Dietary Omega-3 Deficiency from Gestation Increases Spinal Cord Vulnerability to Traumatic Brain Injury-Induced Damage

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Abstract

Although traumatic brain injury (TBI) is often associated with gait deficits, the effects of TBI on spinal cord centers are poorly understood. We seek to determine the influence of TBI on the spinal cord and the potential of dietary omega-3 (n-3) fatty acids to counteract these effects. Male rodents exposed to diets containing adequate or deficient levels of n-3 since gestation received a moderate fluid percussion injury when becoming 14 weeks old. TBI reduced levels of molecular systems important for synaptic plasticity (BDNF, TrkB, and CREB) and plasma membrane homeostasis (4-HNE, iPLA2, syntaxin-3) in the lumbar spinal cord. These effects of TBI were more dramatic in the animals exposed to the n-3 deficient diet. Results emphasize the comprehensive action of TBI across the neuroaxis, and the critical role of dietary n-3 as a means to build resistance against the effects of TBI.

Results

Synaptic Proteins (Fig. 1)

BDNF levels were significantly reduced in the animals fed the n-3 deficient diet (n=3, n=3, n=3, p<0.01, n=3) as compared to the animals fed adequate n-3 (n=3 adq/sham, n=6) (Fig. 1A). FPI reduced levels of BDNF in the n-3 deficient group (n=3, n=3, n=5, p<0.01, n=5). Although FPI reduced BDNF levels in the n-3 def rats (p<0.02, n=7), these levels were still higher than the n-3 def group (p<0.05, Fig. 1A). The n-3 deficient diet reduced the levels of pTrkB/TrkB when compared to the n-3 adq/sham group (p<0.01, Fig. 1B). Although FPI reduced levels of pTrkB/TrkB in the group receiving adequate levels of n-3, pTrkB/TrkB levels were still higher than the n-3 def group (n=3, n=3, n=3, p<0.05, Fig. 1B). Levels of pCREB/CREB were reduced in the animals exposed to the n-3 deficient diet (n=3, n=3, n=3, p<0.05, Fig. 1C). pCREB/CREB levels even further (Fig. 1C). Fig. 1D showed the representative western bands corresponding to protein markers and animal groups.
Membrane Homeostasis (Fig. 2)

We assessed levels of 4-HNE which is a suitable marker of plasma membrane lipid peroxidation. Results showed that the n-3 def diet increased levels of 4-HNE in the spinal cord as compared to n-3 adq diet (p<0.01, Fig. 2A, 2B). FPI elevated 4-HNE levels even further in the n-3 def animals (p<0.01, Fig. 2A, 2B). Although FPI also elevated levels of 4-HNE in the n-3 adq group, 4-HNE levels were lower than in the n-3 def group (p<0.01, Fig. 2A, 2B).

We measured iPLA2 levels based on its involvement in the metabolism of membrane phospholipids [11] FPI significantly reduced the levels of iPLA2 in the animals fed n-3 deficient diet (n-3 def/FPI vs. n-3 def/sham, p<0.01). FPI had no effects on levels of iPLA2 in the n-3 adq group suggesting a counteractive effect such that levels of iPLA2 in the n-3 def rats exposed to FPI rats were significantly lower than their counterpart in the n-3 adq group (p<0.01, Fig. 2A, 2B).

Although the exposure to the n-3 deficient diet did not affect levels of syntaxin-3 in the sham rats relative to the adq group, FPI strongly reduced syntaxin-3 levels in the n-3 def group (n-3 def/FPI group as compared to n-3 adq/FPI group (P<0.05) and n-3 def/sham (P<0.05) groups (Fig. 2D).

Fatty Acid in Spinal Cord (Fig. 3)

Levels of docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) were measured in the spinal cord region using gas chromatography. Results showed that the levels of DHA significantly decreased in animals fed on n-3 deficient diet (n-3 def/sham). FPI did not affect levels of DHA in the n-3 def group (n-3 def/FPI) or the n-3 adq group (Fig. 3A). In turn, levels of AA were increased significantly in the sham and FPI groups exposed to the n-3 deficient diet (p<0.01, Fig. 3B). FPI also increased AA levels in the n-3 adq rats (P<0.01) (Fig. 3B).

Discussion

We found that brain concussive injury reduces molecular substrates of plasticity in the spinal cord, and these effects were dependent on the availability of DHA in the diet. These results emphasize the comprehensive action of TBI across the neuroaxis, and the critical role of diet as a means to build resistance against the effects of TBI. According to our results, proper exposure to n-3 fatty acids during gestation and throughout maturation of the CNS is crucial for building neural resilience during adulthood. The effects of diet and TBI were observed on levels of molecules...
associated with the function of BDNF on synaptic plasticity, and plasma membrane homeostasis in the spinal cord.

