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TITLE: Neural Resilience to Traumatic Brain Injury: Identification of Bioactive Metabolites of Docosahexaenoic Acids Involved in Neuroprotection and Recovery

PRINCIPAL INVESTIGATOR: Hee-Yong Kim

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<b>14. ABSTRACT</b> A mouse model of TBI has been established with behavioral test parameters to evaluate functional deficits. Dietary conditions to generate extreme and moderate n-3 fatty acid depletion in the mouse brain have been established for testing TBI outcome. Significant retardation of spontaneous recovery from TBI-induced motor and cognitive deficits in mice raised on an n-3 deficient diet where brain DHA was depleted by over 70%. Degradation of alpha spectrin, a marker of TBI induced injury, was elevated in the cortex of TBI-inflicted DHA-deficient mice with less NeuN-positive neurons, indicating exacerbated injury in these animals. Moderate depletion of DHA (by 30%) also showed the same trend in behavioral outcome, and biochemical characterization is in progress. Effects of DHA and synaptamide on axon growth have been established in cortical neuron cultures. Software for the isotope assisted mass spectrometric identification of metabolites has been developed which is being applied to DHA-derived metabolites formed by cortical neurons in cultures as well as TBI-inflicted brain homogenates. A nanosymposia presentation and a manuscript have been generated from this project.					
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## ***Introduction***

Military personnel in combat deployments are afflicted with high rates of traumatic brain injury (TBI) causing lifelong neurological and cognitive impairments, especially in learning and memory. Numerous studies have shown that docosahexaenoic acid (DHA) is essential for proper brain development and function [1,2], although the underlying mechanisms are still unfolding. Under normal conditions, DHA is present in esterified form in membrane phospholipids, especially the aminophospholipids, phosphatidylethanolamine (PE), and phosphatidylserine (PS). Despite tight regulation to maintain membrane phospholipid homeostasis, DHA enrichment can expand the PS pool in the neuronal membranes [3], as DHA-containing phospholipids serve as the most favored substrate for PS biosynthesis in mammalian tissues [4]. On the contrary, depletion of DHA has been shown to decrease PS levels significantly in brain tissues [3, 5-7]. Since PS is known to participate in key signaling events supporting cell survival and differentiation, DHA-dependent PS modulation is an important aspect of neuroprotection [8]. Following brain injury, polyunsaturated fatty acids including DHA and arachidonic acid (AA, 20:4n-6) are released from neural membranes and metabolized to many bioactive derivatives. Some of the AA-derived eicosanoids are known to be pro-inflammatory, exacerbating the initial injury [9,10]. In contrast, some DHA-derived docosanoids has been shown to ameliorate or resolve inflammatory processes [11]. Furthermore, N-docosahexaenoylethanolamide (synaptamide), a DHA metabolite of a separate class, has been recently identified as a potent neurotogenic and synaptogenic agent [12]. In this regard, the DHA content of the brain may be an important variable to consider in devising a strategy to improve neuroprotection and recovery outcome after brain injuries.

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## *Objective*

- The major goals of this project are to develop strategies to improve neural resilience to traumatic brain injury and facilitate recovery through mechanism-based optimization of the nutritional DHA or metabolite status in neuronal tissues.
- Aim 1. To determine if diets rich in DHA afford protection to the nervous system against traumatic brain injury in animal models
- Aim 2. To identify bioactive DHA metabolites formed in the brain that are involved in neuronal survival, neurite development, learning and memory
- Aim 3. To determine if DHA-derived mediators improve recovery after traumatic brain injury in animal models
- Aim 4. To devise therapeutic approaches for improving DHA status and/or administering specific bioactive metabolites that facilitate recovery from traumatic brain injury.

## *Statement of Work*

### **Year 2**

Task 1: Testing effects of DHA status on TBI injury outcome using animal and dietary models established (months 9-24)

Task 2: Testing bioactivity of identified DHA metabolites in cell culture systems (months 13-24)

Task 3: Developing software in consultation with an instrumental company software team to establish a method to profile a broader range of DHA metabolites (months 6-18)

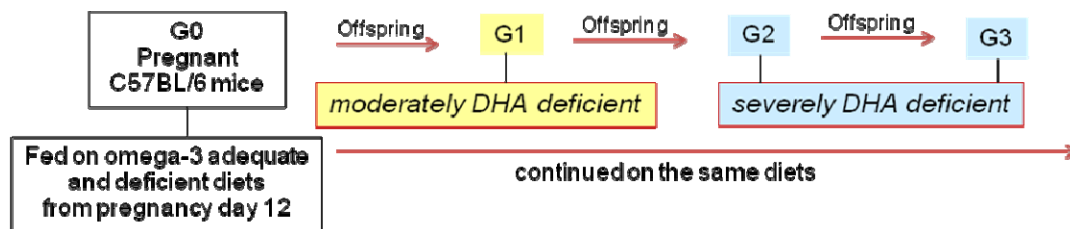
- Milestone 1: Effects of DHA status evaluated in an extreme case of DHA-deficiency in comparison to DHA-adequate controls.
- Milestone 2: Bioactivity of major DHA metabolites tested for survival, neurite development and synapse formation in neuronal culture.
- Milestone 3: More DHA metabolites identified
- Milestone 4: Publication 1 on identification of brain DHA-metabolites by isotope-assisted metabolomics approach

## Report

Task 1: Testing effects of DHA status on TBI injury outcome using animal and dietary models established (months 9-24)

During this report period, we established the effects of DHA status on the recovery from TBI-induced motor deficit using the CCI-model established during the last period. We also established moderately DHA-deficient model in addition to the extreme DHA-deficient model.

