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Selective Reduction of Brain Docosahexaenoic Acid after Experimental Brain Injury and Mitigation of Neuroinflammatory Outcomes with Dietary DHA

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Abstract

Background Docosahexaenoic acid (DHA) is an omega-3 fatty acid that is important for brain development and function, but the interactions of dietary DHA with fatty acid profiles, sensory sensitivities, and inflammation that may change after traumatic brain injury (TBI) are poorly understood. It is also unknown whether DHA alters experimental TBI outcomes measured more than 2 weeks after injury. The current study investigated whether dietary DHA, provided before (PreDHA) or after (PostDHA) experimental TBI, would improve outcomes for up to 24 days after injury.

Methods Rats consumed predetermined diets for 28 days prior to midline fluid percussion injury (mFPI) or to sham surgery. The effects of PreDHA, TBI, and PostDHA on comprehensive fatty acid profiles, neuroinflammation, sensory sensitivity, and spatial learning were then evaluated.

Results The results provided novel evidence that TBI selectively reduced brain DHA content, as injury did not decrease any other fatty acid that was measured. Furthermore, PreDHA and PostDHA attenuated injury-induced increases in sensory sensitivity as well as in tumor necrosis factor-α (TNF-α), interleukin-10, and interleukin-1β in the somatosensory cortex. However, [³H]PK11195 autoradiography showed that PostDHA was more effective than PreDHA in reducing microglial/macrophage activation in the somatosensory cortex, hippocampus, and substantia nigra. Spatial learning outcomes

Keywords

► traumatic brain injury
► docosahexaenoic acid
► neuroinflammation
► post-concussive syndrome

were largely unaffected by diet or injury, but PostDHA was associated with shorter swimming distances in the Morris water maze (MWM) at 15 days post-injury.

Conclusion   Overall, sufficient DHA intake may be necessary to replace DHA that is lost to TBI and may improve some symptoms of post-concussive syndrome (PCS) over an extended period through inflammation-related mechanisms.

Introduction

Over 1.7 million traumatic brain injuries (TBI) are documented in the United States alone because of their association with hospitalization.[1] This figure may grossly underestimate the true number of brain injuries because many diffuse TBI, concussions, and other subconcussive injuries are considered "mild" and go unreported because hospitalization does not seem necessary. However, such lower grade TBIs can still have long-term effects on brain health.[2] The long-term symptoms of TBI can include sensory sensitivity or dysfunction,[3,4] impaired cognitive or emotional ability,[5–7] and loss of neurological function.[6]

TBI is induced by mechanical forces that initiate a protracted cascade of secondary injury processes, including inflammation and oxidative stress.[8] Accordingly, molecules such as interleukin (IL)-6, IL-10, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) can be significantly elevated in the cerebrospinal fluid and blood serum for up to 22 days after TBI in humans.[9] Thus, many therapies have attempted to target specific factors in these secondary processes in the hours to days following TBI. This approach hypothesizes that attenuation of secondary signaling would reduce the long-term symptoms of TBI, but receptor-based therapies have had limited success in clinical trials.

The shortcomings of targeting a single, specific TBI factor[10] suggest that pleiotropic agents aimed at the multifaceted pathophysiology of the secondary injury cascade may be more effective. One such approach, investigated in the current study, may be the maintenance of sufficient levels of docosahexaenoic acid (DHA). DHA is the most concentrated omega-3 polyunsaturated fatty acid (PUFA) in the nervous system, and dietary sources of this nutrient include mothers' milk, fish, marine algae, and supplements containing DHA. Randomized, controlled trials in humans indicate that DHA has an excellent safety profile[11] and that elevation of its tissue concentration throughout the body allows for better cognitive function in healthy, aging populations[12,13] and improved neurodevelopment.[14] The positive findings in these clinical trials are likely due to the multiple actions of DHA, which include regulation of apoptotic signaling,[15] decreased inflammatory signaling,[16] and promotion of the function of some G-protein coupled receptors.[17]

While DHA or omega-3s have already shown favorable effects in animal models of TBI that cause gross anatomical damage to the brain,[18–22] the present study was designed with three novel goals. First, comprehensive lipid profiling of the brain has not been reported after diffuse experimental TBI with midline fluid percussion injury (mFPI), which, like concussion and subconcussive injury, does not cause gross anatomical damage resulting in overt tissue loss. Therefore, this study examined the brain, red blood cells (RBCs) and plasma for changes in 27 fatty acids 23 to 24 days after mFPI so that the longer term effects of diffuse TBI on the lipid profile could be better understood.

The second novel goal of this study focused on the premise that dietary administrations of DHA before or after TBI represent two equally valid scenarios of prophylaxis and therapy, respectively, which may differ in long-term efficacy. Thus, this work employed DHA administration before and after diffuse TBI within the same study using a defined DHA-enriched diet rather than direct administration of DHA-containing oil. Such a design evaluates three sets of likely circumstances and answers specific questions. For example, the DHA intake in most westernized societies is not sufficient[23]; therefore, this study evaluated the effects of deficient and sufficient DHA intake prior to brain injury on longer term outcomes (*i.e.* beyond 2 weeks after diffuse TBI). Second, the effects of TBI on the ability to eat or the medical care received after TBI may result in reduced DHA intake; therefore, this study also examined the effects of lowered DHA status after TBI on brain-related outcomes regardless of prior intake.

Conversely, the third circumstance modeled by this study involves the typically low DHA intake by Western societies and the possibility that medical care or new knowledge gained by a person affected by brain injury will result in sufficient DHA intake soon after TBI.

Another unique focus of this study is based on the fact that the most common and "mild" form of brain injury (*i.e.* concussion) can cause long-lasting post-concussive syndrome (PCS), in which affected individuals have an abnormally low tolerance for normal levels of sensory input.[24–26] Thus, the whisker nuisance task (WNT) was used as an outcome that models PCS[27] and as an assessment of the effects of DHA on long-term sensory sensitivity after diffuse TBI.

Longer timelines for behavioral and cytokine endpoints provided the opportunity to evaluate the longer term, post-TBI conditions of cognitive impairment, PCS, and neuroinflammation that have been noted in humans.[3–7,9,25] Each study arm tested spatial learning with the Morris water maze (MWM) at 15 to 18 days post-injury and sensory sensitivity with the WNT at 22 days post-injury. Neuroinflammation was then determined by the degree of microglial/macrophage activation and by the protein levels of IL-1β, IL-6, IL-10, TGF-β, and TNF-α at 23 to 24 days post-injury. We hypothesized that both pre- and post-injury DHA administration would increase brain DHA content, reduce post-traumatic neuroinflammation, and improve long-term sensory and cognitive outcomes of TBI.

Materials and Methods

Animals

Adult male Sprague–Dawley rats were obtained from Harlan, Inc. (Indianapolis, IN) and housed in pairs on a 12 h:12 h light schedule with *ad libitum* access to rat chow and water. The ordering and receipt of the animals were coordinated with the study design that called for 28 days of diet administration prior to injury and a standard injury weight of 350 g. All animals were between 84 and 96 days of age at the time of injury. All animal procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Diets

The animal diets were designed to provide either 0% (control) or ∼1% DHA, expressed as a weight percentage of the total fatty acids in the diet. The diets were based on the AIN-93G standard, but their fat contents consisted of controlled blends of coconut oil, high-oleic safflower oil, TBHQ-free soy oil (Dyets, Inc.), and DHASCO oil (DSM Nutritional Products). The oils were analyzed as fatty acid methyl esters (FAME) prior to diet formulation by Dyets, Inc., and the diets were similarly analyzed prior to their use in the study. Thus, the oil blends allowed for a balancing of all fatty acids between diets except for DHA content and total omega-3 content (►**Table 1**). Each diet had been fed to separate, randomized sets of animals for 28 days prior to sham surgery or brain injury (see below). Directly thereafter, the animals were either maintained on a DHA-deficient diet (controls), switched to DHA-deficient diets (Preinjury DHA; PreDHA), or switched to DHA-sufficient diets (Post-injury DHA; PostDHA; see ►**Fig. 1** for study design). The animals were randomized to the diets, and the researchers were blinded from the treatments throughout all experimental procedures.

Midline Fluid Percussion Injury

A total of 75 rats were subjected to mFPI consistent with methods that have been described previously.[27–30] Rats were anesthetized using 5% isoflurane in 100% oxygen for 5 minutes, and the head of the rat was placed in a stereotaxic frame with continuously delivered 2.5% isoflurane via a nosecone. While anesthetized, body temperature was maintained using a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). A midline incision was made across the top of the head, and the fascia was removed from the surface of the skull. A trephine (4.8 mm outer diameter) was centered on the sagittal suture between bregma and lambda, and the craniotomy was performed without disrupting the dura. A cap was prepared from the female portion of a Luer-Loc needle hub, and the cap was fixed over the craniotomy using cyanoacrylate gel and methyl methacrylate (Hygenic Corp., Akron, OH). The incision was sutured at the anterior and posterior edges, and topical Lidocaine ointment was applied. Rats were then placed in a heated recovery cage and monitored until ambulatory.

