slides for immunohistochemical assays. In order to prevent the detection of false changes between groups due to possible differences in the localization of similar anatomical areas along the anterior to posterior axis, we used hippocampal sections selected from the middle location, ensuring the highest possible correspondence between groups. Alternating sections were used for assessing the markers described below to ensure relevance to the same anatomical localization for all markers. Mitosis in the dentate gyrus (DG) area within the hippocampus was determined using a rabbit antibody against phosphorylated histone H3 (pH3, Ser 10, pH 3, Millipore, Temecula, CA), as previously described (Hendzel et al., 1997). A rabbit antibody against activated Caspase-3 (Cell Signaling Technology, Danvers, MA) was used for apoptosis assessment within the hippocampus. The early differentiation state of neurons within the DG was determined using a goat primary calretinin antibody (N-18, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary fluorescent antibodies were used as it follows: goat anti-rabbit Cy3-conjugated (AP132C, Millipore) for pH 3 and Caspase-3 labeling, and Alexa Fluor 594 chicken anti-goat (Invitrogen, Carlsbad, CA) for detection of calretinin detection. The following common protocol was used for all immunohistochemical determinations: slides were deparaffinized in xylene, twice for 5 min each, followed by incubation for 5 min in xylene–ethanol 1:1. Rehydration was performed successively in 100%, 95%, 70%, and 50% ethanol, respectively, each for 5 min with gentle agitation on a horizontal shaker. Antigen retrieval was performed for 5 min at 98 °C using a Decloaking Chamber Pro (Biocare Medical, Concord, CA), in a Tris–EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Triton

X-100, pH 9.0). Blocking was performed for 2 h at room temperature, using either 5% goat serum (for pH 3 or activated Caspase-3 labeling) or 5% bovine serum albumin (BSA) for calretinin labeling, in phosphate buffer saline (PBS) with 0.1% Triton X-100. Incubation with the primary antibody (1/200 in blocking buffer) was performed overnight at 4 °C, followed by 3 washes for 15 min each (0.1% Triton X-100 in PBS) and 2 h incubation with the secondary fluorescent antibody (1/1000 diluted in PBS). After three final washes (15 min each in 0.1% Triton X-100/PBS), the slides were incubated in diaminophenylindole (DAPI), 0.1 mg/ml in PBS. Slides were mounted in an aqueous medium (Fluoromount) with glass cover slips.

2.3. Extraction and measurement of fatty acids

Total lipids were extracted using the method of Bligh and Dyer (Bligh and Dyer, 1959), from dam livers (30 mg) and serum (200 μl), male P19 brains (30 mg), and from three diet samples (C, S, and D, 30 mg respectively), at the UNC Nutritional Biochemistry and Molecular Biology Core. In brief, the lower (chloroform) phase was transferred to a clean tube and evaporated to dryness under nitrogen. The residual lipids were saponified and the fatty acids trans-methylated by sequential 1 ml addition of 4.25% NaOH in $CHCl_3$:MeOH (2:1, v/v) and 1 N HCl in saline (Tacconi and Wurtman, 1985). The samples were mixed vigorously then centrifuged at 1500 rcf for 5 min. The lower phase containing the fatty acid methyl esters was carefully transferred to a clean, dry tube and evaporated to dryness under nitrogen. Fatty acid methyl esters were then resuspended in 50 μl undecane, and analyzed using capillary gas chromatography (GC). Fatty acid methyl esters were analyzed by Fast GC on a Perkin Elmer AutoSystem XL Gas Chromatograph (Shelton, CT), split injection, with helium as the carrier gas. The methyl esters were separated on a capillary column coated with 70% cyanopropyl polysilphenylene – siloxane (10 m × 0.1 mm ID – BPX70 0.2 μm; SGE, Austin, TX); injector 240 °C and detector 280 °C. Data was analyzed with the Perkin Elmer Totalchrom Chromatography Software, version 6.2. Heptadecanoic acid (17:0) was added to the samples as an internal standard to correct for recovery and quantitation. Individual fatty acids were identified by comparing their retention with authentic standards (Nu Chek Prep, Elysian, MN).

2.4. Image and statistical analysis

Image analysis of postnatal brain sections was performed using a Zeiss Axio Imager A1 Microscope (Carl Zeiss, Thornwood, NY) with Plan-Neofluar 20× and 40× objectives. Images were collected and analyzed as it follows: for the assessment of mitosis and apoptosis within the dentate gyrus (DG), pH3 or caspase-3 positive cells, respectively, were counted in both hemispheres on three alternating sections and the values were averaged to obtain a single value per region and animal. For calretinin assessment, since its abundance within the DG did not clearly allow the counting of positive cells, we used the integrated optical density (IOD) as proxy marker for protein levels, as previously described (Niculescu et al., 2006), using the ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). Statistical testing was performed using one-way ANOVA followed by the Tukey test for multiple comparisons, and statistical significance considered for $p < 0.05$ (JMP 7.0.1, SAS Institute, Cary, NC).

