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# Omega-3 fatty acids deprivation affects ontogeny of glutamatergic synapses in rats: Relevance for behavior alterations

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ABSTRACT

Essential omega-3 polyunsaturated fatty acids (ω3) are crucial to brain development and function, being relevant for behavioral performance. In the present study we examined the influence of dietary ω3 in the development of the glutamatergic system and on behavior parameters in rats. Female rats received isocaloric diets, either with ω3 (ω3 group) or a ω3 deficient diet (D group). In ontogeny experiments of their litters, hippocampal immunocontent of ionotropic NMDA and AMPA glutamatergic receptors subunits (NR2 A\B and GluR1, respectively) and the alpha isoform of the calcium-calmodulin protein kinase type II (αCaMKII) were evaluated. Additionally, hippocampal [$^3$H]glutamate binding and uptake were assessed. Behavioral performance was evaluated when the litters were adult (60 days old), through the open-field, plus-maze, inhibitory avoidance and flinch-jump tasks. The D group showed decreased immunocontent of all proteins analyzed at 02 days of life (P2) in comparison with the ω3 group, although the difference disappeared at 21 days of life (except for αCaMKII, which content normalized at 60 days old). The same pattern was found for [$^3$H]glutamate binding, whereas [$^3$H]glutamate uptake was not affected. The D group also showed memory deficits in the inhibitory avoidance, increased in the exploratory pattern in open-field, and anxiety-like behavior in plus-maze. Taken together, our results suggest that dietary ω3 content is relevant for glutamatergic system development and for behavioral performance in adulthood. The putative correlation among the neurochemical and behavioral alterations caused by dietary ω3 deficiency is discussed.



## 1. Introduction

Dietary omega-3 (ω3) polyunsaturated fatty acids (PUFAs) have a great relevance to brain health. It has been established that they are critical for proper infant growth and neurodevelopment (Marszalek and Lodish, 2005). Among the ω3 PUFAs, DHA is the most important ω3 with physiological significance for brain function (Marszalek and Lodish, 2005; Bourre, 2004), especially during prenatal brain development when it is incorporated into nerve growth cones during synaptogenesis (Martin and Bazan, 1992). The human brain growth spurt that takes place from the third trimester of pregnancy until 18 months post birth correlates with DHA accretion into brain phospholipids (Lauritzen et al., 2001). Insufficient dietary supply of ω3 PUFAs during pre- and postnatal development decreases the levels of DHA in neural tissue with a reciprocal increase of docosapentanoic acid (DPA, C22:5 ω6) (Schiefermeier and Yavin, 2002), leading to behavioral deficits in animal models (Lim et al., 2005; Moriguchi et al., 2000). Accordingly, dietary DHA supplementation in breastfeeding has been shown to improve mental development in human children (Hibbeln et al., 2007; Birch et al., 2000; Willatts et al., 1998).

Studies have shown that omega-3 dietary deficiency affect the glutamatergic, dopaminergic and serotoninergic systems (Moreira et al., 2010; Zimmer et al., 2000; Delion et al., 1996). Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS), involved in brain plastic processes, such as learning/memory, brain development and ageing (Tzingounis and Wadiche, 2001; Danbolt, 2001; Segovia et al., 2001; Ozawa et al., 1998); however, overstimulation of the glutamatergic system may be highly neurotoxic. The main process responsible for maintaining extracellular glutamate concentration below toxic levels is the

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glutamate uptake by the transporters GLT1, GLAST (astrocytic) and EAAC1 (neuronal) (Sheldon and Robinson, 2007; Maragakis and Rothstein, 2004). Thus, the balance of the physiological/excitotoxic glutamatergic tonus is modulated by the activity of these proteins, especially the astrocytic transporters.

Glutamate exerts its physiological or toxic effects via ionotropic (iGluRs) and/or metabotropic (mGluRs) receptors. iGluRs are cation-specific ion channels, classified as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and *N*-methyl-D-aspartate (NMDA) receptors, whereas mGluRs are G-proteins-coupled receptors which modulate the production of intracellular messengers (Ozawa et al., 1998; Kew and Kemp, 2005).

