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Metabolomics uncovers dietary omega-3 fatty acid-derived metabolites implicated in anti-nociceptive responses after experimental spinal cord injury

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Abstract

Chronic neuropathic pain is a frequent comorbidity following spinal cord injury (SCI) and often fails to respond to conventional pain management strategies. Preventive administration of docosahexaenoic acid (DHA) or consumption of a diet rich in omega-3 polyunsaturated fatty acids (O3PUFAs) confers potent prophylaxis against SCI and improves functional recovery. The present study examines whether this novel dietary strategy provides significant antinociceptive benefits in rats experiencing SCI-induced pain. Rats were fed control chow or chow enriched with O3PUFAs for 8 weeks before being subjected to sham or cord contusion surgeries, continuing the same diets after surgery for another 8 more weeks. The paw sensitivity to noxious heat was quantified for at least 8 weeks post-SCI using the Hargreaves test. We found that SCI rats consuming the preventive O3PUFA-enriched diet exhibited a significant reduction in thermal hyperalgesia compared to those consuming the normal diet. Functional neurometabolomic profiling revealed a distinctive deregulation in the metabolism of endocannabinoids (eCB) and related N-acyl ethanolamines (NAEs) at 8 weeks post-SCI. We found that O3PUFAs consumption led to a robust accumulation of novel NAE precursors, including the glycerophospho-containing docosahexaenoyl ethanolamine (DHEA), docosapentaenoyl ethanolamine (DPEA), and eicosapentaenoyl ethanolamine (EPEA). The tissue levels of these metabolites were significantly correlated with the antihyperalgesic phenotype. In addition, rats consuming the O3PUFA-rich diet showed reduced sprouting of nociceptive fibers containing CGRP and dorsal horn neuron p38 MAPK expression, well-established biomarkers of pain. The spinal cord levels of inositols were positively correlated with thermal hyperalgesia, supporting their role as biomarkers of chronic neuropathic pain. Notably, the O3PUFA-rich dietary intervention reduced the levels of these metabolites. Collectively, these results demonstrate the prophylactic value of dietary O3PUFA against SCI-mediated chronic pain.

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Keywords

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Chronic neuropathic pain is one of the most important determinants in the perceived quality of life of spinal cord injury (SCI) patients (Anderson, 2004). Unfortunately, current therapeutics to treat this condition lack necessary efficacy and are limited in scope by unwanted side effects and poor tolerance. These shortcomings could be partly overcome with the use of preventive approaches that can provide resilience to damage prior to irreversible biochemical alterations have occurred in the perturbed cord. Trauma to the spinal cord triggers a robust secondary pathophysiological response, leading to cell death, inflammation, and dysfunction (Hulsebosch, 2002; Norenberg et al., 2004). Neuroinflammation is regarded as a hallmark mechanism underlying injury progression and pain processing (Christensen and Hulsebosch, 1997a; Hulsebosch et al., 2009), and thus represents an attractive target for therapeutic strategies (Kwon et al., 2011a; Kwon et al., 2011b).

Dietary-essential omega-3 polyunsaturated fatty acids (O3PUFAs), such as docosahexaenoic acid (DHA), are integral components of neural membrane phospholipids and play crucial roles in anti-inflammatory responses (Calder, 2008). Longstanding studies have demonstrated that dietary PUFAs are mediating factors in pain processing, as evidenced by increased threshold for thermal pain and neuropathic pain in rats fed with high omega-3 to omega-6 PUFA ratios (Yehuda and Carasso, 1993). Recent studies have shown that O3PUFAs and their derivatives can exert strong antinociceptive effects against thermal and chemical stimulation in various animal models (Nakamoto et al., 2010; Xu et al., 2010; Tokuyama and Nakamoto, 2011). Given this evidence, it would seem reasonable to consider that dietary O3PUFAs may also play important roles in SCI-induced pain.

In a recent report, we showed that SCI causes a robust PUFA deregulation and leads to a marked DHA deficiency, which was associated with impaired recovery and dysfunction (Figueroa et al., 2013). Notably, administration of O3PUFAs maintained the cord PUFA homeostasis, conferred neuroprotection, prevented dysfunction and facilitated recovery after acute and chronic SCI, even when administered in a prophylactic manner (Figueroa et al., 2012; Figueroa et al., 2013). These findings led us to hypothesize that a preventive diet enriched in O3PUFAs modulates behavioral responses implicated in pathological nociception in rats. This idea is supported by studies showing that the diet type at the time of injury can affect pain behaviors associated with nerve lesions (Shir and Seltzer, 2001; Alloui et al., 2003; Hargraves and Hentall, 2005; Li et al., 2005; Estebe et al., 2006). Despite this evidence, diet remains a largely unexplored therapeutic avenue to ameliorate pain in SCI.

This study is an initial attempt to assess the effects of dietary O3PUFAs on thermal pain stimuli in SCI rats. Because N-Acylated ethanolamines (NAE) and related endocannabinoids (eCBs) are bioactive lipids implicated in pain processing (Beltramo et al., 2006; Petrosino et al., 2007; Hama and Sagen, 2011), we focused on identifying the involvement of dietary O3PUFAs in their local modulation following SCI. Here, the eCB metabolome has been expanded to include the ethanolamines, glycerides, and metabolic precursors, intermediates, and derivatives. These metabolites have been implicated in regulating anti-inflammatory responses and can exert cannabimimetic actions as endogenous agonist of cannabinoid receptors (Devane et al., 1992; Hanus et al., 1993; Priller et al., 1995; Brown et al., 2010), but whether dietary PUFAs impact the levels of these bioactive lipids in SCI has not been comprehensively evaluated. To address this issue, we employed both LC/MS and GC/MS-based metabolomics on cord samples collected from sham and contusion SCI operated

Sprague-Dawley rats that received either control or O3PUFA-enriched diets. Deciphering the neurometabolomic profile that distinguishes pain-like behaviors may have important clinical implications for pain management and allow for improved prognosis in SCI.

2. Experimental Procedures

2.1. Animals

All animal studies were performed in compliance with the Loma Linda University School of Medicine regulations and institutional guidelines consistent with the NIH Guide for the Care and Use of Laboratory Animals. Female Sprague-Dawley rats were received from Charles River Laboratories (Portage, MI) and housed in individual cages on alternating 12 h light/dark cycles. It is worth noting that in this study we used two independent cohorts of animals. Although both cohorts received the same dietary and surgical interventions, and behavioral testing, there were differences in the time allowed for survival (cohort 1: at least 4 animals per diet group, allowed to survive until 12 weeks post-injury; cohort 2: at least 13 animals per diet group, allowed to survive until 8 weeks post-injury). Animals in cohort 2 were also used to determine the effect of dietary O3PUFAs in sensorimotor and autonomic dysfunction after SCI (Figueroa et al., 2013).

2.2. Diet Composition

Custom AIN-93-based diets were prepared with modifications to the fat composition as described previously (Figueroa et al., 2013). Briefly, dietary fats were approximately 6% of the pellets dry weight and were supplied as either soybean oil (control chow) or menhaden fish oil (O3PUFA-enriched chow: DHA = 12.82-gm and EPA = 6.91-gm per 100 gm of diet). Diets were matched for cholesterol content.

2.3. Surgical and Post-Operative Procedures

Eight weeks after the dietary pretreatment, animals were deeply anesthetized with a mixture of ketamine/xylazine (80 mg/kg and 10 mg/kg, respectively). The spinal cord injuries were generated using the well-characterized New York University (NYU) Impactor (Gruner, 1992). Notably, trauma caused using this device induces below-level pain that is well developed and longstanding, suggesting that the model is suitable for chronic pain research (Jung et al., 2008). To produce the contusion, the skin and the muscles overlying the spinal column were cut. A laminectomy was performed at the T9-T10 level and the T8 and T12 spinal processes were clamped to the Impactor, and the exposed dorsal surface of the cord was subjected to weight drop impact using a 10-g rod released from a height of 12.5-mm. Sham animals received only a laminectomy. The animals body temperature was maintained at 37°C during the procedure. After operation, muscle layers were sutured and skin layers closed. The bladders of injured rats were expressed using the Crede's maneuver three times a day until voiding reflexes were restored. Cefazolin (Bristol Myers Squibb, New York, NY; 25 mg/kg, s.q.) and Buprenex® (buprenorphine; Reckett and Colman Pharmaceuticals, Inc. Richmond, VA; 0.05 mg/kg, s.c.) were given to all rats for 5 and 3 consecutive days, respectively. Animals were allowed to survive for 8 or 12 weeks post-operation and the spinal cord tissue dissected for metabolomics and immunohistochemical analyses, respectively.

2.4. Nociceptive Testing

Thermal hyperalgesia (TH) was assessed using the well-established Hargreaves withdrawal test to thermal noxious stimulus (Hargreaves et al., 1988). This behavior has been found to be a sensitive and reproducible behavioral test to investigate chronic neuropathic pain and is exhibited approximately 28 days following contusion injury (Resnick et al., 2004; DomBourian et al., 2006; Rajpal et al., 2007; Cramer et al., 2008). In the week prior to the

baseline recordings, the animals were habituated to the behavioral testing apparatus by undergoing 5 different daily testing sessions. Once plantar paw placement was re-established, rats were evaluated weekly until animals were euthanized. Briefly, the animals were placed in a Plexiglas enclosure that rested on an elevated glass floor (Plantar Test, UGO BASILE, Biological Research Apparatus, Comerio, Italy). After allowing the animals to acclimate to the chamber for 30 min, a movable focused infrared emitter was placed under the animal's paw. A photocell automatically turned the emitter off when the animal moved its paw and the latency time for the animal to withdraw its paw was recorded. Strength of stimulation was adjusted to produce hindpaw baseline latencies close to 12 seconds (approximately 50–60 °C). A safety cutoff of 20 sec was used to prevent prolonged exposure to the noxious heat. Five different trials were performed per paw with at least 5 min allowed between each trial. The instrument operators were blinded to the treatment assignations. Minimum and maximum latency values were excluded from each paw analysis at each time point.