During the last report period, we have successfully established the CCI-model for TBI. During this period, we further refined behavioral test parameters to evaluate the functional deficit associated with TBI. We also established dietary conditions to generate moderate and extreme DHA depletion in the mouse brain by feeding mice with an omega-3 deficient special diet for one to three consecutive generations (G1-G3) (Scheme 1). The brain DHA level in the second or third generation omega-3 deficient animals was lowered by over 70%, which was mostly compensated by the increase of docosapentaenoic acid (DPA, 22:5n-6) (Fig. 1).



Scheme 1. Generation of moderate and severely DHA-deficient mice

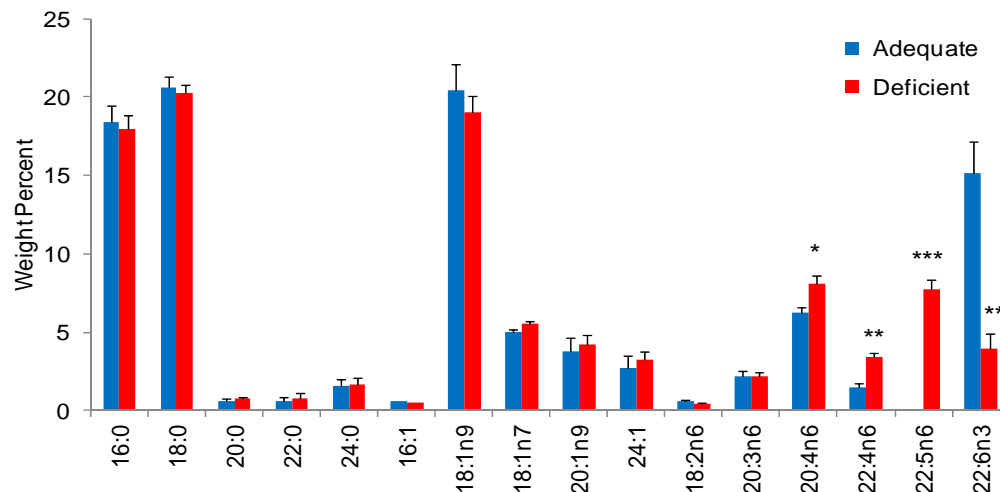


Fig. 1. Fatty acid composition of the brains from omega-3 fatty acid adequate and severely deficient mice. Data are expressed as mean  $\pm$  SD (n=3 for each group). \*p<0.05, \*\*p<0.01, and \*\*\*p<0.001 compared to the adequate group

Using these animal models, we investigated the effect of DHA-depletion on the TBI outcome. Age and gender matched mice at 10-12 weeks from adequate and severely deficient groups were subjected to the CCI procedure and the TBI-induced motor and cognitive deficits were evaluated using accelerating rotarod, beam walk and novel object recognition (NOR) tests. For the accelerating rotarod test, the mice were pre-trained for the rotarod apparatus for three days and a baseline reading of the mice was recorded on the day prior to the surgery. For the beam walk test, the mice were trained to traverse a narrow beam before the surgery and the number of hind foot slips was observed after surgery and compared to sham operated animals. The rotarod and beam walk

tests were further performed from the first day after injury and each day during spontaneous recovery until day 7 after TBI. The two diet groups showed a significant difference in spontaneous recovery of motor function (Fig. 2). By day 3 after TBI the rotarod and beam walk performances of omega-3 adequate animals was recovered significantly. In contrast, the deficient group showed prolonged motor deficits until day 7 after TBI. Statistical significance between two groups was reached with 8 animals from each group.

