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TBI and Sex: Crucial role of progesterone protecting the brain in an omega-3 deficient condition

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Abstract

We assessed whether the protective action of progesterone on traumatic brain injury (TBI) could be influenced by the consumption of omega-3 fatty acids during early life. Pregnant Sprague Dawley rats were fed on omega-3 adequate or deficient diet from 3rd day of pregnancy and their female offspring were kept on the same diets up to the age of 15 weeks. Ovariectomy was performed at the age of 12 weeks to deprive animals from endogenous steroids until the time of a fluid percussion injury (FPI). Dietary n-3 fatty acid deficiency increased anxiety in sham animals and TBI aggravated the effects of the deficiency. Progesterone replacement counteracted the effects of TBI on the animals reared under n-3 deficiency. A similar pattern was observed for markers of membrane homeostasis such as 4-Hydroxynonenal (HNE) and secreted phospholipases A2 (sPLA2), synaptic plasticity such as brain derived neurotrophic factor (BDNF), syntaxin (STX)-3 and growth associated protein (GAP)-43, and for growth inhibitory molecules such as myelin-associated glycoprotein (MAG) and Nogo-A. Results that progesterone had no effects on sham n-3 deficient animals suggest that the availability of progesterone is essential under injury conditions. Progesterone treatment counteracted several parameters related to synaptic plasticity and membrane stability reduced by FPI and n-3 deficiency suggest potential targets for therapeutic applications. These results reveal the importance of n-3 preconditioning during early life and the efficacy of progesterone therapy during adulthood to counteract weaknesses in neuronal and behavioral plasticity.

Keywords

Anxiety; Neuroplasticity; Omega-3 fatty acid; Progesterone; Traumatic Brain Injury

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Introduction

Although the outcome of traumatic brain injury (TBI) is clearly influenced by sex (Wohltmann et al., 2001), the mechanisms involved are poorly understood. It is known that the functions of gonadal steroids, such as progesterone, extend well beyond reproduction (Camacho-Arroyo and Montor, 2012; Kinsley et al., 2012), playing roles, for instance, in recovery after injury. Cycling females typically show less cerebral edema than males and pseudo-pregnancy in females gives even greater protection (Roof et al., 1993). Progesterone has a neuroprotective role improving survival and outcome in animal models of TBI (Roof and Hall, 2000; Stein, 2001), and is in phase III clinical trials for the treatment of TBI (Stein and Wright, 2010). The fact that the concentration of progesterone fluctuates in females across the menstrual cycle poses a challenge for the efficacy of treatments for TBI. Diet is a vital aspect of daily living which has demonstrated capacity to influence brain plasticity (Gomez-Pinilla, 2008), may be instrumental to alter the course of progesterone-based TBI treatments.

Based on the action of the omega-3 fatty acid in protecting the brain against the effects of TBI (Mills et al., 2011; Bailes and Mills, 2010), we focused our current studies on the influence of n-3 fatty acids on progesterone treatment for TBI. The action of n-3 fatty acids ranges from supporting learning (Fedorova et al., 2009) to counteracting behavioral impairments caused by TBI (Wu et al., 2011). For instance, low plasma levels of n-3 fatty acids, particularly DHA in humans has been associated with increased risk of suicide in a population with high risk of trauma (Lewis et al., 2011). Recent reports also suggest that lower consumption of DHA increases likelihood of anxiety disorders particularly in females (Jacka et al., 2013) and rodents studies have shown that low consumption of n-3 diet increases anxiety-like behavior (Harauma and Moriguchi, 2011) and depression (Takeuchi et al., 2003). It also appears that the action of n-3 fatty acids in psychiatric disorders may be sex related. For example, cross-sectional epidemiological survey suggest that low dietary n-3 fatty acid intake is associated with an elevated risk of depression in females (Timonen et al., 2004). Recent reports show that DHA is significantly reduced in the postmortem prefrontal cortex (PFC) of female, but not male, patients with major depression (McNamara et al., 2007). It is also known that the incidence of major psychiatric illnesses in women increases during periods of ovarian hormonal fluctuations such as the postmenopausal period (Deecher et al., 2008). This implies that the actions of progesterone and n-3 fatty acids may influence each other, making it is crucial to determine how progesterone can influence the TBI pathology during n-3 fatty acids deficient condition.

We assessed selected molecular systems important for plasticity and behavior in the hippocampus since the effects of TBI have been well characterized in this region (Ariza et al., 2006) and recent studies have shown the involvement of dentate gyrus in controlling specific features of anxiety (Kheirbek et al., 2013). The hippocampus also contains progesterone receptors (Bali et al., 2012) and is susceptible to the effects of n-3 fatty acids (Kang and Gleason, 2013). In the hippocampus, we studied brain-derived neurotrophic factors (BDNF) because of its described involvement on cognitive function and emotions (Croll et al., 1998; Hall et al., 2000). Neuronal plasticity is supported by neurotrophic factors such as BDNF (Cowansage et al., 2010), and limited by the growth inhibitory myelin

proteins such as myelin-associated glycoprotein (MAG) and Nogo-A (Cai et al., 1999). Growth-associated protein-43 (GAP-43) is highly expressed in the areas of adult rat brain with ongoing structural plasticity, and modulates the formation of new connections (Emery et al., 2003). Syntaxin is a protein that is profusely expressed in synaptic terminals, and the action of DHA on promoting neurite outgrowth and membrane expansion has been shown to rely on STX-3 (Darios and Davletov, 2006). Thus, we analyzed these molecular markers and anxiety like behavior to determine whether n-3's and progesterone interact within the context of TBI.

MATERIALS AND METHODS

Animals and diets

Female Sprague–Dawley rats (250–280 gm) were obtained from Charles River Laboratories (Wilmington, MA, USA) on 3rd day of pregnancy and were kept under standard housing condition (22–24 °C) with 12-h light/dark cycle in two dietary groups. One group of pregnant female rats was fed an n-3 adequate diet (n-3 diet) and the other group was fed an n-3 deficient diet (n-3 def) through gestation and lactation. The diets were provided as powder in a bowl and animals had free access to food and water. Assessments of food intake showed no differences in food consumption among groups (data not shown). The custom diets used were based on the composition of American Institute of Nutrition Diet (AIN-93G) and prepared commercially (Dyets Inc. Bethlehem, PA). Both diets had the same basal macronutrients, vitamins, minerals and basal fats (hydrogenated coconut and safflower oils) (Table 1). The differences between the two diets were the amount of n-3 fatty acids, which was achieved by adding 0.5% of flaxseed (source of 18:3*n*-3; ALA), 1.2% of docosahexaenoic acid, 0.24% EPA and 0.1% other n-3 fatty acids (Procured from Nordic Naturals, Inc., Watsonville, CA, USA as ProDHA capsule) of total n-3 adequate diet. The n-3 "deficient" diet contains only 0.05% (w/w) n-3 fatty acids (Table 2).