It is well-recognized that the testing season, the climate (humidity and temperature), the time of day, the cage density, the animal weight, the locomotor behavior, the number of instrument operators, and order of testing have a significant impact on the results of pain studies in rodents (Mogil et al., 2000; Mogil and McCarson, 2000; Le Bars et al., 2001; Chesler et al., 2002). Furthermore, the repetitive nature of the Hargreaves test makes it very susceptible to learning phenomena (Kocevski and Tvrdeić, 2008). Dietary lipids, including DHA, are known modulators of learning and sensitization processes, which could introduce unwanted confounding effects and affect the outcome of sensory results (Ikemoto et al., 2001; Hashimoto et al., 2002; Lim et al., 2005; McNamara et al., 2008; Joseph et al., 2012). On the basis of this evidence and to facilitate data interpretation, the thermal latencies are represented as percent change from baseline and were normalized to changes observed in sham animals as previously reported (Figueroa et al., 2013). Briefly, the nociceptive phenotype was determined by the following equation:

$$\mathbf{HWL}_{\%} = [(\mathrm{HWL_{ib}} - \mathrm{HWL_{ix}})/\mathrm{HWL_{ib}}] - [(\mathrm{HWL_{sb}} - \mathrm{HWL_{sx}})/\mathrm{HWL_{sb}}]\,(\times 100) \quad \text{EQ.1}$$

HWL% = hindpaw withdrawal latency percent change from baseline normalized to week-to-week changes in sham animals; **ib** = latency from injured animal in diet *A* at baseline; **ix** = latency from injured animal in diet *A* at time *x*; **sb** = averaged latency from all sham animals in diet *A* at baseline; **sx** = averaged latency from all sham animals in diet *A* at time *x*.

2.5. Metabolomic Profiling

Unbiased metabolic profiling was performed as previously described (Figueroa et al., 2013). Animals were deeply anesthetized and transcardially perfused with ice-cold PBS to limit blood contamination. Spinal cord samples (75–100 mg) were flash frozen in liquid nitrogen and immediately stored at −80°C. Samples were homogenized in water at the time of analyses. The protein was precipitated with methanol containing four standards to report on extraction efficiency. The resulting supernatant was split into equal aliquots for analysis on the three platforms. Aliquots were dried under nitrogen and vacuum-desiccated. The metabolomics profiling platform employed for this analysis was based on a combination of three independent platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS^2) optimized for basic species, UHPLC/MS/MS^2 optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Controls were analyzed concomitantly with the experimental samples. For instance, aliquots of a well-characterized human plasma pool served as technical replicates throughout the data set, extracted water samples served as process blanks, and a cocktail of standards spiked into

every analyzed sample allowed instrument performance monitoring. Experimental samples and controls were randomized across platform run days. The metabolites were identified by automated comparison of the ion features in the experimental samples and compared to a reference library of chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as associated MS spectra. The neurometabolomics features were curated by visual inspection for quality control using software developed at Metabolon (Dehaven et al., 2010). Archived mass spectrometry data from our previously reported study, which was curated for only identified 'named' compounds in Metabolon's chemical reference library (Figueroa et al., 2013), was re-curated to further investigate the unidentified compounds that were detected in the study.

Of particular importance, inositols features have been implicated as osmolytes and clinical metabolic markers of inflammation (Brand et al., 1993; Schuhmann et al., 2003), SCI-mediated chronic pain (Pattany et al., 2002), and recently as a marker of SCI progression (Erschbamer et al., 2011). Since SCI-induced edema and water disturbances may introduce bias in the quantification of osmolytes, the relative levels of Ins were quantified relative to creatine levels (Ins/Cr ratio).

2.6. Metabolomics Analyses

An estimate of the false discovery rate (FDR), which is given by the *q*-value, was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies as previously reported (Storey and Tibshirani, 2003b, a). For example, when analyzing 200 compounds, it is expected to see about 10 compounds meeting the $p \leq 0.05$ cut-off by random chance. Thus, the *q*-value describes the false discovery rate; a low *q*-value ($q < 0.10$) is an indication of high confidence in a result.

The partial least square-discriminant analysis (PLS-DA) is a supervised method that uses multivariate regression techniques to extract via linear combination of original variables (X) the information that can predict the class membership (Y). This regression was performed using the *plsr* function provided by *R pls* package. The classification and cross-validation were performed using the corresponding wrapper function offered by the *caret* package. To assess the significance of class discrimination, prediction accuracy during training and the separation distance permutation test were performed. In each permutation, a PLS-DA model was built between the data (X) and the permuted class labels (Y) using the optimal number of components determined by cross validation for the model based on the original class assignment. The variable importance in projection (VIP), which is a weighted sum of squares of the PLS loadings and takes into account the amount of explained Y-variation in each dimension was used to measure the impact of each metabolite in the model. Generally, features with high impact have VIP values higher than 1.

2.7. Immunodetection

Immunofluorescence methods has been described previously (Figueroa et al., 2012). Briefly, spinal cord sections were dried at room temperature for 10–15 min, washed with PBS, and post-fixed with 4% PFA for 10 min. The sections were blocked and incubated at 4°C ON in 20% normal donkey serum with 0.1% Tween-20 with rabbit anti-phosphorylated-p38, p-p38 (1:200; R&D Systems, Minneapolis, MN) and mouse anti-NeuN monoclonal antibody (1:250; Millipore, Billerca, MA). Additional experiments used mouse anti-CD11b (OX42, 1:100; AbD Serotec, Raleigh, NC) to examine reactive microglial cells. Alternatively, sections were incubated with rabbit anti-GAP43 (1:500; Abcam, Cambridge, MA) and sheep polyclonal calcitonin gene-related peptide (CGRP; 1:500; Abcam). The sections were then washed with PBS and incubated in secondary antibodies [Alexa Fluor® 594-conjugated donkey anti–rabbit or donkey anti-sheep (1:500; Invitrogen, Carlsbad, CA) and Alexa

Fluor® 488-conjugated donkey anti–mouse or donkey anti-rabbit (1:500; Invitrogen)]. Primary antibody omission and normal serum controls were used to confirm the specificity of the immunoreaction. Slides were examined with an Olympus Optical Fluoview FV1000 confocal microscope. Unbiased stereological methods were followed as previously reported (Figueroa et al., 2012). Two blinded observers quantified the immunoreactivity in lamina I to III, which were identified by superimposing photomicrographs with spinal cord diagrams from the Watson, Paxinos, and Kayalioglu spinal cord atlas. For each animal, the p-p38-positive neurons were counted manually in at least 4 randomly selected areas of the superficial dorsal horns. The mean number of p-p38-expressing neurons was then tabulated for each animal and group. For fiber sprouting analyses, the CGRP-GAP43 double labeling immunoreactivity was quantitated by inverting merged images into black (marker-positive) and white in the NIH Image J program for measurement of positive pixels/area in the dorsal horns laminae I to III. Results were obtained by averaging measurements made by blinded investigators.

2.8. Statistical Analysis

Statistical analyses were performed using SPSS version 20.0 (IBM: SPSS, Armonk, New York), Prism 5 software v5d (GraphPad Software Inc., San Diego, CA), the “R” program (http://cran.r-project.org/), and metaboanalyst (Xia et al., 2009; Xia et al., 2012). Two-Way Analysis of Variance (ANOVA) followed by Bonferroni post-hoc comparisons was used to determine the effect of the diet type, injury, and time on hindpaw thermal withdrawal latencies and differences within and between groups. To determine the antinociceptive effects of dietary O3PUFAs in time we calculated the area under the nociception versus time curve (AUC) and subjected AUCs to t-tests as previously described (Svensson et al., 2005; Buczynski et al., 2010; Morisseau et al., 2010). ANOVA contrasts were used to identify features that differed significantly between tested groups. All other data were assessed by Mann-Whitney U test. The Kolmogorov-Smirnov and Shapiro-Wilk normality tests together with the Grubbs’ method, also known as ESD (extreme studentized deviate; www.graphpad.com), were used to investigate outliers and spread. Spearman’s rank correlation tests were used to explore associations between detected metabolites and the sensory phenotype. Data are presented as mean ± SEM. Statistical differences were considered significant at $p < 0.05$ unless otherwise specified.

3. Results

3.1. General conditions and summary of previously published findings related to this study

Previously, we reported that experimental spinal cord injury (SCI) leads to a marked docosahexaenoic acid (DHA) deficiency and motor and autonomic deficits, which were corrected by dietary omega-3 polyunsaturated fatty acids (O3PUFAs) prophylaxis (Figueroa et al., 2013). Here, we hypothesized that this dietary prophylactic intervention would reduce thermal hypersensitivity in SCI. We characterized the antihyperalgesic effects of this dietary strategy and investigated the extent to which dietary O3PUFAs impact the levels of bioactive metabolites and cellular targets associated with nociception and inflammation in the injured cord.

