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Effects of Omega-3s and Vitamin E Prophylactic Diets and the Implications of Lipid Transport in Spinal Cord Injury

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in conjunction with the
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Effects of Omega-3s and Vitamin E Prophylactic Diets and the Implications of Lipid
Transport in Spinal Cord Injury

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
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ABBREVIATIONS

ROS	Reactive Oxygen Species
HOCl	Hypochlorous Acid
MPO	Myeloperoxidase
Cl^-	Chloride Ion
H_2O_2	Hydrogen Peroxide
C	Cytosine
HOC5	Hydroxycytosine
HOU5	Hydroxyuracil
ClC5	Chlorocytosine
ClU5	Chlorouracil
ClG8	Chloroguanosine
ClA8	Chloroadenosine
PMN	Polymorphonuclear neutrophil
Tyr-192	Tyrosine – 192 amino acid of protein
Lys-195	Lysine – 195 amino acid of protein
His-193	Histidine – 193 amino acid of protein
5mC5	Methylcytosine
DNMTDNA	Methyltransferase
CNS	Central nervous system
SCI	Spinal cord injury
ω3PUFAs	Omega-3 polyunsaturated fatty acids

FAT/CD36	Fatty acid translocase/membrane cluster of differentiation 36
APC	Adenomatous Polyposis Coli Protein
DHA	Docosahexaenoic Acid
PUFA	Polyunsaturated Fatty Acid
AA	Arachidonic Acid
TBARS	Thiobarbituric acid reactive substances
CPGs	Central Pattern Generators
NAE	N-Acylated Ethanolamines
eCBs	Endocannabinoids
DSD	Dyssynergia
tcMMEPs	Transcranial Magnetic Motor Evoked Potentials
VLF	Ventrolateral Funiculus
CREB	Cyclic AMP responsive element binding protein
BBB scale	Basso-Beattie-Bresnahan
dpi	Days post injury
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
IR	Immunoreactivity
TdT	Terminal deoxynucleotidyl transferase
CST	Corticospinal tract
VGM	Ventral gray matter
µg	Microgram

dT	Oligo
Akt	Protein kinase B
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Ct	Crossing threshold
ESD	Extreme studentized deviate
LFB	Luxol fast blue
NF-H	Neurofilament
OPC	Oligodendrocyte precursor cell
dCST	Dorsal corticospinal tract
ED1	Marker for monocytes/macrophages/microglia
GFAP	Glial fibrillary acidic protein/marker for astrocytes
CD68	Membrane cluster of differentiation 68/ marker for macrophages
CD11b	Membrane cluster of differentiation 11b/ marker for microglia
wpo	Weeks post-operation
EPA	Eicosapentaenoic acid
LA	Linolenic acid
GC/MS gas	Chromatography/ mass spectrometry
ESI	Electrospray ionization
LIT	Linear ion-trap
MVDA	Multivariate data analysis

PLS-DA	Partial least squares projections to latent structures discriminant analysis
BMR	Basal metabolic rate
wpi	Week post-injury
PC1	Principal component 1
PC2	Principal component 2
DGLA	Dihomo-γ-linolenic acid
GLA	γ-linolenic acid
DPA	Docosapentaenoic acid
SDA	Stearidonic acid
PG	Prostaglandins
LT	Leukotrienes
TX	Thromboxanes
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HODE	Hydroxyoctadecadienoic acid
DHEA	Docosahexaenoyl ethanolamine
DPEA	Docosapentaenoyl ethanolamine
EPEA	Eicosapentaenoyl ethanolamine
TH	Thermal hyperalgesia
HWL	Hindpaw withdrawal latency
UHPLC/MS/MS^2	Ultrahigh performance liquid chromatography/tandem mass spectrometry

GAP43	Growth-associated protein 43
CGRP	Calcitonin gene-related peptide
GP-NAEs	Glycerophospho- containing N-acyl ethanolamines
LEA	Linoleyl ethanolamine
AEA	Arachidonoyl ethanolamine
EG	Eicosenoyl glycerol
2-AG	2 arachidonoyl gylcerol
2-PG	2-palmitoyl glycerol
1-OG	1-oleoyl glycerol
PEA	Palmitoyl ethanolamine
PLD	Phospholipidase D
Abh4	Phospolipidase A/B or α-β-hydrolase 4
GDE1	Glycerophosphodiesterase
NAPE	N-acyl phosphatidyl ethanolamine
G3P	Glycerol-3-phosphate
LPA	Lysophosphatidic acid
PA	Phosphatidic acid
FDR	False discovery rate
VIP	Variable importance in protection
OEA	Oleoyl ethanolamine
Ins/Cr	Inositol-to-creatine levels
BSA	Bovine Serum Albumin
NeuN+	Neuronal nuclei positive

TBI	Traumatic brain injury
NG2	Neural/glial antigen 2, Chondroitin sulfate proteoglycan
CTL	Control
INJ	Injury

ABSTRACT OF THE DISSERTATION

Effects of Omega-3s and Vitamin E Prophylactic Diets and the Implications of Lipid
Transport in Spinal Cord Injury
by

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Doctor of Philosophy, Graduate Program in Physiology
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Dr. Marino De León, Chairperson

Traumatic injury to the central nervous system (CNS) presents a devastating

problem to our society. Although current interventions are promising, no effective

treatments are available. The neural membrane fatty acid composition is markedly altered

during the first few days after spinal cord injury (SCI). SCI results in autonomic

dysfunction, paralysis, spasticity, and significant chronic neuropathic pain. The trauma

leads to significantly increased levels of free radical and oxidative stress that contribute

to obstructing tissue healing and recovery. A growing body of evidence shows that

administration of hydrophobic molecules such as Vitamin E and long-chain omega-3

polyunsaturated fatty acids (ω3PUFAs) are capable of attenuating secondary damage,

while promoting functional recovery after SCI. The precise molecular mechanisms

coupling ω3PUFAs and Vitamin E to pro-restorative targets, is not well understood.

Increasing attention is now being paid to understand the cellular targets of these

hydrophobic molecules and how they are being transported after CNS trauma. The

present study investigates the impact pre-treatment with ω3PUFAs and Vitamin E (alpha-

tocopherol) in key functional outcomes observed during the acute phase of SCI.

Additionally, we explored potential mechanisms through which these hydrophobic

molecules were exerting its beneficial effects such as the survival of motor neurons and oligodendrocytes and the transport of ω3PUFAs and Vitamin E. Due to the hydrophobicity of ω3PUFAs and Vitamin E, membrane transport may be required to facilitate their mobilization and meet the increased metabolic demand at the injury site. The fatty acid translocase/membrane cluster of differentiation 36 (FAT/CD36) is a B class scavenger receptor, which has been implicated in the uptake and signaling of hydrophobic molecules, including ω3PUFAs and Vitamin E. Female adult Sprague-Dawley rats were received tail injections with ω3PUFAs (i.e. DHA) or were fed either with a normal diet or a regiment supplemented with ω3PUFAs and Vitamin E for eight weeks. Following the treatment animals were exposed to a contusion SCI or Sham. All animals were examined using standard functional behavior analysis. We report that pre-treatment with DHA injections improved locomotion, bladder recovery, and survival of both neurons and oligodendrocytes. Dietary ω3PUFAs pre-treatment yielded the same results as DHA injections and in addition it decreased neuropathic pain as measured through thermal hyperalgesia and allodynia. Dietary Vitamin E improved locomotion, improved H-reflex depression (indirect measure for spasticity), accelerated bladder recovery, and significantly increased the numbers of oligodendrocytes, but did not seem to preserve neurons in the ventral horn of injured rats. Further, dietary Vitamin E also increased the levels of supraspinal serotonin immunoreactivity. FAT/CD36 mRNA levels were increased at 7 days post-injury and its protein levels remained constant despite neuronal and oligodendrocyte loss. We observed the highest IR in motor neurons of the ventral gray matter and mature oligodendrocytes expressing the adenomatous polyposis coli protein (APC). Quantitative analysis of FAT/CD36 expression showed only Vitamin

E upregulated this protein in neurons after SCI. Our findings support the complementary use of ω3PUFAs and Vitamin E to ameliorate motor, sensory, and autonomic dysfunctions observed following SCI. Lastly, given the beneficial roles of ω3PUFAs and Vitamin E in ameliorating functional recovery, FAT/CD36 may be a contributor to basic protection mechanisms in the injured spinal cord. Future pharmacological studies will confirm the role of FAT/CD36 in cell survival after SCI.