Experimental design

A total of 12 litters (6/diet group) were included in the study. There was no significant difference observed in litter sizes among the diet groups (n-3 diet, 12.0 ± 1.03; n-3 def, 11.33 ± 0.80). At weaning on postnatal day (PND) 21, female offspring were randomly divided in a way that each offspring within an experimental group (n=6) was chosen from different litter. The offspring were weaned to the same diet as their dams up to the age of 15 weeks and body weights were measured in both n-3 adequate or deficient diet groups in every two weeks since weaning. There were no significant changes observed in body weight and weight gain of animals exposed to the experimental diet (Fig. S1). At the age of 12 weeks, ovariectomies were performed in female offspring of each dietary group to eliminate endogenous ovarian steroid production. Ovariectomized females were subjected to either sham or fluid percussion injury (FPI) after 2 weeks of ovariectomy (i.e. at the age of 14 weeks) to ensure a uniform period of hormonal depletion before progesterone replacement and to recover from surgical stress. Progesterone (16 mg/kg, sc) (Barha et al., 2011) or vehicle (50% DMSO+50% Saline) (Gibson et al., 2011) was administered daily, to the animals fed an n-3 deficient diet for 7 days, starting from the day of injury till 1 hr prior to the behavior. The animals in n-3 diet groups received only vehicle treatment. All animals

were handled by the experimenters daily in a similar manner to rule out the possibility of any behavior alteration due to animal handling. Rats were habituated to the testing room for at least 1 h prior to behavioral assessments. All animals were subjected to the elevated plus maze (EPM) test to assess the anxiety-like behavior after 1 week of FPI and then sacrificed immediately for tissue collection (Fig. 1). All experiments were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of California at Los Angeles Chancellor's Animal Research Committee.

Ovariectomy

At the age of 12 weeks, all female offspring were subjected to the ovariectomy. The surgery was preceded by a small midline dorsal skin incision under anesthesia, approximately half way between the middle of the back and the base of the tail. To access the peritoneal cavity, bilateral muscle incisions were made to the sides of the abdominal wall in the region of a periovarian fat pad. The ovaries were exteriorized through the muscle incision by grasping periovarian fat with tissue forceps, and the pedicle was ligated and excised between the uterine horn and the fallopian tube. After ovaries removal, both incisions in the abdominal musculature were sutured with absorbable sutures and skin wound was closed with nylon suture. Neomycin was applied on the suture and the rats were placed in a heated recovery chamber before being returned to their home cages.

Fluid percussion injury

Two weeks after ovariectomy, fluid percussion injury was performed as previously described (Wu et al., 2011). In brief, a 3.0-mm-diameter craniotomy was made over the left parietal cortex, 3.0 mm posterior to bregma and 6.0 mm lateral (left) to the midline with a high-speed drill (Dremel, Racine, WI). A plastic injury cap was placed over the craniotomy with silicone adhesive and dental cement. When the dental cement hardened, the cap was filled with 0.9% saline solution. Anesthesia was discontinued and the injury cap was attached to the fluid percussion device (AmScien Instruments, Richmond, VA). At the first sign of hind-limb withdrawal to a paw pinch, a mild fluid percussion pulse (1.5 atm) was administered to the epidural space. Immediately upon responding to a paw pinch, anesthesia was restored and the skull was sutured. Neomycin was applied on the suture and the rats were placed in a heated recovery chamber before being returned to their cages. The severity of injury was determined by measuring the loss of consciousness time, which is defined as the time from the injury to the return of a hind-limb withdrawal reflex. According to the reported fluid percussion injury severity data, the loss of consciousness time for < 45 sec is considered for mild injury (Giza et al., 2002; Gurkoff et al., 2006). All the animals included in the study were selected on the basis of loss of consciousness time for < 45 sec (n-3 diet/FPI/VEH: 22.67± 2.9, n-3 def/FPI/VEH: 19.83±2.7, n-3 def/FPI/PRO: 25.1 ±3.2). Sham animals underwent an identical procedure with the exception of the fluid pulse.

Elevated plus maze (EPM)

We analyzed the anxiety like behavior in rats based on the time spent in open arms of EPM, which is considered as one of the most commonly used test for analyzing anxiety like behavior (Carobrez and Bertoglio, 2005). After one week post FPI, elevated plus maze

(EPM) test was performed according to the (Walf and Frye, 2007). Briefly, the EPM apparatus made of laminated wood consisted of 2 opposing open arms (10 × 50 cm) and 2 opposing closed arms (10 × 50 cm with 30 cm high walls). The maze was placed 60 cm above the floor. White curtains surrounded the maze and behavior was recorded by a video camera over a period of 5 min. The intensity for lights in the behavior testing room was 60 lux by using 4 × 25W overhead lights and apparatus was placed in the centre of room. Each rat was placed in the middle of the maze facing the open arm that faced away from the experimenter. The time spent and the number of entries in each arm was measured using AnyMaze video tracking software (San Diego Instruments, San Diego, CA). The total number of times rats entered in arms during the EPM test was calculated to account for differences in general motor activity in the maze. After the plus maze test the animals were sacrificed by decapitation and the fresh brains were dissected out, frozen in dry ice and stored at −70 °C until use.

Immunobloting

The hippocampal tissue ipsilateral to the craniotomy were homogenized in a lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl pH 8.0, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.1 mM benzethonium chloride and 0.5 mM sodium vanadate. We selected to study hippocampal tissue based on reports that the hippocampus is susceptible to the action of progesterone (Bali et al., 2012), TBI (Ariza et al., 2006) and DHA (Kang and Gleason, 2013). The homogenates were then centrifuged, the supernatants were collected and total protein concentration was determined according to MicroBCA procedure (Pierce, IL, USA), using bovine serum albumin (BSA) as standard. Briefly, protein samples were separated by electrophoresis on 10% polyacrylamide gel and electrotransferred to a PVDF membrane (Millipore, Bedford, MA). Non-specific binding sites were blocked in Tris-buffered saline (TBS), pH 7.6, containing 5% non-fat dry milk. Membranes were rinsed in buffer (0.05% Tween-20 in TBS) and then incubated with anti-BDNF, anti-Syntaxin 3, anti-GAP-43, anti-Nogo-A, anti-iPLA2, anti-sPLA2, anti-actin, (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NPY1R (1:1000; Alpha Diagnostic Intl. Inc., TX, USA) and anti-MAG (1:1000; Chemicon International), followed by anti-rabbit or anti-goat or anti-mouse IgG horseradish peroxidase-conjugate (1:10,000; Santa Cruz Biotechnology). After rinsing with buffer, the immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the manufacturer's instructions. The signals were digitally scanned and then quantified using ImageJ software. Actin was used as an internal control for western blot such that data were standardized according to actin values.

Fatty acid analysis

Fatty acids were measured by gas chromatography in hippocampal tissue-extracted lipids. For extraction of lipids, frozen tissues were homogenized in chloroform/methanol (2:1 vol/vol), containing 50 μg/ml of butylated hydroxytoluene to prevent lipid oxidation during the procedure. Tricosanoic acid methylester (C23:0) was used as an internal standard. Tissues were grounded to powder under liquid nitrogen and subjected to extraction of total lipids.

Fatty acid methylation was done by heating with 14% (w/v) boron trifluoride–methanol reagent at 90°C for 1 hr.

Extracted lipids were analyzed on Clarus 500 gas chromatograph (GC; PerkinElmer) equipped with a built-in Autosampler and flame ionization detector (FID). An Elite-WAX column (60m, 0.32-mm internal diameter, PerkinElmer) was used, with hydrogen as the carrier gas. GC oven temperature was initially held at 140°C for 2 min and raised with a gradient of 5°C min^{-1} until 250°C and held for 10 min. The total run time is 34 min. The injector and detector were maintained at 250°C and 300°C, respectively. A 1μl sample of Fatty acid methyl esters (FAME) was injected in split injection mode with a 100:1 split ratio. Peaks of resolved fatty acid methyl esters were identified and quantified by comparison with standards (Supelco 37-component FAME Mix).