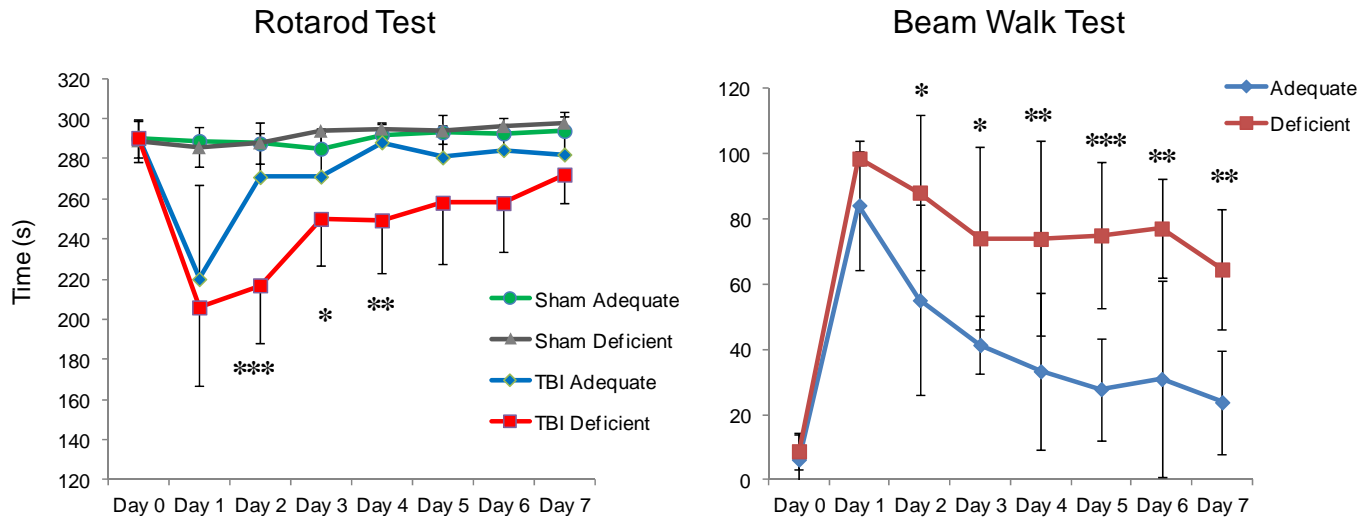


Fig. 2. Effects of dietary omega-3 fatty acids on spontaneous recovery of TBI-induced motor deficits evaluated by rotarod and beam walk tests. Data are expressed as mean  $\pm$  SD (n=8 for each group). \* $p$ <0.05, \*\* $p$ <0.01, and \*\*\* $p$ <0.001 as compared to the TBI adequate group.

The novel object recognition (NOR) test was performed on day 7 after TBI. Mice were individually acclimatized to the test arena daily for 10 min for three days prior to the testing. On the third day, the animals were exposed to two objects for 10 min each and subsequently tested for memory after 2 hours. In sham animals, the NOR performance between omega-3 adequate and severely deficient groups was not statistically different. Nevertheless, NOR performance was significantly impaired in TBI-inflicted severely omega-3 deficient animals compared to the corresponding adequate group (Fig. 3). This data suggests that DHA-depleted animals are particularly susceptible to TBI-induced cognitive deficit and adequate omega-3 fatty acid provision may have a role in preventing such deficit.

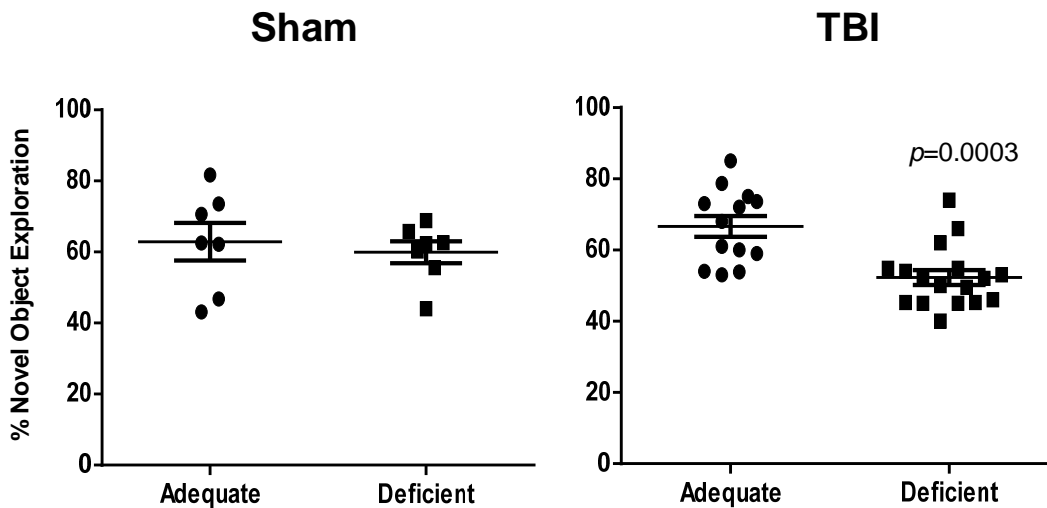


Fig. 3. Effects of dietary omega-3 fatty acids on the TBI-induced cognitive deficit evaluated by novel object recognition test. Data are expressed as mean  $\pm$  SE (n=7-17 for each group).  $p=0.0003$  as compared to the TBI adequate group.