For injury induction, rats were re-anesthetized (60–90 min after surgery) with 5% isoflurane delivered for 5 minutes. The dura was inspected through the needle hub to ensure it was

Table 1 Diet compositions

Ingredient (wt% diet)	No DHA diet	DHA diet
Casein	20.00	20.00
L-cystine	0.30	0.30
Sucrose	10.00	10.00
Cornstarch	39.75	39.75
Dyetrose[a]	13.20	13.20
Cellulose	5.00	5.00
Mineral Mix 210025[b]	3.50	3.50
Vitamin Mix 310025[c]	1.00	1.00
Choline	0.25	0.25
Fat composition	(wt% diet)	
Total	7.00	7.00
Coconut oil	2.34	2.30
Safflower oil	3.82	3.68
Soybean oil[d]	0.84	0.84
DHASCO oil[e]	0.00	0.18
Fatty acid composition	(wt% total fatty acids)	
ΣSat	41.06	41.14
ΣMono	41.71	41.14
18:2n – 6	15.88	15.45
18:3n – 3	1.29	1.28
20:4n – 6	0.02	0.01
22:6n – 3	0.00	0.92
ΣPUFA	17.30	17.78
Σn – 3	1.37	2.29
Σn – 6	15.92	15.49
n – 6:n – 3	11.59	6.76

Abbreviations: ΣSat, summation of saturated fatty acids; ΣMono, summation of monounsaturated fatty acids; ΣPUFA, summation of polyunsaturated fatty acids; Σn – 3, summation of omega-3 fatty acids; Σn – 6, summation of omega-6 fatty acids; n – 6:n – 3, omega-6:omega-3 fatty acid ratio; DHA, docosahexaenoic acid.
[a]Carbohydrate composition (%): monosaccharides, 1; disaccharides, 4; trisaccharides, 5; tetrasaccharides and higher, 90.
[b]AIN-93G mineral mix (mg/100 g diet): calcium, 500; phosphorus, 156.1; potassium, 360; sodium, 101.9; chloride, 157.1; sulfur, 30; magnesium, 50.7; iron, 3.5; copper, 0.6; manganese, 1; chromium, 0.1; iodine, 0.02; selenium, 0.02; fluoride, 0.1; boron, 0.05; molybdenum, 0.02; silicon, 0.5; nickel, 0.05; lithium, 0.01; vanadium, 0.01.
[c]AIN-93VX vitamin mix (units/100 g diet): thiamin, 0.6 mg; riboflavin, 0.6 mg; pyridoxine, 0.7 mg; niacin, 3 mg; pantothenate, 1.6 mg; folate, 0.2 mg; biotin, 0.02 mg; cyanocobalamin, 2.5 mg; vitamin A, 400 IU; vitamin E, 7.5 IU; vitamin D3, 100 IU; vitamin K1, 0.08 mg.
[d]TBHQ-free.
[e]Provided by DSM Nutritional Products.

intact with no debris. The hub was then filled with normal saline and attached to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). An injury of moderate severity for the model employed (2.0 atm) was administered
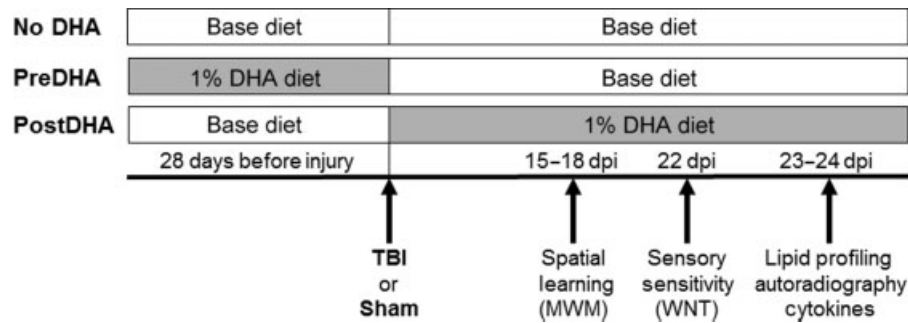
Fig. 1 Experimental design. All rats received base diet containing No DHA (clear bars) or an equivalent diet containing DHA as ~1% of the total fatty acids (gray bars). The No DHA group received base diet for 28 days prior to surgery/injury and base diet for the remaining 23 to 24 days of the study. The PreDHA group received the DHA diet for 28 days prior to injury/surgery (TBI or Sham) followed by base diet for the remainder of the experiment. The PostDHA group received base diet prior to injury/surgery, followed by DHA diet for the rest of the study. Each experimental arm included both TBI and Sham animals. Evaluations of spatial learning in the MWM occurred at 15 to 18 dpi, and assessments of sensory sensitivity (WNT) occurred at 22 dpi. Tissues for lipid profiling, autoradiography and cytokine analyses were then collected at 23 or 24 dpi. See text for details. DHA, docosahexaenoic acid; dpi, days post-injury; PostDHA, post-injury DHA; PreDHA, pre-injury DHA; TBI, traumatic brain injury; WNT, whisker nuisance task.

by releasing the pendulum of the fluid percussion device from a calibrated height and onto the fluid-filled cylinder. Sham-injured rats underwent the same procedure, but the pendulum was not released. Rats were monitored for the presence of a forearm fencing response, and righting reflex times were recorded for the injured rats as indicators of injury severity.[31] The injury hub was removed, and the brain was inspected for uniform herniation and integrity of the dura through the craniotomy. The incision was cleaned using saline and closed using sutures. Moderately brain-injured rats had righting reflex recovery times >5 minutes and a positive fencing responses, and the injury severities were comparable amongst all brain-injured diet groups. Sham-injured rats recovered the righting reflex within 20 seconds. After spontaneously righting, rats were placed in a heated recovery cage and were monitored until ambulatory (~5 to 15 minutes) before being returned to their cage. Adequate measures were taken to minimize pain or discomfort. Mortality was not encountered as a result of surgery or TBI.

Morris Water Maze

Spatial learning ability was assessed in all 75 rats using a MWM testing paradigm similar to those used in other experimental models of TBI.[32–38] The chief difference in our longer term approach was that testing occurred on days 15 to 18 post-injury, while others have typically tested within the first 2 weeks after injury. Briefly, animals were tested in a circular pool (127-cm diameter × 56-cm height) containing a circular platform (13.5-cm diameter) that was submerged 1 cm below the waterline in a single quadrant of the pool. Black, non-toxic powdered paint was added to obscure the platform. The lighting of the room and its spatial cues were constant throughout the testing period. On days 15 to 18 after injury or sham surgery, the rats were tested in four trials per day (total of 4 days of testing and 16 acquisition trials). Each trial lasted 60 seconds, and there was a 5-minute inter-trial interval.[32] Rats started from one of the four pool quadrants, and the starting point varied for each animal and each testing day. If an animal did not find the platform within 60 seconds during a

given trial, it was placed on the platform by the handler and was allowed to rest for 15 seconds. Four hours following the last acquisition trial, the platform was removed, and a 30-second retention trial was performed. If an effect on retention was detected at this 4-hour time point, the retention testing was repeated 24 hours after the last acquisition trial. Every trial was recorded and analyzed with Accuscan Instruments EzVideoDV Automated Tracking System (Columbus, OH), allowing for the quantification of swimming distance, escape latency, and swimming speed.

Whisker Nuisance Task

Sensory sensitivity was assessed by the WNT that has been described previously.[27,39] A darkened plastic test cage (16.5 × 38.1 × 55.9 cm) lined with an absorbent pad was used. On day 22 post-injury, a time point that was chosen specifically to allow for the largest and established dynamic range in this measure, rats were acclimated to the test cage for 5 minutes prior to testing. Testing involved manual stimulation of the whiskers of both mystacial pads with a wooden applicator stick for 3- and 5-minute periods with an inter-trial interval of ≤1 minute. Animals were tested individually, the test cage was cleaned after every test, and a fresh absorbent pad and wooden applicator stick was used for each animal. For each trial, observations were recorded as discontinuous, categorical data for (1) movement, (2) stance and body position, (3) breathing quality, (4) whisker position, (5) whisking response, (6) evading stimulation, (7) response to stick presentation, and (8) grooming response. Normal behavior for each category was equivalent to that seen in uninjured animals during stimulation and received a score of zero. For example, an animal that was relaxed and looking upward during stimulation was given a score of 0 for stance and body position. Abnormal behaviors expressed in response to whisker stimulation were assigned scores of 1 to 2 depending on the degree of expression. For example, an animal that cowered and exhibited a guarded position was given a score of 2 for stance and body position. The maximum whisker nuisance score was 16 (two points for each of eight

categories), where each 2-point increase indicated the expression of 1 to 2 behaviors. Higher scores indicate abnormal responses to the stimulation overall, in which the rat freezes, becomes agitated or is aggressive. Lower scores indicate normal responses, in which the rat is either soothed or indifferent to the stimulation. A 5-minute period of non-stimulation was scored immediately following the last trial as a control for spontaneously occurring behaviors.[27,39]

Tissue Extraction
On day 23 or 24 post-TBI, tissues were harvested after rapid decapitation for lipid profiling, receptor autoradiography, and cytokine protein levels. Directly after decapitation, the brain was extracted, flash frozen in isopentane, chilled on dry ice, and the headspace of each sample tube was purged with nitrogen. Brains were then stored at –80°C until use. During the brain extraction, a separate technician handled the remaining carcass and collected ∼3 mL of whole, trunk blood. Each whole blood sample was split equally and centrifuged at 100g for 15 minutes at 4°C. Plasma was transferred to a fresh tube, the tube's headspace was purged with nitrogen gas, and the plasma sample was then flash frozen on dry ice and stored at –80°C until use. The remaining RBC pellet was resuspended in two volumes of isotonic saline solution and was centrifuged again. The supernatant was removed, and the pellet was resuspended again in two volumes of isotonic saline. The pelleted RBCs and the isotonic saline supernatant were then flash frozen on dry ice, the tube's headspace was purged with nitrogen gas, and the sample was stored at –80°C until use.