CHAPTER ONE

INTRODUCTION

Traumatic spinal cord injury (SCI) is an incapacitating disorder which affects millions of people and results in motor, sensory, and bladder dysfunction. Traumatic SCI is a main cause of disability among young adults. Epidemiologic studies show SCI affects more than 12,000 people per year in the US. Not included in these counts, are the incidences of non-traumatic SCI caused by congenital, developmental, inflammatory, and degenerative disorders [1]. Patients undergoing surgery of the spinal cord [2-4], athletes [5], and military personnel[6] are particularly susceptible and at higher risk for incurring SCI.

Initial mechanical injury to the spinal cord initiates multiple cascades of secondary events responsible for neurological dysfunction and associated comorbidities. These events are pronounced within the first few days and up to a week following injury, and include metabolic alterations [7-15], oxidative stress, inflammation[16-26], demyelination/axonal damage [26-31], apoptosis [32-35], neurogenic shock, vascular insults, excitotoxicity, and calcium-mediated injury [18, 36-37].

Our studies investigate preventive approaches that confer resistance to damage in the spinal cord and promote physiological responses that improve recovery following injury. In many cases SCI cannot be prevented, but we aim to ameliorate the secondary damage responsible for neurological dysfunction in affected patients. There are very few studies focusing on SCI susceptibility and, therefore, an urgent need to provide clinically translational methods to prevent dysfunction. Studies performed in the last decade show

that detrimental outcomes in SCI are not dependent on the presence of set factors, but rather the balance between restorative and destructive molecular signals. [38]. As mentioned before, the secondary events responsible for neurological dysfunction in SCI occur within the first few days and up to a week after injury. For this reason, our studies focused on investigating the neuroprotective effects of prophylaxis with docosahexaenoic acid (DHA; 22:6 n-3) and Vitamin E during this acute injury phase.

SCI causes prominent dysregulation of lipid homeostasis, specifically in polyunsaturated fatty acids (PUFAs). Following injury, there is a preferential breakdown of arachidonic acid (AA, O6PUFA) from cell membrane components and marked peroxidation of docosahexaenoic acid (DHA, O3PUFA [8, 10, 41-45]. As a result, there is a deficiency in O3PUFAs as O6PUFAs are metabolized, thus changing the homeostatic ratio of O3PUFAs/ O6PUFAs in the spinal cord. PUFAs are thought to be involved in nerve cell responses to injury. DHA has been correlated with improved functional recovery [46, 51-56] while AA has been implicated in the secondary events leading to neurological dysfunction [46-50]. We attribute these correlations, in part, to the pro-inflammatory and anti-inflammatory properties of AA and DHA, respectively. DHA is anti-inflammatory [57-59], neuroprotective [60-62], and able to upregulate survival proteins such as Akt and CREB, which are in involved in pathways leading to myelin stabilization and neuroprotection [67-70]. Evidence to the advantages of DHA has been shown in various models of neurological dysfunction [71-74] that include SCI [46, 51-53]. Furthermore, studies have shown that treatment with DHA improves neurological dysfunction by targeting multiple cascades involved in the pathophysiology of SCI [57-66].

In addition to DHA, our studies investigated the prophylactic effects of Vitamin E. Dietary Vitamin E supplementation improves locomotor recovery and [75-78] ameliorates nerve conduction deficits following SCI [75, 76]. Although it has been proposed that Vitamin E protects neural tissue by decreasing lipid peroxidation of products such as thiobarbituric acid reactive substances (TBARS) [75] and malondialdehyde [79], there is still a need to understand the mechanisms underlying its protective effects. Our aim is to provide evidence that prophylactic dietary intervention with O3PUFAs and Vitamin E will improve resiliency and functional recovery following SCI. We are specifically interested in prophylactic interventions because of their potential impact in populations at high risk for neurological injury (i.e., surgery, neurodegenerative/congenital disorders, athletes, and military personnel). Also, our findings will help in identifying key biomarkers contributing to the susceptibility of the spinal cord to secondary injury and dysfunction (locomotor dysfunction, neuropathic pain, bladder dysfunction, and spasticity).

It is well documented that the spinal cord circuitry contributes to the control of movement [80, 81]. For example, stretch reflexes are single-phased motor responses to sensory input that are produced by the spinal cord. After SCI, there is disruption and, eventually, a deficiency in the mechanistic components that inhibit lower extremity H-reflexes — the electrical analogues of stretch reflexes. Inhibition of these reflexes during the gait cycle is important for normal walking, and disruption of these during SCI explains why paralysis or abnormal gait occurs. There is dysfunctional reflex inhibition following injury which dysregulates the motor output necessary for normal gait[82-86].

The spinal cord is also able to generate more complex rhythmic behaviors on its own (without supraspinal or proprioceptive signals from the central nervous system) through spinal central pattern generators (CPGs). CPGs are an organizational system in charge of producing basic motor patterns. Higher centers activate the appropriate sets of spinal CPGs to modify them. Thus, spinal CPGs have the intrinsic capacity to produce movement without proprioceptive or supraspinal input [81, 87-90]. The control of motion does need supraspinal influence for the initiation and termination of locomotion, but, due to the significant automaticity and well-integrated nature of its sensorimotor responses, the specific millisecond to millisecond control of the musculature performing locomotor functions is accomplished by the spinal circuitry [91]. Lastly, the spinal cord processes and integrates complex sensory information which serves to generate motor responses for a particular group of sensory input [92, 93].

When the spinal cord is lesioned, the amount of fibers spared in the ventrolateral/dorsolateral funiculi (where the corticospinal, rubrospinal, and reticulospinal tracts are located) positively correlate with increased locomotor scores in open field and grid tests [94]. Additional studies also show that the severity of dysfunction after SCI is closely linked to the amount of white matter spared in the lesioned area [95-100]. These findings demonstrate the importance of the spinal cord for proper locomotion. Moreover, following weeks of body weight support exercises, the spinal cord can potentiate motor patterns in the lower limb muscles. Lumbosacral coordination of lower limb motor pools can occur when enough proprioceptive information related with weight support stepping is relayed through the lumbosacral spinal cord to the neural control circuitry. Complete injuries to the spinal cord are unable

to activate the motor pools necessary for weight support stepping due to an inability to activate enough motor units. This may be due to either insufficient proprioceptive input or muscle atrophy [101-105]. Certainly, the spinal cord, its organization, and associated structures are crucial for locomotion. An additional focus of our study is to determine the prophylactic effect of O3PUFAs and Vitamin E on locomotor recovery in different SCI models.

In addition to locomotor and sensory dysfunction, SCI predisposes patients to chronic neuropathic pain, a condition which adversely impacts their quality of life [106]. The existing therapies aimed at treating chronic neuropathic pain are not efficient enough, have considerable side effects, and have low tolerance. We propose that prophylactic therapies such as O3PUFAs (i.e. DHA) can ameliorate the development of chronic neuropathic pain by increasing the resiliency of the spinal cord prior to injury. PUFAs have been shown to mediate pain processing. For example, rats on diets with high omega-3 to omega-6 PUFA ratios have increased thresholds for thermal and neuropathic pain [107]. Also, O3PUFAs and their metabolites decrease thermal and chemical stimulation in animal models [108-110]. Interestingly enough, there are studies where pain behaviors related to nerve injuries are influenced by diet when the injury occurs [111-115]. These studies lead us to hypothesize that a prophylactic diet with O3PUFAs can play a significant role in abrogating/ameliorating chronic neuropathic pain after SCI. We specifically assessed if O3PUFAs are effective in decreasing thermal pain stimuli after SCI and the modulators responsible for their potential effects. A group of bioactive lipids, N-Acylated ethanolamines (NAEs) and endocannabinoids (eCBs) have been associated to pain[116-118], regulate anti-inflammatory processes, and act on

cannabinoid receptors as endogenous agonists [119-122]. Due to the association of NAEs and eCBs to pain, inflammation, and cannabinoid receptors, we studied if O3PUFAs are involved in their modulation after SCI. Our goal was to find the metabolomic profiles that would increase chronic pain and the one profile that would ameliorate it.