Statistical Analysis

The results are represented as mean ± standard error of the mean (SEM). Two separate ANOVA (two-way) analyses were performed to show the main effects and interaction of two independent factors. First ANOVA (two-way) analysis was conducted in n-3 diet/Sham/VEH, n-3 diet/FPI/VEH, n-3 def/Sham/VEH, n-3 def/FPI/VEH groups, based on the diet (n-3 def vs. n-3 diet) and injury (FPI vs. Sham) factors. In another 2-way ANOVA, only the n-3 deficient fed diet groups (n-3 def/Sham/VEH, n-3 def/Sham/PRO, n-3 def/FPI/VEH and n-3 def/FPI/PRO) were included, to show the interaction between hormonal treatment (PRO vs. VEH) and injury (FPI vs. Sham) factors. For multiple comparisons, Tukey's post hoc analyses were performed separately in the above-mentioned two sets of treatment groups, to determine the significance of difference among various groups. The changes in body weight were analyzed using repeated measure ANOVA. The p-value < 0.05 was considered to be statistically significant.

RESULTS

Progesterone ameliorate the effect of TBI on anxiety-like behavior in n-3 deficiency

Two-way ANOVA analysis showed a significant effect of diet (n-3 def vs. n-3 diet; $F_{1,20} = 32.869$, $p<0.01$) and injury (FPI vs. Sham; $F_{1,20} = 9.0$, $p<0.01$) on the percentage of time spent in open arms. The Tukey's multiple comparison tests resulted in a marked reduction in the percentage of time spent in open arms with dietary n-3 fatty acid deficiency (n-3 def/Sham/VEH Vs n-3 diet/Sham/VEH, $p<0.05$; Fig. 2A), which was further aggravated after TBI (n-3 def/FPI/VEH Vs n-3 def/Sham/VEH, $p<0.05$). However, TBI had no effect on these variables in n-3 fed animals (n-3 diet/FPI/VEH Vs n-3 diet/Sham/VEH, $p>0.05$). To evaluate the therapeutic potential of ovarian hormones, we assessed the impact of progesterone treatment on anxiety-like behavior in dietary n-3 fatty acid deficient conditions. The significant effect of injury (FPI vs. Sham; $F_{1,20} = 12.369$, $p<0.01$), hormonal treatment (PRO vs. VEH; $F_{1,20} = 22.606$, $p<0.01$) as well as their interaction ($F_{1,20} = 8.618$, $p<0.01$) were observed on the percentage of time spent in open arms by two way ANOVA analysis. The post-hoc analysis revealed that progesterone treatment was not effective to reverse the effect of alone n-3 fatty acid deficiency (n-3 def/Sham/PRO Vs n-3 def/Sham/VEH, $p>0.05$). However, the progesterone treatment reversed the effect of brain injury on

anxiety in n-3 deficient animals as evidenced by an increase of time spent in open arm (n-3 def/FPI/PRO Vs n-3 def/FPI/VEH, $p<0.01$). The general motor activity, as measured by total number of entries during the EPM test, was similar among all the groups, thus excluding the possibility of interference by motor activity (Fig. 2B).

We assessed the levels of the Neuropeptide-Y1 Receptor (NPY1R) based on the demonstrated role of NPY on anxiety-like behavior (Primeaux et al., 2005). The levels of NPY1R were significantly affected by diet ($F_{1,20} = 67.822$, $p<0.01$), injury ($F_{1,20} = 10.924$, $p<0.01$), and a significant interaction between diet and injury ($F_{1,20} = 7.123$, $p<0.05$) was also observed by two-way ANOVA. The post-hoc analysis using Tukey's multiple comparison tests showed that the dietary n-3 deficiency reduced NPY1R levels (n-3 def/Sham/VEH Vs n-3 diet/Sham/VEH, $p<0.01$; Fig. 2C), which was further reduced after TBI (n-3 def/FPI/VEH Vs n-3 def/Sham/VEH, $p<0.01$). However, prior exposure to dietary n-3 supplementation preserved the levels of NPY1R in animals subjected to TBI (n-3 diet/FPI/VEH Vs n-3 diet/Sham/VEH, $p>0.05$). Two way ANOVA analysis resulted in a significant effect of hormonal treatment ($F_{1,20} = 10.679$, $p<0.01$) and its interaction ($F_{1,20} = 15.003$, $p<0.01$) with injury on the levels of NPY1R. Tukey's multiple comparison test showed that the treatment with progesterone reversed the effect of TBI on NPY1R in n-3 deficient group (n-3 def/FPI/PRO Vs n-3 def/FPI/VEH, $p<0.01$) while progesterone was not effective to ameliorate the effect of n-3 deficiency alone (n-3 def/Sham/PRO Vs n-3 def/Sham/VEH, $p>0.05$).

Effects of DHA and progesterone on proteins involved in synaptic plasticity

To analyze the alterations in synaptic functioning, we have assessed the levels of BDNF, syntaxin-3 and GAP-43 in ovariectomized female animals subjected to TBI and progesterone under altered dietary n-3 fatty acid conditions. Two-way ANOVA analysis showed a significant effect of diet ($F_{1,20} = 13.279$, $p<0.01$) and injury ($F_{1,20} = 6.606$, $p<0.05$) for the levels of BDNF. The post-hoc analysis for multiple comparisons showed that TBI reduces BDNF level during n-3 fatty acid deficiency only (n-3 def/FPI/VEH Vs n-3 def/Sham/VEH, $p<0.05$; Fig. 3A), while n-3 deficiency alone has no effect on BDNF level (n-3 def/Sham/VEH Vs n-3 diet/Sham/VEH, $p>0.05$). The effect of hormonal treatment ($F_{1,20} = 17.818$, $p<0.01$) and its interaction ($F_{1,20} = 10.612$, $p<0.01$) with injury was significant for the levels of BDNF as analyzed by two-way ANOVA. Treatment with progesterone reverses the effect of traumatic injury on BDNF level under n-3 deficient condition (n-3 def/FPI/PRO Vs n-3 def/FPI/VEH, $p<0.01$). Progesterone treatment was not able to reverse the effect of n-3 fatty acid deficiency alone (n-3 def/Sham/PRO Vs n-3 def/Sham/VEH, $p>0.05$) as shown with the Tukey's post-hoc test.

We assessed the levels GAP-43, a growth associated protein, which provides an index of growth or plasticity as it is expressed at high levels in neuronal growth during development and axonal regeneration. With regards to the levels of GAP-43, two-way ANOVA revealed a significant effect of diet ($F_{1,20} = 32.334$, $p<0.01$), injury ($F_{1,20} = 4.668$, $p<0.05$) as well as interaction ($F_{1,20} = 6.832$, $p<0.05$) between diet and injury for the levels of GAP-43. The post-hoc test showed that GAP-43 levels were not altered under the n-3 deficiency (n-3 def/Sham/VEH Vs n-3 diet/Sham/VEH, $p>0.05$; Fig. 3B), while TBI reduced the levels of

GAP-43 during n-3 fatty acid deficiency only (n-3 def/FPI/VEH Vs n-3 def/Sham/VEH, $p<0.05$). There was a significant effect of hormonal treatment ($F_{1,20} = 6.666$, $p<0.05$) and its interaction ($F_{1,20} = 7.629$, $p<0.05$) with injury for the levels of GAP-43 as revealed by two-way ANOVA analysis. Tukey's post-hoc test for multiple comparisons showed that the treatment with progesterone counteracted the TBI induced changes in GAP-43 under the n-3 deficient condition (n-3 def/FPI/PRO Vs n-3 def/FPI/VEH, $p<0.01$).