We have also evaluated the anxiety-like behavior using open field test (Fig. 4). Animals with severe omega-3 deficiency showed significantly less time in the center zone compared to the adequate animals, indicating that DHA-deficiency alone can cause increased anxiety. TBI significantly increased anxious behavior in both adequate and deficient groups; however, the significant difference between two groups remained after TBI. These data suggest that the TBI-induced anxious behavior can be exacerbated by severe omega-3 deficiency.

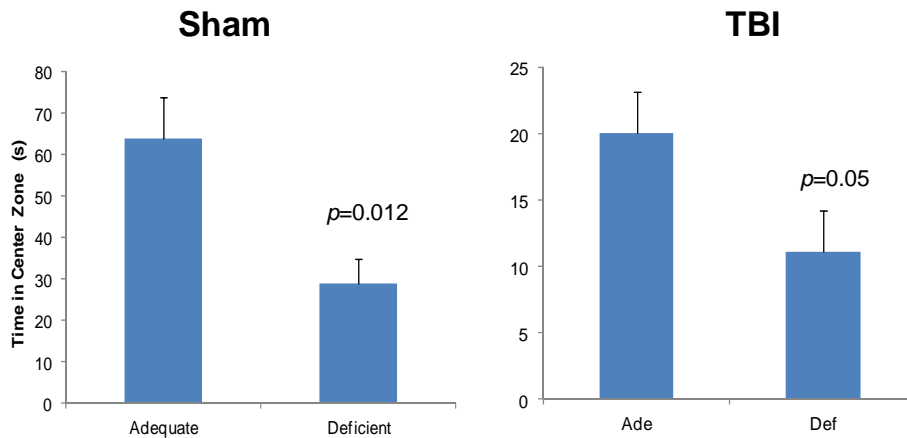


Fig. 4. Effects of dietary omega-3 fatty acids on the TBI-induced anxious behavior evaluated by open field test. Data are expressed as mean  $\pm$  SD (n=7-8 for each group).  $p=0.012$  and  $0.05$  as compared to the corresponding TBI adequate group.

Upon completion of the behavioral tests, brains were harvested (on day 8 after TBI), and histological and biochemical evaluation was performed including cavity size, NeuN staining and western blotting for spectrin alpha. Although statistical insignificance was not reached, the cavity size of the TBI-inflicted deficient brains showed an increasing trend compared to the adequate group (Fig. 5). Western blot analysis indicated that degradation of alpha spectrin, a marker of TBI induced injury, was elevated in the cortex of TBI-inflicted DHA-deficient mice after 24 h of injury (Fig. 6). Immunohistochemical analysis of NeuN, a neuronal marker protein, showed a reduction of NeuN-positive cells in the injured hemisphere in comparison to uninjured hemisphere (40-60% reduction). The NeuN positive cells in the injured hemisphere were further decreased in DHA-depleted brains in comparison to the DHA-adequate brains (Fig. 7). While  $p$  value was  $0.06$ , only 3 animal samples have been tested so far, and it is likely that statistical significance will be reached when additional samples are analyzed. These biochemical and histological results consistently supports the behavioral outcome where omega-3 adequate mice recover better than deficient animals.

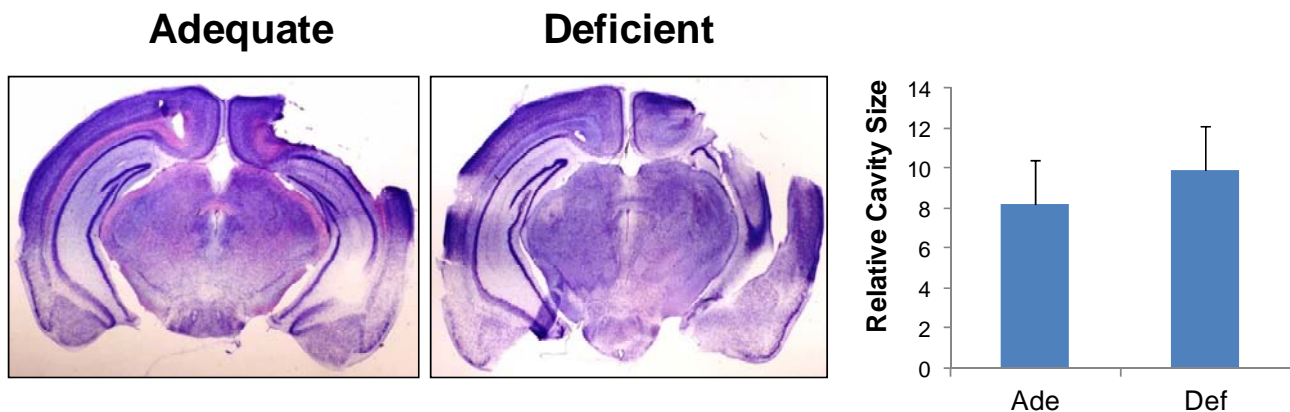


Fig. 5. Cresyl violet staining of brain sections and quantification of brain cavities in TBI-inflicted omega-3 fatty acid adequate and severely deficient mice. Data are expressed as mean  $\pm$  SD (n=5 for each group).