SCI causes areflexia and muscle weakness leading to paralysis and chronic pain, both of which are negatively affected by the development of spasticity[123-126] as the nerve cell network recovers. Spasticity results from increased excitatory and decreased inhibitory signals in the neuronal circuitry as the injured nervous tissue tries to compensate for descending and motor-sensory input loss [123, 127, 128]. It is characterized by increased resistance to passive stretch, reduced joint range of motion, and voluntary muscle activity such as spasms, hyperreflexia, clonus, co-contraction [124, 129, 130]. Involuntary muscle spasms (i.e., prolonged contractions) and tone in extensor muscles can facilitate walking, standing, and transfers [131-133], but excessive spasticity will hinder functional recovery. We wanted to investigate whether Vitamin E and O3PUFAS could improve the excessive spasticity seen after SCI. Our rationale for Vitamin E usage is derived from studies showing that Vitamin E supplementation improved the recovery of spinal evoked potentials after injury [75, 76]. Vitamin E is a powerful antioxidant and O3PUFAs are highly anti-inflammatory which makes both hydrophobic molecules particularly helpful in the context of SCI. Decreased ROS and inflammation may decrease the damage done to the neuronal network after SCI, which contributes to the development of spasticity. O3PUFAs have been shown to be neuroprotective in the context of SCI and its effects may extend to the amelioration of spasticity.

In addition to paralysis and chronic pain, SCI patients also experience life-threatening secondary complications associated with autonomic bladder dysfunction [134, 135]. Unfortunately, there is no safe or effective treatment to treat bladder dysfunction. Although electrical modulation of spinal command centers involved in controlling micturition behaviors is a selective and potentially safe treatment option, there is an urgent medical need for studies identifying safe pharmacological interventions. Bladder dysfunction initially presents as areflexia and then develops into bladder hyperreflexia [136, 137], dysfunctional relaxation of the bladder neck, abnormal contraction of the external urethral sphincter [138], and detrusor-sphincter dyssynergia (DSD) [136] after the acute stage. The pathophysiological change from areflexia to hyperreflexia and abnormal relaxation/contraction of bladder neck and sphincters is attributed to (i) the loss of supraspinal descending regulation of lumbosacral autonomic neurons, (ii) the emergence of reorganized spinal micturition reflex pathways after injury, (iii) and the activation of mechano-insensitive unmyelinated C-fibers that usually do not respond to bladder distention to mechano-sensitive fibers in addition to the normally mechano-sensitive A-delta fiber afferents [139, 140].

Current interventions to ameliorate bladder dysfunction after SCI include cholinergic muscarinic receptor antagonists [141-147], chemical blockade of C-fiber afferent neurotransmission with capsaicin or resiniferatoxin[148-154] and alpha1-AR receptor antagonists[155-161]. Additional interventions include suppression of motor-neuron or interneuron excitation in the spinal cord by glycine, GABA agonists, and baclofen which is currently being used to treat dysfunctional contraction of the external urethral sphincter[162-168]. Botulin toxin, a presynaptic neuromuscular blocker, is now

FDA-approved to treat bladder hyperreflexia and DSD by inducing reversible muscle weakness [169-174]. Notably, antioxidant therapy is a promising therapy to improve bladder function following SCI. For example, treatment with quercetin improved bladder contractility, while decreasing reactive oxygen species, plasma cytokines, and caspase 3, and prevented depletion of free radical scavengers after SCI in rats [175]. Another study showed decreased urinary tract infections when SCI patients were treated with anti-oxidant cranberry extract supplements for at least 6 months [176]. Due to its ample anti-oxidant properties, it seemed reasonable to investigate if Vitamin E prophylaxis can improve bladder function. Additionally, we investigated if O3PUFAs improved bladder function due to their anti-inflammatory properties and neuroprotective effects in various models of SCI.

In addition to functional recovery our project focused on potential mechanisms through which O3PUFAs and Vitamin E are protective in the context of spinal cord injury. Not only were we interested in knowing if O3PUFAs and Vitamin E are major modulators of repair mechanisms following SCI, the molecular mediators and targeted cell types coupling these hydrophobic molecules to functional recovery after SCI were studied.

The first molecular mediator we were interested in is the fatty acid translocase membrane cluster of differentiation 36 (FAT/CD36). FAT/CD36 is a B class scavenger receptor and lipid transporter highly expressed in models of neurological and cardiovascular injury and implicated in the uptake and signaling of fatty acids and Vitamin E [177-180]. FAT/CD36 have been shown to be beneficial or detrimental after injury [181-186]. One of the long-term goals of our laboratory is to delineate the

contribution of lipid transport proteins such as FAT/CD36 to differential outcomes following neurotrauma. The objectives of this application were to characterize the spatiotemporal expression and functional roles of CD36 after SCI and find out if prophylactic diets enriched with O3PUFAs and Vitamin E modulate the expression of this protein in specific cell types important for functional recovery (i.e. mature oligodendrocytes and motor neurons). (references in my proposal). As previously mentioned cell death, neuroinflammatory processes, and deregulated lipid metabolism are markedly increased during the first week of injury in rats. These events lead to detrimental alterations in spinal cord tracts and results in neuronal dysfunction.

Functional neuronal cells and oligodendrocytes in the spine are essential for locomotion and proper conduction. For this reason, we investigated whether, in addition to improving functional recovery, a prophylactic diet enriched with O3PUFAs and Vitamin E decreased neuronal and oligodendrocytic cell death within the spinal cord. Previous work has found that intravenous administration of O3PUFAs right before injury (30 minutes before) increases neuronal and oligodendrocyte survival [52]. There is less published work about the effects of dietary Vitamin E prophylaxis compared to O3PUFAs prophylaxis in the context of SCI. Vitamin E is powerful antioxidant that may decrease neuronal and oligodendrocyte death in the context of SCI. Oligodendrocytes are extremely susceptible to reactive oxygen species (ROS) [187, 188] and Vitamin E has been shown to decrease the accumulation of intracellular ROS in rat brain oligodendrocytes and pre-OLs in vitro [189-192]. Increased oligodendrocyte survival in Vitamin E fed rats may result in better conduction by way of increased myelination.

Increased myelination ,then, would lead to enhanced preservation of the neuronal tracts responsible for locomotion and conduction [31, 193-198].

In summary, this study investigated the impact of O3PUFAs and Vitamin E (alpha-tocopherol) dietary prophylaxis in key functional outcomes observed during the acute phase of SCI. Additionally, we also investigated potential mechanisms through which these hydrophobic molecules were exerting their beneficial effects, i.e. survival of motor neurons survival of oligodendrocytes, and the role of FAT/CD36 in the pathophysiology of SCI. Our findings support the complementary use of O3PUFAs and Vitamin E to improve motor, sensory, and autonomic dysfunctions observed following SCI. Given the beneficial roles of O3PUFAs and Vitamin E in assisting functional recovery, its transport, potentially by FAT/CD36, may be a contributor to basic protection mechanisms in the injured spinal cord.

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CHAPTER TWO

DOCOSAHEXAENOIC ACID PRETREATMENT CONFERS PROTECTION AND FUNCTIONAL IMPROVEMENTS AFTER ACUTE SPINAL CORD INJURY IN RATS

By

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Abstract

Currently, few interventions have been shown to successfully limit the expansion of secondary damage pathways that are initiated during the acute phase of spinal cord injury (SCI). Docosahexaenoic acid (DHA, C22:6 n-3) is neuroprotective when administered following SCI, but its potential as a pretreatment modality has not been addressed. This study used a novel DHA pretreatment experimental paradigm that targets acute cellular and molecular events during the first week after SCI in rats. We found that DHA pretreatment reduced functional deficits during the acute phase of injury, as shown by significant improvements in BBB locomotor scores and the detection of transcranial magnetic motor evoked potentials (tcMMEPs) responses when compared to vehicle-pretreated animals. Histopathological analyses of DHA-pretreated rats showed increased white matter tissue preservation and more axons immunoreactive for phosphate-dependent neurofilament heavy domains. We found a significant increase in the survival of $NG2^+$, APC^+, and $NeuN^+$ cells in the ventrolateral funiculus (VLF), dorsal corticospinal tract, and ventral horns and dorsal horns, respectively. Interestingly, these DHA protective effects were observed despite the lack of inhibition of high expression of inflammatory markers for monocytes/macrophages and astrocytes, ED1/OX42 and GFAP. Further, DHA pretreatment induced levels of Akt and cyclic AMP responsive element binding protein (CREB) mRNA and protein. This study shows for the first time that DHA-pretreatment ameliorates functional deficits, increases tissue sparing, and precursor cell survival. DHA induction of pro-survival/anti-apoptotic pathways may be independent of its anti-inflammatory properties.

