

Original/Investigación animal

# Dietary ratios of n-6/n-3 polyunsaturated fatty acids during maternal pregnancy affect hippocampal neurogenesis and apoptosis in mouse offspring

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## Abstract

**Objective:** although n-3 polyunsaturated fatty acids (PUFAs) play crucial roles in brain development and function, neither the optimal level of n-3 PUFAs nor the optimal ratio of n-6/n-3 PUFAs in the maternal diet are well defined. In this study, we investigated the effects of dietary n-6/n-3 PUFA ratios during pregnancy on neurogenesis and apoptosis in the brains of mouse offspring.

**Methods:** female C57BL/6J mice were fed one of three diets with high, medium and low ratios of n-6/n-3 PUFAs (15.7:1, 6.3:1, 1.6:1), as well as a high fish oil diet with a n-6/n-3 ratio of 1:5.7; an n-3 PUFA-deficient diet served as control. The feeding regimens began two months before mouse conception and continued for the duration of the pregnancy. The neurogenesis and apoptosis of hippocampal CA3 area in the offspring were detected.

**Results:** compared to the n-3 PUFA-deficient diet, n-3 PUFA-containing diets, particularly those with n-6/n-3 PUFA ratios of 6.3:1 and 1.6:1, significantly increased both phosphorylation of histone H3 at ser 10 (p-H3ser10) and calretinin-positive cells in hippocampus CA3 of the offspring. Furthermore, increased expression of Bcl2 protein, decreased expression of Bax protein, and reduced caspase 3 activity and numbers of TUNEL apoptotic cells were found in the three diets with high, medium and low n-6/n-3 PUFA ratios. However, there were no differences in any of these parameters between the high fish oil diet group and the n-3 PUFA-deficient diet group.

**Conclusions:** these data suggest that a higher intake of n-3 PUFAs with a lower ratio of n-6/n-3 PUFAs of between about 6:1 to 1:1, supplied to mothers during pregnancy, may benefit brain neurogenesis and apoptosis in

## LAS RATIOS NUTRICIONALES DE ÁCIDOS GRASOS POLIINSATURADOS N-6/N-3 DURANTE LA GESTACIÓN AFECTAN A LA NEUROGÉNESIS HIPOCAMPAL Y A LA APOPTOSIS EN LAS CRÍAS DE RATONES

## Resumen

**Objetivo:** a pesar de que los ácidos grasos poliinsaturados n-3 (PUFAs por sus siglas en inglés) desempeñan un papel fundamental en el desarrollo y en las funciones cerebrales, aún no está bien definido el nivel óptimo de PUFAs n-3 ni la ratio óptima de PUFA n-6/n-3 en la dieta materna. En este estudio hemos investigado los efectos de las ratios nutricionales de PUFA n-6/n-3 durante la gestación sobre la neurogénesis y la apoptosis en el cerebro de crías de ratón.

**Métodos:** se alimentó a hembras de ratón C57BL/6J con una de las tres dietas de estudio: ratio alta, media y baja de PUFA n-6/n-3 (15,7:1, 6,3:1, 1,6:1). También se añadió una dieta rica en aceite de pescado con una ratio n-6/n-3 de 1:5,7; como control se empleó una dieta deficitaria en PUFA n-3. Los regímenes alimenticios se iniciaron dos meses antes de la concepción de los ratones y continuó durante todo el embarazo. Se detectó la neurogénesis y apoptosis del área hipocampal CA3 en las crías.

**Resultados:** en comparación con la dieta deficitaria en PUFA n-3, las dietas con PUFA que contienen n-3, particularmente aquellas con ratios PUFA n-6/n-3 de 6,3:1 y 1,6:1, aumentaron significativamente la fosforilación de histona H3 en la Ser10 (p-H3ser10) y las células calretinina positivas en el área hipocampal CA3 de las crías. Además, se detectó un aumento de la expresión de proteína Bcl2, una reducción de la expresión de proteína Bax, y una reducción de la actividad de caspasa 3, así como de las cifras de células apoptóticas TUNEL en las tres dietas, con ratios altas, medias y bajas de PUFA n-6/n-3. Sin embargo, no se observó diferencias en ninguno de estos parámetros entre el grupo de dieta rica en aceite de pescado y el grupo de dieta deficitaria en PUFA n-3.

**Conclusiones:** estos datos sugieren que una ingesta más elevada de PUFA n-3 con una ratio más baja de PUFAs n-6/n-3 entre 6:1 y 1:1 aproximadamente, administrada a las madres durante la gestación, podría ser beneficiosa para la neurogénesis cerebral y la apoptosis de las crías.

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**offspring. However, excessive maternal intake of n-3 PUFAs may exert a negative influence on brain development in the offspring.**

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Key words: *n-3 polyunsaturated fatty acids. Neurogenesis. Apoptosis. Hippocampus. Mice.*

## Introduction

The n-3 polyunsaturated fatty acids (n-3 PUFAs), especially docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), play crucial roles in brain development and function. Emerging evidence has shown that inadequate intake of n-3 PUFAs decreases DHA concentrations in the brain and leads to poorer development of visual acuity and lower indices of neural development<sup>1-2</sup>, whereas DHA supplementation during fetal or early postnatal periods results in long-term enhancement of spatial learning ability in the offspring and offers resistance against neuropsychiatric disorders; these effects persist into adulthood<sup>3-4</sup>. Moreover, n-3 PUFA availability reverses age-related changes and maintains learning memory performance in humans and animals<sup>3-5</sup>. The positive effects of DHA on neurodevelopment include promotion of hippocampal neurite growth and maturation as well as differentiation and growth of neural stem cells (NSCs) into neurons, enhancing synaptic plasticity by increasing long-term potentiation and modulating synaptic protein expression to stimulate dendritic arborization and new spine formation<sup>6-7</sup>. Furthermore, n-3 PUFAs have anti-oxidative, anti-inflammatory and anti-apoptotic effects, leading to neuron protection in the aged, damaged, and Alzheimer's disease brains<sup>8</sup>.