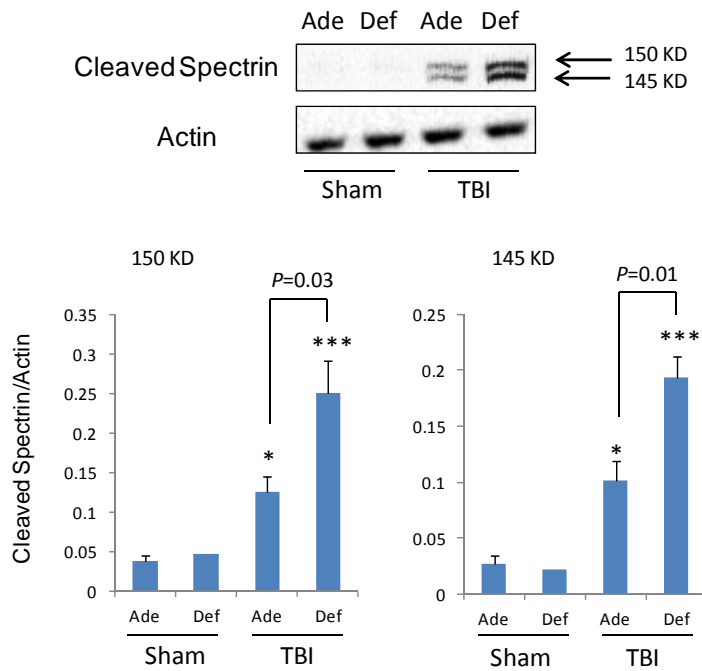


Fig. 6. Western blot analysis of cleaved spectrin in TBI-inflicted omega-3 fatty acid adequate and severely deficient mouse brains. Data are expressed as mean  $\pm$  SD (n=4 for each group).

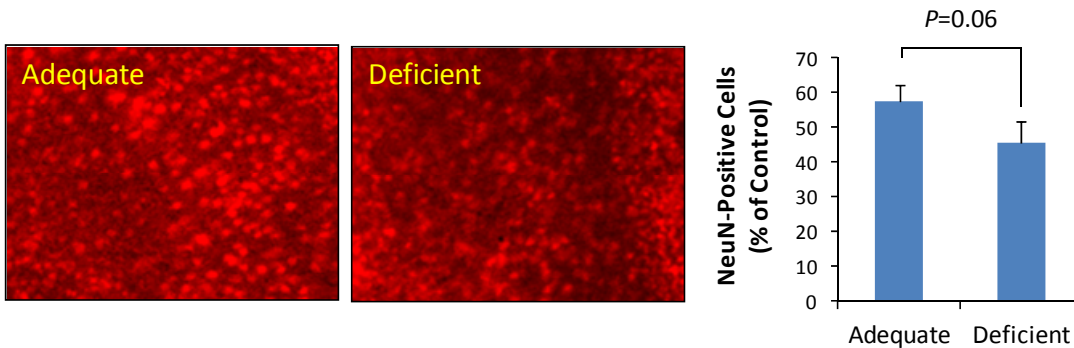


Fig. 7. NeuN immunostaining of brain sections and quantification of NeuN-positive cells in TBI-inflicted omega-3 fatty acid adequate or severely deficient mice. % of NeuN-positive cells in the injured hemisphere was calculated against NeuN-positive cells in the uninjured hemisphere. Data are expressed as mean  $\pm$  SD (n=3 mice with 3 sections/mouse for each group).

Using the extreme case of DHA depletion, we established that DHA-adequate mice recover better from TBI compared to the DHA-deficient mice. We have extended this study to a model of moderate DHA deficiency which commonly occurs in humans. The moderately DHA-depleted mice (G1) were generated by feeding pregnant mice a special omega-3 deficient diet from the gestation day 12 (Scheme 1) throughout pregnancy and lactation period and offspring mice were continued on the same diet until the time of experiments (10-12 weeks old). Such deprivation of dietary omega-3 fatty acids effectively lowered brain DHA by 30% compared to the omega-3 adequate group (Fig. 8).