Introduction

Traumatic spinal cord injury (SCI) is a devastating disorder affecting several million people worldwide. Interestingly, the prevalence of non-traumatic SCI as consequence of congenital, developmental, inflammatory, and degenerative disorders suggest that the number of SCI patients would more than quadruple if non-traumatic causes were included [1]. The spinal cord is also at significant risk of injury in numerous surgical interventions [2, 3], sports [4], and military conflicts [5]. Although SCI may prove difficult to prevent in most instances, there is a need to further investigate effective preventive strategies to ameliorate the physiological outcomes in the event of damage. This could have important clinical implications in situations where injury is an unavoidable risk.

Injury to the spinal cord results in immediate mechanical primary damage, which is followed by secondary injury mediated by a complex cascade of molecular events [6]. These immediate and acute secondary mechanisms include neurogenic shock, vascular insults, excitotoxicity, calcium-mediated injury, inflammation, cell death, and additional cellular and molecular disturbances [7]. Based on evidence that proposes that the major occurrence of apoptosis, white matter degeneration, and inflammatory responses occur during the first week after SCI [8-11], we focused this study on investigating the neuroprotective efficacy of a prophylactic docosahexaenoic acid (DHA; 22:6n-3) treatment during the acute injury phase. DHA is a promising intervention to prevent neurodegeneration because it can simultaneously target several pathways that contribute to acute SCI pathophysiology. For instance, DHA and its derivatives show anti-inflammatory [12-14] and neuroprotective attributes [15-17]. Moreover, DHA activates

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both Akt and CREB, which have been associated with neuroprotection and myelin stability in vitro and in vivo [18-21]. These beneficial effects have been reported in a number of neurological studies [22-25], including SCI [26-29]. However, there is a lack of consensus regarding the primary neuroprotective mechanisms of DHA and whether the protection is primarily due to direct activation of pro-survival pathways or to modulation of inflammation.

Initial experiments from our lab showed that two low dose tail injections of DHA administered one week apart in advance of SCI significantly improved important functional outcomes without inhibiting microglial activation at one week after SCI. Based on these findings, we focused this study on the characterization of these protective prophylactic effects to provide strong support for the idea that DHA pretreatment may target various secondary injury processes associated with acute neurological impairments after SCI. This study further supports our initial observations and demonstrates, for the first time, that DHA is neuroprotective, increases functional white matter tissue preservation, and ameliorates locomotor and conduction deficits in the presence of an inflammatory environment when administered in a prophylactic manner in a rodent model of SCI. Our results suggest that this neuroprotection may be attributable, at least partially, to the activation of Akt and CREB signaling pathways and support the use of DHA pretreatment to successfully target the secondary injury response associated with acute SCI.

Materials and Methods

Experimental procedures were performed in compliance with Loma Linda University and University of Puerto Rico School of Medicine regulations and institutional guidelines consistent with the NIH Guide for the Care and Use of Laboratory Animals.

Animals

Young adult (200-250 grams) female Sprague-Dawley rats were used based on their behavioral and anatomical traits; females are normally less aggressive and less prone to urinary tract infections than their male counterparts. Animals were obtained from Hilltop Lab (Scottsdale, PA) and Charles River Laboratories (Portage, MI). Animals were housed with a light/dark cycle of 12/12 h, and fed ad libitum. Following preliminary experiments to investigate dose- and time-dependent responses, four groups of animals received a tail vein injection of either vehicle or docosahexaenoic acid (DHA; Compound ID: 445580; Source: Sigma-Aldrich; IUPAC: 4Z, 7Z, 10Z, 13Z, 16Z, 19Z-docosa-4, 7,10,13,16,19-hexaenoic acid; MW: 328.488280 g/mol; MF: $C_{22}H_{32}O_2$) at a dose of 250 nmol/kg (~82 μg/kg) in a volume of 5 mL/kg before undergoing contusive spinal cord injury (SCI) or spinal cord laminectomy only (sham). The DHA stock was dissolved at 1 M in ethanol, diluted with sterile saline, adjusted to pH 7.4, and used immediately after preparation. This bolus administration has been shown to lead to a concentration of ~3.8 μM of DHA in the blood (50 nmoles DHA/13 mL blood) and is neuroprotective while promoting recovery in rodents at 7 days and 6 weeks when administered 30 min after contusive SCI [27-29]. We performed preliminary experiments to evaluate the effects of

multiple DHA doses, routes of administration, and time of intervention. We found that neither a single DHA injection administered one week before nor one hour after SCI was sufficient to ameliorate the locomotor functional impairments when compared to vehicle controls (data not shown). As a consequence, the results reported herein include animal groups that were pretreated one week and one hour before undergoing trauma to the spinal cord (See Figure 1). Animals did not receive further DHA administration after injury. The number of animals per group was 4-6 in the sham and preliminary study groups and 11-12 rats in the SCI groups discussed in this manuscript.

Surgical Procedures

To perform the injury to the spinal cord, animals were deeply anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). It is noteworthy that ketamine has been shown to induce metabolic changes and increased lipid turnover [30]. However, this finding seems to be significant only at higher doses than the one used in this study. The spinal cord was injured at thoracic level 10 (T10) using the NYU/MASCIS impactor device; a reliable and consistent model, which exhibits a progressive secondary injury expansion, including apoptotic cell death and activation of inflammatory pathways [31, 32]. Briefly, the skin and muscles overlying the spinal column were incised and a laminectomy performed at T10, leaving the dura intact. The T8 and T12 spinal processes were clamped to the Impactor and contusion injuries were produced by releasing a 10-g rod from 12.5-mm onto the exposed T10 cord. Analysis of impact parameters was used to identify potential outliers. Behavioral analyses were also used to corroborate differences in injury severity within groups. Two were excluded from the study based on these

criteria. Sham animals received only a laminectomy to expose the spinal cord. After surgery, muscle layers were sutured and skin layers closed with wound clips.

Post-injury Care

Each animal received 5 mL of sterile saline injected subcutaneously. 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. The bladders of injured rats were expressed using the Crede's maneuver (abdominopelvic compression) three times a day until voiding reflex was restored. All animals survived for 7 days after surgery and behavioral testing was performed during this acute phase. Severe urinary tract infections, autophagy, mortality and morbidity are some of the major problems in post operative care of spinal cord injured animals. Three out of fifty-two animals were excluded from the data analysis because of the aforementioned reasons.

Analysis of Locomotion Function

Rats' spontaneous open-field locomotion was evaluated using the 22-point (0-21) BBB scale (Basso-Beattie-Bresnahan; [33, 34]. Briefly, animals were acclimatized to the open field environment during several sessions before testing. Subsequently, two double-blinded and trained observers assessed the locomotive function, joint movement, paw placement and rotation, coordination, and tail and trunk position and stability for four minutes. In this scale, a completely paralyzed rat scores 0, a rat with increasing joint movements but without weight support scores between 1 and 8, a rat with abnormal

locomotion but with weight supported steps (plantar or dorsal) and graded coordination patterns scores between 9 and 20, and a normal (and sham) rat scores 21.

Because our data was focused on the behavioral effects of DHA during the acute injury phase, locomotor scores were transformed to help assure that the data were amendable to parametric analyses and to increase statistical power [35]. This transformation produces a continuous distribution by pooling together scores from 2-4 and thus avoids potentially suspect measures of performance in the lower end of the scale. Additional statistical power was also achieved by obtaining a measure of locomotor performance during earlier injury stages at 1, 3, and 5 days post injury (dpi). This provides a behavioral index of the injury extent that is correlated with long-term recovery (approximate values of $r > 0.40, 0.60, 0.70$, respectively $p < 0.05$, [36].

*** Electrophysiological Assessment of Recovery ***

Analyses of axonal conductance were performed using transcranial motor evoked potential (tcMMEPs) studies using electrophysiological methods described previously [32, 37, 38]. Animals were sedated with a cocktail containing ketamine (i.m. 40 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and acepromazine (i.m. 2.5 mg/kg; Vetus Animal Health, Rockville Center, NY). Once restrained, the magnetic transducer was placed over the skull and a short magnetic pulse (70 μsec; clockwise direction) was delivered using a Maxim 2002 stimulator (Magstim Company Limited, Spring Gardens, Whitland, United Kingdom) attached to a 50-mm-diameter hand-held magnetic transducer. Magnetically evoked EMG responses were recorded using monopolar needle electrodes placed on both gastrocnemius muscles. Data was amplified using Magstim

Neurosign 100 (Magstim Company Limited, Spring Gardens, United Kingdom).