3. Results

3.1. Body weight

No differences in the body weight of adult females were detected during the gestation and lactation periods (data not shown). At the end of the study period (postnatal day 19, P19), there was increased body weight in the C–S group of pups (8.46 ± 0.24 g standard error of the mean, SEM, $n = 30$) when compared to the D–S group (7.55 ± 0.19 g SEM, $n = 28$, Fig. 1), for both sexes, with no differences between sexes for the same dietary treatment, and no interaction between the exposure factor (gestation vs lactation) and the diet factor, as assessed by two-factor ANOVA analysis.

3.2. Immunohistochemistry

Within the DG area mitosis, as assessed by pH3 staining, was increased only in the C–S group when compared to all other groups (Fig. 2a). Within the C–S group the number of mitotic cells was 36% higher than in the C–C group (9.44 ± 0.55 SEM positive cells per DG vs 6.94 ± 0.69 SEM, respectively). Apoptosis assessment using activated caspase-3 (Fig. 2b) indicated an increased number of positive cells in the D–D group vs all other groups (9.05 ± 0.92 SEM in the D–D group vs 5.61 ± 0.68 SEM in the C–C group, respectively). The assessment of calretinin levels (IOD values, see Section 2, Fig. 2c) indicated a significant increase in the C–S group when compared to all other groups (4.33 ± 0.37 SEM in the C–S group vs 3.05 ± 0.28 SEM in the C–C group, respectively).

3.3. Fatty acids (FA) profiling

FA profiling of maternal livers (Table 2) indicated, among other changes, higher concentrations of the ω-3 fatty acids 18:3n3 (linolenic, ALA), 20:5n3 (eicosapentaenoic, EPA), and 22:5n3 (docosapentaenoic, DPA) in the C–S and D–S groups, when compared to the C–C and D–D groups. The most physiologically abundant ω-3 species, 22:6n3 (docosahexaenoic, DHA) was decreased in the D–D group as compared to all other groups. Within the ω-6 species, 18:2n6 (linoleic) and 20:3n6 (dihomo γ-linolenic) were increased in the C–C and D–D groups when compared to the C–S and D–S groups. The arachidonic acid concentration (20:4n6) was different between each two group comparisons, with the highest value in the D–D group (16.56 ± 0.85 nmol/mg SEM), and the lowest in the D–S group (0.36 ± 0.04 nmol/mg SEM).

Within the maternal serum (Table 3) ALA was similarly increased in the C–S and D–S groups when compared to the C–C and D–D groups. Interestingly, its ω-3 products of elongation and desaturation (EPA, DPA, and DHA) were variably changed between various groups. EPA was the highest in the D–S group when compared to all other groups, while it was also increased in the C–S group when compared to either C–C or D–D groups. DPA was increased mostly in the D–S group, followed by the C–S group. The DHA serum concentration differed only between the D–D group (88.17 ± 34.7 nmol/μl SEM) and the D–S group (244.28 ± 23.32 nmol/μl SEM). Within the ω-6 fatty acid species, linoleic and dihomo γ-linolenic acids were decreased in the C–S group compared to C–C. The arachidonic acid concentration was decreased in the C–S and D–S groups as comparison with either C–C or D–D groups.

In the P19 brains ALA was detected only in the groups supplemented with flaxseed oil during lactation (C–S and D–S, Table 4). EPA and DPA had increased levels in the C–S and D–S groups when compared to C–C and D–D groups, but the content of the final product of ω-3 elongation/desaturation (docosahexaenoic, DHA) did not vary between groups. Less changes than in the maternal liver and serum were observed within the ω-6 group of fatty acids, with only dihomo γ-linolenic acid being changed. Its concentration was higher in the C–S group than in both C–C and D–D groups. Additionally, the D–S group had higher concentration of dihomo γ-linolenic than the D–D group.

4. Discussion

In the present study we tested the hypothesis that the maternal supplementation with flaxseed oil (containing more than 50% ALA) during lactation, alters hippocampal neurogenesis in the offspring at the end of lactation period (postnatal day 19, P19). In addition, we tested whether maternal flaxseed oil availability, prior and during gestation, modulates this outcome. After birth the dentate gyrus (DG) remains an active site of neurogenesis in mice (Kempermann, 2002; Kempermann et al., 2006). In rodents, increased postnatal neurogenesis within the DG was associated with improved performance on spatial learning task and increased long-term potentiation (LTP, correlated with memory acquisition) (Kempermann et al., 1997; van Praag et al., 1999a,b). Moreover, alterations of postnatal hippocampal neurogenesis (i.e. loss of plasticity) may play an important role for the onset of clinical depression (reviewed in Kempermann, 2002).