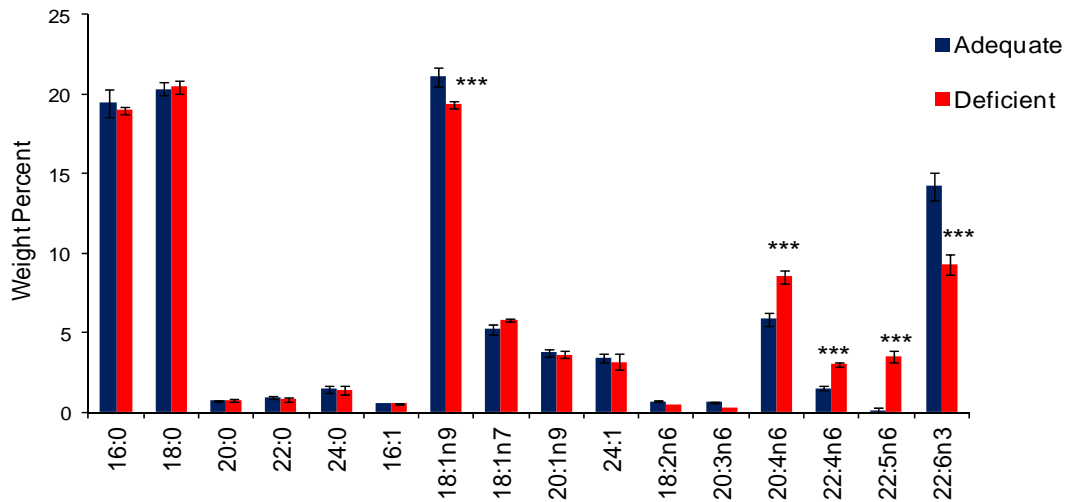


Fig. 8. Fatty acid composition of the brains from the first generation offspring mice fed on omega-3 fatty acid adequate and deficient diets. Moderate DHA-depletion was induced. Data are expressed as mean  $\pm$  SD (n=3 for each group). \*\*\*p<0.001 compared to the adequate group.

Moderate DHA-depletion also had a similar impact on the TBI outcome, although the extent was not as severe as the extreme case of DHA-depletion. Both rotarod and beam walk tests indicated significant differences between adequate and deficient groups with the latter showing slower recovery (Fig. 9). In addition, cognitive function and anxious behavior were also adversely affected by the moderate omega-3 fatty acid deficiency in TBI-inflicted mice (Fig. 10).

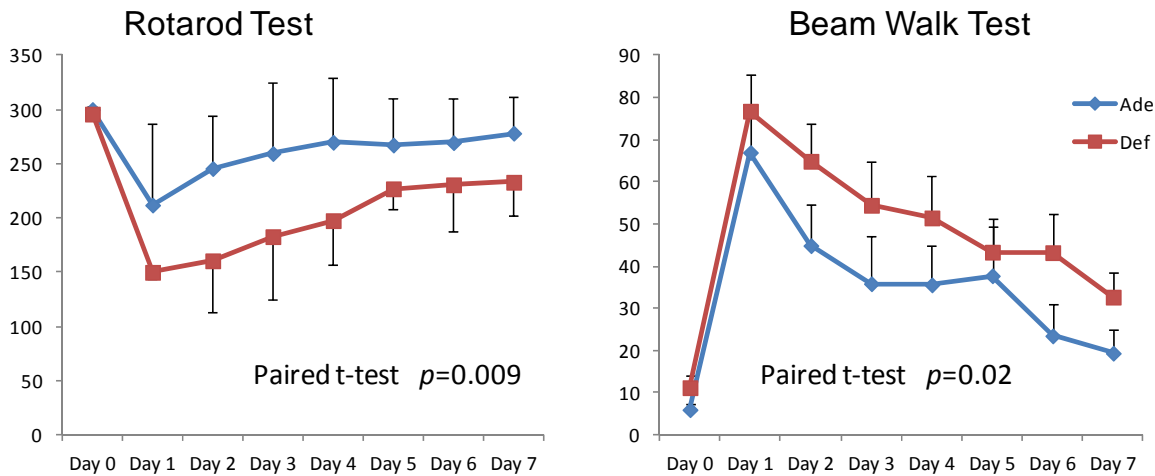


Fig. 9. Effects of moderate omega-3 fatty acid deficiency on spontaneous recovery of TBI-induced motor deficits evaluated by rotarod and beam walk tests. Data are expressed as mean  $\pm$  SE (n=8 for each group). Paired t-test indicates significant difference between two groups.

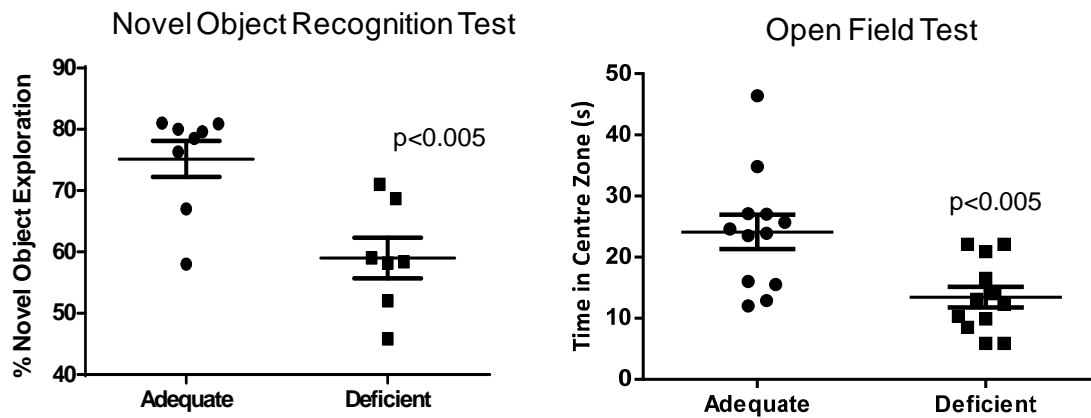


Fig. 10. Effects of moderate omega-3 fatty acid deficiency on the cognitive function and anxiety in TBI-inflicted mice evaluated by novel object recognition and open field tests, respectively. Data are expressed as mean  $\pm$  SE.