Afterward, the signal was converted and analyzed using the Digidata 1322A (Molecular

Devices Corporation, Whipple Road Union City, CA) and Axoscope 8.2/Axograph X

software, respectively (Molecular Devices Corporation). Only responses with amplitude

measurements above 130 µV, and latencies shorter than 25 msec appearing at 70%, 85%,

and maximum stimulation intensity were considered. Following our behavioral and

electrophysiological assessment, animals were processed for mRNA and histological

analyses to evaluate acute outcome measures.

Tissue Preparation

To prepare the tissue for histopathological analyses, we followed previously

published procedures [32, 39]. Animals were submitted to fast and humane euthanasia

with Fatal-Plus™ (Vortech; Dearborn, MI) and perfused transcardially with phosphate

buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate

buffer. During rostro-caudal tissue harvest, the area of visible bruising of the cord from

rod impact at T10-11 was marked. The spinal cords were removed and post-fixed for 3

hours in 4% PFA, cryoprotected in 30% sucrose for 12-16 hours at 4°C, embedded in

Tissue-Tek® O.C.T.™ compound (Sakura, Torrance, CA), and immediately frozen on

dry ice. A series of transverse 20-µm coronal cryodissections were cut on a Richart-Jung

Cryocut 1800 cryostat (Leica, Deerfield, IL) kept at -20°C. Every 5th section was

collected and placed on consecutive microscope slides (Superfrost Plus; Fisher Scientific,

Pittsburgh, PA). Thus, each slide contained sections 1 mm apart. The slides were stored

at -20°C until use.

Myelin Staining and Quantification of White Matter Spared

To study the integrity of myelin and cell survival after the DHA intervention, histological analysis was performed on coded Luxol fast blue/cresyl violet stained coronal sections, as described before (Figueroa et al., 2006). The stained sections were visualized with an Olympus BX50 microscope and digitized with a SPOT color cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI). Double-blinded morphometric analyses were performed using ImageJ software (US National Institutes of Health, Bethesda, MD; http://rsbweb.nih.gov/ij/index.html) to determine the extent of Luxol fast blue staining.

Immunofluorescence

Immunofluorescence double labeling has been described previously [32]. Spinal cord coronal sections were dried at room temperature for 10-15 min, washed with PBS, and post-fixed with 4% PFA for 10 minutes. The sections were washed, blocked and incubated in either mouse anti-Neun monoclonal antibody (Clone A60; 1:250; Millipore, Billerica, MA), mouse anti-APC-7 monoclonal antibody (Clone CC-1; 1:500; Calbiochem, San Diego, CA), mouse anti-NG2 chondroitin sulfate proteoglycan monoclonal antibody (1:250; Millipore), and mouse anti-neurofilament, heavy tail domain monoclonal antibody (Clone RMO-24; 1:100; Millipore) to examine the immunoreactivity (IR) and cell numbers of neurons, mature oligodendrocytes, oligodendrocyte precursor cells, and phosphate-dependent NF-H domains in axons, respectively. Alternatively, sections were incubated in mouse anti-GFAP (1:200; Millipore), mouse anti-CD11b (OX42; 1:100; AbD Serotec, Raleigh, NC), and mouse

anti-CD68 (ED1; 1:100; AbD Serotec) to examine the infiltration, proliferation, and recruitment of reactive astrocytes, monocytes/macrophages and microglial cells, respectively. For double-labeling immunohistochemistry, sections were concomitantly incubated with either anti-NeuN, anti-APC-7, and anti-NG2 antibodies and either rabbit anti-pAkt or rabbit anti-pCREB (1:100, 1:50, respectively; Cell Signaling, Danvers, MA). The antibody solutions were applied to the sections overnight at 4°C. On the following day, the sections were incubated with Alexa Fluor® 488 or 594-conjugated donkey anti–mouse or anti-rabbit antibodies (1:250; Invitrogen, Carlsbad, CA). Primary antibody omission controls, antibody preabsorption with immunizing peptide, and normal mouse and donkey serum controls were used to further confirm the specificity of the IR. Slides were examined with an Olympus Optical Fluoview FV1000 confocal microscope. The images were prepared for publication with Photoshop CS4 software (Adobe Systems, San Jose, CA).

TdT FraEL Labeling (TUNEL) and Quantification of Cell Death

To investigate the oligoprotective role of DHA after SCI, $NG2^+$ cells were visualized and quantified after being exposed to the terminal deoxynucleotidyl transferase (TdT) FragEL DNA fragmentation detection kit as previously described [32]. Briefly, for double-label immunofluorescence, TUNEL, and $NG2^+$ cell analyses, sections were respectively labeled using Fluorescein-FragEL (Oncogene Research Products, Boston, MA) and a primary antibody against mouse anti-NG2 chondroitin sulfate proteoglycan (1:250; Millipore, Billerica, MA) that was subsequently labeled with Alexa Fluor® 594-conjugated donkey anti–mouse antibody (1:250; Invitrogen, Carlsbad, CA). Sections

were then coverslipped with Hoechst 33342 mounting media and observed under an Olympus FluoView™ FV1000 confocal microscope (Olympus America, Inc., Center Valley, PA). Photomicrographs were captured and analyzed for colocalization by blinded investigators using NIH ImageJ software.

Stereological Methods and Histological Analysis

Our unbiased stereological methods were based on the following steps: 1) estimation of the injury area before quantification. Spinal cords epicenters were marked during tissue harvest and sections were collected in successive slides thus providing a clearer estimation of the spinal cord regions. In addition, investigators used Luxol fast blue-cresyl violet staining and qualitative morphometric analyses to determine the region of interest before proceeding with quantification. The lesion epicenter was defined as the spinal cord sections containing the least amount of spared white matter. 2) Randomization of location and orientation within the ventrolateral funiculus (VLF), corticospinal tract (CST), and ventral gray matter (VGM) using appropriate sampling techniques. 3) Counting of cells, pixel profiles, and areas by a defined test area within an unbiased counting frame. 4) Normalization of time required for image acquisition to prevent photobleaching. 5) Digitalization and image quantification was carried out by blinded observers in at least four animals per group. For staining analyses, a minimum of four images per area per animal was used from the epicenter and 6 areas immediately adjacent to the lesion site. Image analyses of similar areas were averaged and the total sample number used for statistical analysis equaled the number of animals used. 6) Cells identified as positive IR were manually quantified for cells counts. Automated particle

analyses were used after threshold correction to detect the levels of phosphorylated Akt and CREB in spinal cord sections. 7) To assess the extent of white matter content, automated particle detection was performed using ImageJ. Briefly, images were converted to a binary outline image and adjusted to a similar intensity threshold. The number of particles in an unbiased VLF rectangular selection was quantified using the particle analysis tab on ImageJ. 8) Nucleic acid fragmentation in NG2$^+$ cells was quantified using NIH ImageJ, adapted from previous methods [32]. The total FragEL fluorescence and the number of NG2$^+$ cells that co-localized with Fluorescein-FragEL staining were quantified using the colocalization finder plug-in on NIH ImageJ software.