The roles that maternal availability of omega-3 fatty acids have in brain development have been extensively studied (Brenna, 2011). Because DHA is the most abundant omega-3 species in human brain, most of these studies have focused upon its role in brain development (Guesnet and Alessandri, 2011). While the exact mechanisms involved in the beneficial roles of omega-3 fatty acids for brain development have not been completely unraveled, DHA accretion in membrane phospholipids is considered to be a major contributor to establishing the required cellular signaling needed for neurogenesis (Guesnet and Alessandri, 2011). Less is known about the role of ALA in brain development, and particularly in hippocampal neurogenesis during gestation and lactation periods. *In vitro* studies indicated that, besides being a precursor for DHA formation in liver, ALA also has a distinct role in cell signaling (Darios and Davletov, 2006; Ren and Chung, 2007), and its role in the regulation of gene expression is mediated either through peroxisome proliferator activated receptor (PPAR-dependent), PPAR-independent mechanisms (reviewed in Innis, 2003), or by altering the phosphorylation of mitogen-activated protein kinases (MAPKs) involved in cell proliferation, differentiation and apoptosis (Blondeau et al., 2001; Ren and Chung, 2007). ALA inhibits both TNF-α gene expression and NF-kB-dependent transcriptional activity (Ren and Chung, 2007). ALA also has a neuroprotective effect against excitotoxic cell death in granule neurons (Lauritzen et al., 2000), activating the neuronal potassium TREK 1 channel (Heurteaux et al., 2004), and producing cerebral vasodilatation (Blondeau et al., 2007). ALA supplementation induced neurogenesis and synaptogenesis by directly enhancing neuronal brain-derived neurotrophic factor (BDNF) expression in immature neural stem cells and in mature hippocampal neurons (Blondeau et al., 2009). A very recent studies using C57Bl/6 mice revealed that the administration of a

flaxseed oil diet during gestation and lactation increased the expression levels of brain neuron-specific enolase, glial fibrillary acidic protein and myelin basic protein in pup mice at postnatal days 21 and 42, while expression of PPAR-γ in the brains of pup mice was increased only at postnatal day 7 (Tian et al., 2011).

In our study the maternal ALA supplementation from flaxseed oil increased cell proliferation and early neuronal differentiation within the DG of suckling pups (Fig. 2a and b). However, this enhancement effect was offset by the administration of an ALA-low diet prior and during gestation (D–S group), which precluded any increase in neurogenesis due to the post-gestational administration of an ALA-supplemented diet. When a standard diet containing soybean oil (Table 1) was administered during gestation, followed by postnatal ALA supplementation using flaxseed oil (C–S group), the number of cells labeled against phosphorylated histone H3 (pH3) was 36% higher than in the group receiving only a standard diet (C–C group) throughout the entire study. While an ALA-low diet (D–D) did not reduce cell proliferation, the beneficial effects of ALA supplementation in lactating mothers did not occur if they received an ALA-low diet in gestation (Fig. 2a). Similarly, the increased levels of calretinin present in the C–S group did not occur in the D–S group (Fig. 2c). Within the neuronal differentiation process in the dentate gyrus, calretinin is a transient marker for postmitotic neurons in their early differentiation state, being later replaced by calbindin in the mature dentate granule neurons (von Bohlen Und Halbach, 2007). These results indicated that ALA supplementation exerts an active influence upon neurogenesis in both fetal and lactating periods of DG development.

In contrast with the changes in neurogenesis, apoptosis (as determined by activated caspase-3 labeling) was increased only in the D–D group of pups (a 61% increase when compared to the C–C group receiving a standard diet). However, this increase was offset by the maternal postnatal ALA supplementation. Therefore, apoptosis in DG, as measured at postnatal day 19 (P19), seems to exclusively depend upon the ALA intake during lactation. This outcome suggests that ALA availability during gestation has a negligible influence, if any, upon postnatal apoptosis within the DG in offspring at P19.

In order to determine the impact of maternal ALA availability upon the content of fatty acids involved in the ω-3 and ω-6 elongation and desaturation processes, respectively, we determined the concentration of total fatty acids in the maternal liver and serum, and in the brain of P19 pups. Within the maternal liver ALA, EPA, and DPA were increased in both supplemented groups during lactation (Table 2), regardless the ALA availability during gestation. This indicates that the content of these fatty acids reflects the actual dietary maternal intakes (at day 19 of lactation) rather than any historical intakes during gestation. The fact that the elongation/desaturation products EPA and DPA were increased by ALA supplementation suggests that, within the liver of lactating mice, this conversion may have biological significance towards enhancing the pool of ω-3 products. However, these increases did not lead to a similar increase in the DHA liver content. Interestingly, DHA decreased in the D–D group, indicating that ALA intake may, nevertheless, be used for maintaining the DHA levels in the maternal liver, in the case of a DHA-depleted diet, and this finding is in agreement with previous reports (Guesnet and Alessandri, 2011). Therefore, since none of the diets contained any detectable amounts of DHA (Table 1), it is

safe to assume that the DHA decrease in the D–D group is due to lower maternal conversion of DHA from ALA intake during gestation or lactation.

The changes in ω-6 fatty acid species (linoleic, eicosatrienoic, and arachidonic) are also associated with the content of linoleic acid within the ALA-supplemented diet when compared to either the standard or the ALA-deficient diets. The 20:3n6 (8,11,14-eicosatrienoic, dihomo γ-linolenic) and the 20:4n6 (arachidonic) acids are products of elongation and desaturation catalyzed by the same enzymes that control the ω-3 enzymatic processes (Innis, 2007b). However, the increased concentration of dihomo γ-linolenic acid in the brains from the C–S and D–S groups cannot be necessarily considered a result of a hypothetical increase in the elongation/desaturation activity for the omega-6 pathways. As indicated in Table 1, the only diet containing this species was the flaxseed oil diet (S diet). Therefore, it is entirely possible that the reported increase in dihomo γ-linolenic to be caused by the flaxseed oil intake during lactation.