Fatty Acid Profiling
All brain, plasma, and RBC samples directed toward lipid profiling were freeze-dried prior to esterification.[40] Briefly, tricosanoic free fatty acid was added to each sample as an internal standard. Samples were converted to methyl esters using 1.5N Methanolic HCl, at 100°C for 60 minutes. Analysis was done with a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector. The fatty acid methyl esters were separated on a 30-m FAMEWAX capillary column (Restek; 0.25 mm diameter, 0.25 μm coating thickness) using hydrogen at a flow rate of 40 mL/min with a split ratio of 48:1. Chromatographic run parameters included an oven starting temperature of 130°C that was increased at 6°C/min to 225°C, where it had been held for 20 minutes before increasing to 250°C at 15°C/min, with a final hold of 5 minutes. The injector and detector temperatures were constant at 220°C and 230°C, respectively. Peaks were identified by comparison of retention times with reference standard mixtures from NuCheck Prep (Elysian, MN, USA). Standard curves were generated using the ratio of reference standard to internal standard and were then used for quantitation by correlating the sample ratios.

[³H]PK11195 Autoradiography
In the uninjured rat brain, expression of the translocator protein 18 kDa (TSPO) receptor is restricted to the ependymal cells that line the cerebral ventricles and the choroid plexus.

However, after TBI or other neuroinflammatory events in the CNS, expression of the TSPO increases in activated microglia/macrophages. [³H]PK11195 binds selectively to the TSPO and previous studies have used [³H]PK11195 autoradiography to quantify changes in brain neuroinflammation following experimental TBI.[28,41–43] The fresh frozen brains directed toward this measure were cryosectioned at 16 μm and mounted on SuperFrost Plus slides. The sections were incubated in 1 nM [³H]PK11195 (specific activity 85.5 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA, USA) for 2 hours at room temperature, and the sections were then rinsed and dried. RayMax Beta High Performance Autoradiography Film was used to visualize ligand binding. All films were processed using Kodak GBX developer. Binding data were analyzed using NIH image v1.59 with a Sony XC-77 CCD camera via a Scion LG-3 frame-grabber. The cortex (18–24 sections per rat), hippocampus (18–24 sections per rat), thalamus (9–13 sections per rat), and substantia nigra (6 sections per rat) were outlined manually according to their anatomical boundaries,[44] and, for each brain region, sham rat sections were measured to acquire the background intensity level. The threshold intensity value was then set at the highest grayscale value measured for any sham-operated rat, and the percentage of pixels over threshold was recorded for each region.

Cytokine Protein Measurements
The remaining fresh-frozen brain and plasma samples were prepared for the measurement of TNF-α, IL-10, IL-1β, IL-6, and TGF-β by multiplexed, enzyme-linked immunosorbent assay (ELISA). Brain samples were thawed on ice, and the somatosensory cortex and hippocampus were dissected away from the rest of the brain. The samples were then homogenized in ice-cold (4°C) phosphate buffered saline containing 100 μM phenylmethylsulfonyl fluoride with a Precellys-24 homogenizer (1.4 mm ceramic bead kit). Similarly, plasma was thawed and diluted 1:40 in ice-cold (4°C) phosphate buffered saline containing 100 μM phenylmethylsulfonyl fluoride. Samples were centrifuged at 10,000g for 5 minutes, and the supernatants (∼200 to 250 μL) were collected for the ELISA assays.

Chemiluminescent multiplex assay kits for rat TNF-α, IL-10, IL-1β, IL-6, and for human TGF-β were run according to the manufacturer's protocols (Quansys Biosciences; Logan, UT, USA). Assay limits and standard curves were as follows: TNF-α (0.8–28,400 pg/mL), IL-10 (0.2–7,400 pg/mL), IL-1β (0.3–20,000 pg/mL), IL-6 (17.0–28,800 pg/mL), and TGF-β (1.8–29,000 pg/mL). The samples and standards had been incubated in the ELISA plates for 12 hours at 4°C. The chemiluminescent signals were detected with the Quansys Q-View Imager Pro and its associated Q-View Software with an image exposure time of 270 seconds. The ELISA data for each sample were then normalized to the protein concentration of the sample as determined by a Biorad DC Protein Assay Kit (Hercules, CA, USA).

Statistical Analysis
Initial group sizes were determined by power analysis of earlier existing data for each outcome measure (Minitab 17), and the final group sizes are indicated in the figure legends.

The data were graphed using GraphPad Prism 6 and analyzed using GraphPad 6 or SPSS 23.0. Fatty acid profiles were compared by using 2-way analysis of variance (ANOVA; injury × diet) followed by Tukey's *post hoc* test for each fatty acid measured. The data resulting from the autoradiography and the cytokine ELISAs were analyzed by 1-way ANOVA followed by Tukey's *post hoc* test. The WNT data were not continuous; therefore, they were analyzed with the non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison test. Swimming distances and latencies to find the hidden escape platform in the MWM were analyzed by linear regression, by area under the curve, and by 2-way repeated measures ANOVA (injury × diet), with testing day as the repeated measure, followed by Tukey's *post hoc* test. Swimming speeds and memory recall probe tests in the MWM were analyzed with 1-way ANOVA followed by Tukey's *post hoc* test. In all analyses, statistical significance was assigned when the α value (p) was <0.05.

Results

Sham Outcomes

DHA administration increased DHA brain content in uninjured sham animals [$F_{(2,12)} = 7.78$, $p = 0.007$; ►Fig. 2A]. Therefore, uninjured shams treated with No DHA, preoperative DHA (PreDHA) and postoperative DHA (PostDHA) were kept as separate treatment groups for the lipid profiling analyses. In contrast, administration of DHA to uninjured sham rats did not affect [^{3}H]PK11195 binding [somatosensory cortex, $F_{(2,14)} = 0.15$, $p = 0.87$; hippocampus, $F_{(2,14)} = 1.49$, $p = 0.26$; thalamus, $F_{(2,14)} = 0.33$, $p = 0.73$; substantia nigra, $F_{(2,14)} = 0.23$, $p = 0.80$], cytokine levels in somatosensory cortex [TNF-α, $F_{(2,12)} = 1.24$, $p = 0.33$; IL-10, $F_{(2,11)} = 0.44$, $p = 0.66$; IL-1β, $F_{(2,11)} = 0.75$, $p = 0.50$; IL-6, $F_{(2,12)} = 0.25$, $p = 0.78$], WNT scores [$H_{(2)} = 4.40$, $p = 0.11$], latency to find the platform in the MWM [$F_{(2,27)} = 0.36$, $p = 0.70$], total distance to find the platform in the MWM
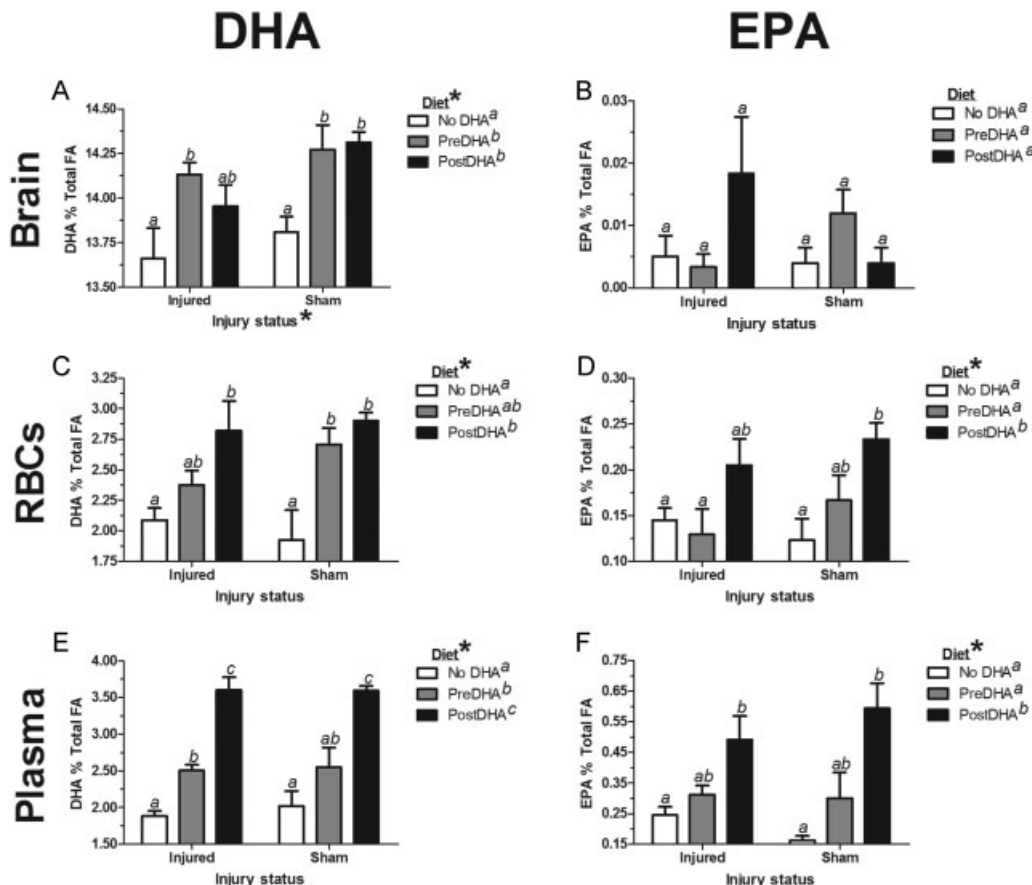