DHA accretion in neural tissues peaks during the brain growth spurt in the last trimester of pregnancy and early postnatal life<sup>9</sup>. In mammals, n-3 PUFAs cannot be synthesized *de novo* and must be obtained largely from dietary sources. Thus, the maternal diet needs to include an appropriate quantity of n-3 PUFAs and this, together with the ratio of n-6 to n-3 PUFAs, is likely to be an important determinant of DHA accretion in the brain and optimal function in offspring. Evidence from studies on the evolutionary aspects of diet indicate that, in the modern Western diet, an increased intake of n-6 PUFAs and/or a reduced intake of n-3 PUFAs has resulted in an increased ratio of n-6/n-3 PUFAs to between 15:1 and 25:1, which is much higher than the ratio of 1-2:1 thought to pertain during early human evolution and their genetic patterns establishment<sup>10</sup>. This may have contributed to the current prevalence of many chronic diseases including depression and Alzheimer's disease<sup>11</sup>.

There are existing WHO and FAO recommendations on the quantities of n-3 PUFA intake that are thought appropriate for pregnancy, as well as for in-

**Sin embargo, una ingesta excesiva de PUFA n-3 puede ejercer una influencia negativa sobre el desarrollo de las crías.**

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Palabras clave: *Ácidos grasos poliinsaturados n-3. Neurogénesis. Apoptosis. Hipocampo. Ratones.*

fants and young children, in the context of brain and cardiovascular development<sup>11</sup>. However, the optimal ratios of n-6/n-3 PUFAs for brain development and function, and the upper limit of n-3 PUFAs in maternal diets that should be observed to avoid potential damage to the brain in the offspring, are still at issue. In our previous study aimed at addressing these issues, we fed pregnant mice on diets with variable n-3 PUFA contents (i.e. ratios of n-6/n-3 PUFAs ranging from 15.7:1 to 1.6:1) until the end of lactation. We found that DHA concentrations and the expression levels of neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) in pup mice increased with decreasing dietary ratios of n-6/n-3 PUFAs, in a somewhat dose-dependent manner<sup>12</sup>. This suggests that a higher intake of n-3 PUFAs, resulting from a low ratio of n-6/n-3 PUFAs of about 1-2:1 is beneficial for early brain development. In the current study, we used maternal diets similar to those in our previous work<sup>12</sup> to investigate their effects on hippocampal neurogenesis and apoptosis in the offspring of mice.

## Materials and Methods

### *Diets*

Two n-3 PUFA dietary regimes with different n-6/n-3 PUFA ratios, as well as an n-3 PUFA-deficient diet, were used. For each of the n-3 PUFA-containing diets, flaxseed and fish oils (the main sources of n-3 PUFAs) were combined with other oils to yield three different n-6/n-3 PUFA ratios of 15.7:1, 6.3:1 and 1.6:1, which represent the ratio in the modern Western diet, the recommended ratio and the ratio in our ancestors' diet, respectively. Additionally, to determine the potential side effects of a high intake of n-3 PUFAs, particularly EPA and DHA, a high fish oil diet comprising fish oil mixed with lard oil was produced to yield a n-6/n-3 PUFA ratio of 1:5.7. All diets were designed to contain 6% fat (wt/wt) (14% total energy) with the same baseline consisting of 200 g casein, 20 g lard oil, 388 g corn starch, 100 g maltodextrin, 150 g sucrose, 35 g mineral mix, 10 g vitamin mix, 47 g cellulose, 40 g calcium phosphate and 2.5 g choline per 1 kg. Fatty acid methyl esters (FAMES) from each diet was prepared according to a modified method of Lepage and Roy<sup>13</sup> and measured by gas chromatogra-

phy (Agilent 6890N GC, 30 m×0.32 mm id×0.25 μm DB, P/N 19091J-433). The fat contents and fatty acid compositions are shown in table I. The n-3 PUFAs in the five diets provided 0.67%, 2.92%, 6.56%, 17.85% and 23.93% of total fatty acids (0.09%, 0.41%, 0.92%, 2.50% and 3.84% of the total energy), respectively. All diets were prepared by the Institute of Laboratory Animal Sciences at the Chinese Academy of Medical Science and stored at -20°C before use.

### Animals

C57BL/6J mice (three to four weeks old, female) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences of China and were housed at the animal facilities in the Center under a 12-hour (h) light/12-h dark cycle with free access to food and water. After one week of recovery from transportation, the mice were randomly divided into five groups and fed one of five types of diet. C57 BL/6J male mice were fed the same diet with n-6/n-3 PUFA ratio at 6.3:1. After 10 weeks feeding, the female mice in each group were mated and continued on its

own diet throughout gestation and the 7 days of lactation. Seven days after birth, pups were anesthetized by intra-peritoneal injection of Avertin (2,2,2-tribromoethanol) (T-4840-2, Aldrich Chemical) (125 mg/kg) and then sacrificed immediately by decapitation. Six to seven pups were chosen for each group bred by different dams. The skulls were opened and the brains were fixed by immersion in 10% paraformaldehyde or snap-frozen in liquid nitrogen and stored at -80°C. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of National Administration Regulations on Laboratory Animals of China. The protocol was approved by the Committee on the Ethics of Animal Experiments of Hospital 304 affiliated to the General Hospital of the People's Liberation Army in China.