3.2. Preventive dietary O3PUFAs attenuate the development of below-level thermal hyperalgesia after injury to the spinal cord

To examine the effect of dietary O3PUFAs on the onset and maintenance of neuropathic pain after SCI, we used the paw thermal sensitivity to noxious heat (Hargreaves testing). We found no differences in the average baseline hindpaw withdrawal latencies between groups (10.61 ± 0.59 s for control-fed animals and 11.34 ± 0.44 s for animals receiving O3PUFA

diets; mean ± SEM, $p > 0.05$). Because of differences in survival times and missing data points between animal cohorts, we used two-way ANOVA to identify the effect of the diet type and operation in the thermal latencies differences. We found that both diet type and the surgical intervention were significant sources of variation in our data set [for the diet effect $F(3,49253) = 40.22$, $p = 0.0001$, $n = 8–18$ rats per group]. Post hoc analysis revealed significant differences in thermal thresholds between sham and injured animals receiving control diets from week 8 to week 12 post-operation ($p < 0.05$). A novel finding of this study is that the animals consuming diets rich in O3PUFAs showed no significant alterations in their hindpaw withdrawal latencies when compared to their sham counterparts ($p > 0.05$). Post hoc comparisons between injured groups revealed that the most significant differences in nociception occurred between after 7 wpi and hence the focus period of this current investigation ($p < 0.05$; Fig. 1A). Sham-operated rats receiving O3PUFA-rich diets did not show significant changes in their thermal thresholds when compared to animals being fed with control diets ($p > 0.05$).

Calculation of the area under the thermal withdrawal latency change versus time curve (AUC) showed a potent antihyperalgesic effect of dietary O3PUFAs in SCI rats (Fig. 1B; Mann Whitney U rank test $p = 0.0008$; $n = 18$).

3.3. Metabolomic profiling reveals distinctive endocannabinoid signatures associated with chronic SCI and dietary O3PUFAs

We used an untargeted metabolomics approach (Figueroa et al., 2013) to investigate the neurochemical consequences of O3PUFAs consumption on the endocannabinoid (eCB) metabolome. Figure 2A summarizes the four groups analyzed in this study and the metabolomic interactions between them. A total of 275 named metabolites and 76 unnamed biochemical were detected and analyzed. The diet rich in O3PUFA significantly changed more than 20% of the detected metabolic features (74 total altered features: 40 upregulated, 34 downregulated when compared to animals receiving control diets). Of significance, more than 40% of these altered metabolites were associated with the endocannabinoid metabolome and are the focus of the present study. In this study, data was protected against false positives using the false discovery rate q value. We first sorted the metabolomic data by the p-value and chose the cutoff for significance at $p < 0.05$. We found that the false discovery rate of various diet-derived metabolites with significant p values were extremely low ($q < 0.1\%$) when comparing injured animals, validating the significance of our results (see below).

The partial least square-discriminant analysis (PLS-DA) score plot was obtained using the variation scores of the first two principal components, component 1 (22.7%) and component 2 (44.5%). These analyses revealed distinctive endocannabinoid-related profiles between groups (Fig. 2B). Each plot mark corresponds to an animal subject and the variability in relative metabolite levels detected for that animal. Hotelling's T^2 confidence ellipse, at a significance level of 0.05, showed no outliers. Permutation analyses validated the class discrimination and neurometabolomic separation (observed test statistic $p < 0.01$). Consequently, more than 67% (component 1 + component 2) of the metabolomics differences can be explained with certainty by the generated PLS model. For simplicity, only the prediction accuracy during training result is shown (Fig. 3A).

We found that both chronic SCI and the preventive diet enriched in O3PUFAs had a significant impact in the levels of acyl glycerol class endocannabinoids and in the metabolism of N-acyl ethanolamines (Figure 3B). Also, the cord of injured animals receiving the O3PUFAs showed higher levels of eicosenoyl, palmitoyl, arachidonoyl, and oleoyl glycerols when compared to control fed injured animals. In general, the diet rich in O3PUFAs skewed the metabolomic profile towards increased levels of long-chain N-acyl

ethanolamines. In particular, we identified a selective group of glycerophospho-containing N-acyl ethanolamines (GP-NAEs). These molecules showed the strongest influence (highest variable importance in projection, VIP, values) to the observed metabolomics differences between groups.

3.4. Dietary O3PUFA leads to a marked accumulation of diet-derived N-acyl ethanolamine (NAEs) precursors

The beneficial neurological effects of O3PUFAs are partly related to their anti-inflammatory properties, however the exact mechanisms behind these actions are unknown. A putative mechanism could be via their conversion to related N-acyl ethanolamines (NAEs). Here, we present new evidence demonstrating the significant impact of diet in the regulation of these bioactive lipids after chronic SCI. In agreement with previous studies(Wood et al., 2010), we found that dietary O3PUFAs lead to a robust accumulation N-acyl ethanolamines glycerophospholipids containing DHA, DPA, and EPA fatty acids (Fig. 4A). Interestingly, metabolomic analysis revealed very low abundance of these lipids in the cord of animals receiving control diets.

Figure 4B illustrates the current knowledge on the NAEs biosynthetic pathways (Simon and Cravatt, 2006, 2008). Briefly, it has been proposed that NAEs are biosynthesized from their corresponding N-acyl phosphatidyl ethanolamines (NAPEs) occur through a single NAPE-PLD-dependent pathway (NAPE-PLD). Alternatively, NAPE-PLD-independent multi-step processes have been recently reported and involve alpha/beta-hydrolase 4 (ABDH4 or Abh4) and the glycerophosphodiesterase, GDE1. The results presented herein suggest a marked activation of NAPE-PLD-independent pathways following chronic SCI.

The effects of the diet and chronic SCI on the detected metabolites associated with the endocannabinoid and related NAEs are summarized in Table 1. ANOVA contrasts were performed to determine statistical differences in metabolite relative amounts between groups (differences were considered significant when $p < 0.05$; n = at least 8 rats per group). The false discovery rate showed very low q-values for features associated with the diet-derived O3PUFAs (EPEA = 8×10^{-15}; DPEA = 2.40×10^{-9}; DHEA = 0.012), supporting the significance of the observations.

Spearman's correlation analyses were used to explore the linear trends between cord metabolite levels and changes in thermal thresholds. The relative levels of NAEs containing 22 carbons N-acyl chains and the glycerophospho NAEs of O3PUFAs were positively correlated with reduced thermal withdrawal latency changes (Fig. 5A–D; Spearman r values > 0.50; $p < 0.05$). We also found a significant positive correlation between the relative levels of palmitoyl ethanolamine (PEA) and non-hyperalgesic responses, supporting its anti-inflammatory roles in SCI (Esposito et al., 2011; Esposito and Cuzzocrea, 2012). Together, our findings suggest that these diet-derived metabolites may play significant roles in antinociception.

3.5. Functional metabolomics implicate the NAEs biosynthetic pathways in SCI-induced neuropathic pain

The biochemical basis and etiology underlying chronic neuropathic pain remains poorly understood and has limited the development of effective interventions. To characterize the unique lipidomic changes underlying neuropathic pain-like behaviors after contusive SCI, we first used the K-means clustering method to assign the animals to three groups according to their thermal threshold changes (Fig. 6A). This partitioning method identified a group of animals that showed no significant alterations in their thermal threshold (normal behavior, non-hyperalgesic; $p > 0.05$). Another cluster was shown to exhibit significantly reduced

latencies when compared to their baseline values (hyperalgesic behavior; $p < 0.05$). The algorithm also identified animals with increased thermal latencies at 8 wpi when compared to baseline values (hypoalgesic behavior; $p < 0.05$). The mean baseline values did not differ significantly between clustered groups ($p > 0.05$), demonstrating that the different phenotypes developed after SCI.

Notably, the glycerophospho N-acyl ethanolamines (GP-NAEs) derived from the O3PUFA-rich diet were the most relevant metabolites for explaining the differences between non-hyperalgesic and hyperalgesic animals (Fig. 6B). The metabolomics analyses revealed decreased levels of the O3PUFA-derived GP-NAEs in the hyperalgesic animals, suggesting a potential role for these in neuropathic pain.

Although additional factors contribute to the development of pain following SCI, it is well established that the extent of cord damage is a major determinant (Lindsey et al., 2000; Yezierski, 2000). To determine the relative contribution of tissue spared to the observed metabolomics differences, we assessed the cord damage between the animals exhibiting thermal hyperalgesia and those showing no significant differences in paw withdrawal thresholds when compared to baseline values ($p > 0.05$). Because the spinal cords were used for metabolomics studies, we could not determine the extent of injury using stereological techniques. However, the Basso, Beattie and Bresnahan (BBB) locomotor score provides a reliable indirect measure of the injury magnitude (Basso et al., 1996). We found that the BBB locomotor scores were not significantly different between clustered groups, indicating that the extent of injury (and repair) was similar ($p > 0.05$; Fig. 6C). This observation validates that additional processes such as chronic neuroinflammation and hyperexcitability play major roles in pathological nociception after SCI.

3.6. Animals fed with a diet rich in O3PUFAs exhibit reduced levels of p38 MAPK expression in dorsal horn neurons following SCI

To examine the anti-inflammatory effects of the O3PUFA-enriched diet, we determined the mRNA levels of key cytokines, chemokines, and receptors that have been associated with inflammatory pain (e.g., IL-1β, IL-6, TNF-α, CCL2, CCR2, CX3CL1, and CX3CR1). We found that the O3PUFA diet did not reduce the mRNA levels of these pro-inflammatory factors in below-level cord segments when compared to control-fed animals at 8 wpi ($p > 0.05$; data not shown). Histological analyses showed no significant differences in the dorsal horn immunoreactivity (IR) to OX42 between treatment groups (Fig. 7A–C). These results do not contradict the well-established roles of dietary O3PUFAs but rather suggest that these anti-inflammatory effects may occur during the initial inflammatory trigger in a time- and context-dependent manner. This observation also points to the involvement of additional mechanisms. For instance, although we did not observe significant differences in the expression of these biomarkers, qualitative differences were noticeable in cell morphology. In particular, we found microglia with morphological features typically implicated in activated states in the spinal cords of animals receiving control chow (e.g., hypertrophied cell bodies and thick processes) (Fig. 7D). Interestingly, a few animals receiving the preventive dietary intervention rich in O3PUFAs exhibited microglial cells with small soma containing thin and radially projecting processes (Fig. 7E), confirming previous observations on the DHA-elicited immunomodulatory effects in microglia (Ebert et al., 2009).