Syntaxin-3 is the protein responsible for transporting vesicles which participate in exocytosis and also plays important role in the growth of neuritis (Martín-Martín et al., 1999). Two-way ANOVA analysis showed a significant effect of diet ($F_{1,20} = 59.029$, $p<0.01$) and injury ($F_{1,20} = 11.329$, $p<0.01$) for the levels of STX-3. Tukey's multiple comparisons showed the dietary deficiency of n-3 fatty acid reduces the level of syntaxin-3 (n-3 def/Sham/VEH Vs n-3 diet/Sham/VEH, $p<0.01$; Fig. 3C), which was further exacerbated by brain injury (n-3 def/FPI/VEH Vs n-3 def/Sham/VEH, $p<0.01$). However, no changes were observed in STX-3 after TBI in n-3 diet group (n-3 diet/FPI/VEH Vs n-3 diet/Sham/VEH, $p>0.05$). We observed a significant hormonal treatment ($F_{1,20} = 10.212$, $p<0.01$) effect and its interaction ($F_{1,20} = 12.090$, $p<0.01$) with injury on STX-3 levels by two-way ANOVA analysis. The post-hoc test for multiple comparisons showed that progesterone treatment counteracts the n-3 fatty acid deficiency and TBI induced changes in Syntaxin-3 (n-3 def/FPI/PRO Vs n-3 def/FPI/VEH, $p<0.01$). However, progesterone treatment was not able to counteract the effect of n-3 deficiency alone (n-3 def/Sham/PRO Vs n-3 def/Sham/VEH, $p>0.05$).

Progesterone normalized the levels of MAG and Nogo-A induced after injury

Given the powerful growth inhibitory action of MAG and Nogo-A on axonal growth, we evaluated the capacity of progesterone and n-3 fatty acid to influence MAG and Nogo-A levels in TBI. Two-way ANOVA analysis showed a significant effect of diet ($F_{1,20} = 4.654$, $p<0.05$) and its interaction with injury ($F_{1,20} = 5.985$, $p<0.05$) on the levels of MAG. With regards to the levels of Nogo-A, there was a significant effect of injury ($F_{1,20} = 5.754$, $p<0.05$) and interaction between diet and injury ($F_{1,20} = 5.465$, $p<0.05$). Multiple comparisons revealed a significant increase in both MAG (n-3 def/FPI/VEH Vs n-3 def/Sham/VEH, $p<0.05$; Fig. 4A) and Nogo-A (n-3 def/FPI/VEH Vs n-3 def/Sham/VEH, $p<0.05$; Fig. 4B) levels after TBI under n-3 deficiency while no effect from TBI was observed in n-3 diet groups (n-3 diet/FPI/VEH Vs n-3 diet/Sham/VEH, $p>0.05$). Given the beneficial effects of progesterone on CNS function, we evaluated the capacity of progesterone to reduce these myelin-derived proteins after FPI. A significant effect of hormonal treatment ($F_{1,20} = 26.617$, $p<0.01$) and interaction effects ($F_{1,20} = 16.581$, $p<0.01$) were observed for the levels of MAG by ANOVA (two-way) analysis. For the level of Nogo-A, a significant interaction for injury vs. hormonal treatment ($F_{1,20} = 7.20$, $p<0.05$) was observed. One week of progesterone treatment overcame the injury-related increase in MAG (n-3 def/FPI/PRO Vs n-3 def/FPI/VEH, $p<0.01$) and Nogo-A (n-3 def/FPI/PRO Vs n-3 def/FPI/VEH, $p<0.05$). The treatment with progesterone was not able to ameliorate the effect of n-3 deficiency alone (n-3 def/Sham/PRO Vs n-3 def/Sham/VEH, $p>0.05$) as resulted with Tukey's post-hoc test for multiple comparisons.

Influence of DHA and progesterone on molecules associated with membrane homeostasis

We examined the lipid peroxidation marker 4-Hydroxynonenol (HNE) and found a significant effect of diet ($F_{1,20}$ = 8.839, p<0.01) and injury ($F_{1,20}$ = 6.658, p<0.05) by two-way ANOVA analysis. Multiple comparisons showed the elevated 4HNE levels with TBI under n-3 deficiency only (n-3 def/FPI/VEH Vs n-3 def/Sham/VEH, p<0.05; Fig. 5A). Two-way ANOVA analysis resulted in a significant effect of hormonal treatment ($F_{1,20}$ = 17.813, p<0.01) and its interaction with injury ($F_{1,20}$ = 13.280, p<0.01) for 4HNE levels. Tukey's post-hoc test showed that the treatment with progesterone was able to counteract the effect of brain injury on 4HNE (n-3 def/FPI/PRO Vs n-3 def/FPI/VEH, p<0.01). Further we analyzed the effect of n-3 fatty acid and TBI on fatty acid metabolizing molecules such as Ca^{2+} independent PLA2 (iPLA2) and secreted phospholipases A2 (sPLA2). Two-way ANOVA analysis showed a significant diet effect for iPLA2 ($F_{1,20}$ = 26.168, p<0.01), levels of sPLA2 were significantly affected by diet ($F_{1,20}$ = 60.297, p<0.01) and injury ($F_{1,20}$ = 13.051, p<0.01). The post-hoc test for multiple comparisons showed that the dietary n-3 deficiency elevated the levels of sPLA2 (n-3 def/Sham/VEH Vs n-3 diet/Sham/VEH, p<0.01; Fig. 5C) and reduced the level of iPLA2 (n-3 def/Sham/VEH Vs n-3 diet/Sham/VEH, p<0.05; Fig. 5B), in comparison to the n-3 diet group. TBI further increased the levels of sPLA2 (n-3 def/FPI/VEH Vs n-3 def/Sham/VEH, p<0.01), whereas no changes were observed in n-3 diet supplemented group after TBI (n-3 diet/FPI/VEH Vs n-3 diet/Sham/VEH, p>0.05). ANOVA (two-way) analysis revealed a significant effect of hormonal treatment ($F_{1,20}$ = 27.887, p<0.01) and its interaction with injury ($F_{1,20}$ = 17.077, p<0.01) for the levels of sPLA2. Tukey's post-hoc test showed that the treatment of progesterone counteracted this effect by normalizing the levels of sPLA2 (n-3 def/FPI/PRO Vs n-3 def/FPI/VEH, p<0.01). However, progesterone treatment was not effective to reverse the effect of n-3 fatty acid deficiency alone on both iPLA2 and sPLA2 (n-3 def/Sham/PRO Vs n-3 def/Sham/VEH, p>0.05).

Changes in fatty acid levels in hippocampal tissue after traumatic brain injury and progesterone treatment

We assessed the fatty acid levels by using gas chromatography in the hippocampal tissue. ANOVA (two-way) analysis showed a significant effect of diet for DHA (C22:6n-3) ($F_{1,20}$ = 63.834, p<0.01) and arachidonic acid (AA; C20:4n-6) ($F_{1,20}$ = 12.232, p<0.01) levels whereas significant interaction between diet and injury ($F_{1,20}$ = 6.877, p<0.05) was observed only for DHA levels. Tukey's post-hoc test for multiple comparisons showed that exposure to n-3 fatty acid deficient diet resulted in a significant decrease in DHA levels (n-3 def/Sham/VEH Vs n-3 diet/Sham/VEH, p<0.01; Fig. 6A) and increase in AA levels (n-3 def/Sham/VEH Vs n-3 diet/Sham/VEH, p<0.05; Fig. 6B) as compared to the animals having n-3 diet. We observed a significant effect of injury on DHA ($F_{1,20}$ = 5.503, p<0.05) and AA levels ($F_{1,20}$ = 4.948, p<0.05) by two-way ANOVA analysis, while a significant interaction between injury and treatment was observed only in DHA ($F_{1,20}$ = 4.385, p<0.05). Multiple comparisons showed that TBI and treatment with progesterone did not affect the levels of DHA and AA either in the n-3 diet or deficient groups.