Our results indicated that the mice from both severe and moderate DHA-deficiency groups had slower recovery of vestibulomotor functions as assessed by the rotarod and beam walk tests. Unlike the n-3 adequate controls, the brain injured n-3 deficient mice also failed to discriminate between the familiar and novel objects in the object recognition test, indicating impaired memory. Histological analysis revealed an increased cavity volume in the n-3 deficient group along with less NeuN-positive neurons, indicating exacerbated injury. Western blot analysis indicated that degradation of alpha spectrin, a marker of TBI induced injury, was elevated in the cortex of TBI-inflicted DHA-deficient mice after 24 hours of injury. These results consistently indicate that DHA-adequate mice recover better from TBI. Considering that modern diets have low n-3 fatty acids and DHA deficiency is common in humans, results from this study present strong possibility of using nutritional remediation as a tool to enhance recovery from brain injury.

#### Task 2: Testing bioactivity of DHA metabolites in cell culture systems (months 13-24)

*We have previously identified N-docosahexaenylethanolamide (synaptamide) as a potent neurotogenic and synaptogenic metabolite of DHA formed in the hippocampal and cortical neuronal cultures. In addition to dendrite extension, we tested the bioactivity of synaptamide on axon growth in cortical neurons during this period. The cortical neuron cultures were chosen, since the cortical region would be most significantly inflicted by the CCI procedure.*

Axon growth of cortical neurons was promoted by DHA dose-dependently in the 0.1-1  $\mu$ M range. DHA-derived synaptamide was even more potent in stimulating axon growth. Significant effects were observed at a concentration as low as 5 nM while other fatty acid ethanolamines including DPAn-6 ethanolamide (DPEAn-6), anandamide (AEA) and oleylethanolamide (OEA) exerted no effects at 10 nM (Fig. 11).

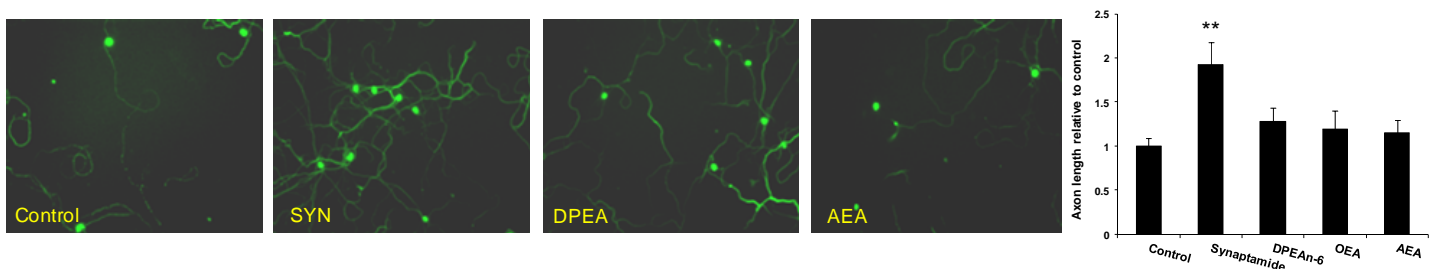


Fig. 11. Effect of polyunsaturated fatty acid ethanol amides on cortical axon growth. Cortical neuron cultures were treated with 10 nM fatty acid amides for 3 days, immunostained for axon specific marker SMI-312 and axon length was quantified by Metamorph software. \*\*,  $p < 0.01$  vs. control.

To monitor the effects of DHA and its metabolites on axon repair, we are in the process of establishing an *in vitro* axon injury model using a microfluidic culture platform. In this model, cortical neurons are seeded in one side of a culture chamber and axons are allowed to grow through multiple grooves to reach the other side of the chamber (Fig. 12). At the end of the grooves (dotted line), axons are severed by aspiration of the media from the axonal compartment for 5 sec, and regrowth of axon is monitored using axon specific markers such as SMI-312. This device was also used to confirm the positive effect of synaptamide on axon growth (Fig. 13). Once the model is established, we will examine the effects of DHA or specific DHA metabolites on axon repair.