Fig. 2 TBI selectively reduced brain DHA, while dietary DHA increased brain DHA, but not brain EPA. (**A**) Diffuse brain injury significantly reduced brain levels of DHA, and dietary DHA significantly increased brain DHA. (**B**) Brain EPA levels were not affected by TBI or dietary DHA. (**C**) Dietary DHA provided after TBI or sham surgery significantly increased DHA levels in RBC. The effects of preinjury DHA on RBC DHA were intermediate, and TBI did not affect this measure. (**D**) Dietary DHA increased RBC levels of EPA, this effect was most pronounced in PostDHA sham animals, and TBI did not change RBC EPA levels. (**E**) DHA provided both before and after TBI significantly increased plasma DHA, similar effects were noted in sham animals, but TBI did not alter plasma DHA. (**F**) Dietary DHA administered after TBI and sham surgery increased plasma EPA levels significantly, but injury did not affect plasma EPA. *, significant main effect ($p < 0.05$) of injury or diet. Non-matching letters indicate significant differences ($p < 0.05$) in Tukey's *post-hoc* tests. Error bars represent the SEM. Sample sizes: Injured brain, $N = 6$; Sham brain, $N = 5$; Injured RBC and plasma, $N = 4$–6; Sham RBC and plasma, $N = 3$. See text for statistical outputs. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; PostDHA, post-injury DHA; PreDHA, pre-injury DHA; RBCs, red blood cells; SEM, standard error of the mean; TBI, traumatic brain injury.

$[F_{(2,27)} = 0.13, \ p = 0.80]$, or swim speed in the MWM $[F_{(2,27)} = 0.06, \ p = 0.94]$. Therefore, the three sham groups were merged into a combined sham group for analysis of these measures. Subsequently, the sham group size was made comparable to the injured group sizes by using a random number generator method (Microsoft Excel) that selected sham animals randomly for each analysis.

Fatty Acid Profiling

Diffuse brain injury had a main effect of selectively reduced DHA levels in the brain as determined by 2-way (injury × diet) ANOVA [F(1,27) = 5.01, p = 0.034; ►Fig. 2A]. In each dietary treatment group, the brain DHA levels of the injured animals were, on average, lower than the DHA levels in the uninjured sham animals (No DHA Injured = 13.66% vs. No DHA sham = 13.81%; PreDHA injured = 14.13% vs. PreDHA sham= 14.27%; PostDHA injured = 13.96% vs. PostDHA sham = 14.31%). No other fatty acid was affected by injury, although a total of 27 fatty acids were analyzed (►Supplementary Tables S1–S3 (online only) for the complete fatty acid profiles of the brain tissue, RBCs and plasma, respectively).

Diet also had a positive, main effect on brain DHA levels $[F_{(2,27)} = 9.29, \ p < 0.001]$. Within the injured group, PreDHA rats had significantly higher brain DHA levels than No DHA rats, with PostDHA rats exhibiting an intermediate phenotype. Within the sham group, PreDHA and PostDHA animals both had significantly increased levels of brain DHA compared with the No DHA animals. However, brain levels of eicosapentaenoic acid (EPA), a precursor to and retroconversion metabolite of DHA,[45] were not affected by diet or injury (►Fig. 2B).

In contrast to the results in brain tissue, dietary DHA increased the levels of both DHA and EPA in RBCs and plasma, while injury had no effect on these measures. Within the RBCs of the injured group, PostDHA rats had significantly higher DHA levels than No DHA rats had, with PreDHA rats exhibiting an intermediate phenotype. Within the RBCs of the sham group, both PreDHA and PostDHA animals had significantly increased DHA levels when compared with the No DHA animals $[F_{(2,19)} = 9.32, \ p = 0.002; \ $►Fig. 2C]. In general, DHA provided after injury was significantly better at increasing RBC levels of DHA and EPA. However, PostDHA was effective in only driving RBC levels of EPA significantly higher in sham animals, while PreDHA had little effect in these measures $[F_{(2,19)} = 5.11, \ p = 0.017; \ $►Fig. 2D].

The effects of dietary DHA on plasma levels of DHA and EPA were similar to those in RBCs, but they were more pronounced. Dietary DHA had a positive, main effect on plasma DHA $[F_{(2,19)} = 52.54, \ p < 0.001; \ $►Fig. 2E], and both PreDHA and PostDHA generally caused a significant increase in plasma DHA levels when compared with levels in No DHA controls. However, the changes in plasma DHA noted in PreDHA animals were only significantly different from No DHA animals in the injured group, but not in the sham group. Significant increases in plasma EPA were only detected in PostDHA animals $[F_{(2,19)} = 14.74, \ p < 0.001; \ $►Fig. 2F].

The levels of other fatty acids in the brain, RBCs and plasma were also altered significantly by dietary DHA, but not by injury (►Table 2). A small, but significant, increase of $18{:}2n-6$ in the brain was driven by DHA intake. In contrast, brain levels of $22{:}4n-6$ and $22{:}5n-6$ were pushed significantly lower by dietary DHA. The initial 2-way ANOVA for $22{:}5n-6$ reported a main effect of diet $[F_{(2,27)} = 72.46, \ p < 0.001]$ and no effect of injury, but a significant interaction between the two was detected $[F_{(2,27)} = 4.09, \ p = 0.028]$ and called for a separation of these analyses. One-way ANOVA for dietary effects on brain $22{:}5n-6$ resulted in the parameters reported in ►Table 2, and a similar analysis for injury continued to find no significant effect on brain levels of $22{:}5n-6$. The RBC and plasma levels of $22{:}4n-6$ and $22{:}5n-6$ were also decreased by dietary DHA. However, plasma $22{:}5n-3$ was increased by dietary DHA, and this effect was significant in Tukey's *post hoc* test when PostDHA sham animals were compared with No DHA sham animals. No other significant changes in the fatty acid profiles of the three tissues were noted.

Table 2 Other fatty acids in brain, red blood cells and plasma that were affected by dietary DHA[a]

Brain	No DHA injured	PreDHA injured	PostDHA injured	No DHA sham	PreDHA sham	PostDHA sham	Main effect of DHA Diet	p
$18{:}2n-6$	0.57 ± 0.02^a	0.64 ± 0.03^b	0.63 ± 0.02^{ab}	0.57 ± 0.02^a	0.60 ± 0.01^{ab}	0.64 ± 0.02^{ab}	$F_{(2,27)} = 5.39$	0.011
$22{:}4n-6$	3.68 ± 0.06^a	3.51 ± 0.03^b	3.52 ± 0.03^b	3.63 ± 0.04^a	3.40 ± 0.02^b	3.50 ± 0.04^b	$F_{(2,27)} = 14.47$	<0.001
$22{:}5n-6$	0.73 ± 0.02^a	0.46 ± 0.02^b	0.53 ± 0.02^c	0.66 ± 0.01^a	0.46 ± 0.03^b	0.58 ± 0.02^c	$F_{(2,30)} = 63.80$	<0.001
RBC	No DHA injured	PreDHA injured	PostDHA injured	No DHA sham	PreDHA sham	PostDHA sham	Main effect of DHA diet	p
$22{:}4n-6$	2.00 ± 0.10^a	1.46 ± 0.07^b	1.61 ± 0.15^b	1.95 ± 0.10^a	1.59 ± 0.04^{ab}	1.73 ± 0.07^{ab}	$F_{(2,19)} = 6.74$	0.006
$22{:}5n-6$	0.57 ± 0.02^a	0.34 ± 0.02^b	0.34 ± 0.03^b	0.53 ± 0.06^a	0.38 ± 0.04^b	0.39 ± 0.05^b	$F_{(2,18)} = 59.13$	<0.001
Plasma	No DHA injured	PreDHA injured	PostDHA injured	No DHA sham	PreDHA sham	PostDHA sham	Main effect of DHA diet	p
$22{:}4n-6$	0.34 ± 0.02^a	0.29 ± 0.01^{ab}	0.24 ± 0.02^b	0.28 ± 0.03^{ab}	0.31 ± 0.02^{ab}	0.27 ± 0.04^{ab}	$F_{(2,19)} = 3.53$	0.049
$22{:}5n-3$	0.32 ± 0.02^a	0.32 ± 0.02^a	0.35 ± 0.03^a	0.25 ± 0.04^a	0.29 ± 0.02^a	0.43 ± 0.03^b	$F_{(2,19)} = 7.35$	0.004
$22{:}5n-6$	0.34 ± 0.04^a	0.24 ± 0.02^a	0.12 ± 0.03^b	0.34 ± 0.02^a	0.25 ± 0.04^{ab}	0.15 ± 0.04^b	$F_{(2,19)} = 17.21$	<0.001

Abbreviations: DHA, docosahexaenoic acid; RBC, red blood cell.
[a]Data are presented as the mean percentage of total fatty acids ± standard error of mean. Non-matching letters indicate significant differences ($p < 0.05$) in Tukey's *post-hoc* tests. Sample sizes are the same as in ►Fig. 2.

Microglial/Macrophage Activation

The effect of DHA on mFPI-induced microglial/macrophage activation has not been evaluated previously. ►**Fig. 3** indicates that TBI increased [³H]PK11195 binding qualitatively and that both PreDHA and PostDHA reduced that increase. However, the effect of DHA was most evident in PostDHA rats. All brain-injured groups exhibited regions of increased microglial activation, particularly in the somatosensory cortex, hippocampus, and thalamus. All three of these brain regions are in the path of the mFPI-induced percussion wave that first passes through the sagittal midline of the brain from superior to inferior. The wave then reflects laterally off the base of the skull at ~45° in both directions.[46] Thus, the pattern of [³H]PK11195 binding in the injured animals matches the path of the percussion injury wave (►**Fig. 3A–C**). Conversely, uninjured sham rats displayed minimal glial activation, with [³H]PK11195 binding being limited to areas lacking an intact blood–brain barrier (a normal phenomenon; ►**Fig. 3D**).