Because LC/MS and GC/MS-based metabolomics provide a more sensitive method to assess inflammatory biomarkers, we measured the levels of inositol (Ins; see Experimental Procedures). Interestingly, the averaged relative levels of the major omega-3 PUFA-derived GP-NAEs were negatively associated with Ins relative levels (Spearman $r = -0.68$, $p < 0.0001$; Fig. 7A). Further, dietary O3PUFAs significantly reduced the cord Ins levels (Fig.

7B; $p < 0.0001$, $n = 10$). Altogether, these data support an anti-inflammatory and antinociceptive role for O3PUFAs in chronic SCI.

A number of pharmacological studies implicate the spinal p38 mitogen-activated protein kinase, p38 MAPK, as one important underlying mechanism of nociception and neuronal hyperexcitability in SCI (Crown et al., 2008; Gwak et al., 2009). Here, we used immunohistochemistry to examine the expression of this established pain biomarker in rats treated with dietary O3PUFAs relative to animals receiving control diets. We found that the cords of animals receiving O3PUFAs had decreased phosphorylated p38-positive neurons in the superficial dorsal horns relative to controls at 12 wpi (Fig. 8A–G; $p < 0.05$; n = at least 4 animals). Not surprisingly, we found a significant positive correlation between the expression levels of neuronal p-p38 MAPK and the hyperalgesic behaviors (data not shown).

3.7. Dietary O3PUFA reduces the sprouting of CGRP-containing fibers in chronic SCI

The growth-associated protein 43 (GAP43) and calcitonin gene-related peptide (CGRP) have been widely used as biomarkers of nociceptive fiber sprouting and neuropathic pain (Nahin et al., 1994; Christensen and Hulsebosch, 1997b; Ondarza et al., 2003). Thus, we tested whether the preventive O3PUFA-enriched diet reduces the sprouting of CGRP-containing primary afferents following chronic SCI. Laser confocal microscopy showed CGRP and GAP43 colocalization in spinal cord sections obtained from regions 3–5 mm caudal to the injury site (Fig. 9A). CGRP (Fig. 9B, F) and GAP43 (Fig. 9C, G) labeled photomicrographs from animals receiving control chow (Fig. 9B–E) or O3PUFA-rich (Fig. 9F–I) diets were merged (Fig. 9D, H) and subsequently converted to binary format to facilitate automated analyses (Fig. 9E, I). Quantitative double labeling immunofluerescence revealed decreased sprouting of CGRP-positive primary afferents at 12 wpi (Mann Whitney U test $p < 0.05$, n = at least 4 animals) (Fig. 9J).

4. Discussion

This study shows that a preventive diet rich in omega-3 polyunsaturated fatty acids (O3PUFAs) reduces thermal hyperalgesia in rats experiencing chronic spinal cord injury (SCI). This antihyperalgesic effect was directly correlated with the levels of a series of novel glycerophospho ethanolamines containing O3PUFA acyl chains. The anti-inflammatory effects of this O3PUFA-enriched diet were evident by a significant reduction in levels of inflammatory biomarkers, including cord inositols and the phosphorylated p38 MAPK in dorsal horn neurons.

Chronic neuropathic pain is a debilitating co-morbidity associated with SCI and persists even at the later stages of recovery and rehabilitation. This condition often manifest as evoked pain, including hyperalgesia (amplified pain response to noxious stimuli) and/or allodynia (painful response to innocuous stimuli). The intensity and frequency of neuropathic pain is particularly influenced by trauma-induced neurochemical and neuroanatomical changes in synaptic circuitry and dorsal horn neuron hyperexcitability (Crown et al., 2008; Gwak et al., 2009; Gwak et al., 2010). Current approaches to treat neuropathic pain include behavioral, pharmacological, and surgical modalities, however, none of these interventions are regarded as highly effective. This could be partly due to patients being treated after considerable and perhaps irreversible changes have developed. There is thus a need to develop preventive approaches to build resilience to damage. Complementary with current strategies, this type of approach may be particularly important in individuals at high risk of traumatic injuries like those actively participating in contact sports, selected surgeries, first responders, and our men and women in the military service. The promise of using preventive approaches to treat chronic neuropathic pain is supported

by studies showing that central pain can be prevented by pre-administration of opiates (Dickenson and Sullivan, 1987), anti-inflammatory molecules (Plunkett et al., 2001), or by prophylactic cell transplantation strategies (Brewer and Yezierski, 1998; Yu et al., 1998). Although it may seem unreasonable to use these approaches in individuals undergoing SCI due to potential and unwanted side effects, prophylactic treatment with dietary O3PUFAs offers an excellent profile of clinical safety and may be beneficial in preventing pain and dysesthesias in individuals at risk (Bays, 2006; Mayer et al., 2006).

O3PUFAs, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are ubiquitous lipid messengers that regulate crucial neural processes in health and disease. We recently reported that dietary O3PUFAs exert a stringent control over phospholipid production and are principal determinants of the cord lipid composition following chronic SCI (Figueroa et al., 2013). Further, when the spinal cord is enriched with O3PUFAs before SCI, these lipids mediate robust neuroprotection, recovery, and activate pro-restorative responses (Figueroa et al., 2012; Figueroa et al., 2013; Lim et al., 2013). Several recent studies have shown the importance of PUFAs in nociception, supporting the hypothesis that dietary lipids are key elements of the nociceptive pathways (Yehuda and Carasso, 1993; Nakamoto et al., 2010; Xu et al., 2010; Tokuyama and Nakamoto, 2011; Veigas et al., 2011). Consistent with this idea, the present study shows that consumption of a diet rich in O3PUFAs produces significant thermal anti-hyperalgesia when compared to animals receiving control diets after contusion SCI. In contrast, thoracic contusion to the cord resulted in a significant reduction in below-level thermal withdrawal latencies in animals receiving control chow. Importantly, we reported that control-fed injured rats exhibited significant sensory deficits to mechanical stimulation on their hindpaws after SCI (Figueroa et al., 2013). Together, these findings discard the idea that thermal hyperalgesia was due to a hyperreflexic responses, a common occurrence in contusive SCI (Christensen and Hulsebosch, 1997a). This observation further suggests that both neurodegenerative and pro-inflammatory responses may play important roles in the development of neuropathic pain after SCI (Krassioukov et al., 1999; Baumgärtner et al., 2002).

Several lines of evidence demonstrate that the level of spinal cord damage play significant roles in the development and maintenance of pain-like behaviors (Lindsey et al., 2000; Yezierski, 2000; Park et al., 2007), suggesting that pain may be amenable to neuroprotective approaches. In agreement with others, evidence from our lab has shown that O3PUFAs confer potent neuroprotection against SCI (Figueroa et al., 2012; Figueroa et al., 2013). Together, these data suggest that neuroprotection may be an underlying mechanism involved in the anti-nociceptive responses mediated by the dietary O3PUFAs. However, neuroprotective approaches do not always result in anti-nociceptive responses (Mills et al., 2002). We found comparable open-field locomotor scores when animals were grouped based on their thermal withdrawal phenotypes. While neurodegenerative differences between clusters may be subtle or undetectable as measured by the BBB locomotor scores, we interpret this finding as evidence supporting additional underlying causes of neuropathic pain.

The neurometabolomic etiology of neuropathic pain remains poorly understood and this gap in the literature has limited the development of effective therapeutics. N-Acylated ethanolamines (NAE) and related endocannabinoids (eCBs) are a large class of naturally occurring lipids that exhibit diverse bioactivities, including neuroprotective (Jackson et al., 2005) and antinociceptive (Beltramo et al., 2006; Petrosino et al., 2007; Hama and Sagen, 2011) responses. Notably, it has been shown that SCI rats exhibit profound alterations in the metabolic pathways associated with these lipids (Garcia-Ovejero et al., 2009). These lipids are produced on demand from membrane phospholipids and glycerophospho-linked precursors by a series of intracellular enzymatic reactions, followed by immediate signaling

and metabolism (Berger et al., 2001; Artmann et al., 2008; Wang and Ueda, 2009; Banni and Di Marzo, 2010; Wood et al., 2010; Brown et al., 2012). The findings reported in the present study support and expand on these observations by showing that chronic SCI results in a marked deregulation in the metabolic pathways of NAEs and related eCBs. In particular, we found reduced levels of palmitoyl ethanolamine (PEA) in the chronically injured cord, which were correlated with hyperalgesic behaviors in SCI rats. PEA has been implicated in anti-inflammatory responses and functional recovery after SCI (Genovese et al., 2008; Esposito et al., 2011; Esposito and Cuzzocrea, 2012) and reduces pain-like behaviors in experimental models of neuropathic pain (Costa et al., 2008; Buczynski et al., 2010). Notably, dietary prophylaxis with O3PUFAs sustained the levels of PEA after SCI. Further, the O3PUFA-derived NAEs identified in this study are precursors of docosahexaenoyl ethanolamine (DHEA; synaptamide) and eicosapentaenoyl ethanolamine (EPEA), which bind to cannabinoid receptors in rats (Sheskin et al., 1997) and may contribute to the beneficial effects mediated by dietary O3PFAs (Balvers et al., 2010; Meijerink et al., 2011; Shinohara et al., 2012). Although the metabolic pathways involved in NAE biosynthesis remain unclear, our results strongly suggest that NAPE-PLD-independent (N-acyl phosphatidyl ethanolamine phospholipase D) pathways are activated in chronic SCI and represent a promising therapeutic target (Tsuboi et al., 2013). Because NAEs can modify the response to nociceptive stimuli and are tightly regulated by diet, O3PUFAs could have important implications for chronic pain management.