RNA Extraction and Real-Time PCR

Animals were submitted to humane euthanasia with Fatal-Plus™ (Vortech; Dearborn, MI) and perfused transcardially with ice-cold 0.01 M phosphate buffered saline (PBS) solution, pH 7.4 (Sigma-Aldrich, St. Louis, MO) as previously described [32]. Spinal cord segments containing the laminectomy/injury area (5 mm) were collected at 7 days post operation (dpo). Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions and the RNA concentration was determined on a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). One microgram (μg) of total RNA from each sample was used for the first strand cDNA synthesis. The first-strand cDNA synthesis was primed using oligo (dT) based on the SuperScript II First-Strand synthesis kit (Invitrogen, Carlsbad, CA). The synthesized cDNA was used as template for relative quantification of target transcription in spinal cord tissue by real-time PCR. The cDNA was amplified by PCR

using a pair of primers specific for protein kinase B (Akt, Akt1 primers were used in this study; FWD: 5'-TAC CAT GAA CGA CGT AGC CA-3' and REV: 5'-AGG TGC CAT CAT TCT TGA GG-3') and the cyclic AMP responsive element binding protein (CREB; FWD: 5'- CAT GGA CTC TGG AGC AGA CA-3' and REV: 5'-GGG CTA ATG TGG CAA TCT GT 3'). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; FWD: 5'-TGC CAC GAA GAC TGT GG-3' and REV: 5'- TTC AGC TCT GGG ATG ACC TT-3') and β-actin (FWD: 5'- GGG AAA TCG TGC GTG ACA TT-3' and REV: 5'-GCG GCA GTG GCC ATC TC-3') served as internal controls for data normalization. Real-time PCR amplification and analysis were carried out on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). SYBR green I (P/N 4309155; Applied Biosystems, Foster City, CA) was used as a fluorescent reporter dye for the presence of double stranded DNA. After optimization of PCR conditions, 25 μl reactions containing cDNA, SYBR Green PCR Master Mix, and forward/reverse primers were prepared in triplicates. Each PCR run was repeated twice. Cycles consisted of 10 seconds at 95°C for denaturation and 30 seconds at 60°C for annealing/extension. A negative (mock) control reaction without reverse transcription was also performed to verify for genomic DNA contamination in our samples. The specificity of real-time PCR products was confirmed by melting curve analysis. A single melting curve peak was used to determine specificity for each gene target. Since the PCR efficiencies of our target and control genes were similar, the relative levels of target mRNAs were calculated using the comparative Ct (crossing threshold) values as previously described [32, 40, 41].

Statistical Analysis

Data are presented as mean ± SEM. Two-way ANOVA followed by Bonferroni post hoc comparisons was used to determine the effect of our intervention and time on open field locomotion scores. Fisher's exact test was used to determine the effects of our DHA pretreatment on the tcMMEP conduction latencies, followed by a relative risk estimate of the data. All other data were assessed by *t-test*. Statistical analyses were performed using Prism 5 Software (GraphPad Software Inc., San Diego, CA). Outliers were identified using the Grubbs' method, also known as ESD (extreme studentized deviate). Alternatively, values falling more than 2 SD from the mean were excluded from all statistical analyses. Only two animals were excluded from the study after using these exclusion methods. Statistical differences were considered significant at $P < 0.05$.

Results

Effects of DHA Pretreatment on Hindlimb Recovery During the Acute Phase of Injury

After a five-day training and behavioral habituation period, female Sprague-Dawley rats were given two DHA injections; one at one week and an additional challenge dose at one hour before contusive spinal cord injury (SCI). Our rationale for the pretreatment schedule is based on longstanding pharmacokinetic analysis showing that intravenous administration of DHA results in fast incorporation [42] and accumulates in neurons and glia up to 7 days after injections [43]. In addition, interventions for inflammatory conditions, such as the use of methotrexate for rheumatoid arthritis, are administered weekly and in low doses [44]. Animals were allowed to survive for a full week after injury while behavioral data was collected at 1, 3, 5, and 7 days post-injury (dpi). At the

end of the study, spinal cords were harvested and tissues were used for inmmunohistochemical studies, RNA and protein analyses (Fig 1A summarizes the study timeline).

The Basso, Beattie, and Bresnahan (BBB) locomotor grading scale was used to assess the effects of DHA pre-administration in the locomotive behavior of injured rats. Analysis of BBB scores during the acute injury phase demonstrate significant improvements in locomotor behavior in DHA-pretreated rats (Fig. 1B; untransformed data means $\pm$ SEM: 21 ± 0, 1.5 ± 0.8, 4.5 ± 0.9, 7.1 ± 0.7, at baseline, 1, 3, 5, and 7 dpi, respectively; $n = 10$) when compared to animals treated with vehicle injections at 1, 3, 5, and 7 dpi (untransformed data means $\pm$ SEM: 21 ± 0, 0 ± 0, 0.4 ± 0.2, 2.3 ± 0.6, 3.5 ± 0.7 at baseline, 1, 3, 5, and 7 dpi, respectively; $n = 9$) [F(3,100) = 373.19, MSE = 2289, **P < 0.01 at 1, 3 and 5 dpi, ***P < 0.0001 at 7 dpi].

To validate our acute behavioral results, we used a simple post hoc transformation that improves the metric properties of the lower portion of the BBB scale [35]. Transformation (pooling) of BBB scores 2–4 removes the discontinuity in the grading scale to obtain an ordered scale and improves statistical power. Two-way ANOVA analysis followed by Bonferroni post hoc test after employing this approach demonstrated very significant differences in locomotor behavior between treatment groups. Our data transformation and analysis revealed improved BBB scores in the DHA-pretreated group (Fig. 1C; transformed data means $\pm$ SEM: 12 ± 0, 1.1 ± 0.6, 2.1 ± 0.5, 3.8 ± 0.8, 5.8 ± 0.7 at baseline, 1, 3, 5, and 7 dpi, respectively; $n = 10$) when compared to controls (transformed data means $\pm$ SEM: 12 ± 0, 0 ± 0, 0.3 ± 0.2, 1.9 ± 0.5, 2.4 ± 0.4 at baseline, 1, 3, 5, and 7 dpi, respectively; $n = 9$) [F(3,100) = 176.5, MSE = 687.6, **P <

0.01 at 3 and 5 dpi, ***$P < 0.0001$ at 7 dpi].

A video recording of each subject's performance in the open field was obtained at 1, 3, 5, and 7 dpi. Still images from representative vehicle subjects show limited movement, dragging of hindlegs (Fig. 1D), and slight movement of hindlimb joints (Fig. 1E) at 7 dpi. Remarkably, DHA-pretreated animals began to show signs of intermediate locomotor recovery indicated by their ability to produce extensive movements of their joints with dorsal stepping patterns (Fig. 1F). Further, end-point BBB score analysis revealed a group of DHA-pretreated animals with scores at or above 8, as evidenced by weight supported locomotive behavior (Fig. 1G).
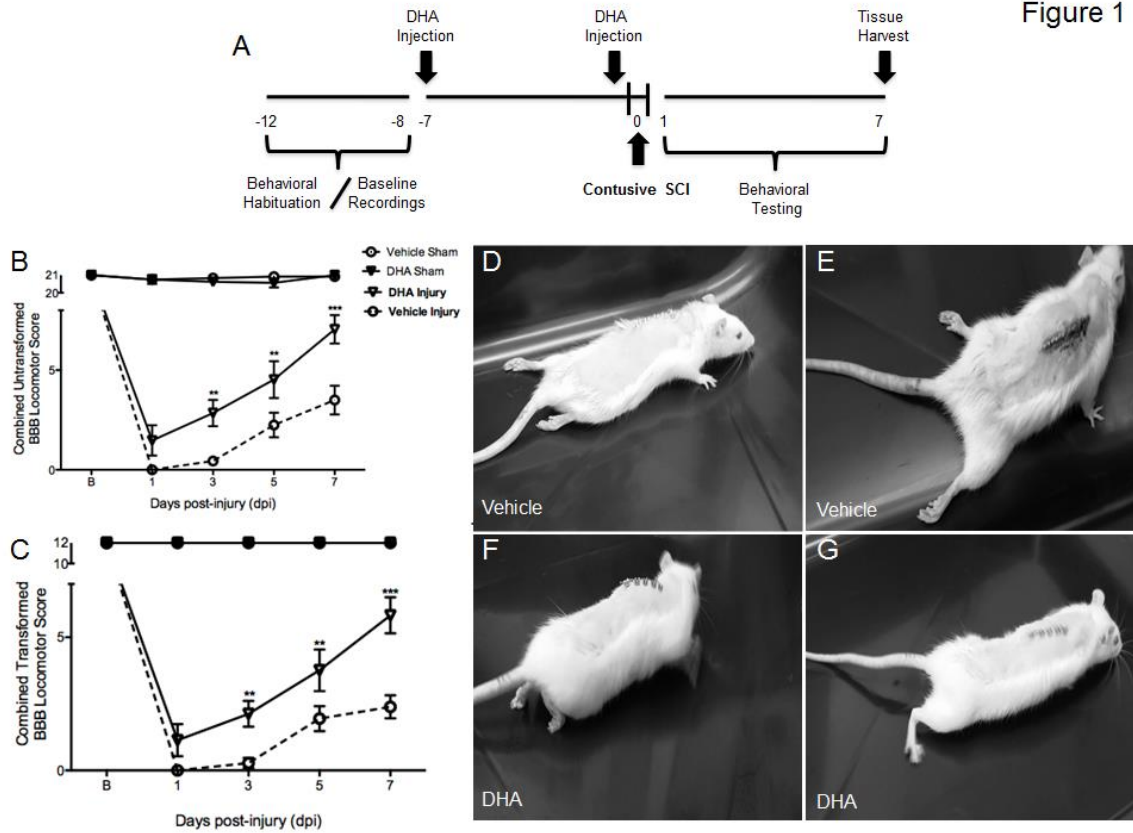
Figure 1. Acute beneficial effects of DHA pretreatment on the hindlimb neurological function of rats after a moderate injury, assessed by the BBB Locomotor Rating Scale. (A) Timeline showing the DHA injection schedule and the timepoints of behavioral assays, surgical procedures, and tissue sample collection. (B-G) Effect of DHA-pretreatment on locomotor function. Time (in days) following pretreatment and spinal cord compression (injury) or laminectomy (sham) is shown on the horizontal axis and untransformed (B) and transformed (C) Basso, Beattie, and Bresnahan (BBB) locomotor rating score is shown on the vertical axis. Animals pretreated with DHA had higher BBB scores at day 3, 5, and 7 when compared to controls. Open-field locomotion still images from vehicle- (D, E) and DHA-pretreated animals (F, G) at 7 dpi. Mean DHA-pretreatment scores revealed that most animals exhibited extensive movements of three joints. DHA-pretreated animals also showed plantar and dorsal weight supported steps at 7 dpi (8-10 untransformed BBB scores). Error bars indicate SEM; vehicle vs. DHA **$P < 0.01$, ***$P < 0.001$; sham DHA, $n = 4$; sham vehicle, $n = 6$; injury vehicle, $n = 9$; injury DHA, $n = 10$ animals.