### Immunohistochemical analysis

The CA3 area in the hippocampus is the major efferent site for neurons of the dentate gyrus, which is one of the few areas of the brain that continues to produce neurons postnatally. To detect neurogenesis in CA3,

**Table 1.**  
*Fat content and fatty acid composition of the experimental diets.*

	<i>n-3 PUFA deficient</i>	<i>n-6/n-3 PUFA ratios</i>			<i>High fish oil</i>
		<i>High</i>	<i>Medium</i>	<i>Low</i>	
<b><i>Fat (g/kg diet)</i></b>					
Lard oil	20	20	20	20	20
Sunflower oil	44	41	36	20	-
Flaxseed oil	-	2	5	16	-
Fish oil	-	1	3	8	44
<b><i>Fatty acid (% of total fatty acids)</i></b>					
Saturated	23.33	22.86	23.2	24.62	36.51
Mono-unsaturated	27.33	26.94	27.16	28.04	30.87
C18:2n-6	48.67	47.02	42.51	28.64	4.02
C18:3n-3	0.67	2.4	5.25	13.72	0.89
C20:4n-6	-	0.01	0.01	0.02	0.14
C20:4n-3	-	0.07	0.17	0.54	3.03
C20:5n-3	-	0.28	0.7	2.22	12.38
C22:5n-3	-	0.05	0.14	0.43	2.41
C22:6n-3	-	0.19	0.47	1.48	8.25
Total n-3 PUFAs	0.67	2.92	6.56	17.85	23.93
DHA	-	0.19	0.47	1.48	8.25
Energy from n-3 PUFAs (%)	0.09	0.41	0.92	2.50	3.84
Energy from DHA (%)	-	0.03	0.07	0.21	1.18
n-6/n-3 PUFA ratios	72.6:1	15.7:1	6.3:1	1.6:1	1:5.7

two biomarkers, phosphorylation of histone H3 at serine 10 (p-H3ser10) and calretinin were used: p-H3ser10 is a marker of mitosis and is essential for chromosome condensation, while calretinin, a calcium-binding protein, is normally expressed in neuronal cells and plays a central role in directing the differentiation of neuronal progenitor cells<sup>14-15</sup>. To determine the degree of apoptosis, levels of the antiapoptotic protein Bcl2 and the proapoptotic protein Bax were assessed<sup>16</sup>.

Serial sections, 12  $\mu\text{m}$  thick, of paraffin-embedded brain were mounted on Colorfrost Plus glass slides (Fisher Scientific, USA). The sections were deparaffinized, rehydrated, and then washed in 0.1 M PBS (pH 6.0). A microwave antigen-retrieval step was performed with 0.01 M sodium citrate buffer (pH 6.0). After cooling, the sections were treated with 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase and then rinsed in PBS. Then they were blocked with normal horse serum in 0.5% BSA solution for 30 minutes at room temperature and incubated with one of the following primary antibodies overnight at 4°C: rabbit anti-p-H3ser10 (1:200, Santa Cruz, USA), rabbit anti-calretinin (1:200, abcam, USA), rabbit anti-Bax (1:200, abcam, USA), and rabbit anti-Bcl2 (1:200, abcam, USA). The next day, the sections were rinsed several times in PBS, and immunoreactivity was visualized using a biotinylated anti-rabbit IgG, the avidin-biotin-peroxidase (ABC) detection system

(Vectastain ABC Elite Kit, Vector Laboratories) and the chromogen 3,3'-diamino-benzidine. The reaction was stopped by rinsing with  $\text{dH}_2\text{O}$ . Image analyses of brain sections were performed using a Leica Qwin V3 system (Leica Inc. Germany) to measure the average optical density (OD) and the numerical density on area (NA) of cells positive for p-H3ser10, calretinin, Bcl2 and Bax in the hippocampus CA3 areas.

#### TUNEL staining

The TUNEL method was performed to detect neuronal DNA fragmentation that results from apoptotic signaling cascades. In brief, paraffin-embedded sections were deparaffinized and dehydrated. After washing in PBS, sections were treated with 20  $\mu\text{g}/\text{mL}$  proteinase K for 20 min. The sections were permeabilized by 0.1% Triton X-100 for FITC end-labeling of the fragmented DNA of apoptotic cells using a TUNEL cell apoptosis detection kit (Beyotime Institute of Biotechnology, China). The FITC-labeled TUNEL-positive cells were imaged using fluorescence microscopy (excitation: 488 nm; emission: 530 nm). Three different areas were selected at random from CA3 regions of each section, and the number of TUNEL-positive cells in these areas was counted. The neuronal apoptosis rate = (number of TUNEL-positive cells/total cells)  $\times$  100%<sup>17</sup>.