The extent of area exhibiting [³H]PK11195 binding was analyzed in the somatosensory cortex, hippocampus, thalamus, and substantia nigra (►**Fig. 4A–D**). Main effects of treatment were detected in the somatosensory cortex [$F_{(3,33)} = 13.40$, $p < 0.001$; ►**Fig. 4A**] and hippocampus [$F_{(3,33)} = 13.93$, $p < 0.001$; ►**Fig. 4B**] with No DHA or PreDHA rats having significantly greater binding in the cortex and hippocampus compared with uninjured sham rats. PostDHA rats exhibited binding in these two regions that was equivalent

to sham rats and significantly lower than both other injured groups. A main effect of treatment was also found in the thalamus [$F_{(3,33)} = 4.16$, $p = 0.01$; ►**Fig. 4C**] and substantia nigra [$F_{(3,33)} = 7.15$, $p = 0.001$; ►**Fig. 4D**] with No DHA rats having significantly higher binding area compared with uninjured sham rats. In the substantia nigra, the binding in No DHA rats versus PostDHA rats also differed significantly.

Cytokine Levels

The activation data acquired through [³H]PK11195 binding suggested that accordant changes in cytokine levels might also occur. Therefore, the protein expression levels of TNF-α, IL-10, IL-1β, IL-6, and TGF-β in the remaining samples of somatosensory cortex, hippocampus, and plasma were determined by multiplexed ELISA. Since the effect of DHA on [³H]PK11195 binding was more profound in the hippocampus than in the thalamus, the hippocampus was selected over the thalamus. As ►**Fig. 5** illustrates, main effects of treatment were detected in the somatosensory cortex for TNF-α [$F_{(3,16)} = 6.68$, $p = 0.004$; ►**Fig. 5A**], IL-10 [$F_{(3,19)} = 5.68$, $p = 0.006$; ►**Fig. 5B**], and IL-1β [$F_{(3,19)} = 3.41$, $p = 0.039$; ►**Fig. 5C**], but not for IL-6 (►**Fig. 5D**). No DHA rats had significantly higher levels of TNF-α and IL-10 in the somatosensory cortex compared with PreDHA, Post DHA, and uninjured sham rats, and PreDHA and PostDHA rats exhibited levels of these cytokines that were equivalent to sham rats. However, IL-1β levels in No DHA rats were only
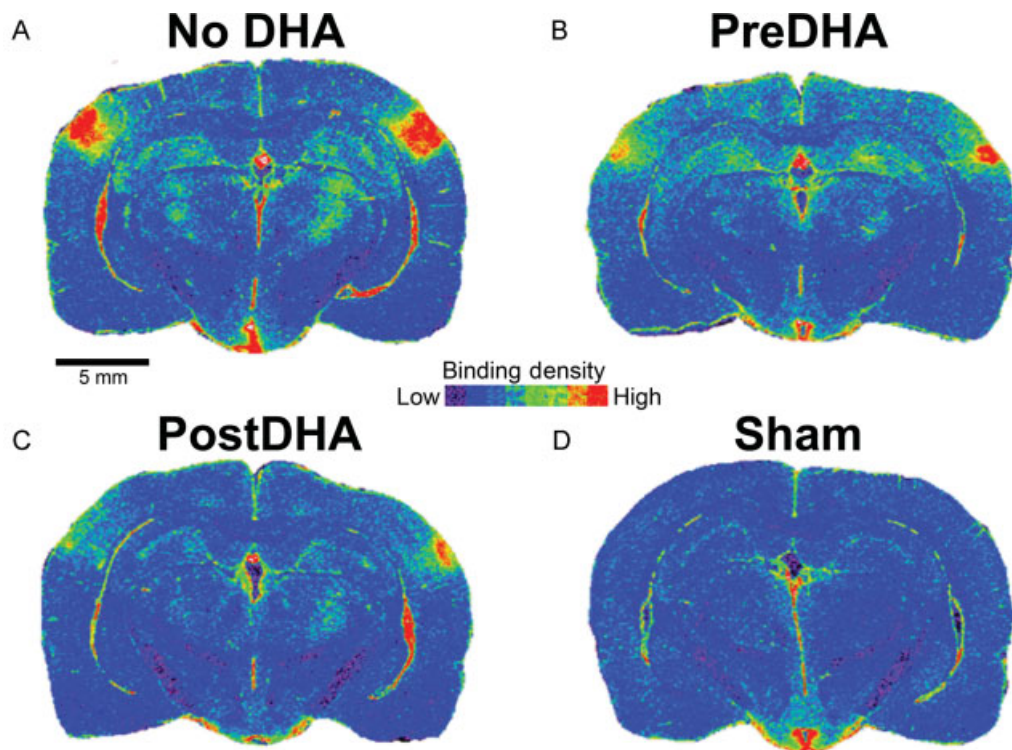

Fig. 3 Dietary DHA reduced microglial/macrophage activation following TBI. [³H]PK11195 autoradiography was used to label activated microglia/macrophages at 23 to 24 days post-injury. Representative coronal sections from (**A**) No DHA rats, (**B**) PreDHA rats, (**C**) PostDHA rats and (**D**) Uninjured sham rats labeled by [³H]PK11195 radioligand binding, with relative binding densities ranging from minimal (blue), to moderate (yellow, green), to maximal (red). All brain-injured groups exhibited focal regions of increased activation, while uninjured sham rats displayed minimal [³H]PK11195 binding. The 5-mm scale bar in (A) also applies to all other panels. DHA, docosahexaenoic acid; PostDHA, post-injury DHA; PreDHA, pre-injury DHA.
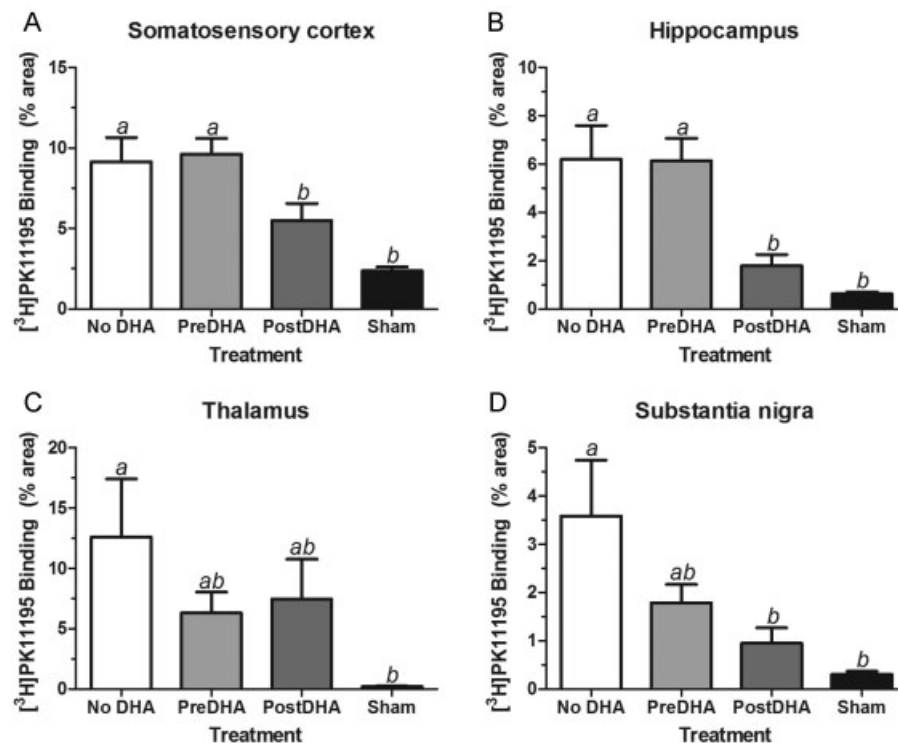
Fig. 4 Post-injury DHA quantitatively reduced [³H]PK11195 binding in key brain regions. In both cortex (**A**) and hippocampus (**B**), brain-injured rats given No DHA or PreDHA had significantly larger areas of binding than sham rats. In contrast, the extent of binding in PostDHA rats was equivalent to sham rats and significantly lower than both of the other injured groups. (**C**) In the thalamus, No DHA rats had significantly higher binding area than sham rats, while rats receiving PreDHA or PostDHA had binding that was equivalent to that in the shams and the No DHA controls. (**D**) In the substantia nigra, No DHA rats had significantly higher binding than sham and PostDHA treated rats, with PreDHA rats exhibiting an intermediate phenotype. Non-matching letters indicate significant differences ($p < 0.05$) in Tukey's *post hoc* tests. Error bars represent the SEM. Sample sizes: No DHA, $N = 9$; PreDHA, $N = 8$; PostDHA, $N = 8$; combined sham, $N = 9$. See text for statistical outputs. DHA, docosahexaenoic acid; PostDHA, post-injury DHA; PreDHA, pre-injury DHA; SEM, standard error of the mean.

significantly higher when compared with sham rats, with IL-1β in PreDHA and PostDHA rats at intermediate levels and not differing significantly from either No DHA rats or sham rats. The level of IL-6 in the somatosensory cortex was equivalent between all four groups, while TGF-β was not detectable in any of the brain samples (*data not shown*). All five cytokines were detectable at lower levels in the hippocampus, but there were no significant effects of treatment (*data not shown*). Cytokine analysis was performed in plasma for the sake of completeness. However, as expected,[47] all cytokines had recovered to baseline levels, and group differences were not evident (*data not shown*).