There is now strong evidence linking the neuroinflammatory milieu after SCI to changes in sensory electrical activity and pain-related behaviors (Hulsebosch, 2008). For instance, accumulating evidence implicates the activation of the spinal p38 mitogen-activated protein kinase, p38 MAPK, as a molecular mechanism underlying neuronal hyperexcitability and pain after SCI (Crown et al., 2006; Crown et al., 2008; Gwak et al., 2009). Remarkably, animals consuming dietary O3PUFAs exhibited reduced numbers of dorsal neurons expressing p-p38, indicating a potential molecular link between dietary lipids and pain. This finding is supported by studies demonstrating that both DHA and EPA alone attenuate the activation of p38 in endothelial cells stimulated by TNF-α (Xue et al., 2006). More recent studies showed that DHA also impairs p38 MAPK signaling in microglial cultures (Antonietta Ajmone-Cat et al., 2011). In agreement with these findings on the potential anti-inflammatory and antinociceptive roles of dietary O3PUFAs, this study shows a marked reduction in the levels of inositols and CGRP-positive sprouting fibers in the chronically injured cord. Importantly, the p38 MAPK has been linked to the regulation of inositol levels in human peripheral blood monocytes and macrophages (Denkert et al., 1998) and to the expression of calcitonin gene-related peptide in rats (Wang et al., 2011). This study supports the dynamic interplay among these biomarkers of the nociceptive system. Further studies are necessary to evaluate these molecular interactions and to investigate their potential as useful biomarkers to discriminate pain severity in SCI-related neuropathic pain.

Although experimental contusion SCI is a well-established and validated animal model for evaluating the development of pain and analgesic strategies (Resnick et al., 2004; DomBourian et al., 2006; Rajpal et al., 2007; Cramer et al., 2008; Jung et al., 2008), there are limitations in extrapolating findings from experimental animal models and to humans. It has been suggested that the mechanisms underlying evoked responses in SCI animals may differ from those of underlying spontaneous neuropathic pain in patients (Craig, 2009). The proper interpretation of animal pain-like behaviors thus remains a challenge and warrants further research. Given the significant contribution of supraspinal structures in pain processing (King et al., 2003; Baastrup et al., 2010; Davoody et al., 2011), futures studies should employ integrated pain-associated behaviors to facilitate data interpretation and to gain better understanding of the antinociceptive targets of dietary O3PUFAs.

4.1. Conclusions

In summary, this study shows for the first time that dietary O3PUFAs prophylaxis attenuates the development of thermal hyperalgesia following SCI, possibly by providing a better bioavailability for anti-inflammatory lipid mediators. Even though recent advances in pain research suggest that combinatorial strategies to both prevent and combat chronic neuropathic pain are a feasible goal (Hama and Sagen, 2012), identifying targets with the intention of preventing pain is an enormous conceptual challenge that has so far stymied drug discovery. The study presented herein supports the use of preventive alternative approaches in individuals at risk of developing neuropathic pain and identifies diet as a potential risk factor for poor outcome. Although further research is needed, our findings have remarkable public health implications to reduce the burden of pain, particularly in populations at risk. Because dietary O3PUFAs are safe, well tolerated, and confer robust protection against experimental SCI they should be favored for early pain management in human SCI.

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Abbreviations

SCI	Spinal cord injury
DHA	docosahexaenoic acid
O3PUFAs	omega-3 polyunsaturated fatty acids
eCBs	endocannabinoids
NAEs	N-acyl ethanolamines
DHEA	docosahexaenoyl ethanolamine/synaptamide
DPEA	docosapentaenoyl ethanolamine
EPEA	eicosapentaenoyl ethanolamine
TH	thermal hyperalgesia
HWL	hindpaw withdrawal latency
UHPLC/MS/MS^2	ultrahigh performance liquid chromatography/tandem mass spectrometry
GC/MS	gas chromatography/mass spectrometry
GAP43	growth-associated protein 43
CGRP	calcitonin gene-related peptide
PLS-DA	partial least square-discriminant analysis
GP-NAEs	glycerophospho-containing N-acyl ethanolamines
BBB	Basso, Beattie and Bresnahan
LEA	linoleyl ethanolamine
AEA	arachidonoyl ethanolamine

EG	eicosenoyl glycerol
2-AG	2-arachidonoyl glycerol
2-PG	2-palmitoyl glycerol
1-OG	1-oleoyl glycerol
PEA	palmitoyl ethanolamine
PLD	phospholipase D
Abh4	phospholipase A/B or α-β-hydrolase 4
GDE1	glycerophosphodiesterase
NAPE	N-acyl phosphatidyl ethanolamine
G3P	glycerol-3-phosphate
LPA	lyso-phosphatidic acid
PA	phosphatidic acid

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Highlights

- Thermal hyperalgesia was reduced in SCI rats consuming O3PUFA-rich diets.
- O3PUFA-derived ethanolamines are associated with the antihyperalgesic behaviors.
- Dietary O3PUFAs reduced the levels of pain biomarkers in the injured spinal cord.

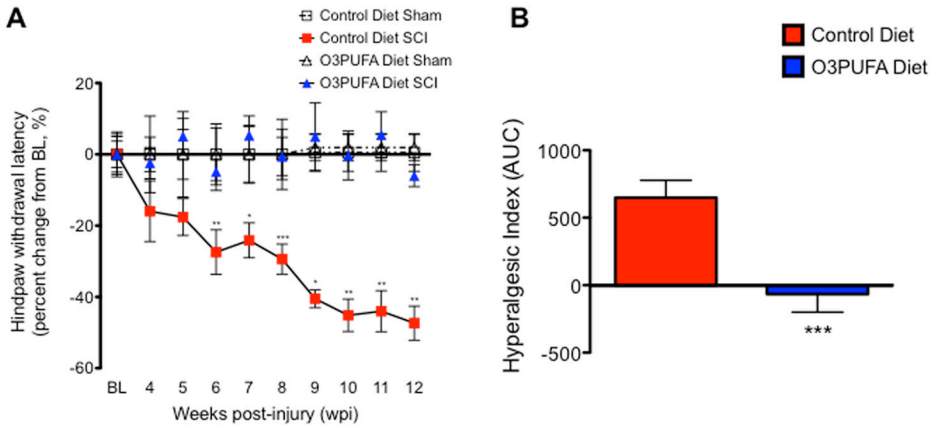

Figure 1.

Responsiveness to thermal stimulation in animals receiving control and O3PUFA-enriched diets. (A) Thoracic contusion to the spinal cord leads to below-level thermal hyperalgesia in animals receiving control diets. Hindpaw withdrawal latencies (averaged percent change from baseline) are plotted versus time (weeks post-injury, wpi). For each timepoint, the individual latencies were adjusted to the percent change from baseline observed in sham animals receiving the same diet using equation 1 (see Materials and Methods section). No significant latency alterations were observed between sham animals. Notably, dietary O3PUFAs prevented the development of thermal hyperalgesia ($p > 0.05$ when compared to sham animals). TW-ANOVA identified the diet type and surgery as significant sources of variation [for diet/surgery $F(3,49253) = 40.22$, $p = 0.0001$, $n = 8–18$]. Bonferroni's post hoc analyses showed significant thermal withdrawal latency changes when comparing injured animals receiving the different diet types ($p < 0.05$). (B) To investigate the overall effect of O3PUFA in thermal hindpaw sensitivities, the hyperalgesic index was generated using the area under the curve (AUC). Analyses of the AUC revealed that the O3PUFA diet had a significant antihyperalgesic effect (Mann Whitney *U* rank test; $p < 0.001$). Each bar represents mean ± SEM; $n = 18$.

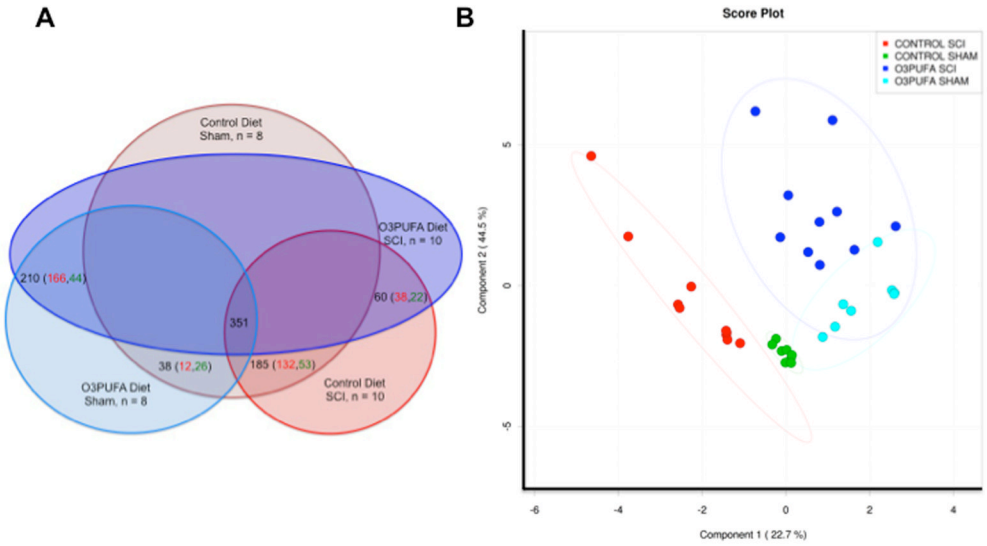

Figure 2.