DHA Pretreatment Significantly Improves Axonal Conduction at 7 Days Post-Contusive Spinal Cord Injury

Transcranial magnetic motor evoked potential (tcMMEPs) *in vivo* electrophysiology was used to evaluate the integrity of thoracic ventrolateral spinal myelinated tracts, which are involved in rodent locomotion [37, 45]. Electrophysiological responses were recorded before lesion (baseline) and at 7 days post injury (dpi). Figure 1 shows representative tcMMEP traces for baseline (Fig. 2A) and treatment groups (Fig. 2B, C). Animals receiving vehicle injections displayed no tcMMEP responses at 7 dpi (Fig. 2B). Remarkably, 67% of the DHA-pretreated animals showed latencies below 25 msec and amplitudes above 130 μV after transcranial magnetic stimulation at 7 dpi (Fig. 2C). Data from baseline and treated animals is summarized in Fig. 2D-E. Contingency table and statistical analyses revealed a *P* value of 0.0303 using Fisher's exact test (*n* = 12). Strength of association analysis demonstrated an odds ratio of 23.40 CI (0.8925 to 613.5) with a sensitivity of 1.000 CI (0.5407 to 1.000) and a specificity of 0.6667 CI (0.2228 to 0.9567).

Figure 2
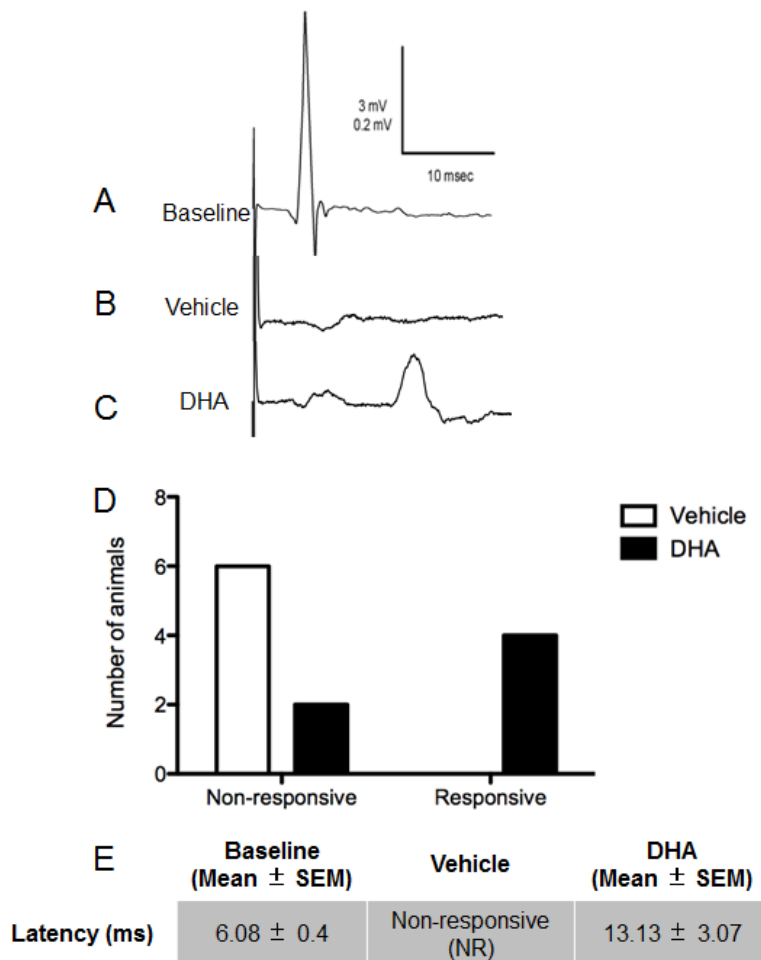
Figure 2. DHA administration before SCI reduces some of the conduction blockade associated with SCI at 7 dpi. Transcranial magnetic motor evoked potential (tcMMEP) responses recorded bilaterally from gastrocnemius muscles at 7 dpi. (A) Baseline; (B) Vehicle; and (C) DHA-pretreated animal at 7 dpi. (D) Sixty-seven percent (67%) of the pretreated animals showed improved nerve conduction and were considered responsive. All vehicle treated animals were non-responsive at 7 dpi. (E) Summary of tcMMEP conduction latencies (ms) averages on responsive animals. Contingency table and statistical analyses revealed a P value of 0.0303 using Fisher's exact test, $n = 12$.

DHA Pretreatment Increased the Density of the Ventrolateral Funiculi (VLF) Spared