Similarly to liver, the measurement of total fatty acid content in serum indicated that maternal ALA intake during the lactation period increases the concentration of omega-3 fatty acids. Conversely, ALA supplementation decreased the concentration of linoleic and arachidonic acids, probably as a direct result of the dietary content in these fatty acids of the ALA-supplemented diet.

Fewer changes were detected within the whole P19 brains (Table 4). Notably, ALA was detected in the supplemented groups (C–S and D–S), with similar increases in its metabolites EPA and DPA when compared to either C–C or D–D groups. However, no differences were detected for DHA levels between dietary treatments. Within the ω-6 species, only 20:3n6 (dihomo γ-linolenic) acid was altered. Interestingly, its higher content in the flaxseed oil supplemented groups (C–S and D–S) is inversely correlated with its maternal levels, indicating that its incorporation into the P19 brains is not necessarily correlated with maternal synthesis or dietary levels. Published information about the correlation between ALA-rich diets and dihomo γ-linolenic levels in brain is scarce. In a very recent study Ander et al. (2010) using flaxseed oil supplementation in rabbits, reported higher dihomo γ-linolenic levels in kidneys but no changes in the brain of young rabbits. However, numerous studies indicated the beneficial roles of dihomo γ-linolenic acid against various pathological conditions, including cell proliferation in cancer (Hou, 2008).

In the present study the outcomes induced by maternal flaxseed oil supplementation to the development of the DG in the suckling pups did not necessarily correlate with the actual fatty acid levels in the P19 brains. For instance, increased cellular proliferation and early neuronal differentiation was reported only in the C–S group, while increased omega-3 fatty acid levels were found in both supplemented groups (C–S and D–S). Moreover, no differences in DHA content were found between any of the dietary groups of pups. These results indicated that ALA is required continuously during both fetal and postnatal stages of brain development, and that supplementation only during lactation may not be sufficient for its established outcomes if the maternal organism is depleted during gestation. Additionally, as mentioned above, ALA may act as a signaling molecule for the activation of multiple pathways, independent of its role as DHA precursor.

We also report changes in body weight between the C–S and D–S groups (Fig. 1). While the two-factor ANOVA testing for the interaction between gestation and lactation did not yield statistical significance, one might speculate that gestational intake of ALA could be a contributing factor in the observed changes. However, it is unclear why these changes were observed only in the groups receiving flaxseed oil during lactation. A recent study performed in rats indicated that flaxseed oil supplementation associated with less weight gain in weaned rats, but with no indication regarding the ALA intake during gestation and lactation (Ferreira Costa Leite et al., 2011).

This study has important limitations. The absence of a supplemented or a control group during gestation, followed by postnatal deficiency, cannot determine whether postnatal ALA deficiency is deleterious when ALA is available during fetal development. In addition, since neurogenesis was determined only at the end of the dietary exposures, it is impossible to ascertain the timing of the described outcomes.

Presently, there are no dietary recommendations regarding the human intake of ALA. Similarly, the ALA content of the customized diets for rodents depends on the type of fat used rather than on optimal intakes of omega-3 fatty acids that have yet to be established. Further studies are required to determine the optimal requirements and timing regarding the administration of α-linolenic acid during both gestation and lactation, and the degree of relevance when using animal models for defining human requirements.

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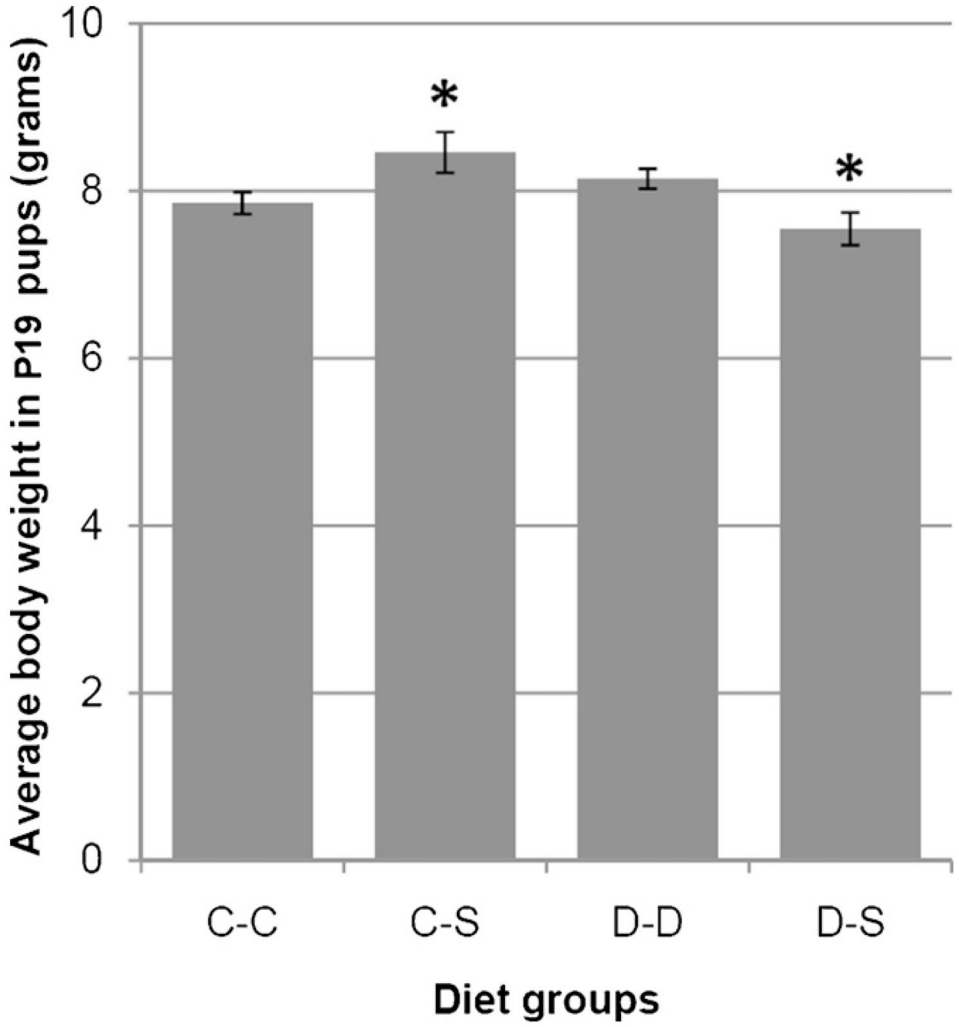