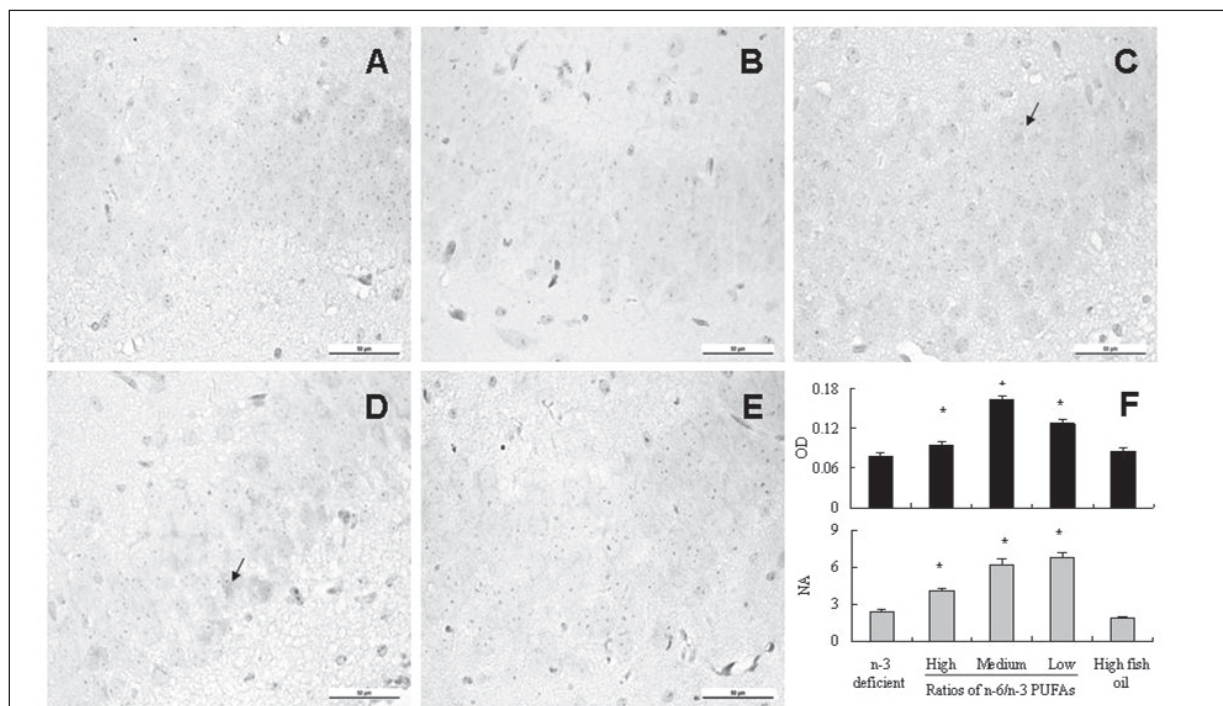


Fig. 1.—Changes in cell proliferation in the hippocampus CA3 area of mouse offspring after supplementation of maternal diet with n-3 PUFAs during pregnancy (original magnification 400 $\times$ ). Immunohistochemical staining was conducted according to the procedures in “Materials and Methods”. Cells that are positive for phosphorylation of histone H3 at ser 10 (p-H3ser10) are colored brown (an example is indicated by an arrow). A: n-3 PUFA-deficient diet; B-D: n-3 PUFA-containing diets with high, medium and low ratios of n-6/n-3 PUFAs, respectively; E: high fish oil diet. F: quantification of p-H3ser10. Values are means  $\pm$  SD (n=6-7 for each group). \*  $P < 0.05$ , compared to the n-3 PUFA-deficient diet group.

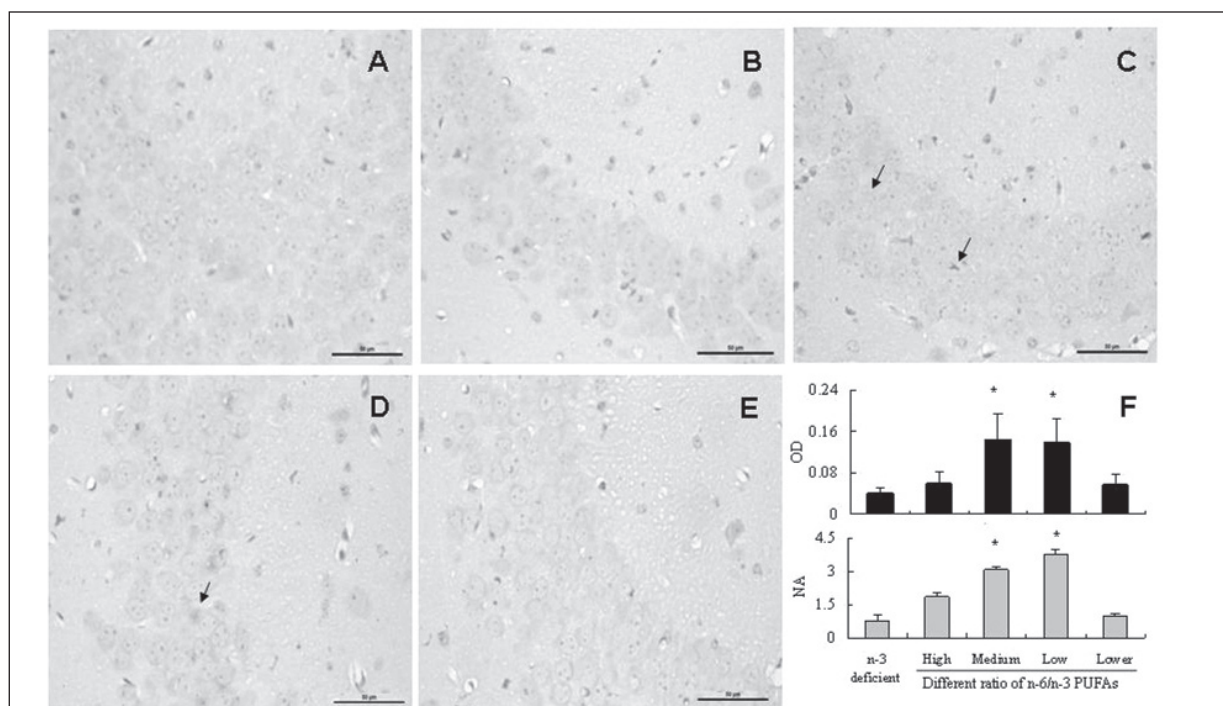


Fig. 2.—Changes in cell differentiation in the hippocampus CA3 area of mouse offspring after supplementation of maternal diet with n-3 PUFAs during pregnancy. Immunohistochemical staining was conducted according to the procedures in “Materials and Methods”. Calretinin-positive cells are colored brown (an example is indicated by an arrow). A: n-3 PUFA-deficient diet; B-D: n-3 PUFA-containing diets with high, medium and low ratios of n-6/n-3 PUFAs, respectively; E: high fish oil diet. F: quantification of calretinin expression. Values are means  $\pm$  SD (n=6-7 for each group). \*  $P < 0.05$ , compared to the n-3 PUFA-deficient diet group.