Sensory Sensitivity

We have previously reported increased sensory sensitivity in rats following mFPI as a late-onset, injury-induced morbidity using the WNT.[27] In the current study, a main effect of treatment was detected on sensory sensitivity [$H_{(3)} = 10.80$, $p = 0.013$; ►Fig. 6] with No DHA rats exhibiting significantly higher nuisance scores compared with the sham rats. However, both PreDHA and PostDHA rats were not significantly different from the shams or from the No DHA group. There were no significant differences in sensory sensitivity when the animals were scored without whisker stimulation immediately following the testing period (*data not shown*).

Spatial Learning

The distance traveled and the latency to find the submerged platform in the MWM were measured to assess spatial learning acquisition on days 15 to 18 post-injury. Swimming speed was also evaluated as a control for any potential gross motor dysfunction. As ►Fig. 7A illustrates, two-way, repeated measures ANOVA indicated that all groups had significant, time-dependent improvements in the distance traveled to the hidden platform [$F_{(3,168)} = 33.81$, $p < 0.001$], but there was no main effect of treatment. Similarly, linear regression analysis and area under the curve analysis did not detect group differences in the slopes or areas plotted by the data. However, the *post hoc* tests in the RMANOVA and a significant main effect in the *Y* intercepts [$F_{(3,235)} = 2.90$, $p = 0.036$] calculated by the linear regressions found that PostDHA animals swam significantly shorter distances than No DHA animals at 15 days post-injury. The Y-intercept for the No DHA group was 4289 ± 661.3 cm and differed significantly [$F_{(1,117)} = 8.56$, $p = 0.004$] from the Y-intercept of the PostDHA group (3342 ± 492.4 cm). No other group differences were found.

Analyses of the latencies to the find the hidden platform detected the same time-dependent improvements in performance [$F_{(3,168)} = 36.82$, $p < 0.001$; *data not shown*], but, regardless of the analysis used, no group differences were
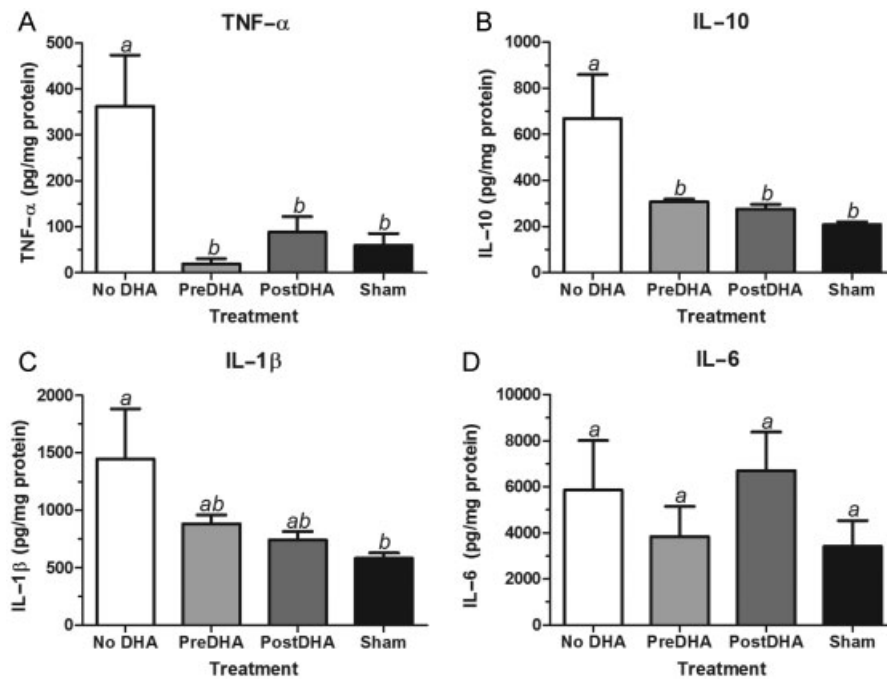
Fig. 5 Both pre- and post-injury DHA reduced injury-related cytokines in the somatosensory cortex. The protein levels of (**A**) TNF-α and (**B**) IL-10 in No DHA rats were significantly higher when compared with those in PreDHA, PostDHA and sham rats. Similar effects were noted for (**C**) IL-1β. However, only sham rats differed significantly from No DHA rats, while PreDHA and PostDHA rats exhibited intermediate phenotypes. (**D**) Treatment did not affect IL-6 levels in the somatosensory cortex. Non-matching letters indicate significant differences ($p < 0.05$) in Tukey's *post hoc* tests. Error bars represent the SEM. Sample sizes: No DHA, $N = 5$; PreDHA, $N = 5$ to 6; PostDHA, $N = 5$ to 6; combined sham, $N = 5$ to 6. See text for statistical outputs. DHA, docosahexaenoic acid; PostDHA, post-injury DHA; PreDHA, pre-injury DHA; SEM, standard error of the mean.

found. Furthermore, neither the probe trial for memory recall at 4 hours after the last acquisition trial found significant effects of treatment (*data not shown*), nor were there

Fig. 6 Dietary DHA mitigated injury-induced increases in sensory sensitivity. Post-injury sensory sensitivity in the No DHA group was significantly higher in comparison to uninjured shams. In contrast, the PreDHA and PostDHA groups exhibited intermediate phenotypes that did not differ significantly from the uninjured shams or the No DHA controls. Non-matching letters indicate significant differences ($p < 0.05$) in Dunn's *post hoc* tests. Error bars represent the SEM. Sample sizes: No DHA, $N = 15$; PreDHA, $N = 13$; PostDHA, $N = 13$; Combined Sham, $N = 15$. See text for statistical output. DHA, docosahexaenoic acid; PostDHA, post-injury DHA; PreDHA, pre-injury DHA; SEM, standard error of the mean.

any significant differences when swim speed was analyzed (►**Fig. 7B**). Therefore, memory recall trials were not performed at 24 hours after the last acquisition trial, and swim distance was used as the measure of learning acquisition.

Discussion

The current study was designed with the goals of (1) directly comparing the efficacy of dietary DHA before, versus after, diffuse brain injury on translational outcomes that were measured more than 2 weeks after injury, (2) establishing a comprehensive fatty acid profile detailing the influences of DHA and its interactions with TBI, and (3) contributing to the understanding of the role of DHA in trauma-induced neuroinflammation. This study clearly demonstrated that DHA was selectively reduced in the injured brain and that dietary DHA alleviated this loss. In addition, dietary DHA reduced cytokine levels and mitigated sensory sensitivity that were both increased by TBI. These effects were measurable ∼3 weeks after TBI, the longest period, to our knowledge, that has been used to assess the specific role of DHA in TBI. Nonetheless, the findings were somewhat asymmetrical in that PostDHA generally showed greater efficacy than PreDHA.

The comprehensive lipid profiling indicated that no other brain fatty acid but DHA was reduced after diffuse TBI. This finding is consistent with earlier work[48] showing that brain DHA decreased after lateral fluid percussion injury. However, this earlier work involved a harsher injury model that induced gross anatomical damage, and it did not provide details on any
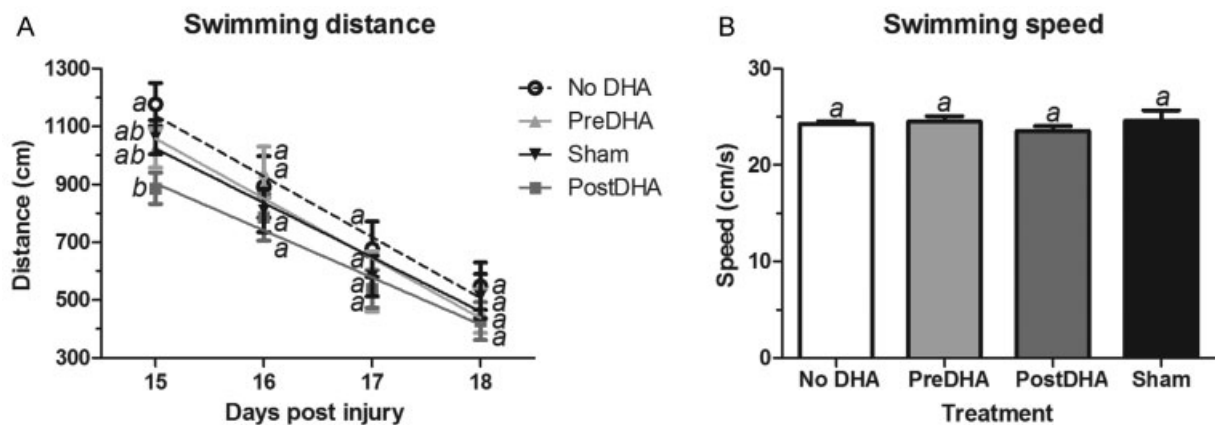
Fig. 7 Longer term spatial learning was not affected by brain injury or dietary DHA. (**A**) A significant, time-dependent shortening in the distances travelled to the platform indicated that all animals learned the task. There were no main effects of diet or injury, but *post hoc* tests and linear regression detected a significant difference between the distances traveled by No DHA rats versus PostDHA rats at 15 days post-injury. (**B**) Average swim speed was not affected by diet or injury status. Non-matching letters indicate significant differences ($p < 0.05$) in *post hoc* tests and in the Y intercepts determined by linear regression analysis. Error bars represent the SEM. Sample sizes: No DHA, $N = 15$; PreDHA, $N = 15$; PostDHA, $N = 15$; Combined Sham, $N = 15$. See text for statistical outputs. DHA, docosahexaenoic acid; PostDHA, post-injury DHA; PreDHA, pre-injury DHA; SEM, standard error of the mean.

other fatty acids except $22:5n – 3$, which increased in their hands. Given that mFPI does not result in gross anatomical damage, the reduction in brain DHA after any brain injury is, therefore, likely, regardless of the TBI model used.