Fig. 1.

Body weight assessment of pups at the end of lactation period (P19). At the end of the suckling period (postnatal day 19, P19) the body weight of pups was recorded, prior to their termination. Pups from both sexes were included, as no between-sex differences were present. Body weights were averaged initially within each litter, then within each group. For each group, $n = 6$ litters were analyzed, with the exception of the D–S group ($n = 5$, see Section 2). After testing for equal variances, statistical testing was performed using the Tukey test for multiple comparisons. An asterisk indicates statistical significance between the two groups. C–C, group of pups from mothers receiving a C diet during gestation and lactation periods, respectively; C–S, group receiving a C diet during gestation and a S diet during lactation; D–D, group receiving a D diet during both gestation and lactation periods; D–S, group receiving a D diet during gestation, and a S diet during lactation, respectively. Error bars represent standard error.

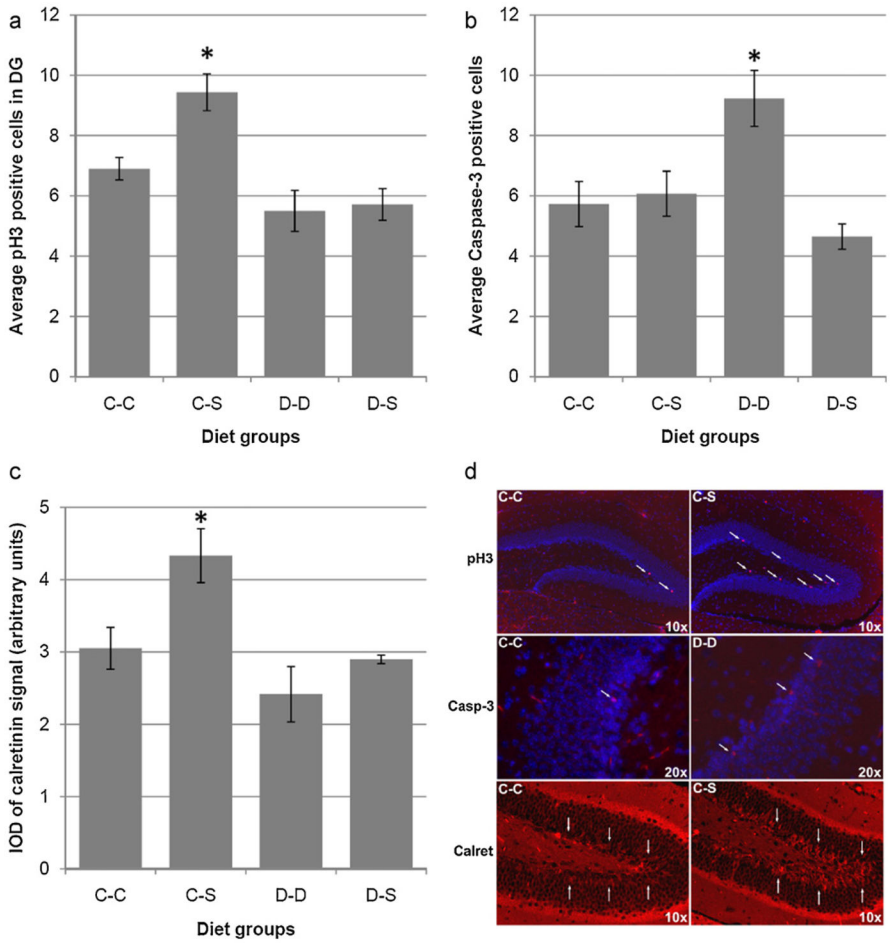

Fig. 2.