Discussion

We found that long-term n-3 deficiency during brain formation reduced several parameters of brain plasticity and behavior in female rats deprived of gonadal steroids. These animals also showed higher vulnerability to the effects of TBI during adult life. Interestingly, progesterone treatment counteracted the effects of TBI in the animals exposed to the n-3 deficient diet, in terms of behavioral performance and most of the molecular parameters. These results reveal the risk imposed by the n-3 deficiency in coping with the consequences of TBI. Results also suggest that progesterone is particularly effective for providing protection against TBI during the weaknesses imposed by a diet deficient in n-3 fatty acids. Progesterone replacement therapy normalized several of the events that had been demoted by the injury during n-3 deficiency, i.e., alterations of synaptic molecules such as syntaxin-3, GAP-43, BDNF and molecules important for the metabolism of fatty acids such as sPLA2.

Progesterone counteracts a TBI-related reduction in molecules related to synaptic plasticity and growth

We found that TBI aggravated the effects of dietary n-3 fatty acid deficiency on molecular systems important for the maintenance of synaptic plasticity and repair such as BDNF, STX-3 and GAP-43. Syntaxin-3 is a member of the syntaxin family of cellular receptors involved in transport of vesicles during exocytosis (Martín-Martín et al., 1999) with important roles in neurite outgrowth (Darios and Davletov, 2006). Our results showed that the pre-exposure to n-3 fatty acid deficient diet reduced the levels of STX-3 and these levels were further reduced by TBI. A similar pattern was observed for BDNF and GAP-43 under n-3 deficiency, which levels were further reduced by TBI. GAP-43 is an intracellular membrane-associated phosphoprotein, present in growth cones and pre-synaptic terminals important for formation of neuronal connections, and synaptic remodeling following traumatic insult (Benowitz et al., 1990). Progesterone treatment counteracted the TBI-induced reductions in BDNF, STX-3 and GAP-43 during n-3 deficient condition, suggesting an action of progesterone on synaptic plasticity and growth. There are previous indications for the involvement of gonadal hormones in axonal growth as observed by increases in GAP-43 mRNA in GnRH neurons during proestrus phase (Prevot et al., 2000).

Progesterone ameliorates TBI- induced axonal injury markers

The limited regenerative capacity of the injured CNS is largely due to the nonpermissive growth properties of the neuronal environment (Yiu and He, 2006) such as the presence of growth inhibitory substances in the myelin (Filbin, 1995). Nogo-A and MAG produced by oligodendrocytes have been shown to inhibit axonal growth by causing growth cone collapse (Chen et al., 2000; Shibata et al., 1998) and by reducing the growth capacity of axons and dendrites (Schwab, 2004). We found that TBI significantly elevated MAG and Nogo-A only in the n-3 dietary deficiency condition. Increased levels of Nogo-A after injury may also contribute to alter neuronal function since Nogo-A is expressed in neurons as well (Schwab, 2010). The results showing that dietary n-3 deficiency restrains the post-injury regenerative potential of the CNS in ovariectomized rats motivated us to determine whether these effects are dependent on the hormonal status of the rats. We observed that

progesterone treatment counteracted the effects of TBI in the n-3 deficient rats, as evidenced by a reduction in the levels of the growth inhibitory molecules MAG and Nogo-A. There is substantial evidence for the role of progesterone preventing axonal damage in the peripheral and the central nervous system (De Nicola et al., 2006; Melcangi et al., 2005). Progesterone has been shown to moderate axonal injury when given to ovariectomized female and male rats (O'Connor et al., 2007). Progesterone can also stimulate myelinogenesis of injured peripheral nerves and prevent neuronal loss following brain contusion, ischemia and edema (Betz and Coester, 1990; Roof et al., 1992). The overall results indicate that progesterone protects against the drawback imposed by n-3 deficiency during brain development on the outcome of TBI.

Restoration of membrane homeostasis with progesterone after TBI

The action of n-3 fatty acids in our paradigm is likely related to the fate of n-3 in the phospholipidic composition of plasma membranes. CNS membranes are enriched in PUFAs, which metabolism is stringently controlled by PLA2 (Sun et al., 2004), and as supported by our results, the hippocampus is a site of prominent expression of PLA2 (Kishimoto et al., 1999; Molloy et al., 1998). Our results showed that n-3 deficiency elevated the levels of sPLA2 and these effects were aggravated by TBI, with important implications for brain plasticity. Recent studies show that increased PLA2 activity plays a central role in acute inflammatory responses in brain, associated with neurological disorders such as ischemia, Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Kalyvas and David, 2004; Phillis and O'Regan, 2004; Sun et al., 2004). PLA2 contributes to the pathogenesis of these disorders by interacting with neural membrane phospholipids to release proinflammatory lipid mediators such as prostaglandins, leukotrienes, and thromboxanes, and by generating 4-HNE. The release of arachidonic acid from membrane phospholipids and subsequent initiation of the arachidonic acid cascade are a well-documented observation following transient global ischemia (Hsu et al., 1989; Rehncrona et al., 1982).

Our results showed that n-3 deficiency elevated the levels of 4-HNE, and these effects were further aggravated by TBI. 4-HNE has been reported to cause a number of deleterious effects in cells including inhibition of DNA synthesis, disturbance in calcium homeostasis, and inhibition of mitochondrial respiration (Feng et al., 2004; Mark et al., 1997; Miller et al., 2013). The increase in 4 HNE and sPLA-2 only in the n-3 deficient group exposed to TBI indicates that dietary n-3 fatty acids can reduce the disruptive effects of TBI on membrane homeostasis. In turn, the counteractive effects of progesterone treatment are in general agreement with studies showing antioxidant effects of progesterone after cortical contusion (Roof et al., 1994; Roof et al., 1997). Administration of progesterone, even post-injury, has been shown to reduce brain edema (Roof et al., 1996), secondary neuronal loss (Roof et al., 1994), and necrotic tissue loss (Shear et al., 2002). The effects of progesterone, and its metabolite allopregnanolone, have been associated with improved neurologic outcome (Djebaili et al., 2005). We observed that progesterone counteracts the action of TBI on 4HNE and sPLA2, which suggest the potential of progesterone in restoring membrane homeostasis after TBI and n-3 deficiency. It is known that iPLA2 is responsible for the release of DHA from phospholipids in the membrane (Basselin et al., 2010), thus it is not surprising that iPLA2 levels remained unchanged after n-3 deficiency, reflecting the fact that

brain DHA levels were already reduced in the n-3 deficient rats. The post-injury treatment with progesterone was not able to counteract the reduction in iPLA2 levels, which is consistent with the inability of progesterone to alter levels of DHA.