The reduction of brain DHA by mFPI was significant overall, but the magnitudes of these changes were small. The reducing effect of injury on brain DHA was between 0.14 and 0.35% of total fatty acids, which amounted to an average, relative reduction of ~1% across all three treatment groups when compared with their respective sham groups. The nervous system has a remarkable ability to retain DHA,[49] which provides some explanation for these small, but consistent, differences after TBI. However, arguably smaller differences in DHA status have had dramatic impacts on outcomes in other models of nervous system injury. For example, a single, intravenous injection of DHA (80 μg/kg bodyweight), provided 30 minutes after spinal cord injury in rats, increased functional motor recovery.[50–52] Similarly, a single, intravenous injection of DHA (5 mg/kg bodyweight) improved outcomes after the induction of experimental stroke in rats.[53–55] Such treatment regimens could not be expected to significantly change DHA levels in the tissue.[56] Importantly, the addition of dietary DHA for 6 weeks after spinal cord injury increased functional recovery beyond that conferred by a single DHA injection alone.[57] Thus, large changes in brain DHA status may not be required for significant effects on some phenotypes, and the sustained availability of DHA, either already in the body or in the diet, was important to the significant changes that we have reported here.

More refined time courses would aid in understanding the interaction between brain injury and brain DHA content, but the experiments reported here and elsewhere provide some guidance. Omega-3 sufficiency in adult rodents results in brain DHA levels that are in the range of 10 to 16%, and a minimum of 3 to 4 weeks on an omega-3-deficient diet is required to induce a significant reduction in DHA. Conversely, the half-time for DHA repletion is ~2.9 weeks in a fully omega-3-deficient, adult

rat.[56,58,59] The current study focused on DHA, and the diets used had a significant amount of $18:3n – 3$ (α-linolenic acid) in an effort to model omega-3 sufficiency, with or without DHA. This diet composition, and the observation that rodents convert $18:3n – 3$ to DHA significantly better than humans do,[60–63] contributed to the high levels of brain DHA observed, even in the No DHA group. Thus, the small, but significant, group differences in diet-induced DHA levels were expected. In addition, the brains of the PreDHA animals in this study were not sampled until 22 to 23 days after the cessation of DHA administration, while the PostDHA animals received 22 to 23 days of DHA prior to brain sampling. As such, the ~3-week, post-injury period was near the minimum amount of time to expect any diet-induced changes in the brain fatty acid profiles in terms of PreDHA effects beginning to diminish and of PostDHA effects accumulating. These circumstances had the result of making the selective, injury-related effects on brain DHA that much more compelling.

Several possible mechanisms could explain the reduction of brain DHA. For example, phospholipase A2 is important for cleaving DHA and other fatty acids from membrane phospholipids.[64] Post-injury activation of phospholipase A2 does occur,[65] perhaps through IL-1β signaling.[47] When these activities are coupled with a compromised blood–brain barrier,[46] it would be consistent with human evidence that DHA levels are increased in the cerebrospinal fluid soon after TBI.[66] Other potential explanations include decreased brain DHA uptake, as seen in models of Alzheimer's disease,[67] or higher rates of brain DHA utilization after TBI since damaged brain cells require more energy.[68] Less conversion of the DHA precursor, $18:3n – 3$ to DHA is also possible, as the enzymes responsible for this conversion may be downregulated by TBI.[48] However, a healthy brain only has a conversion efficiency of ≤1%, most $18:3n – 3$ is converted in the liver, and the brain conversion rates are unchanged by omega-3 deficiency.[60] More data are needed to support these possible explanations, but they are

consistent with the decreased expression of sirtuins, which are metabolic regulators, after TBI.[21]

The type of tissue being sampled also affected the dynamic ranges and power of the fatty acid measurements. As expected from previous work,[56,58,69] the brain was the most refractory to diet-induced changes in the fatty acid profile, followed by the RBCs, and then the plasma. In the periphery, the RBC and plasma fatty acid profiles were also modified by dietary DHA, but TBI had no effect. The finding that brain EPA was not affected by diet lends some support to an independent role of DHA in the current study. Nonetheless, benefits for the brain that may have been conferred through the effects of EPA on peripheral and/or cerebral blood flow cannot be ruled out.[70] Similarly, the significant effects of diet on $18:2n-6$, $22:4n-6$, $22:5n-3$, and $22:5n-6$ are consistent with previous dietary work showing that omega-3 intake regulates the fatty acid desaturase system in a manner that results in these four fatty acids serving as precursor pools or less optimal replacements for arachidonic acid or DHA.[58,59,71,72] Indeed, it may be that alteration of the efficiency of the desaturase system by a single-nucleotide polymorphism may ultimately affect how well a human responds to DHA or omega-3 administration.[61–63]

One limitation of the measures used to detect changes in the brain fatty acid profiles is that the samples were not fixed with microwave irradiation prior to harvesting. Such methods are arguably important when determining the short-term kinetics of fatty acid uptake into the brain.[73] Nonetheless, the rapid harvesting and freezing methods that we employed have proven more than adequate for numerous other studies,[18,20,48,56,59,69,72] particularly when used consistently across an entire experiment. If the current study had used the microwave method, we speculate that the data variance would have been reduced further and that the selective effect of injury on brain DHA would have been more pronounced.

Recent reports suggest that the neuroprotective effects of omega-3 fatty acids may be, in part, due to specialized pro-resolving mediator (SPM) derivatives,[74] including the resolvins,[75] lipoxins,[76] and protectins.[77] To this effect, our group tested the therapeutic efficacy of two SPMs in a mouse TBI model. The DHA-derived SPM, aspirin-triggered resolvin D1, preserved motor and cognitive function, but the EPA-derived SPM, resolvin E1, did not.[75] Similarly, the DHA-derived SPM, aspirin-triggered neuroprotectin D1, reduced neuroinflammation[78] and improved neurological function after experimental stroke in rats.[77] These data are consistent with our lipid profile findings in that dietary DHA primarily increased brain DHA although both DHA and EPA increased in the RBCs and plasma. Our results are also consistent with the rapid metabolism and β-oxidation of EPA by the brain.[79] Overall, these data add further support to the notion that DHA may play an important role as a precursor for bioactive lipid mediators in the context of acute brain injury.

Following TBI, inflammation is a pivotal, secondary injury process, with evidence for its contribution to both beneficial and detrimental outcomes.[47,80] TBI results in the acute expression of inflammation-mediating cytokines,[81–83] which elicit responses that range from immune activation to pro-

grammed cell death.[84] Furthermore, some cytokine signaling can persist into sub-acute and chronic stages of injury.[9]

Omega-3 fatty acids inhibit the production of cytokines in response to insults both in vitro[85,86] and in vivo.[87] In the current study, dietary DHA suppressed microglial/macrophage activation as measured by [3H]PK11195 autoradiography and by ELISA for inflammation-related cytokines at ~3 weeks after TBI. [3H]PK11195 is a ligand of the translocator protein (TSPO) that is expressed by activated microglia/macrophages, and, to a lesser extent, astrocytes, after brain injury.[28,43,88] The somatosensory cortex and substantia nigra were chosen for TSPO binding because of their vulnerability to mFPI.[28,42] Similarly, the thalamus and hippocampus were chosen for their roles in the WNT and the MWM, respectively.[27,89,90] PostDHA significantly reduced injury-induced TSPO binding in three of the four regions of interest. Furthermore, PreDHA reduced TSPO binding to a point that was neither significantly greater than that of uninjured shams nor significantly lower than that of No DHA rats.

Our TSPO findings are consistent with dietary fish oil effects on the ionized calcium binding adaptor molecule 1 (Iba-1) after TBI. The Iba-1 molecule is similar to TSPO in that both are primarily expressed in activated microglia/macrophages. Immunostaining for Iba-1 was reduced in brain slices derived from mice who had received fish oil diets for 60 days prior to controlled cortical impact (CCI) injury followed by another 35 days of fish oil exposure prior to tissue harvesting (i.e. pre- plus post-injury exposure to omega-3 fatty acids).[91] Overall, these results can be interpreted as a modest reduction in inflammation, but the generalized nature of our [3H]PK11195 binding required that cytokine levels in the brain tissue be evaluated as well.