Maternal flaxseed oil availability during gestation and lactation alters postnatal brain development in offspring. At postnatal day 19 (P19) the pups were sacrificed and their brains fixed and sectioned for immunohistochemistry assessment in the dentate gyrus (cell proliferation, apoptosis, and early neuronal differentiation, see Materials and methods). (a) Assessment of mitosis in the DG using a marker specific for the M-phase (phosphorylated histone H3, pH3). (b) Apoptosis in the DG was determined using the detection of activated caspase-3 positive cells. (c) Early neuronal differentiation was measured using an anti-calretinin antibody, as previously described. (d) Representative images for cell proliferation (phosphorylated histone H3, pH3), apoptosis (activated caspase 3, Casp-3), and calretinin labeling (Calret) are presented, for the groups that are significantly different when compared to the control groups (C–C). For each group, $n = 6$ male brains were analyzed, with the exception of the D–S group ($n = 5$, see Section 2). After testing for equal variances, statistical testing was performed using the Tukey test for multiple comparisons. An asterisk indicates statistical significance in comparison with all other groups. C–C, group of pups from mothers receiving a C diet during gestation and lactation periods, respectively; C–S, group receiving a C diet during gestation and a S diet during lactation; D–D, group receiving a D diet during both gestation and lactation periods; D–S, group receiving a D diet during gestation, and a S diet during lactation, respectively. Error bars represent standard error.

IOD, Integrated Optical Density, see Materials and methods. White arrows indicate the presence of positively labeled cells (pH3 or Caspase-3), or the area where calretinin-positive cells were identified, respectively.

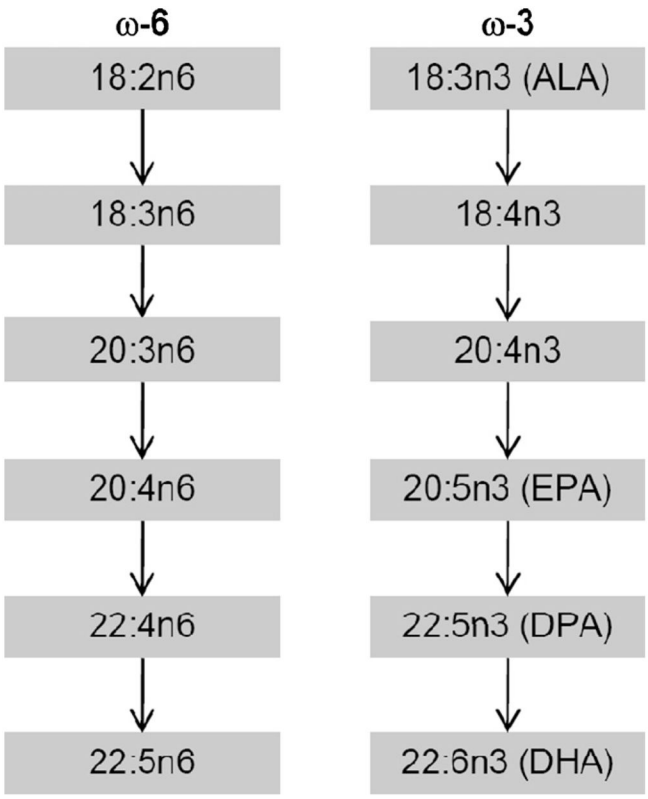

Fig. 3.

Simplified diagram of the omega-3 and omega-6 elongation and desaturation pathways. ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, 7,10,13,16,19-docosapentaenoic acid; DHA, docosahexaenoic acid.

Table 1

Fatty acid composition of diets.

		D diet (nmol/mg diet)	C diet (nmol/mg diet)	S diet (nmol/mg diet)
14:0	Myristic	0.124	0.251	0.151
14:1	Myristoleic	0.011	0.020	0.021
16:0	Palmitic	19.651	19.613	9.628
16:1n7	Palmitoleic	0.245	0.177	0.122
18:0	Stearic	2.910	2.519	2.365
18:1n9	Oleic	34.435	33.229	29.756
18:2n6	Linoleic	87.237	86.938	23.133
18:3n3	Linolenic	1.554	10.827	75.367
20:0	Eicosanoic	0.638	0.795	0.222
20:1n9	11-Eicosenoic	0.352	0.328	0.214
20:2n6	11,14-Eicosadienoic	0.030	0.067	0.049
20:3n6	Dihomo-gamma-linolenic	ND	ND	0.037
20:4n6	Arachidonic	ND	0.009	0.078
20:3n3	11,14,17-Eicosatrienoic	0.187	0.559	0.200
22:0	Behenic	0.012	0.008	ND
22:1n9	Erucic	ND	0.007	ND
20:5n3	Eicosapentaenoic	0.012	0.024	0.013
24:0	Lignoceric	0.228	0.169	0.139
24:1	Nervonic	ND	ND	0.010
22:5n3	7,10,13,16,19-Docosapentaenoic	ND	ND	ND
22:6n3	Docosahexaenoic	ND	ND	ND

ND, not detected.

Table 2

Fatty acid concentrations in maternal livers.