Concerning iGluRs, NMDA receptors are arrangements of the NR1 and NR2A-2D subunits, while AMPA receptors are arrangements of GluR1-GluR4 subunits (Kew and Kemp, 2005; Ozawa et al., 1998). α-Calcium/calmodulin-dependent kinase type II (αCaMKII), a synaptic enzyme that interacts with NMDA and AMPA receptors, is involved in memory modulation, and in hippocampal long-term potentiation (LTP), considered as a model for the cellular and molecular basis of memory (Bevilaqua et al., 2005; Lisman et al., 2002).

The glutamatergic system is the focus of several animal behavioral studies. It is involved in numerous behavioral patterns, such as hyperactivity (Dorval et al., 2007; Fadda et al., 2007), anxiety (Kapus et al., 2008; Bergink et al., 2004) and memory deficits (Vicente et al., 2008; Rotta et al., 2008). There are numerous reports related to the effects promoted by ω3 PUFAs on the glutamatergic system. An *in vitro* study showed that DHA differentially modulate glutamate transporters GLT1, GLAST and EAAC1 (Berry et al., 2005). A dietary supplementation with DHA was capable of restoring LTP and glutamate release in hippocampus of aged rats (McGahon et al., 1999). DHA-enriched diet also reversed the age-related decrease in the GluR2 and NR2B, subunits of AMPA and NMDA receptors respectively, in the forebrain of aged rats (Dyall et al., 2006), and it was protective against MK-801-induced neurotoxicity (Ozyurt et al., 2007). In a previous study, our group demonstrated that ω3 PUFAs were capable of strengthening the glutamatergic response in an ischemic injury model (Moreira et al., 2010).

The purpose of the present study was to investigate the influence of dietary ω3 PUFAs on the ontogeny the glutamatergic system in rat hippocampus and on behavioral performance of adult rats.

## 2. Materials and methods

### 2.1. Animals and diets

The dietary model used in this study has been previously described by Moreira et al. (2010), and it was designed to control the ω3 PUFAs levels in the maternal milk as well as in the offspring diet after weaning. Two weeks before mating, Wistar female rats were housed in a standard animal house with controlled environment (21–22 °C, 12 h dark–light cycle, food and water *ad libitum*). Rats were divided into two groups: ω3 diet (ω3) and ω3 deficient diet (D); the diets prepared at our own laboratory were isocaloric, containing 8% of total fat and differing only in the fatty acids composition (Tables 1 and 2). Female rats received their respective diets throughout the gestation as well as during suckling. After weaning (21 days old), the litters were maintained with the same diet of their dams until 60 days old. Ontogenetic experiments were done with litters at 02, 21 and 60 days old. Behavioral experiments were performed with male adult rats (60 days old). All experiments were in agreement with the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

### 2.2. Neurochemical ontogenetic studies

#### 2.2.1. Western blotting for hippocampal proteins

*2.2.1.1. Hippocampal synaptosomal preparations.* The synaptosomal preparation was obtained as previously described (Dosemeci et al., 2006). All centrifugation steps were performed in a refrigerated (4 °C) centrifuge. Hippocampi of 02, 21 and 60 days old rats were homogenized (motor-driven small capacity Teflon/glass homogenizer in a final volume of 1 mL/hippocampus) in a 25 mM Hepes buffer (pH 7.4) with 0.32 M sucrose, 1 mM $MgCl_2$ and a protease inhibitor cocktail (Sigma). The homogenate was transferred to microfuge tubes (1.5 mL per tube), and centrifuged at 470 × *g* × 2 min using a fixed angle rotor. The resultant supernatant was further transferred to another microfuge tube and centrifuged at 10,000 × *g* × 10 min using the same rotor to obtain a mitochondrial- and synaptosomal-enriched pellet (P2). P2 was resuspended into 0.32 M sucrose (500 μL per tube) and the suspension was layered onto 750 μL of 0.8 M sucrose in a microfuge tube. The samples were centrifuged at 9100 × *g* × 15 min using a swinging bucket rotor. The myelin/light membrane layer at the top of 0.32 M sucrose was removed. Synaptosomal fraction collected at 0.32 M/0.8 M interface was washed twice to remove sucrose excess by centrifugation at 16,000 × *g* × 10 min in 25 mM Hepes (pH 7.4) containing a protease inhibitor cocktail. The final pellet was resuspended in the same solution (200 μL/pellet) for Western blotting analysis.