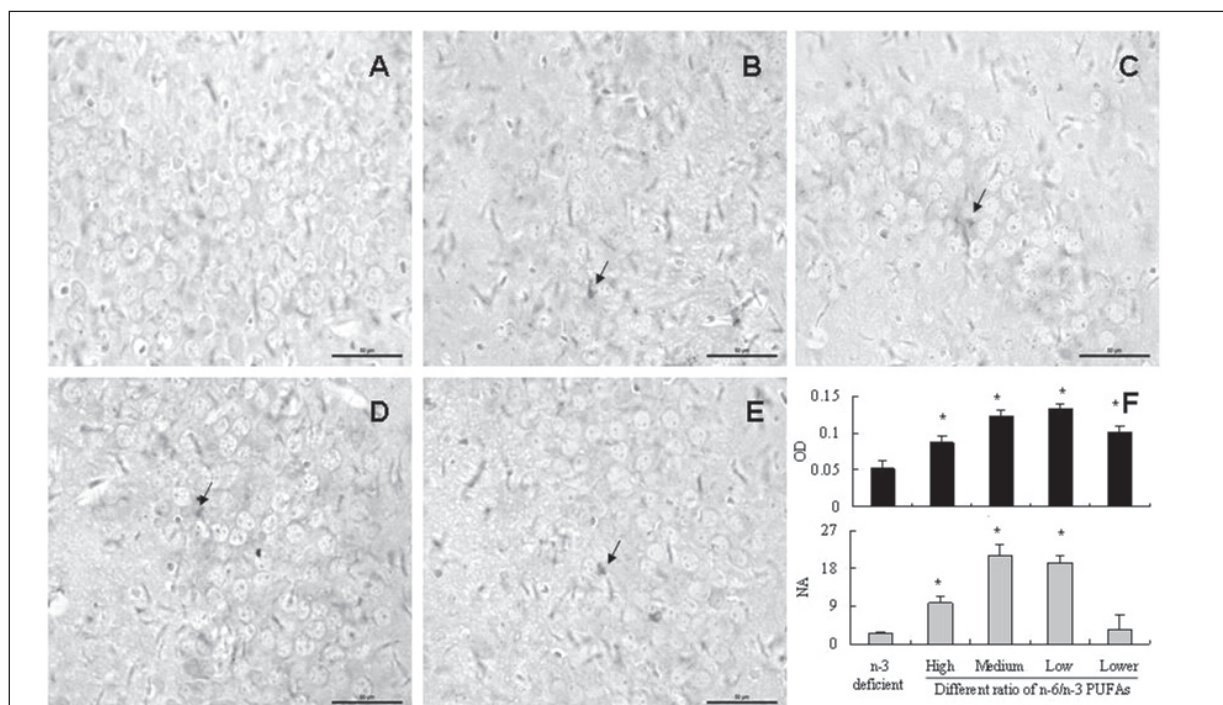


Fig. 3.—Changes in anti-apoptotic protein Bcl2 in the hippocampus CA3 area of mouse offspring after supplementation of maternal diet with n-3 PUFAs during pregnancy. Immunohistochemical staining was conducted according to the procedures in “Materials and Methods”. Bcl2-positive cells are colored brown (an example is indicated by an arrow). A: n-3 PUFA-deficient diet; B-D: n-3 PUFA-containing diets with high, medium and low ratios of n-6/n-3 PUFAs, respectively; E: high fish oil diet. F: quantification of Bcl2 expression. Values are means  $\pm$  SD (n=6-7 for each group). \*  $P < 0.05$ , compared to the n-3 PUFA-deficient diet group.

### Caspase 3 activity assay

Caspase 3 has been identified as a key mediator of DNA fragmentation during apoptosis in neuronal cells. Active caspase 3 proteolytically cleaves and activates other caspases, as well as relevant targets in cells (e.g. PARP), and executes the terminal step in apoptosis<sup>18</sup>. Caspase 3 activity was measured using an assay kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's protocol. The assay is based on the detection of cleavage of the substrate Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide) by caspase 3. Briefly, 10 mg of brain tissue were suspended in lysis buffer and lysates were centrifuged for 5 min at 16000×g. The supernatants (20 μg protein in each sample) were incubated at 37°C for 2 h in 2 mmol/L Ac-DEVD-pNA (2 mM), and the absorbance of yellow pNA cleaved from its precursor was measured using a spectrometer at 405 nm (Tecan GENios, Switzerland). The caspase 3 activity was normalized against total protein concentration in the tissue lysates.

### Statistical analysis

Statistical analysis was performed with SPSS Release 11.5 for Windows. All values were expressed as the mean ± S.D. Significant differences among the groups

were tested by one-way analysis of variance (ANOVA) followed by a Kruskal-Wallis post hoc test. For unequal variances, Games-Howell analyses were used.