		Treatment groups nmol/mg tissue			
		C–C	**C–S**	**D–D**	**D–S**
14:0	Myristic	1.436 ± 0.162[a,b]	1.349 ± 0.178[a,b]	1.796 ± 0.142[a]	1.131 ± 0.070[b]
14:1	Myristoleic	0.034 ± 0.010	0.037 ± 0.010	0.066 ± 0.010	0.033 ± 0.003
16:0	Palmitic	40.125 ± 3.587	38.979 ± 3.865	49.170 ± 4.689	36.349 ± 3.028
16:1n7	Palmitoleic	5.129 ± 0.813	5.574 ± 0.673	6.390 ± 0.746	4.977 ± 0.574
18:0	Stearic	15.592 ± 0.553	15.431 ± 0.676	17.228 ± 0.502	16.306 ± 0.401
18:1n9	Oleic	61.351 ± 9.625[a,b]	66.328 ± 8.602[a,b]	76.980 ± 5.605[a]	43.391 ± 6.256[b]
18:2n6	Linoleic	25.728 ± 2.180[a]	13.549 ± 0.942[b]	27.855 ± 1.148[a]	13.165 ± 1.131[b]
18:3n3	Linolenic	0.911 ± 0.128[b]	7.357 ± 0.96[a]	0.431 ± 0.322[b]	7.141 ± 0.550[a]
20:0	Eicosanoic	0.011 ± 0.008	0.007 ± 0.007	ND	ND
20:1n9	11-Eicosenoic	0.799 ± 0.093[a]	0.679 ± 0.066[a,b]	0.905 ± 0.104[a]	0.415 ± 0.049[b]
20:2n6	11,14-Eicosadienoic	0.258 ± 0.018[a]	0.103 ± 0.011[b]	0.247 ± 0.027[a]	0.096 ± 0.018[b]
20:3n6	Dihomo-gamma-linolenic	1.697 ± 0.105[a]	1.048 ± 0.037[b]	1.672 ± 0.213[a]	0.914 ± 0.061[b]
20:4n6	Arachidonic	13.289 ± 0.656[b]	3.824 ± 0.288[c]	16.561 ± 0.854[a]	0.363 ± 0.042[d]
20:3n3	11,14,17-Eicosatrienoic	ND	0.326 ± 0.046	ND	ND
22:0	Behenic	0.006 ± 0.006[b]	0.058 ± 0.009[a]	ND	0.069 ± 0.015[a]
22:1n9	Erucic	0.014 ± 0.006	ND	0.017 ± 0.005	ND
20:5n3	Eicosapentaenoic	0.764 ± 0.358[b]	9.870 ± 0.798[a]	0.185 ± 0.160[b]	8.915 ± 0.312[a]
24:0	Lignoceric	0.172 ± 0.021	ND	1.823 ± 0.859	0.040 ± 0.010
24:1	Nervonic	ND	ND	ND	ND
22:5n3	7,10,13,16,19-Docosapentaenoic	0.401 ± 0.047[b]	1.228 ± 0.105[a]	0.154 ± 0.054[b]	1.364 ± 0.178[a]
22:6n3	Docosahexaenoic	8.296 ± 0.598[a]	9.300 ± 0.740[a]	3.519 ± 1.042[b]	10.165 ± 0.646[a]

Values are given ± SEM. ND, not detected. Values with different letters in superscript denote statistical significance (Tukey's test, $p < 0.05$).

Table 3

Fatty acid concentrations in maternal serum.