SCI and dietary O3PUFAs modulate the endocannabinoid-related neurometabolome. (A) Venn diagram depicting the numerical interactions among data sets. ANOVA contrasts analyses were used to evaluate the regulation pattern differences between groups. The total number of features detected across 36 spinal cord tissue samples was 351 metabolites. Diagram illustrates the number of total metabolites significantly altered between groups (e.g., O3PUFA diet SCI/Control diet SCI contrast resulted in 60 significantly altered metabolites; 38 upregulated and 22 downregulated; $p < 0.05$). (B) Partial least square discriminant analysis (PLS-DA) distinguished subgroups based on dietary intake at 8 weeks post-injury (wpi). Model was constructed using scaled intensity peaks of the detected features associated with the endocannabinoids (eCBs) system: classic eCBs, eCBs glycerols, and related N-acyl ethanolamines (NAEs) and metabolites. Projections provided statistically significant separations between subgroups.

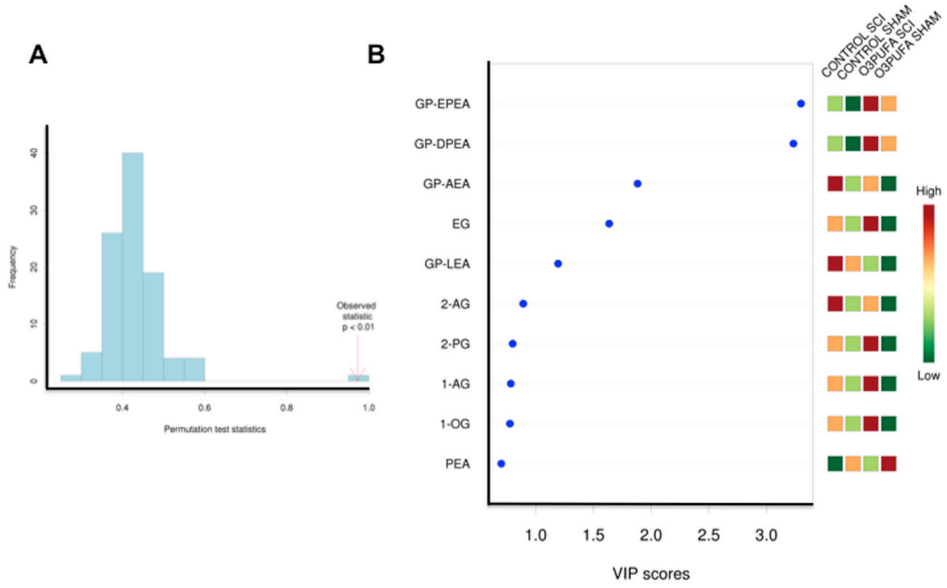

Figure 3.

PLS-DA model validation and metabolite impact. (A) Prediction accuracy training permutation test validate the PLS-DA model by showing a significant observed statistic ($p < 0.01$). (B) The variable influence on projection (VIP) analyses, which reflect the importance of metabolites showed the significant contribution of selective NAEs, endocannabinoids, endocannabinoid glycerols, and O3PUFA-derived glycerophospho ethanolamines (GP-NAEs) in our PLS model. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study. Abbreviations: GP, glycerophospho; EPEA, eicosapentaenoyl ethanolamine; DPEA, docosapentaenoyl ethanolamine; AEA, arachidonoyl ethanolamine; EG, eicosenoyl glycerol; LEA, linoleoyl ethanolamine; 2-AG, 2-arachidonoyl glycerol; 2-PG, 2-palmitoyl glycerol; 1-OG, 1-oleoyl glycerol; PEA, palmitoyl ethanolamine.

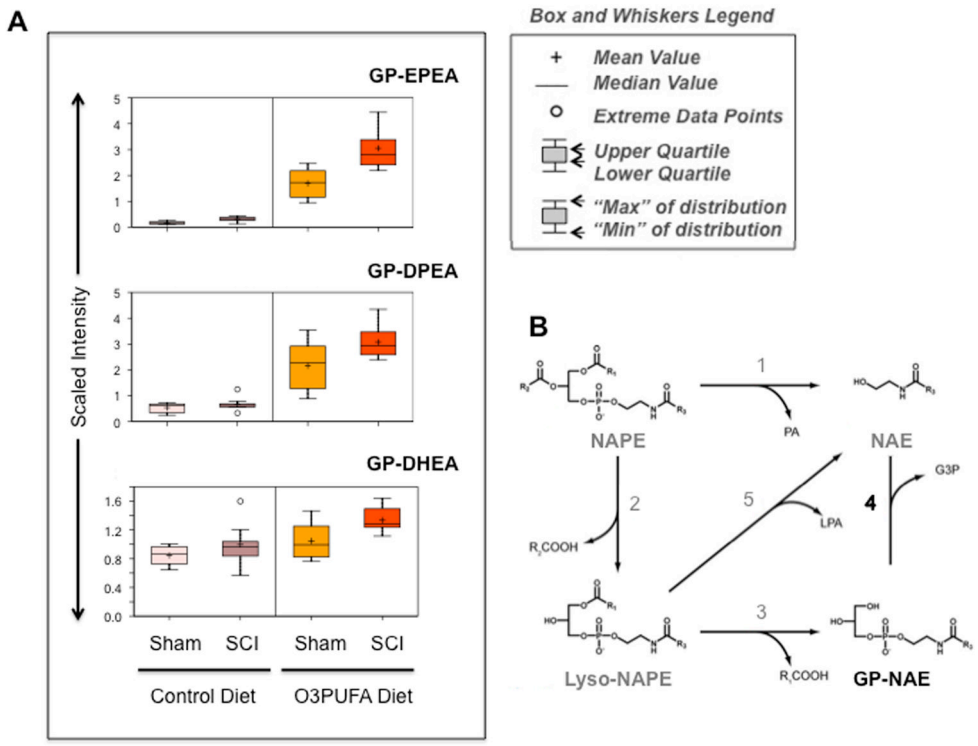

Figure 4.

Chronic O3PUFAs consumption leads to a robust accumulation of diet-derived glycerophospho ethanolamines in the spinal cord. (A) Box and whiskers graphs illustrate a marked increase in the levels of O3PUFA-dervied GP-NAEs (relative to the median metabolite levels in each group). (B) Potential metabolic pathways for the biosynthesis of NAEs. This illustration is adapted and modified from previous reports (Simon and Cravatt, 2006, 2008). Reaction 1 is mediated by an NAPE-selective phospholipase D (PLD). Pathways 2-3-4 and 2–5 are NAPE-PLD independent. Recent studies suggest the involvement of a novel phospholipase A/B, named Abh4, α-β-hydrolase 4 in the NAPE conversion to GP-NAE (reaction 2 and 3). The secretory PLA2 can also release fatty acid from sn-2 position of NAPE (reaction 2). Reaction 4 is catalyzed by a new glycerophosphodiesterase, GDE1. Lyso-PLD catalyzes reaction 5. Abbreviations: DHEA, docosahexaenoyl ethanolamine; DPEA, docosapentaenoyl ethanolamine; EPEA, eicosapentaenoyl ethanolamine; GP, glycerophospho, NAE, *N*-acyl ethanolamine; NAPE, N-acyl phosphatidyl ethanolamine; G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid.

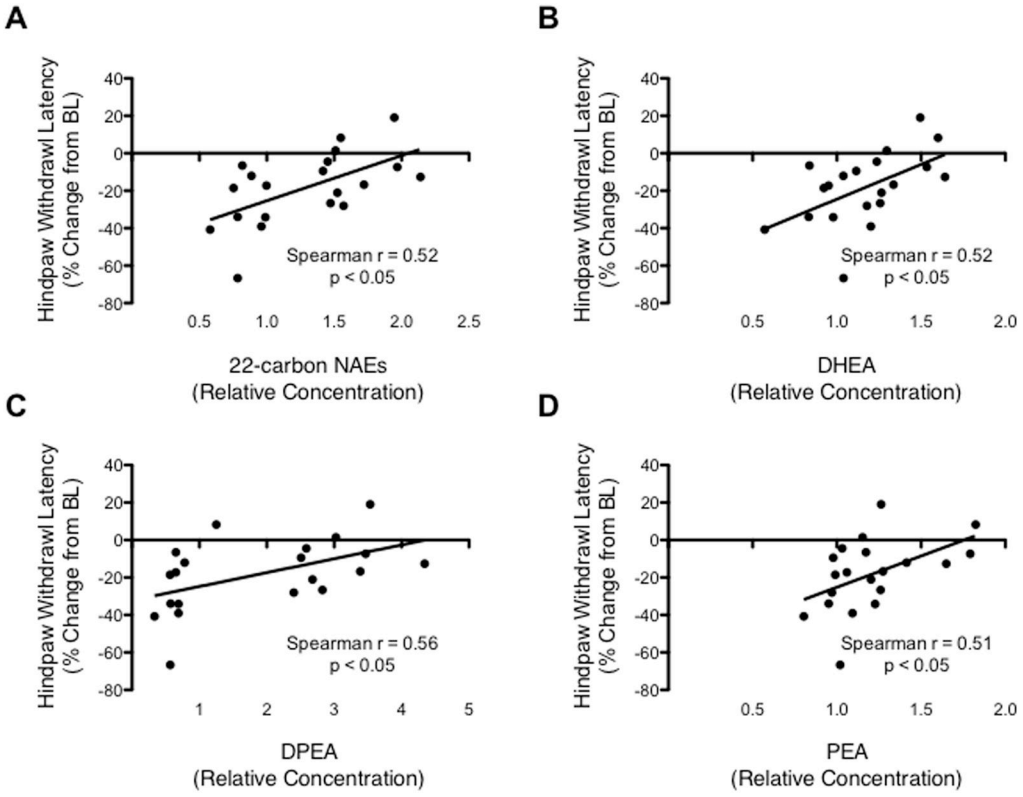

Figure 5.