Progesterone treatment counteracts behavioral alterations after TBI

The higher incidence of neuropsychological disturbances in postmenopausal female has been linked with the deficiency of major n-3 fatty acids, particularly DHA (Robinson et al., 2010). Variations in anxiety-like behaviors occur over the course of the estrous cycle in experimental animals, such that females display reduced anxiety-like behavior during proestrus, when ovarian hormone concentrations are high (Marcondes et al., 2001). Although the influence of ovarian steroids on behavior is well known, there is a paucity of studies examining whether dietary factors modulate the action of steroids on anxiety like behavior. Low dietary intake of n-3 fatty acids has been associated with an elevated risk of anxiety in females (Jacka et al., 2013). Interestingly, long-term n-3 deficiency has also been shown to induce anxiety like behavior in male rats (Tyagi et al., 2013), which supports the importance of proper consumption of DHA for maintaining mental health. There are several studies showing the beneficial influence of dietary n-3 fatty acid supplementation in TBI under varied doses and fatty acid formulations. For example, Bailes and Mills, (2010) demonstrated the behavioral improvement and reduced axonal dysfunction in TBI with dietary supplementation of DHA at 40 mg/kg/day. Dietary supplementation with 6% fish oil for 4 weeks has been demonstrated to improve cognitive performance in a rat model of multiple mild TBIs (Wang et al., 2013). We have also demonstrated the protective influence of dietary supplementation with 8% fish oil for 4 weeks on cognitive performance in TBI animals, in our previous studies (Wu et al., 2007; Wu et al., 2004). In spite of the beneficial effects of dietary n-3 fatty acids, further studies are required to establish the optimal concentration of dietary n-3 fatty acids to cope under challenging conditions.

The increase in anxiety-like behavior, accompanied by a decrease in NPY1R, in the injured n-3 deficient rats suggests that n-3 deficiency can hinder the brain's ability to cope with challenges in adulthood. The reductions in NPY1R levels are in agreement with the protracted behavioral performance as NPY has been associated with the control of stress and anxiety (Bleakman et al., 1992; Giesbrecht et al., 2010). Our results also emphasize a protective influence of progesterone treatment (via daily injections) against anxiety like behavior and reductions in NPY1R levels, such that neurosteroids are considered important modulators of stress (Morrow et al., 1995). Although results of our study are consistent with previous reports, in which, the beneficial effect of progesterone injections has been demonstrated on behavioral performance in brain injury at a dose range 8–16 mg/kg (Goss et al., 2003). However, tapered and steady state progesterone treatment has been suggested to be more effective to counteract the behavioral alterations caused by TBI (Cutler et al., 2006a; Cutler et al., 2006b). Besides our findings, progesterone could also affect behavior by acting on neurotransmitters systems such as GABA (Andrade et al., 2012). The overall results are particularly relevant on the light on population studies showing that the Western diet is deficient in n-3 fatty acids (Simopoulos, 2006). The implication of this research is further significant based on the information that diet is the only source of DHA for the brain, although consumption of DHA is below recommended levels in the western society

(Simopoulos, 2006). The adequate intake of n-3 for general body health varies according to the age and sex among the population, as suggested by the Institute of Medicine (Report 2002/2005).

Conclusion

We found that n-3 deficiency during early life compromises the outcome of TBI in female rats deprived of sex steroids, in terms of lowering molecular systems important for synaptic function, axonal growth, and behavioral performance. Results indicate that progesterone treatment for TBI is particularly efficient under n-3 dietary deficiency. Results also suggest that the n-3 diet and progesterone may help to maintain neuronal signaling by supporting membrane stability and axonal growth. These results have important implications for the management of TBI in the human population with subnormal consumption of n-3 fatty acids during brain development. In particular, our results are significant to understand the capacity of dietary factors acting as a pre-injury condition to influence the ability of the brain to resist TBI and other types of neurological damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Dietary n-3 deficiency aggravates TBI outcome on anxiety and neuronal plasticity
2. Crucial role of progesterone protecting the brain during dietary n-3 deficiency
3. Dietary n-3 during brain development improves outcome after adult TBI

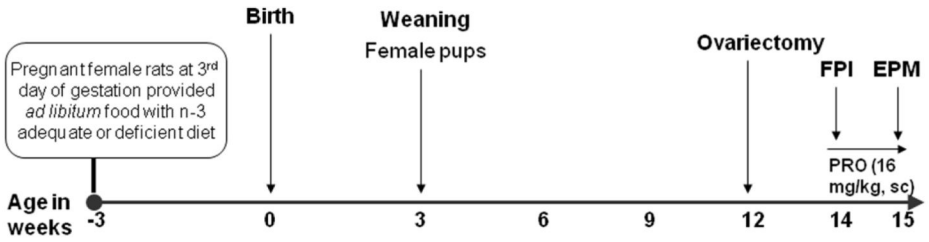

Fig. 1.

Schematic timeline representing experimental design to study the effect of n-3 adequate or deficient diet and progesterone (PRO) on anxiety-like behavior in response to traumatic brain injury.

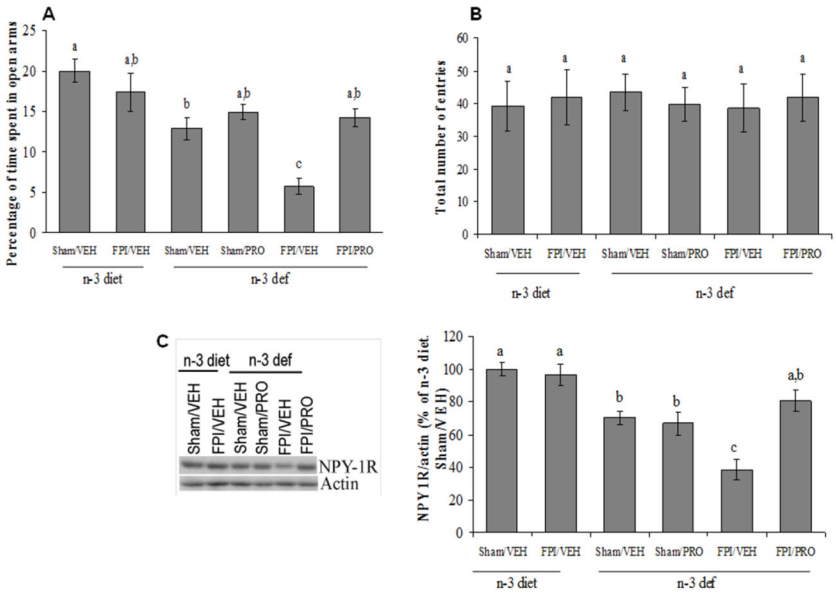

Fig. 2.

(A) Percentage of time spent in open arms, **(B)** Total entries in elevated plus maze (EPM) test and **(C)** Protein levels of NPY1R are shown for the animals treated with n-3 supplemented group (n-3 diet/Sham/VEH), n-3 diet supplemented group receiving lesion (n-3 diet/FPI/VEH), n-3 deficient group (n-3 def/Sham/VEH), n-3 diet deficient group with Progesterone treatment (n-3 def/Sham/PRO), n-3 diet deficient group receiving lesion (n-3 def/FPI/VEH) and n-3 diet deficient group receiving lesion and progesterone treatment (n-3 def/FPI/PRO) groups. Values are expressed as mean ± SEM and statistical analysis was performed in two sets of treatment groups using ANOVA (two-way) and Tukey's multiple comparison post-hoc test. Groups are not sharing a common letter differ significantly at $p<0.05$.