**Synaptic Plasticity**

According to our results, FPI and the diet deficient in DHA reduced protein levels of BDNF and its receptor TrkB in the SC, as well as elements related to the action of BDNF on synaptic plasticity such as syntaxin 3 and CREB, which have recognized roles in synaptic plasticity and learning and memory [12]. These results suggest that FPI reduces the capacity of the SC for plasticity. The action of the BDNF system seems crucial for mediating the action of DHA in the brain as a diet deficient in DHA has been shown to reduce activation of the BDNF TrkB receptors [3], and the capacity of the SC for learning a motorsensory task [13]. Therefore, the reduction of BDNF because of the DHA deficiency or TBI in our study may have negative implications for the potential of the SC to functionally recover after brain or SC injury. On the other hand, the fact that DHA supplementation is related to higher levels of BDNF argues in favor of a therapeutic potential of DHA. Indeed, DHA has shown protective capacity when provided after hemisection or compression spinal cord injury by increasing the survival of neurons and improving locomotor performance [14].

**Membrane Homeostasis**

DHA is a structural component of plasma membrane, and membrane bound DHA supports membrane fluidity [15], which is instrumental for neuronal signaling. The high contents of DHA and other phospholipids in the plasma membranes make the membrane a vulnerable target to lipid peroxidation. Lipid peroxidation has been linked to a disruption in membrane homeostasis and impairment of synaptic plasticity. Here, we found that FPI increased lipid peroxidation in the SC as evidenced by increased levels of 4-HNE. The phospholipase A2 (PLA2) family is involved in the metabolism of membrane phospholipids [11], and the calcium-independent PLA2 (iPLA2) plays an important

Figure 2. Levels of molecules related to plasma membrane homeostasis 4-HNE (A, B), iPLA2 (C), and syntaxin 3 (D) in the lumbar spinal cord of rats exposed to FPI. Results were expressed as mean ± standard error of the mean (SEM), *P < 0.05, **P < 0.01. FPI, fluid percussion injury; n-3 def, omega 3 fatty acids deficient; n-3 adq, omega 3 fatty acids adequate. n-3 def/sham: n = 5; n-3 adq/sham: n = 6; n-3 def/FPI: n = 5; n-3 adq/FPI: n = 7.
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role in synaptic plasticity [16,17]. Therefore, our results showing significant changes in iPLA2 levels in the n-3 def animals undergoing FPI provide an indication for the compromise of membrane homeostasis. In turn, STX-3 is a membrane-bound synaptic protein which function is influenced by DHA [18]. The fact that the diet deficient in DHA increased lipid peroxidation and decreased syntaxin 3, suggests how a lack of membrane DHA promotes membrane instability [19]. Syntaxin 3 is positioned in the presynaptic plasma membrane to detect local changes in PUFA [18] and plays a crucial role in the docking and fusion of vesicles during synaptic transmission [20]. Therefore, our results showing that FPI and dietary n-3 affect levels of 4-HNE, iPLA2, and STX-3, suggest a potential mechanism by which TBI and diet can influence membrane homeostasis required for functional recovery after spinal cord injury.

It is important to consider that although DHA (1.2%) is the most abundant omega-3 fatty acid in our diet, there are other less abundant fatty acids as well such as eicosapentaenoic acid (EPA). EPA has also been reported to support neural repair events such as reducing axonal injury after spinal cord compression; however, its action appears less effective than DHA [21]. Given the large difference in contents of DHA (1.2%) Vs. EPA (0.24%), it is likely that the main effects of the diet are related to DHA.

The effects of the n-3 feeding can also be perceived at the levels of AA and DHA in the spinal cord, as evidenced by results showing that n-3 deficiency increased AA levels but reduced DHA levels. These results are consistent with the possibility that AA could replace DHA in the membrane. A reduction in membrane fluidity can affect transmembrane receptors such as TrkB, and this may explain why the n-3 def diet reduced TrkB activity in our study. DHA modifies the characteristics of lipid rafts by incorporating into raft domains of the membrane and influencing signaling across embedded receptors [22] such as TrkB receptors [23].