## Results

### 1. The effect of maternal dietary n-6/n-3 PUFA ratios on neurogenesis in hippocampus CA3 of the offspring

To study the effect of PUFAs on brain development, we fed pregnant mice diets with variable n-3 PUFA contents and examined neurogenesis in hippocampus area CA3 in pups at 7 days after birth. In general, compared to the mice with an n-3 PUFA-deficient diet, the average optical density and the numerical density on area of p-H3ser10-positive (Fig. 1) and calretinin-positive (Figure 2) cells were greater in mice given the n-3 PUFA diets with high, medium and low ratios of n-6/n-3 PUFAs ( $P < 0.05$ ). However, for mouse pups whose mothers were fed a high fish oil diet with the lowest n-6/n-3 PUFA ratio, there was no significant difference with the group fed a n-3 PUFA-deficient diet for either property ( $P > 0.05$ ; Fig. 1 and 2). Further analysis revealed that, within the dietary ratios of n-6/n-3 PUFAs ranging from the high to the lowest (15.7:1 to 1:5.7), the expression of p-H3ser10 and calretinin manifested an inverse U-shaped pattern with

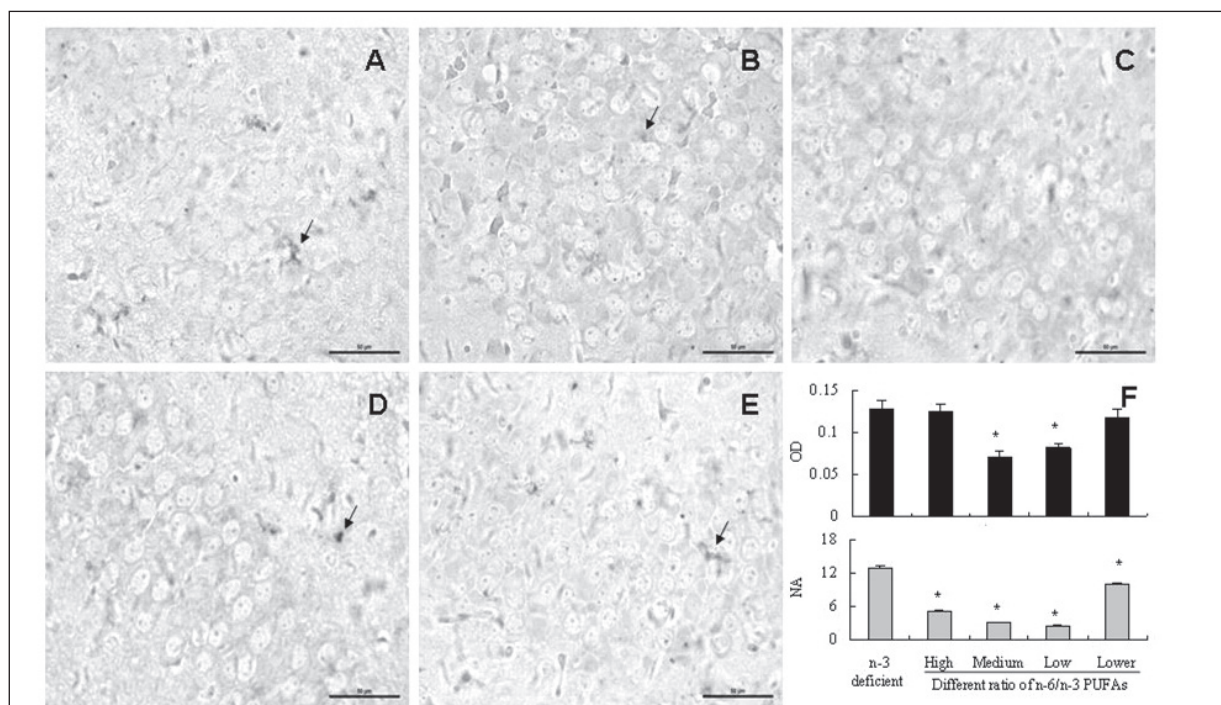


Fig. 4.—Changes in pro-apoptotic protein Bax in the hippocampus CA3 area of mouse offspring after supplementation of maternal diet with n-3 PUFAs during pregnancy. Immunohistochemical staining was conducted according to the procedures in “Materials and Methods”. Bax positive cells are colored in brown (an example is indicated by an arrow). A: n-3 deficient diet; B-D: n-3 PUFA containing diets with high, medium and low ratios of n-6/n-3 PUFA, respectively; E: high fish oil diet. F: quantification of Bax expression. Values are means ± SD ( $n=6-7$  for each group). \*  $P < 0.05$  as compared to the n-3 PUFA deficient diet group.