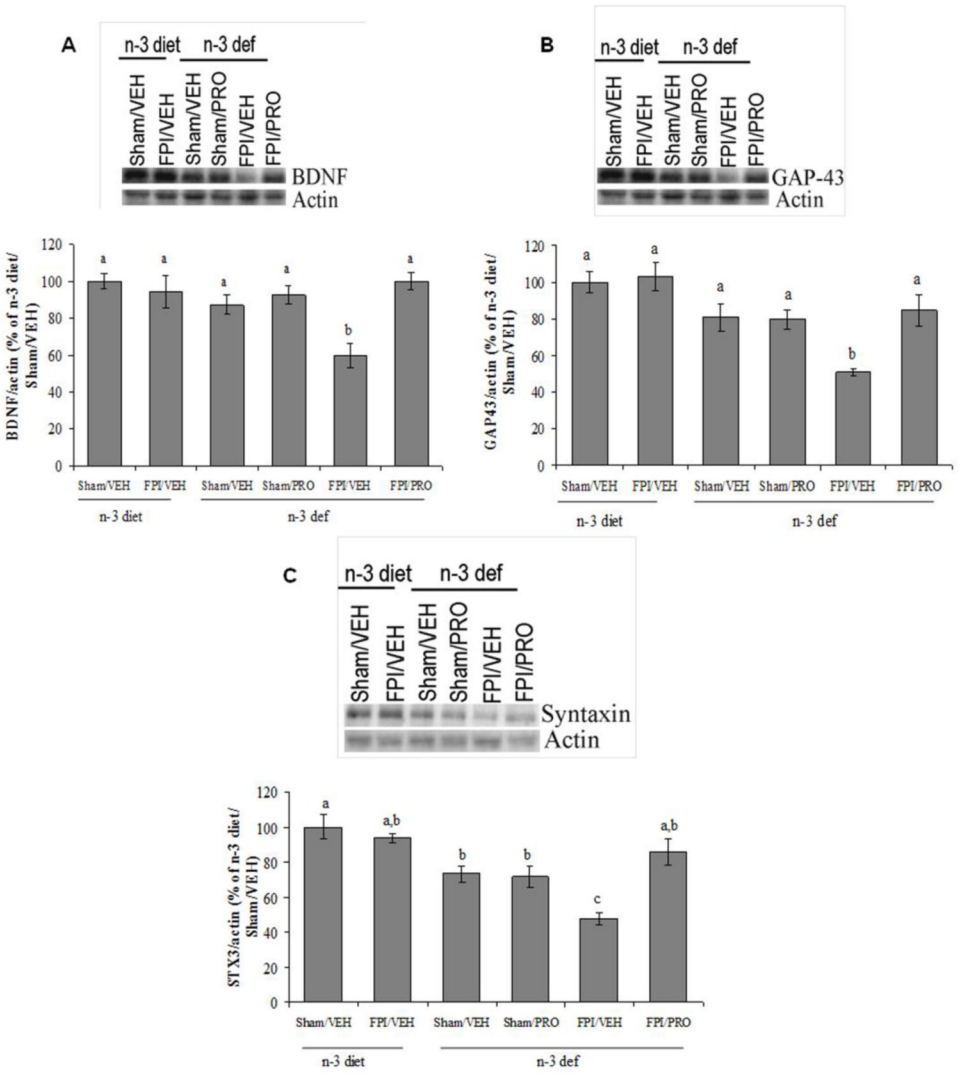

Fig. 3.

(A) Protein levels of BDNF, **(B)** GAP-43 and **(C)** STX-3 are shown for the animals treated with n-3 supplemented group (n-3 diet/Sham/VEH), n-3 diet supplemented group receiving lesion (n-3 diet/FPI/VEH), n-3 deficient group (n-3 def/Sham/VEH), n-3 diet deficient group with Progesterone treatment (n-3 def/Sham/PRO), n-3 diet deficient group receiving lesion (n-3 def/FPI/VEH) and n-3 diet deficient group receiving lesion and progesterone treatment (n-3 def/FPI/PRO) groups. Values are expressed as mean ± SEM and statistical analysis was performed in two sets of treatment groups using ANOVA (two-way) and Tukey's multiple comparison post-hoc test. Groups are not sharing a common letter differ significantly at $p<0.05$.

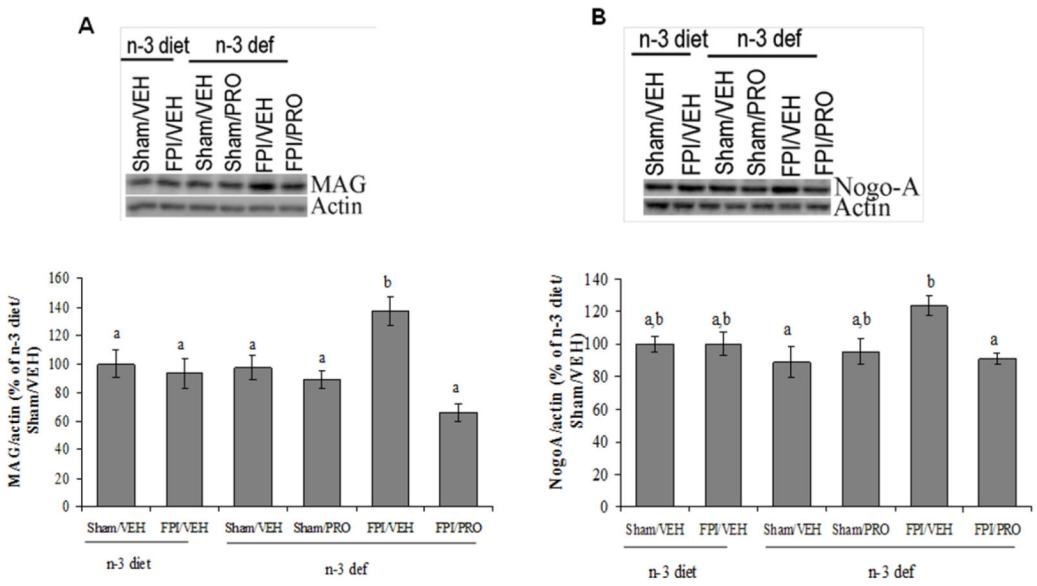

Fig. 4.

(A) Protein levels of MAG and **(B)** Nogo-A are shown for the animals treated with n-3 supplemented group (n-3 diet/Sham/VEH), n-3 diet supplemented group receiving lesion (n-3 diet/FPI/VEH), n-3 deficient group (n-3 def/Sham/VEH), n-3 diet deficient group with Progesterone treatment (n-3 def/Sham/PRO), n-3 diet deficient group receiving lesion (n-3 def/FPI/VEH) and n-3 diet deficient group receiving lesion and progesterone treatment (n-3 def/FPI/PRO) groups. Values are expressed as mean ± SEM and statistical analysis was performed in two sets of treatment groups using ANOVA (two-way) and Tukey's multiple comparison post-hoc test. Groups are not sharing a common letter differ significantly at $p<0.05$.