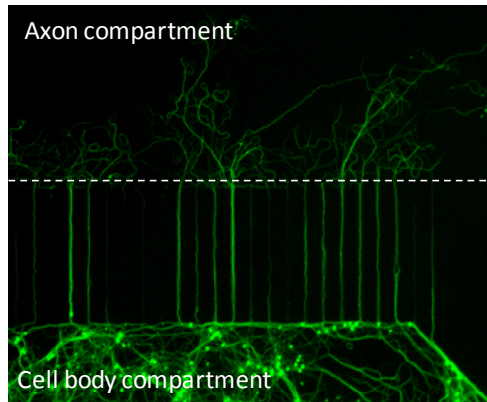


Fig. 12. Axon (SMI-312 positive, green) outgrowth in an axon device observed for cortical neurons after 14 days *in vitro* culture.

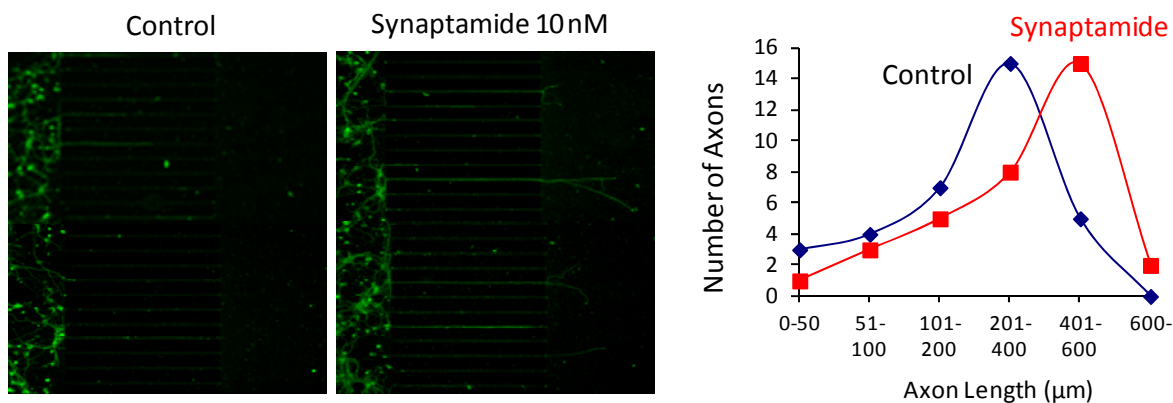


Fig. 13. Effect of synaptamide on axon outgrowth in an axon device after cortical neurons were cultured with 10 nM synaptamide for 72 h.

**Task 3: Developing software in consultation with an instrumental company software team to establish a method to profile a broader range of DHA metabolites (months 6-18)**

Formation of additional DHA metabolites in the cortical cultures was examined using the stable isotope labeling assisted metabolomics approach using the high resolution MS that has been recently installed. A new software “IsotopeLabelFinder” was developed in collaboration with Thermo Instrumental Company using the mass difference between natural and U-C13-labeled DHA metabolites and the distinct isotopic profile of the C13-labeled metabolites. Using this approach, we are in the process of identifying metabolites of DHA formed in the cortical neuron cultures and brain homogenates.

### **Key Research Accomplishments**

1. Dietary conditions to generate extreme and moderate DHA depletion in the mouse brain has been established for testing TBI outcome.
2. Using a mouse model of TBI, we established the adverse effects of both extreme and moderate DHA depletion on spontaneous recovery from injury.
3. Effect of DHA and synaptamide on axon growth has been established in cortical neuronal culture.

4. An axon growth model using a microfluid culture platform has been established for further development into an *in vitro* injury model.
5. An algorithm for stable isotope assisted identification of DHA metabolites has been established.

### ***Reportable Outcomes***

A presentation entitled “Omega-3 Fatty Acid Deficient Diet Worsens Traumatic Brain Injury Outcome” was selected for nanosymposia at the 2012 Society for Neuroscience meeting in New Orleans, LA, and a manuscript has been prepared for submission.

### ***Conclusion***

- Milestone 1: Evaluation of the effects of DHA status in an extreme case of DHA-deficiency in comparison to DHA-adequate controls has been completed.
- Milestone 2: Bioactivity of synaptamide, the major DHA metabolite formed in the cortical neuron cultures, has been tested for axon growth.
- Milestone 3: An algorithm to identify more DHA metabolites has been developed.
- Milestone 4: Publication 1 on ‘Omega-3 Fatty Acid Deficient Diet Worsens Traumatic Brain Injury Outcome’.

During the second year, we have met the above specified milestones approved by the CDMRP. Since the instrument installation was significantly delayed, it was not possible to make the publication 1 on “identification of brain DHA-metabolites by isotope-assisted metabolomics approach”. Instead, we were able to complete the study on effect of the DHA status on TBI outcome and a manuscript is in final preparation for submission to PLoS One. We anticipate acceleration of further metabolomics research to identify new metabolites as we now have developed the software. We will subsequently evaluate their bioactivity on neuronal survival and neurite growth and repair using cortical neuron cultures and axon device. Animal feeding studies will continue in order to further evaluate the injury outcome in a moderately DHA-depleted status. We will continue to pursue the therapeutic dose and time windows of synaptamide treatment using FAAH KO and wild type animals.