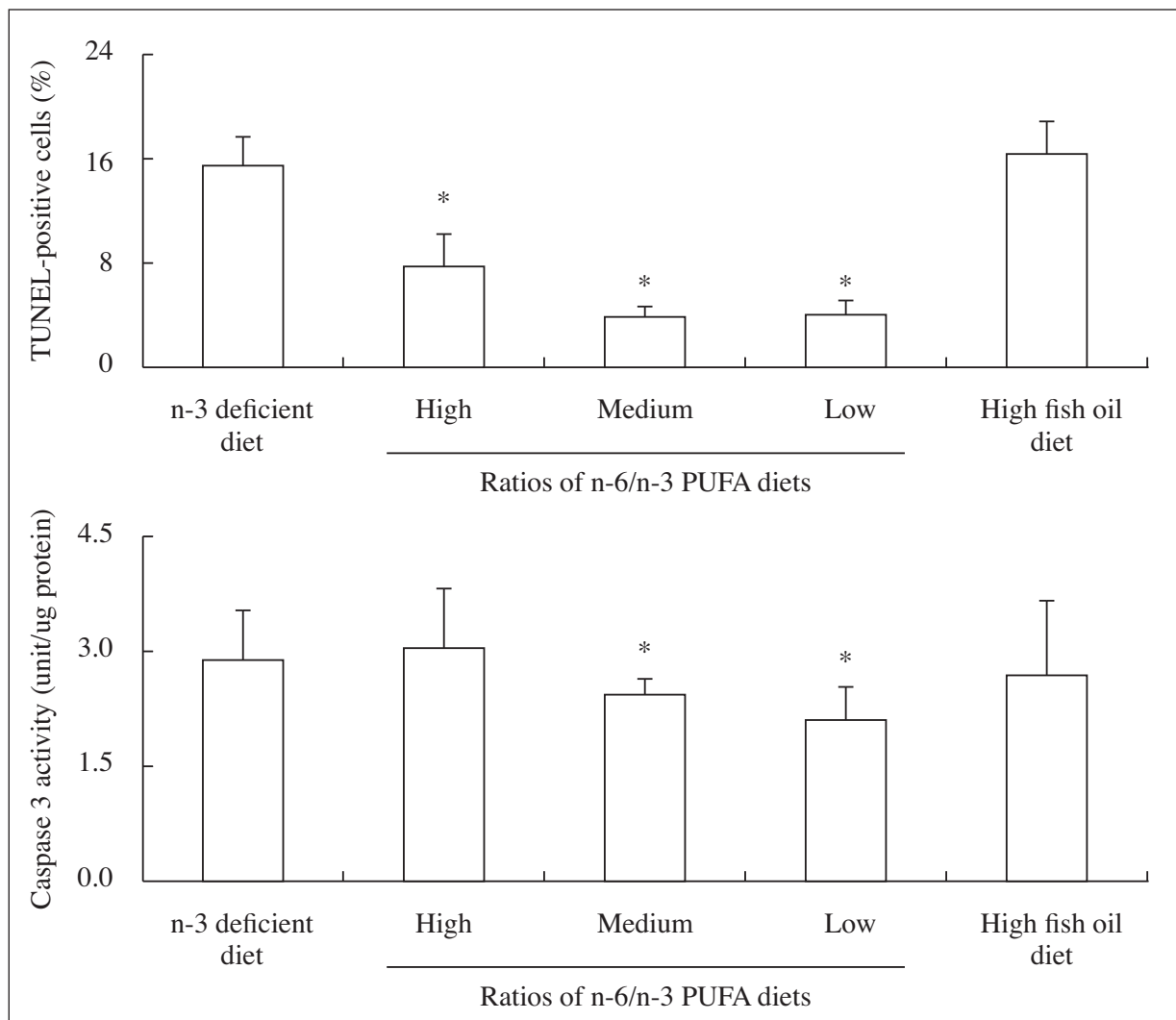


Fig. 5.—Changes in TUNEL-positive cells and caspase 3 activities in the hippocampus CA3 area of mouse offspring after supplementation of maternal diet with n-3 PUFAs during pregnancy. TUNEL staining was conducted according to the procedures in “Materials and Methods”, and the overall proportions of TUNEL apoptotic cells were calculated. Caspase 3 activity was measured according to the procedures in “Materials and Methods”. Values are means  $\pm$  SD ( $n=6-7$  for each group). \*  $P<0.05$ , compared to the n-3 PUFA-deficient diet group.

the peak at groups with n-6/n-3 PUFA ratios of 6.3:1 and 1.6:1. This indicates that excessive intake of n-3 PUFAs may adversely affect neurogenesis.

## 2. The effect of maternal dietary n-6/n-3 PUFA ratios on neuro-apoptosis in hippocampus CA3 of the offspring

The expression of apoptosis-related proteins in hippocampus CA3 was examined in the mouse offspring. Generally, in the three diet groups with high, medium and low n-6/n-3 PUFA ratios, the average optical density and the numerical density on area for Bcl2 (anti-apoptotic; Figure 3) were higher, and those for Bax (pro-apoptotic; Figure 4) were lower, than in the n-3 PUFA-deficient diet group ( $P<0.05$ ). These

data indicate that PUFAs, at appropriate n-6/n-3 ratios, inhibit neuro-apoptosis in developing mouse brain. Again, however, no differences were found in Bcl2 and Bax levels between groups with the n-3 PUFA-deficient diet and the high fish oil diet ( $P>0.05$ ), suggesting that too high an intake of n-3 PUFAs may promote neuronal apoptosis.

## 3. The effect of maternal dietary n-6/n-3 PUFA ratios on TUNEL apoptosis cells and the caspase 3 activity in hippocampus CA3 of the offspring

Figure 5A shows that the overall proportion of TUNEL apoptotic cells in hippocampus CA3 was significantly lower in the three groups with high, medium and low n-6/n-3 PUFA ratios than in the n-3 PUFA-de-

ficient diet group ( $P < 0.05$ ). In the group fed the high fish oil diet, the number of apoptotic cells was the same as in the n-3 PUFA-deficient diet group ( $P > 0.05$ ).

We next measured the caspase 3 activity in the brains of offspring (Figure 5B). The caspase 3 activities in the groups given medium and low n-6/n-3 PUFA ratio diets were lower than those in the n-3 PUFA-deficient diet group ( $P < 0.05$ ), with no changes in the high n-6/n-3 PUFA ratio group and in the high fish oil diet group ( $P > 0.05$ ).

## Discussion

The accretion of DHA induces neurite growth, synaptogenesis, synapsin and glutamate receptor expression and glutamatergic synaptic function, which support the observation that hippocampus-related cognitive function is improved by DHA<sup>19</sup>. In cultures of neuron stem cells or in vivo animal studies, DHA significantly enhances the generation of new neurons and stimulates a more mature morphology in these cells<sup>20-22</sup>. Consistent with these reports, we found in our study that supplementation of the maternal diet with n-3 PUFAs during pregnancy increased the expression of p-H3ser10 and calretinin in offspring hippocampus, reflecting stronger proliferation and differentiation of neuronal stem cells. Furthermore, our results showed that hippocampal neurogenesis was improved by maternal dietary n-6/n-3 PUFA ratios within the range 15.7:1 to 1.6:1, whereas a maternal diet that included high levels of fish oil inhibited neurogenesis in offspring hippocampus. The data suggest that maternal diets with n-6/n-3 PUFA ratios of between 5:1 and 1:1 may be the most beneficial for neurogenesis in mouse pups, which validates our previous findings that a higher maternal intake of dietary n-3 PUFAs with an n-6/n-3 PUFA ratio of between 1:1 and 2:1 promotes better growth and maturation of neurons, astrocytes and myelin in mouse offspring<sup>12</sup>.