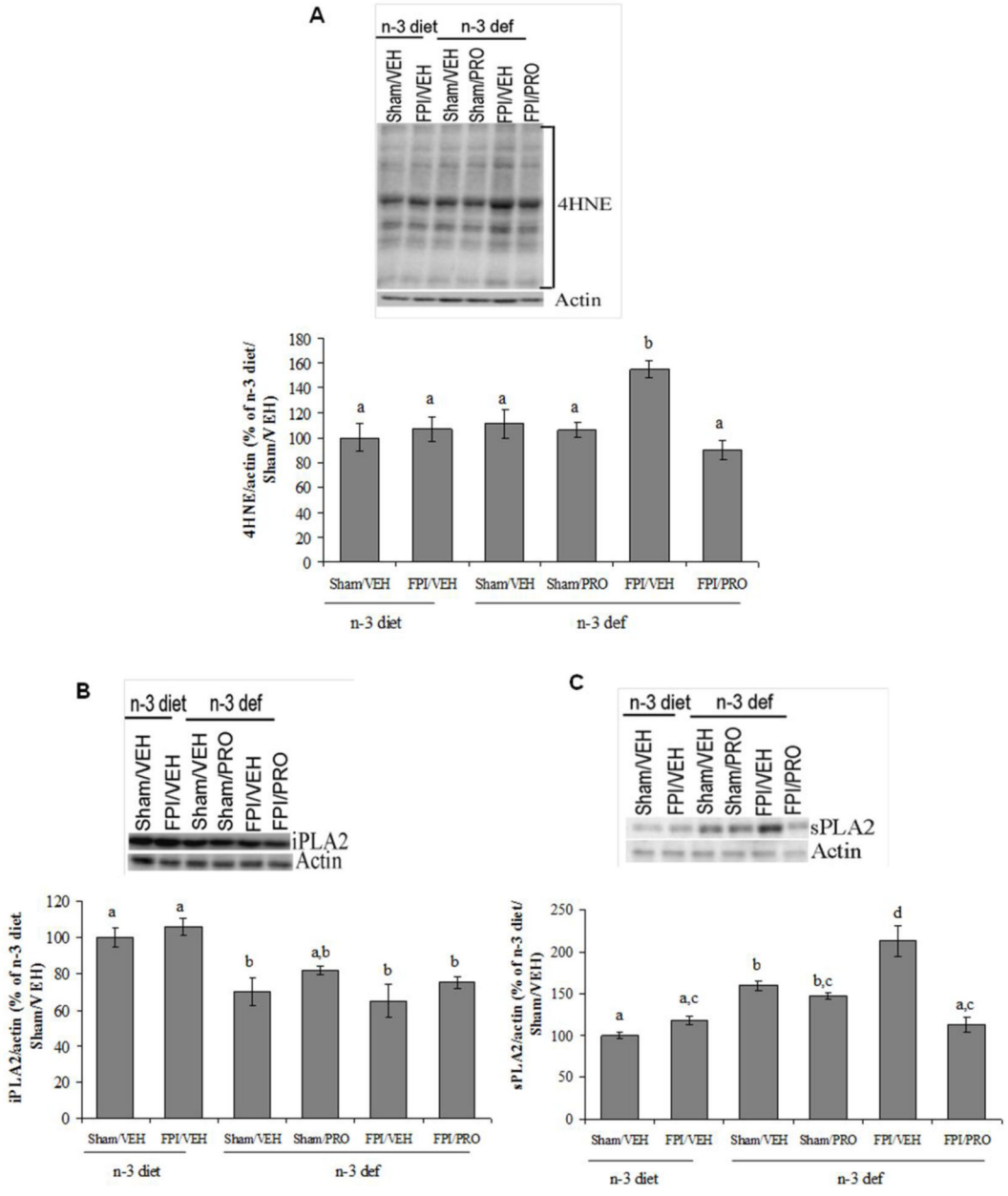

Fig. 5.

(A) 4HNE **(B)** iPLA2 **(C)** sPLA2 levels are shown for the animals treated with n-3 supplemented group (n-3 diet/Sham/VEH), n-3 diet supplemented group receiving lesion (n-3 diet/FPI/VEH), n-3 deficient group (n-3 def/Sham/VEH), n-3 diet deficient group with Progesterone treatment (n-3 def/Sham/PRO), n-3 diet deficient group receiving lesion (n-3 def/FPI/VEH) and n-3 diet deficient group receiving lesion and progesterone treatment (n-3 def/FPI/PRO) groups. Values are expressed as mean ± SEM and statistical analysis was performed in two sets of treatment groups using ANOVA (two-way) and Tukey's multiple comparison post-hoc test. Groups are not sharing a common letter differ significantly at $p<0.05$.

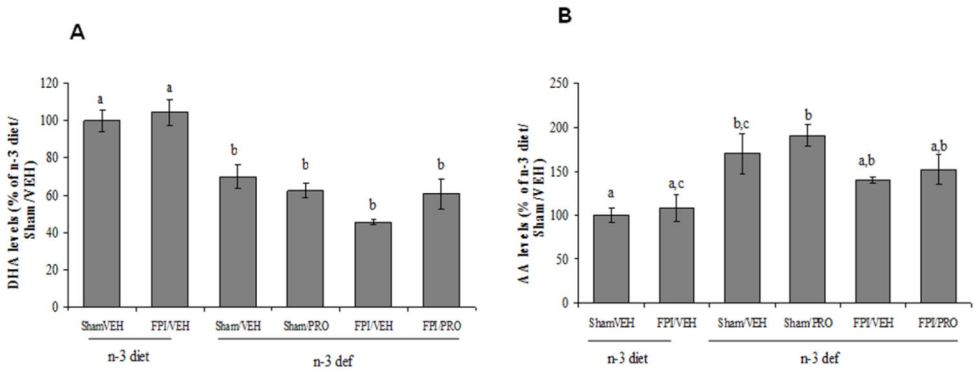

Fig. 6.

(A) Docosahexaenoic acid (DHA; C22:6n-3) and **(B)** Arachidonic acid (AA; C20:4n-6) level are shown for the animals treated with n-3 supplemented group (n-3 diet/Sham/VEH), n-3 diet supplemented group receiving lesion (n-3 diet/FPI/VEH), n-3 deficient group (n-3 def/Sham/VEH), n-3 diet deficient group with Progesterone treatment (n-3 def/Sham/PRO), n-3 diet deficient group receiving lesion (n-3 def/FPI/VEH) and n-3 diet deficient group receiving lesion and progesterone treatment (n-3 def/FPI/PRO) groups. Values are expressed as mean ± SEM and statistical analysis was performed in two sets of treatment groups using ANOVA (two-way) and Tukey's multiple comparison post-hoc test. Groups are not sharing a common letter differ significantly at $p<0.05$.

Table 1

Composition of experimental diets

Ingredient	Amount (g/100 g diet)	
	n-3 adq	n-3 def
Alacid 710, acid casein	20	20
Cornstarch	15	15
Sucrose	10	10
Dextrose	19	19.9
Maltose-dextrin	15	15
Cellulose	5	5
Salt-mineral mix	3.5	3.5
Vitamin mix	1	1
L-cystine	0.3	0.3
Choline bitartrate	0.25	0.25
TBHQ	0.002	0.002
Fat sources:		
Hydrogenated coconut oil	7.45	8.1
Safflower oil	1.77	1.9
Flaxseed oil	0.48	--
DHA[a]	1.2	--
EPA[a]	0.24	--
Other n-3s[a]	0.1	--

Note: Dashes indicate that component was not added.

[a]Procured from Nordic Naturals, Inc., Watsonville, CA, USA as ProDHA capsule that contains 45% (w/w) DHA, 9% (w/w) EPA and 4% (w/w) other n-3s.

Table 2

Fatty acid composition of the diets as percentage of total fatty acids

Fatty acids	n-3 adq	n-3 def
Total saturated	66.49	75.95
Total monounsaturated	3.87	2.18
18:2n-6	13.58	16.67
20:4n6	0.518	ND
18:3n-3	2.71	0.052
20:5n-3	2.82	ND
22:6n-3	9.07	ND

Note: ND indicates that component was not detected.