Metabolic features correlated with pain-like phenotypes. Scatter plot shows the significant relationship between the levels of (A) ethanolamines containing 22-carbon N-acyl chains, (B) GP-DHEA, (C) GP-DPEA, (D) PEA and the hindpaw thermal withdrawal latency change from baseline. For every correlation, the Spearman r was higher than 0.50, with a $p < 0.05$, XY = 20 pairs.

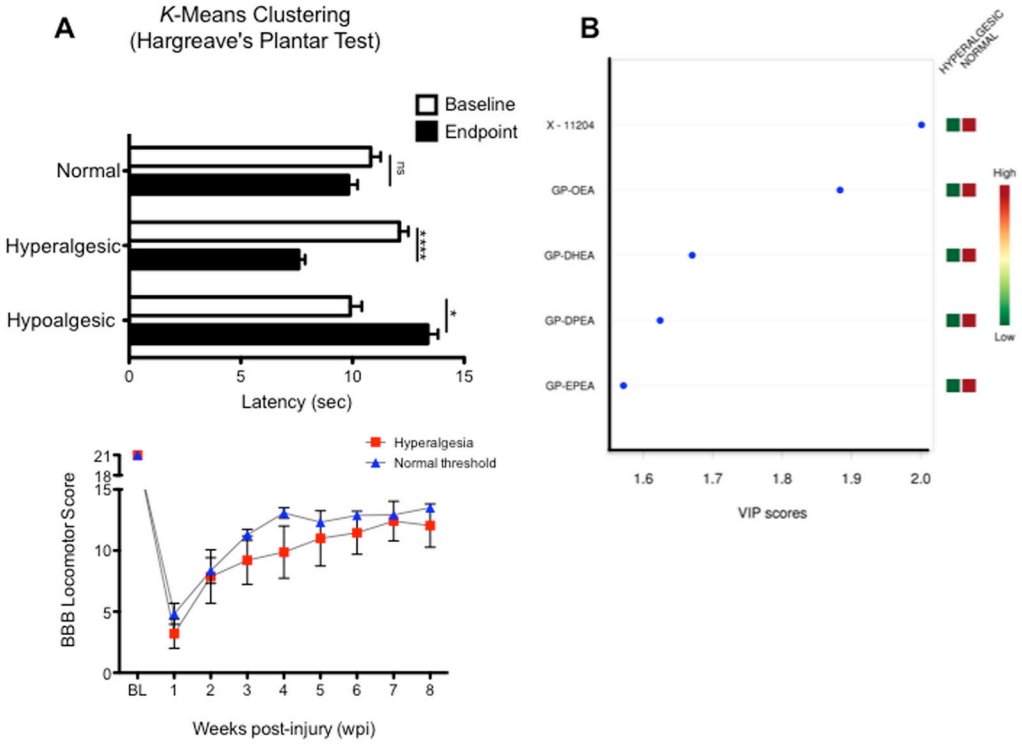

Figure 6.

(A) *K*-means clustering divided animal based on their nociceptive behavior (Δ latency = $\text{latency}_{\text{endpoint}}$ - $\text{latency}_{\text{baseline}}$). A group of animals exhibited no significant Δ latency changes at endpoint ("normal" or non-hyperalgesic cluster). The clustering algorithm identified two additional groups: hyperalgesic rats (significant Δ latency decrease at endpoint) and hypoalgesic (increased Δ latency at endpoint). Data is presented as mean ± SEM. Data was analyzed by TW-ANOVA followed by Bonferroni's post hoc: ns, not significant; (*) = $p < 0.05$; (****) = $p < 0.0001$. (B) Metabolomic analyses using the normal and hyperalgesic clusters confirmed the potential role of the NAE metabolism in pain processing after SCI. In particular, the O3PUFA-derived NAEs were shown to have a significant impact in the metabolic differences observed between thermal pain behaviors. Notably, the animals showing hyperalgesic phenotypes exhibited reduced levels of these metabolites. Abbreviations: X-11204, unnamed compound which has been tentatively identified as an unsaturated hydroxyl fatty acid with an empirical formula of $C_{13}H_{24}O_3$; GP, glycerophospho; OEA, oleoyl ethanolamine; DHEA, docosahexanoyl ethanolamine; DPEA, docosapentaenoyl ethanolamine; EPEA, eicosapentaenoyl ethanolamine. (C) Basso, Beattie and Bresnahan locomotor scores measured in hyperalgesic (n = 6) and non-hyperalgesic (n = 13) rats. Repeated measures ANOVA did not identify pain classification (non-hyperalgesia vs. hyperalgesia) as a significant contributor of the total variance ($p > 0.05$). Pain classification accounted for 1.54% of the total variance (after adjusting for matching) with a *p* value of 0.2669. F statistics (1,66.88) = 1.318, indicating that the extent of the injury was similar in both groups. To determine whether the mean differences were more apparent, the data was analyzed using the Mann-Whitney *U* t-test at the time points showing greatest difference. The analysis revealed a *p* value of 0.2005 at 4 weeks post-injury, which can be interpreted as no reason to reject the null hypothesis. In other words, the locomotor score distributions of both dietary groups are identical at 4 wpi. This observation proposes underlying neurochemical mechanisms that may be independent of the injury severity and to the amount of spared tissue. Data represents mean ± SEM.

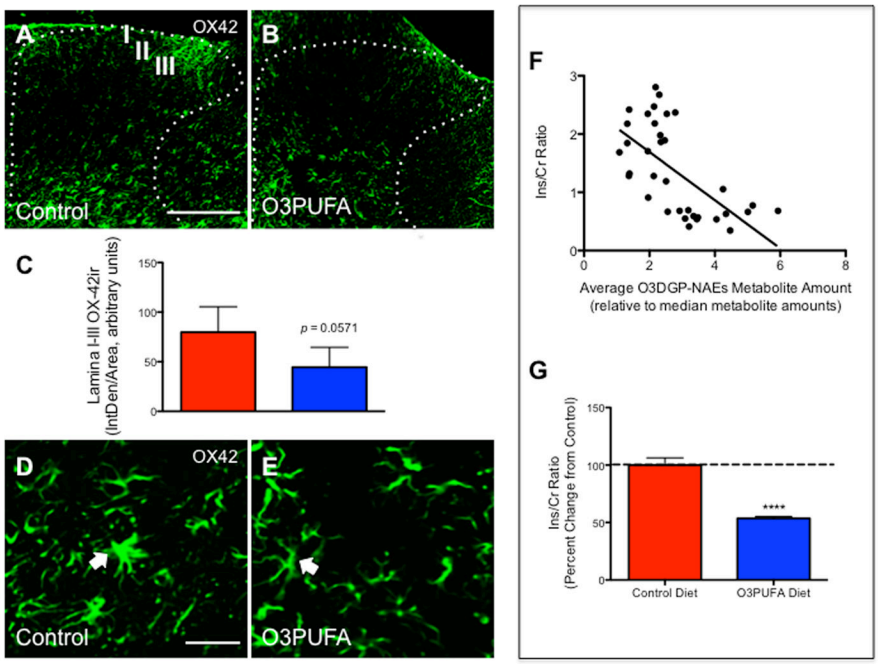

Figure 7.

Dietary O3PUFA did not reduce microglial cell immunoreactivity in superficial dorsal horns following chronic SCI. Representative images from OX42 immunoreacted spinal cord injured caudal sections from animals receiving control (A) or O3PUFA (B) diets. Quantitative analyses showed no significant changes in inflammatory markers immunoreactivity between treatment groups in the dorsal horn laminae I-III at 12 weeks post-injury (C). Closer examination revealed that following chronic SCI, spinal microglia displayed hypertrophied cell bodies and thick processes, which are characteristic of their activated state (D). Interestingly, some animals treated with dietary O3PUFA showed microglial cells with small soma containing thin and radial projecting processes (resting state of microglia, E). Scale bar = A-B, 200 μm; D–E, 20 μm. The arrows indicate OX42-positive cell somata. Dietary O3PUFA-derived GP-NAEs levels are potentially implicated in anti-inflammatory responses. (F) A scatter plot shows the relationship between the levels of the O3PUFA-derived GP-NAEs (O3DGP-NAEs) and the total inositol-to-creatine levels (Ins/Cr). The Spearman r = −0.68, CI(−0.83 to −0.44), $p = 0.0001$, XY = 18 pairs includes sham and injury-operated animals fed with control diet. (G) Preventive administration of dietary O3PUFAs resulted in a significant reduction in the Ins levels (Mann-Whitney *U* test, **** $p < 0.0001$, $n = 10$). Data represents mean ± SEM.

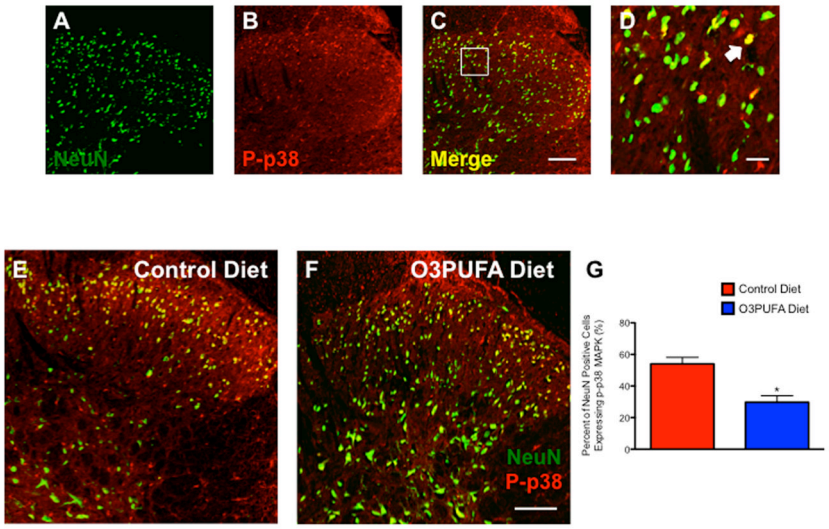

Figure 8.