The levels of IL-1β, IL-6, IL-10, and TNF-α can be altered by omega-3 fatty acids,[85–87] but the time frame after TBI for which these effects can be measured in vivo is not clear. Some reports, including our own in mice,[92] argue that most, if not all, changes in cytokine levels after TBI resolved within 72 hours post-injury.[47] However, elevated levels of IL-6, IL-10, TNF-α, and TGF-β can be detected in the cerebrospinal fluid of brain-injured humans for up to 22 days after TBI.[9] In our hands, both Pre- and PostDHA reduced TNF-α, IL-10, and, to some extent, IL-1β in the somatosensory cortex at ~3 weeks after mFPI. These cytokine findings are consistent with (1) the TSPO binding in these same animals, (2) the effects of a continuous (pre- + post-injury) fish oil diet on Iba-1 immunostaining and protein levels of cyclooxygenase-2 in mice at 35 days post-injury,[91] and (3) that microglial activation can be detected with immunostaining for complement type 3 receptor for up to 28 days after mFPI.[46] If microglial activation is still occurring to a significant degree at such extended post-injury time points, it is logical to hypothesize that cytokine signaling is also still occurring because activated glia release cytokines.[93,94] Given the current results, as well as those of others, it appears that some longer term, secondary neuroinflammatory signals can be detected for at least 21 to 35 days after brain injury in both humans[9,95,96] and rodents.[91,97] Furthermore, these longer term signals can be reduced in

preclinical settings by DHA alone (current study) or by fish oil,[91] which contains DHA and EPA.

The mechanisms by which DHA might regulate injury-induced cytokine levels has been addressed to some extent.[98] One set of possibilities is that PreDHA may enhance the initiation of a beneficial immune response acutely post-injury, while PostDHA may help resolve an ongoing pro-inflammatory response. While this supposition has not been tested directly in the context of TBI in vivo, in vitro pretreatment with DHA increases the phagocytic activity of microglia and promotes the M2, alternatively activated microglial phenotype[99,100] that is more closely associated with tissue repair than with the inflammatory cascades of the M1 phenotype.[101] It, therefore, seems plausible that the intermediate [3H] PK11195 findings we observed for the PreDHA group could have been due to increased phagocytosis being part of an activated microglial phenotype and to DHA being withdrawn from this group directly after injury. In contrast, the Pre- and PostDHA groups both likely benefited from the suppression of M1-related pro-inflammatory cytokines (e.g., TNF-α and IL-1β)[28,101] by DHA or its docosanoid metabolites.[102] These scenarios for both PreDHA and PostDHA would not only explain the differences in [3H]PK11195 binding, but also the reduction in TNF-α, IL-1β, and IL-10 in the somatosensory cortex that was common to both groups.

The IL-10 cytokine is largely considered to be an anti-inflammatory, pro-resolving factor, as it is expressed in response to TNF-α and IL-1β and is involved in reducing their expression.[25,47,93,99,101] Thus, it was expected that IL-10 would be elevated in injured, No DHA animals along with TNF-α and IL-1β. However, in animals exposed to DHA, it appears that the M1-related responses were reduced, which subsequently resulted in significantly less induction of IL-10. Overall, our cytokine results are consistent with a recent TBI study comparing omega-3 sufficient and omega-3 deficient animals for 4 days after controlled cortical impact,[103] except that our cytokine measurements were acquired at ~3 weeks after mFPI and focused on DHA.

There is much left to be understood with regards to cytokine signaling cascades after TBI, their effects on tissue repair and function,[47] and their interactions with DHA.[98] For example, it is not clear why IL-6 was not affected in this study as it is induced by IL-1β and TNF-α,[104] and elevated IL-6 has been reported in the brain at 21 days after controlled cortical impact injury.[105] Two potential explanations exist for this discrepancy. First, the time course of IL-6 expression is shifted to later in the inflammatory cascade, and, second, the IL-6 time course may vary by brain region. In cultures of the cerebral cortex, both IL-1β and TNF-α can induce themselves, and they have upward slopes of expression that do not resolve within 24 hours. In contrast, IL-6 expression peaks in these same cultures at 2 to 6 hours after exposure to IL-1β or TNF-α, followed by a resolving phase that occurs in the presence of high levels of IL-1β and TNF-α.[104] Such conditions would likely contribute to variability in IL-6 levels and result in data that do not differ significantly between treatment groups, particularly if the dynamic range of the response is small and if the tissues from all animals are not harvested at the exact same time. A similar lack of effect on

cortical IL-6 at ~21 days after an inflammatory insult has been reported in several models. In a chemically induced colitis model, IL-6 in the cerebral cortex peaked at 7 days after insult and resolved by 21 to 28 days.[106] In rat models of abdominal surgery and of parasite infection, significant changes in IL-1β and microglia activation were detected in the brain 3 weeks after insult, but changes in IL-6 were not detected.[107,108] Although we did not observe an IL-6 response, it is intriguing that IL-1β,[99,109] IL-6,[110,111] TNF-α,[112,113] IL-10, and TGF-β[114] have all been linked to pain mechanisms or headaches via calcitonin gene-related peptide. Post-traumatic headaches can increase the amount of time needed for cognitive recovery from TBI,[115] are often part of PCS,[26] and can be associated with sensory hypersensitivity[24] and inflammatory cytokines.[25,93]

We have previously reported increased sensory sensitivity in rats following diffuse TBI as a late-onset morbidity[27,39] that generally reflects the clinical symptomology of agitation.[3,4] The current study used the WNT to assess sensory sensitivity 22 days after injury because our previous work had established that this time point is optimal for the dynamic range of the data. Both the PreDHA and PostDHA groups exhibited sensory sensitivity levels not only comparable to the sham group, but also not significantly different from the highest scores in the No DHA group. On average, exposure to DHA before or after TBI attenuated these scores by approximately two points compared with No DHA, injured rats. The magnitude of this effect is meaningful as it indicates that dietary DHA completely ameliorated one adverse reaction to sensory stimulation or reduced the intensity of two different reactions to sensory stimulation. The timing of our DHA administrations cannot delineate the critical period to affect sensory sensitivity; however, histopathology and inflammation data suggest that a window of 7 to 10 days post-injury may be optimal.[28,30,116] Future studies should address this timing aspect as well as the inclusion of a PreDHA + PostDHA study arm, both of which are limitations of not only the current study, but also of most other published studies of TBI and DHA.[18,20–22,48,117] In contrast, omega-3 sufficiency and omega-3 deficiency both before and after TBI have been tested against appropriate controls,[91,103,118] but these studies did not include PreDHA alone or PostDHA alone within the same study as we have done.

Several studies have shown that DHA,[20,119] fish oil (containing both EPA and DHA),[18,21,22,48,91,117] and omega-3 sufficiency[103,118] can attenuate TBI-induced deficits in cognitive function. For example, dietary administration of fish oil after lateral fluid percussion injury preserved cognitive function in the MWM at earlier assessment time points (days 7–12 after TBI).[21,22,48] Earlier work also suggested that deficits in this task would persist for at least 15 days after the mFPI model used in the current study[89] as well as in others.[120] Furthermore, dietary fish oil given to mice for 2 months prior to controlled cortical impact and 35 days thereafter also improved cognitive function in the MWM at 22 to 27 days post-TBI.[91]

Given the above, as well as the interest in longer term, post-TBI effects, we performed MWM testing on post-injury days 15–18. However, the rats in the current study did not exhibit a significant injury-induced cognitive deficit, with only a minor benefit of PostDHA at post-injury day 15. It is

possible that these impairments could have resolved by the time of testing. Such resolution in rats would be consistent with human studies demonstrating that the disruption of verbal memory, visual memory, and reaction time by TBI can generally be resolved in 2 to 3 weeks, unless post-concussive symptoms, such as post-traumatic migraine headache, extend the recovery process.[115] In which case, the observations of DHA benefits for cognition at earlier post-injury time points,[18,20–22,48,103,118] and not the later ones used in this study, are still consistent with DHA improving the rate of cognitive recovery from TBI. It is also possible that alternative paradigms for cognitive testing, such as re-learning or training before the injury and testing after the injury, would be better at resolving the effects of DHA on TBI-induced cognitive deficits at these later time points. Future studies could address such questions.

Two other explanations exist for the water maze data acquired in this study. First, the omega-3 sufficient diet that we used occluded any DHA-specific effects on cognition, but not on the other measures we employed. Such outcomes would be consistent with both the short-term effects[18,20–22,48,103,118] of DHA and/or omega-3 fatty acids on cognition after TBI as well as when either is available throughout an entire, longer term TBI experiment in rodents.[91,108] It should be noted, again, that rodents convert $18:3n - 3$ to DHA significantly better than humans do.[60–63] Another possibility is that lateral FPI, controlled cortical impact, and the impact-acceleration weight-drop models are all known to cause lesions and swelling of the brain parenchyma, which does not typically occur after midline FPI.[121] Thus, as demonstrated in the controlled cortical impact model, it may be that greater injury forces are required to elicit a more sustained cognitive impairment.[91,120] Such circumstances would provide another explanation for the differences between this study and earlier studies[18,20–22,48,103,118,119] with regards to the effects of omega-3 fatty acids on cognition after TBI.

In conclusion, the work reported here clearly demonstrated that TBI resulted in a selective reduction in brain DHA content. Dietary DHA countered the reduction of brain DHA and alleviated injury-related brain inflammation and long-term sensory hypersensitivity. These results indicate that pre-injury supplementation of DHA may offer prophylactic efficacy against trauma-induced deficits, and post-injury supplementation may serve as an intervention to replenish brain DHA content, both of which could lessen the permanent injury burden resulting from TBI. Indeed, recent trials in college football players indicated that prophylactic DHA (2–4 g/d) reduced neurofilament levels in the serum, which is a measure of white matter injury to the brain, after a season of being exposed to subconcussive collisions on the football field.[122]

Conflict of Interests
Christopher M. Butt, Kelly M. Wynalda, and Norman Salem are employees of DSM, a maker of DHA. The studies were conducted at the University of Kentucky and were outside of the control of DSM, with tissue being sent to DSM for the lipid profiling and cytokine studies.

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