It has been demonstrated that n-3 PUFAs have the potential to influence neurogenesis through several distinct mechanisms<sup>1</sup>. They are incorporated into membrane phospholipids, especially phosphatidylethanolamine (PE), and can alter membrane-associated signal transduction pathways (Raf-1 and PI-3 kinase pathways), neurotransmitters (dopamine, serotonin and choline) and receptors, all of which are crucial for neurogenesis<sup>23-25</sup>. DHA is also a ligand for transcription factors such as retinoid X receptors (RXRs), and DHA-RXR signaling can influence neuronal differentiation, neurite outgrowth, synaptogenesis and neurogenesis<sup>22</sup>. The production of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , is decreased by n-3 PUFAs, which can result in marked suppression of hippocampal neurogenesis<sup>26</sup>. Furthermore, n-3 PUFAs can modulate levels of neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and their target receptors, which are

known to promote neuroplasticity, enhance cell survival, and increase cellular excitability and hippocampus neurogenesis<sup>27-29</sup>.

There are several reports showing that n-3 PUFAs can prevent neuronal apoptosis in patients suffering conditions such as Alzheimer's disease<sup>30</sup> and also in animal models under stress conditions. For example, maternal feeding of DHA significantly prevents stress-induced oxidative damage, apoptosis and mitochondrial dysfunction, and attenuates hyperoxia-induced neuronal apoptosis in the developing brain<sup>31-32</sup>. Enrichment of neuronal cells with DHA increases the phosphatidylserine (PS) content and prevents apoptotic cell death by up-regulating Raf-1 translocation and down-regulating caspase 3 activity<sup>33</sup>. As the precursor of neuroprotectin D1 (NPD1), DHA up-regulates the anti-apoptotic genes encoding Bcl-2, Bcl-x1, and Bfl-1(A1)<sup>34</sup>, attenuates caspase 3 activation and decreases compacted nuclei and fragmented DNA<sup>35</sup>. Recently, several publications indicated that EPA-derived E-series resolvins (RvE) and DHA-derived D-series resolvins (RvD) can stimulate PI3K/Akt signaling pathways, thus inhibiting cell apoptosis<sup>36-37</sup>. In the current study, similar to the effects on hippocampal neurogenesis, diets with n-6/n-3 PUFA ratios ranging from 15.7:1 to 1.6:1 increased the level of anti-apoptotic protein Bcl2, and reduced the expression of pro-apoptotic protein Bax, caspase 3 enzyme activity and TUNEL apoptotic cells. Maintaining n-6/n-3 PUFA ratios of between 6.3:1 and 1.6:1 in maternal diets may therefore ensure an optimal anti-apoptotic effect. The high fish oil diet with a 1:6.5 n-6/n-3 PUFA ratio adversely affected Bcl2, Bax, caspase 3 activity and TUNEL apoptotic cells in a similar way to the n-3 PUFA-deficient diet.

Hence, with regard to hippocampal neurogenesis and apoptosis, as determined in this study, the n-6/n-3 PUFA ratio in maternal diets may be optimal in the range 5:1 to 1:1. For diets with a low n-6/n-3 PUFA ratio of about 1.6:1, which mimic our ancestors' diet, total n-3 PUFAs and DHA account for 17.85% and 1.48% of total fatty acids and provide 2.5% and 0.21% of total energy, respectively. This is higher than the recommendation of 0.5-2.0% of total energy from n-3 PUFAs<sup>38</sup>, but lower than the average intake (5% of total energy) in Eskimos, who mostly obtain this dietary component from fish oils<sup>39</sup>. Thus, the dietary intake of n-3 PUFAs needed for brain DHA accretion and brain development may be higher than that recommended currently. It is important to stress the adverse effects of excessive n-3 PUFA intake on brain development, as well as on other organs and systems. Church and his colleagues reported that excessive n-3 PUFA consumption from dietary fish oil (n-6/n-3 PUFA ratio = 1:14) by the rat mother during pregnancy and lactation causes abnormal neurological responses in young adult offspring<sup>40</sup>. The harmful effects of excess n-3 PUFA consumption in fetal and infant brain and sensory functioning may be related to lower AA concentra-



tions and may induce oxidative stress and subsequent cell apoptosis<sup>41</sup>. In the current study, maternal intake of n-3 PUFAs at 23.93% of total fatty acids (3.84% of total energy) or DHA at 8.25% of total fatty acids (1.18% of total energy) exerted a negative influence on neurogenesis and apoptosis in the offspring brain. The data available from intervention trials in human pregnancy indicate that doses of up to 3 g/day of fish oil n-3 PUFAs are safe. This intake contains about 1.2 g/d of DHA, providing 0.3% of total energy<sup>42</sup>, which is higher than the 0.21% of total energy in the diet with a n-6/n-3 PUFA ratio of 1.6:1 in this study. Therefore, based on the current evidence, it is not yet possible to set an estimated average requirement for n-3 PUFAs and DHA in pregnancy.

In conclusion, our data suggest that a higher intake of dietary n-3 PUFAs with an n-6/n-3 PUFA ratio of between 6:1 and 1:1 during mouse pregnancy may be beneficial for hippocampal development by facilitating neurogenesis and inhibiting neuronal apoptosis in the offspring. Either deficiency or excessive amounts of n-3 PUFAs during pregnancy seem to adversely affect brain development.

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