The current results emphasize the pervasive effects of brain trauma impacting CNS regions, which are distant from the lesion. These results have important implications for the design of potential treatments directed to counteract the effects of TBI. Based on results showing the comprehensive effects of brain injury in the brain and spinal cord, it appears that interventions that have the capacity to influence the entire neuroaxis can be particularly effective. As discussed above, the broad spectrum of action of the omega-3 fatty acid DHA positively influencing the brain and spinal cord appears particularly suitable for this purpose. It is critical to complement our molecular data with behavioral studies. It is known that the type of TBI used in the current study promotes deficits in cognition and gait [1] and that post-injury treatment with DHA counteracts some these deficits [8]. A period as short as 12 days of DHA following FPI has been shown to be sufficient to counteract deficits in hippocampal-dependent learning [8]. The unique aspect of our results is the demonstration that dietary DHA during CNS maturation confers resilience to neurological damage in adult life. These results have important implications to appraise the role of diet as a vulnerability factor for the outcome of TBI. It is a common observation that healing after brain or spinal cord injury is not often predictable based on the extent of the neurological damage. This implies that vulnerability factors associated with the environment and genetics have great potential to determine the outcome of CNS injured patients.

Our results show that exposure to n-3 fatty acids during gestation and throughout maturation of the CNS is important for building resilience to neurological damage incurred later on in life. Further studies are required to define whether shorter dietary DHA exposure can confer CNS protection. In conclusion, these results are important to define the broad and positive action of n-3 diet on counteracting the effects of concussive brain injury on the spinal cord.

Materials and Methods

All experimental procedures 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 (UCLA) Chancellor’s Animal Research Committee (ARC).

Experimental Design

Two-day pregnant female Sprague-Dawley rats weighing between 280 and 300 g were obtained from Charles River Laboratories, Inc. (Portage, MI, USA). The animals were housed with 12-h light/dark cycles and a maintained temperature of 22–24°C. Animals were randomly divided into two dietary groups:
Fluid Percussion Injury (FPI)

FPI was performed as previously described [10]. Under deep anesthesia, a 3.0 mm diameter craniotomy was made 3.0 mm posterior to the bregma and 6.0 mm lateral (left) to the midline, and a cap was glued over the craniotomy. The cap was filled with 0.9% saline solution and a mild fluid percussion pulse (1.5 atm) was administered. The severity of injury was confirmed based on the unconscious time (less than 120 seconds) of the animal after FPI, and a mild fluid percussion pulse (1.5 atm) was added. The severity of injury was confirmed based on the unconscious time (less than 120 seconds) of the animal after FPI.

Tissue Collection

One week following FPI, rats were sacrificed by decapitation, and their spinal cords were collected and stored in −70°C for lipids and protein measurements.

Protein Analysis

BDNF, phosphorylated tyrosine kinase (pTrkB), phosphorylated cAMP response element-binding (pCREB), syntaxin-3, calcium-independent phospholipase A2 (iPLA-2), and 4-Hydroxynonenal (4-HNE) proteins of the right lumbar SC were analyzed using western blots as previously described [10]. Tissue was first homogenized in a lysis buffer and the total protein was separated by electrophoresis on a polyacrylamide gel and electrotransferred onto a PVDF (nitrocellulose for BDNF) membrane (Millipore, Bedford, MA). After blocking, the membranes were rinsed with TBS-T and incubated with the primary antibody for actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BDNF (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), pTrkB (1:200, BD Biosciences, Sparks, MD, USA), TrkB (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), pCREB (1:1,000, Millipore, Bedford, MA, USA), CREB (1:200, Millipore, Bedford, MA, USA), syntaxin-3 (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), iPLA-2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or 4-HNE (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunocomplexes were visualized by chemiluminescence using the commercial kit ECL plus (Amer sham Pharmacia Biotech Inc., Piscataway, NJ, USA). Respective protein sizes were compared to the Benchmark pre-stained protein ladder (Invitrogen Technology, Carlsbad, CA, USA). Protein bands were digitally scanned and quantified using the ImageJ software. Actin was used as an internal control. The phosphorylated proteins were normalized to their respective non-phosphorylated partners.

Statistical Analysis

Protein data were expressed as a mean percentage of the control (sham/n-3 adq) group DHA and AA levels were expressed as a ratio of fatty acid (mg/tissue(g). Significance among groups was determined using SPSS software. One-way analysis of variance (ANOVA) was performed followed by Fisher’s Least Significance Difference test. Results were expressed as mean ± standard error of the mean (SEM), n = 5–7/group. Significant difference was considered at P<0.05.

Author Contributions

Conceived and designed the experiments: ZY RA FGP. Performed the experiments: CF RA YZ. Analyzed the data: CF YZ. Wrote the paper: ZY CF FGP.

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