Preventive dietary O3PUFAs reduce the expression of phosphorylated p38 in below-level dorsal horn neurons. At 12 weeks post-injury, laser confocal microscopic evaluation revealed dorsal NeuN-positive neurons (A) containing the phosphorylated p38 MAPK (B). Merged photomicrographs (C) and inset (D) show distinctive neuronal subpopulations expressing this inflammatory marker after chronic SCI. Dorsal horn photomicrographs show noticeable differences in the number of p-p38-containing neurons when comparing dietary groups (control = E vs. O3PUFA = F). (G) Manual cell counts confirmed that the dietary O3PUFA intervention significantly reduced the percent of NeuN-positive cells expressing p-p38 MAPK in below-level dorsal horn superficial laminae ($p < 0.05$; n = at least 4 animals). Scale bars = A–C and E–F, 100 μm; D, 20 μm.

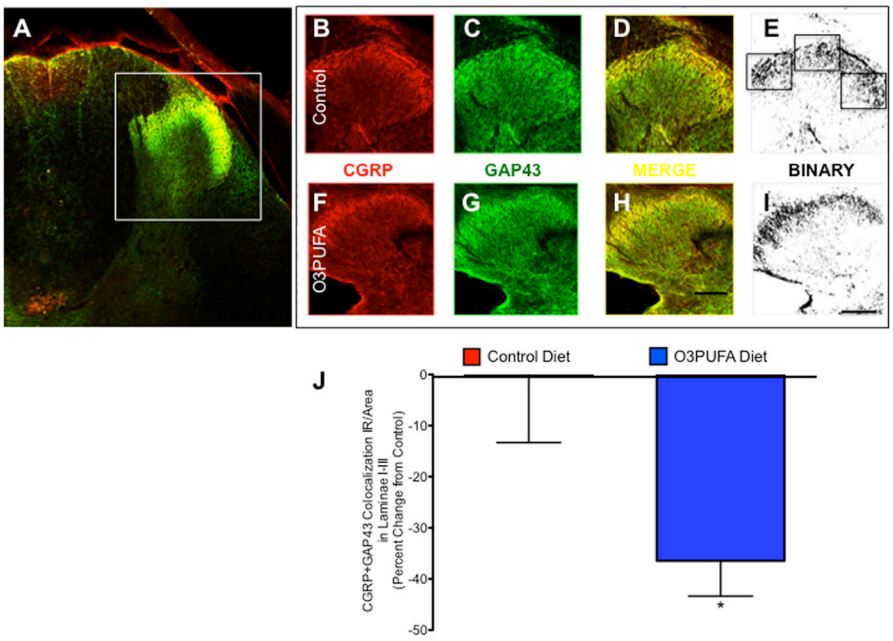

Figure 9.

Dietary O3PUFA-pretreatment reduces nociceptive fiber sprouting following chronic SCI. (A) Double labeled spinal cord section showing calcitonin gene-related peptide (CGRP) and growth-associated protein 43 (GAP43) immunoreactivity (IR) in spinal cord. Confocal photomicrographs showing CGRP (B) and GAP43 (C) immunoreactivity in control chow-fed rat. Images were merged to quantify the immunoreactivity of CGRP-containing sprouting afferent fibers (D). Optical density was most intense in laminae I to III of the dorsal horns. Sections were morphometrically analyzed using stereological methods after thresholding binary images using ImageJ software (E). Representative image from CGRP (F) and GAP43 (G) immunoreactivity in an animal fed O3PUFA-enriched diets. Merged (H) and binary (I) images depict CGRP-containing $GAP43^{+}$ fibers in the dorsal horn (H). Boxes represent areas showing colocalization. Scale bar = 100 μm. (I) Results from quantification of binary particle counts of dorsal horn superficial laminae. Analysis showed that O3PUFA-enriched diet significantly reduced the colocalization of GAP43 and CGRP, suggesting a reduction in nociceptive fiber sprouting. Bars represent means ± standard error of the mean; Mann Whitney U test $*p < 0.05$, n = at least 4 rats.

Table 1

The endocannabinoid (eCB) metabolome is altered following chronic SCI and influenced by dietary O3PUFAs. ANOVA contrasts were performed to determine statistical differences in metabolite relative amounts (differences were considered significant when $p < 0.05$; $n = 8$ rats per sham group and 10 rats per injury group). Comparisons were made between the four studied groups: (1) sham control diet, (2) SCI control diet, (3) sham O3PUFA-rich diet, and (4) SCI O3PUFA-rich diet. Notably, in spinal cord injured animals, the O3PUFA diet decreased the levels of the glycerophospho 2-LEA and 2-AEA and dramatically increased the levels of O3PUFA-derived GP-NAEs. Numbers represent fold of change. Heat map color legend: *red*, significant increase; *green*, significant decrease; *light red* and *green* represent marginal statistical significance. Abbreviations: LEA, linoleyl ethanolamine; AEA, arachidonoyl ethanolamine; GP-NAEs, glycerophospho n-acyl ethanolamines.

		Fold of Change			
		ANOVA Contrasts			
	Biochemical Name	**Ctrl-SCI Ctrl-Sham**	**O3PUFA-SCI O3PUFA-Sham**	**O3PUFA-Sham Ctrl-Sham**	**O3PUFA-SCI Ctrl-SCI**
PUFAs	eicosapentaenoate (EPA; 20:5)	5.09	4.08	6.71	5.37
	docosahexaenoate (DHA; 22:6)	2.87	3.30	1.20	1.38
	arachidonate (AA; 20:4)	1.14	1.03	−1.14	−1.25
eCBs	oleic ethanolamide (OEA)	−1.41	−1.33	−1.03	1.02
	palmitoyl ethanolamide (PEA)	−1.92	−1.61	1.05	1.24
	2-arachidonoyl glycerol (2-AG)	2.21	2.37	−1.18	−1.10
	2-oleoylglycerol (2-OG)	1.66	2.07	−1.30	−1.03
Precursors, Derivatives, Isomers, Candidates	ethanolamine	1.34	1.69	1.08	**1.36**
	phosphoethanolamine	1.92	2.47	−1.19	1.08
	glycerophosphoethanolamine	1.12	1.93	−1.04	1.64
	glycerol	1.02	1.01	−1.04	−1.04
	glycerol 3-phosphate (G3P)	1.41	1.90	0.94	1.26
	1-palmitoyl glycerophosphoethanolamine	1.60	**1.18**	1.38	1.01
	2-palmitoyl glycerophosphoethanolamine	1.10	−1.04	1.05	−1.09
	1-palmitoleoyl glycerophosphoethanolamine	3.26	3.77	1.20	1.38
	2-palmitoleoyl glycerophosphoethanolamine	1.43	1.44	1.24	**1.25**
	1-stearoyl glycerophosphoethanolamine	1.59	1.40	1.22	1.07
	1-oleoyl glycerophosphoethanolamine	2.33	2.36	1.08	1.10
	2-oleoyl glycerophosphoethanolamine	−1.12	−1.20	1.10	1.02
	2-linoleoyl glycerophosphoethanolamine	**1.35**	1.02	−1.47	−1.96

		Fold of Change			
		ANOVA Contrasts			
	Biochemical Name	**Ctrl-SCI Ctrl-Sham**	**O3PUFA-SCI O3PUFA-Sham**	**O3PUFA-Sham Ctrl-Sham**	**O3PUFA-SCI Ctrl-SCI**
	1-arachidonoyl glycerophosphoethanolamine	4.52	4.06	−1.15	−1.28
	2-arachidonoyl glycerophosphoethanolamine	1.20	1.06	**−1.16**	−1.32
	2-docosapentaenoyl glycerophosphoethanolamine (GP-DPEA)	1.26	1.42	4.05	4.56
	2-docosahexaenoyl glycerophosphoethanolamine (GP-DHEA)	1.18	1.28	**1.24**	1.34
	elcosapentaenoyl glycerophosphoethanolamine (GP-EPEA)	1.83	1.8	10.27	10.15
	1-palmitoyl plasmenylethanolamine	2.28	1.78	1.48	1.15
	1-palmitoyl glycerol (1-monopalmitin)	2.53	2.53	1.10	1.10
	2-palmitoyl glycerol (2-monopalmitin)	1.89	2.27	−1.27	−1.04
	1-stearoyl glycerol (1-monostearin)	1.94	2.11	1.01	1.10
	1-oleoyl glycerol (1-monoolein)	2.42	2.58	−1.03	1.04
	1-arachidonyl glycerol	1.70	2.64	−1.56	−1.01
	eicosenoyl glycerol (monoeicosenoin)	3.70	3.22	−1.06	−1.22

Green: indicates significant difference ($p \leq 0.05$) between the groups shown, metabolite ratio of < 1.00

Light Green: narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of < 1.00

Red: indicates significant difference ($p \leq 0.05$) between the groups shown: metabolite ratio of ≥ 1.00

Light Red: narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of ≥ 1.00

Non-colored text and cell: mean values are not significantly different for that comparison