		Treatment groups nmol/µl serum			
		C–C	**C–S**	**D–D**	**D–S**
14:0	Myristic	95.77 ± 12.90	95.99 ±± 6.34	184.00 ± 39.25	128.48 ± 38.20
14:1	Myristoleic	8.95 ± 1.80	9.85 ± 0.25	12.11 ± 2.38	10.82 ± 1.18
16:0	Palmitic	735.97 ± 96.91	493.47 ± 42.70	645.38 ± 113.69	743.88 ± 83.37
16:1n7	Palmitoleic	29.23 ± 4.67	21.86 ± 1.83	28.23 ± 10.03	31.99 ± 3.61
18:0	Stearic	657.62 ± 97.05	430.01 ± 33.84	585.10 ± 104.11	666.42 ± 73.31
18:1n9	Oleic	447.88 ± 64.92	410.03 ± 31.91	447.64 ± 124.82	464.42 ± 60.25
18:2n6	Linoleic	681.31 ± 64.65^{a}	347.28 ± 23.18^{b}	560.63 ± 104.94^{a,b}	489.67 ± 69.19^{a,b}
18:3n3	Linolenic	15.02 ± 8.97^{b}	75.22 ± 12.45^{a}	1.73 ± 1.47^{b}	88.01 ± 11.61^{a}
20:0	Eicosanoic	1.53 ± 0.99	0.64 ± 0.45	1.88 ± 0.68	1.94 ± 0.50
20:1n9	11-Eicosenoic	7.65 ± 1.20	4.83 ± 0.53	6.31 ± 2.19	4.35 ± 1.16
20:2n6	11,14-Eicosadienoic	3.94 ± 1.03	1.64 ± 0.42	4.66 ± 0.68	4.24 ± 0.95
20:3n6	Dihomo-gamma-linolenic	59.74 ± 10.98^{a}	25.40 ± 2.33^{b}	45.85 ± 11.71^{a,b}	35.04 ± 5.39^{a,b}
20:4n6	Arachidonic	379.90 ± 72.14^{a}	65.58 ± 10.00^{b}	399.93 ± 72.46^{a}	112.99 ± 15.58^{b}
20:3n3	11,14,17-Eicosatrienoic	ND	1.79 ± 0.61^{b}	ND	4.34 ± 1.25^{a}
22:0	Behenic	0.36 ± 0.36^{b}	0.71 ± 0.32^{b}	0.88 ± 0.88^{b}	4.98 ± 1.44^{a}
22:1n9	Erucic	ND	ND	ND	ND
20:5n3	Eicosapentaenoic	24.01 ± 9.70^{c}	215.73 ± 20.94^{b}	6.78 ± 6.78^{c}	340.18 ± 37.18^{a}
24:0	Lignoceric	14.32 ± 4.74^{b}	8.64 ± 1.42^{b}	47.99 ± 14.78^{a}	6.08 ± 0.75^{b}
24:1	Nervonic	ND	ND	ND	ND
22:5n3	7,10,13,16,19-Docosapentaenoic	6.78 ± 1.59^{b,c}	10.47 ± 1.14^{b}	2.24 ± 0.01^{c}	20.40 ± 2.76^{a}
22:6n3	Docosahexaenoic	185.79 ± 35.86^{a,b}	139.76 ± 16.14^{a,b}	88.17 ± 34.74^{b}	244.28 ± 23.32^{a}

Values are given ± SEM. ND, not detected. Values with different letters in superscript denote statistical significance (Tukey's test, $p < 0.05$).

Table 4

Fatty acid concentrations in P19 brains.

		Treatment groups nmol/mg tissue			
		C–C	**C–S**	**D–D**	**D–S**
14:0	Myristic	1.028 ± 0.076	1.160 ± 0.149	0.996 ± 0.047	1.072 ± 0.077
14:1	Myristoleic	ND	ND	ND	0.014 ± 0.014
16:0	Palmitic	35.132 ± 3.343	36.683 ± 3.082	33.962 ± 1.968	35.390 ± 0.691
16:1n7	Palmitoleic	1.136 ± 0.138	1.323 ± 0.139	1.074 ± 0.065	1.284 ± 0.087
18:0	Stearic	22.996 ± 2.162	24.057 ± 2.001	22.276 ± 1.388	23.163 ± 0.359
18:1n9	Oleic	11.547 ± 1.156	13.264 ± 1.225	10.976 ± 0.862	12.709 ± 0.485
18:2n6	Linoleic	1.146 ± 0.098	1.371 ± 0.155	1.108 ± 0.088	1.184 ± 0.066
18:3n3	Linolenic	ND	0.038 ± 0.015	ND	0.013 ± 0.013
20:0	Eicosanoic	0.377 ± 0.031	0.416 ± 0.043	0.386 ± 0.043	0.404 ± 0.014
20:1n9	11-Eicosenoic	0.896 ± 0.096	1.049 ± 0.098	0.873 ± 0.097	1.029 ± 0.021
20:2n6	11,14-Eicosadienoic	0.201 ± 0.032	0.225 ± 0.017	0.296 ± 0.027	0.471 ± 0.189
20:3n6	Dihomo-gamma-linolenic	$0.780 \pm 0.055^{b,c}$	1.206 ± 0.108^{a}	0.644 ± 0.065^{c}	$0.997 \pm 0.107^{a,b}$
20:4n6	Arachidonic	14.226 ± 1.216	12.969 ± 1.240	14.697 ± 0.896	13.270 ± 0.631
20:3n3	11,14,17-Eicosatrienoic	ND	ND	ND	ND
22:0	Behenic	0.180 ± 0.014	0.093 ± 0.025	0.160 ± 0.038	0.118 ± 0.025
22:1n9	Erucic	0.112 ± 0.014	0.118 ± 0.012	0.116 ± 0.013	0.117 ± 0.004
20:5n3	Eicosapentaenoic	0.083 ± 0.015^{b}	0.429 ± 0.043^{a}	0.060 ± 0.014^{b}	0.345 ± 0.074^{a}
24:0	Lignoceric	0.123 ± 0.020	0.157 ± 0.019	0.115 ± 0.027	0.154 ± 0.013
24:1	Nervonic	0.020 ± 0.006	0.020 ± 0.008	0.016 ± 0.007	0.027 ± 0.007
22:5n3	7,10,13,16,19-Docosapentaenoic	0.282 ± 0.023^{b}	0.779 ± 0.079^{a}	0.086 ± 0.018^{b}	0.632 ± 0.137^{a}
22:6n3	Docosahexaenoic	19.131 ± 2.231	18.803 ± 1.416	14.485 ± 1.603	20.390 ± 1.561

Values are given ± SEM. ND, not detected. Values with different letters in superscript denote statistical significance (Tukey's test, $p < 0.05$).