**Table 1**
Fatty acids composition of the diet lipids.[a].

| | ω3 diet (%) | D diet (%) |
|---|---|---|
| Saturated | | |
| C16:0 | 10.9 | 11.1 |
| C18:0 | 2.0 | 2.4 |
| C20:0 | 0.5 | 1.3 |
| C22:0 | 0.1 | 2.9 |
| C24:0 | 0.0 | 1.5 |
| Monounsaturated | | |
| C16:1 | 0.2 | 0.2 |
| C18:1 | 25.4 | 46.7 |
| C20:1 | 0.0 | 1.6 |
| Polyunsatutared | | |
| C18:2ω6 | 56.6 | 32.0 |
| C18:3ω3 | 1.2 | 0.0 |
| C20:5ω3[b] | 2.2 | 0.0 |
| C22:6ω3[b] | 3.5 | 0.0 |

[a] According to O'Brien (2004).
[b] According to manufacturer information (Naturalis, Brazil).

*2.2.1.2. Western blotting analysis.* Synaptosomal proteins (30 μg protein/well) were separated in a 7.5% SDS-PAGE mini-gel and transferred to nitrocellulose membrane using a Trans-Blot system (Bio-Rad, Hercules CA). Membranes were processed as follow: (1) blocking with 5% bovine serum albumin (Sigma) for 2 h; (2) incubation with primary antibody overnight: 1:200 anti-αCaMKII (Chemicon International); 1:1000 anti-GluR1 (Upstate Cell Signaling Solutions); 1:5000 anti-NR2A/B (Chemicon International); 1:1000 β-actin (Sigma); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:3000 and mouse 1: 5000 (Amersham Pharmacia Biotech) for 2 h; (4) chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and band intensities were analyzed using Image J software (developed at the U.S. National Institutes of Health and available on the

**Table 2**
Composition of the diets.

| | ω3 diet (%) | D diet (%) |
|---|---|---|
| Casein[a] | 22 | 22 |
| Corn starch | 42 | 42 |
| D-L-Methionin[b] | 0.16 | 0.16 |
| Sucrose | 21 | 21 |
| Fibers | 2 | 2 |
| Mineral salt mix[c] | 4 | 4 |
| Vitamin mix[d] | 1 | 1 |
| Peanut oil | 0 | 8 |
| Corn oil | 7 | 0 |
| Fish oil | 1 | 0 |

Salt and vitamin compositions are according to Horwitz (1980).
[a] Casein, purity 87% (from Herzog, Porto Alegre, Brazil).
[b] D-L-Methionin (from Merk, Rio de Janeiro, Brazil).
[c] Mineral salt mixture: mg/100 g of diet (from Roche, São Paulo, Brazil): NaCl, 557; KI, 3.2; $KH_2PO_4$, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4$–$7H_2O$, 108; $MnSO_4$–$H_2O$, 16; $ZnSO_4$–$7H_2O$, 2.2; $CuSO_4$–$5H_2O$, 1.9; $CoCl$–$6H_2O$, 0.09.
[d] Vitamin mixture: mg/100 g of diet (from Roche, São Paulo, Brazil): vitamin A (retinyl acetate), 4; vitamin D (cholecalciferol), 0.5; vitamin E (DL-α-tocopheryl acetate), 10; menadione, 0.5; choline, 200; PABA, 10; inositol, 10; niacine (nicotinic acid), 4; pantothenic acid (calcium D-pantothenate), 4; riboflavin, 0.8; thiamin (thiamine hydrochloride), 0.5; piridoxine (pyridoxine hydrochloride), 0.5; folic acid, 0.2; biotin (D-(+)-biotin), 0.04; vitamin B12, 0.003.