White Matter at One Week Post-Injury

To evaluate the histopathology of functionally significant ventrolateral white matter tracts and to validate our behavioral and electrophysiological findings, we performed Luxol fast blue (LFB) staining in spinal cord sections of DHA-pretreated and control animals. Figure 3A shows representative gray-scaled LFB stained transverse sections encompassing seven distinct areas at 1mm intervals that extend 3 mm rostrally and caudally from the lesion epicenter. The lesion epicenter (0 mm) was defined as the section exhibiting the largest white matter damage at 7 dpi. Closer examination showed contusion-induced white matter degeneration as evidenced by reduced LFB staining (Fig. 3B-C). The staining density of randomized ventral white matter areas was quantified using automated particle counter analysis on binary photomicrographs from different VLF regions (Fig. 3B'-C'). Quantitative histological analyses revealed increased myelin staining in the epicenter of DHA-pretreated rats when compared to vehicle treated animals at 7 dpi (Fig. 3D; Student's *t*-test, *n* at least 4 animals, *$P < 0.05$). Immunohistochemistry with anti-neurofilament H, NF-H, was used to assess axonal integrity after our DHA intervention. Qualitative analysis of immunostained sections revealed more NF-H immunoreactivity in the ventrolateral white matter of DHA-pretreated animals when compared to our control group (Fig. 3E-F). Control experiments were carried out to determine antibody specificity (Fig. 3G and 3H). Arrows denote NF-H positive axons in white matter.
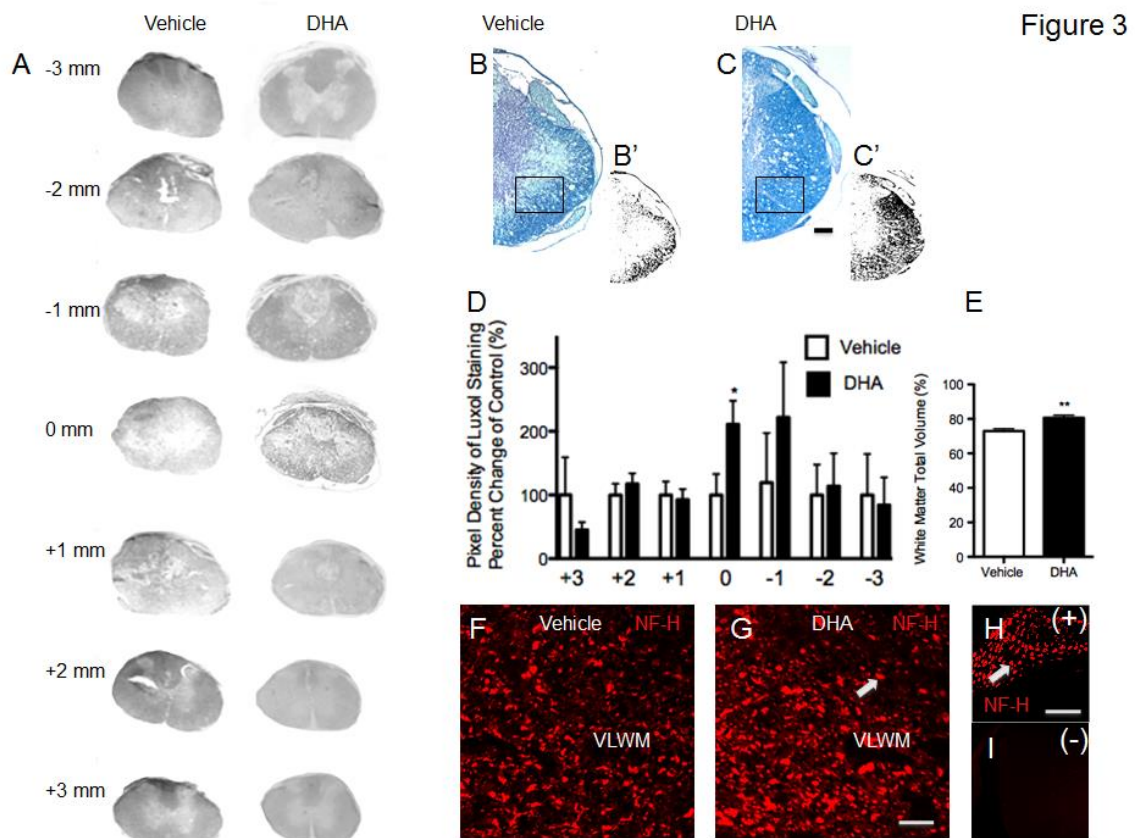
Figure 3. DHA-pretreatment increase white matter sparing of functionally significant areas of the spinal cord at 7 dpi. (A, B) Luxol fast blue (LFB)-stained spinal cord sections from vehicle- (A) and DHA-pretreated rats (B) were taken from the lesion epicenter and adjacent areas of at 7 dpi. (B, C) LFB-stained sections were morphometrically analyzed after tresholding binary images using ImageJ software (B', C'). (D) Results from quantification of particle counts on bilateral analysis of VLF regions (rectangles in B and C) in seven spinal cord areas (ranging from +3 to -3 mm from lesion epicenter (0 mm). Analysis of LFB staining in VLF regions revealed that DHA pretreatment significantly increases white matter spared in the lesion epicenter when compared to vehicle-treated animals at 7 dpi. Student's *t*-test analysis was carried out to determine the statistical significance differences between treatments. (E, F) Laser scanning confocal microscope photomicrographs revealed that DHA-pretreatment increased neurofilament (NF-H) immunoreactivity in ventrolateral white matter regions at 7 dpi. (G) Dorsal roots were used as positive controls for NF-H immunoreaction. (H) Primary antibody omission controls were used to further confirm the specificity of the immunohistochemical labeling. Error bars represent means ± SEM. *$P < 0.05$; $n = 4$-8 animals per area (at least 5 photomicrographs/animal/spinal cord area). Scale bars: B-C, 100 μm; E-F, 50 μm; G-H; 50 μm.

DHA Pretreatment Decreases Apoptotic Cell-death in Functionally Relevant White

Matter Areas and Oligodendrocyte Precursor Cells After SCI

To investigate whether DHA-pretreatment reduces the activation of apoptotic

pathways after SCI, we assessed apoptotic cell death in the white matter using fluorescent

TUNEL. We observed a marked reduction in the number of TUNEL-positive cells in the

WM as compared to those found in vehicle controls at 7 dpi (Fig. 4A; **$P = 0.0043$; $n =$

8 per group). Representative photomicrographs of white matter spinal cord regions

stained with Fluorescein-FragEL and counterstained with DAPI (B-E). Images

demonstrate increased DNA fragmentation in vehicle-treated animals (B, C) when

compared to DHA-pretreated animals (D, E).

To investigate whether DHA decreases the susceptibility of oligodendrocyte

precursor cell (OPC) to undergo apoptosis after SCI, we quantified the DNA

fragmentation in $NG2^+$ cells using immunohistochemical analysis. Examination of

immunoreactivity in the VLF revealed that the number of $NG2^+$ cells undergoing DNA

fragmentation was significantly reduced in our DHA-pretreated group at 7 dpi (Fig. 4A;

*$P = 0.0320$; $n = 4$ per group). In these analyses, apoptotic cells (Fluorescein-FragEL$^+$)

were examined and scored as positive only if they colocalized with $NG2^+$ and DAPI

immunoreactivity (Fig. 4F-G).
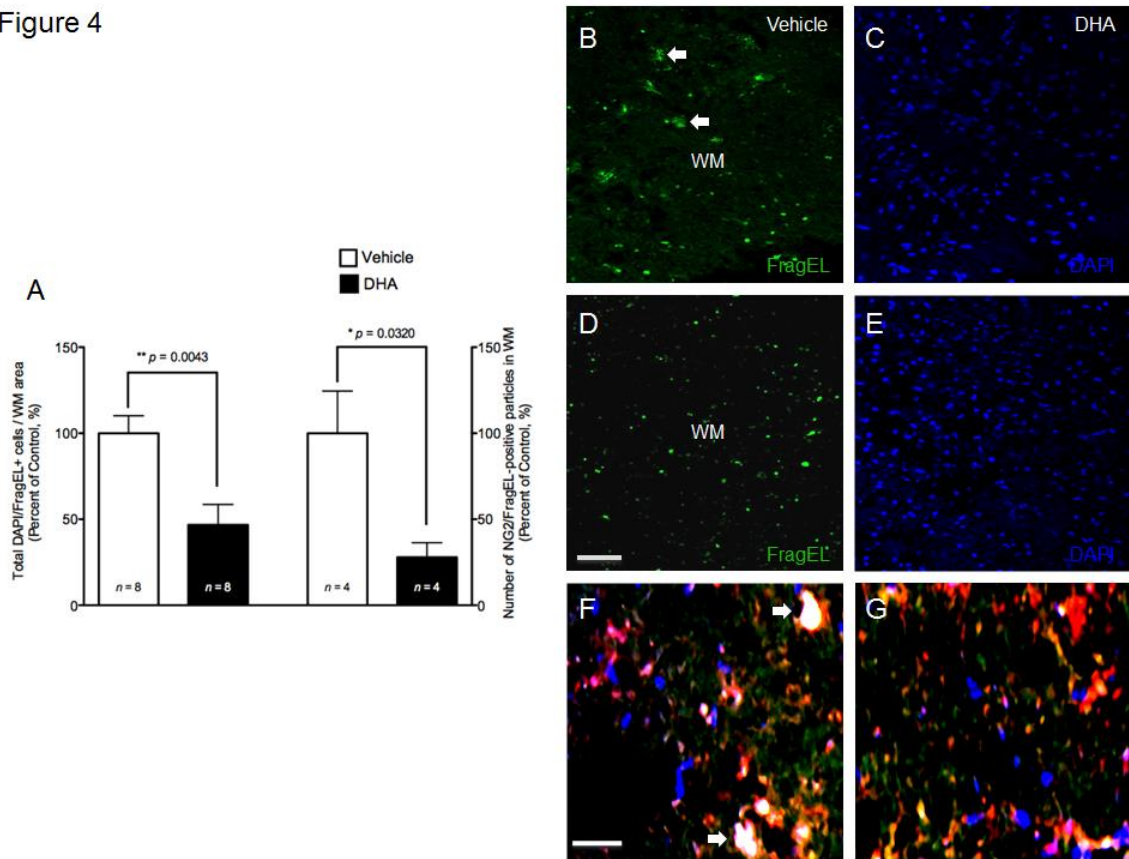
Figure 4. Prophylactic intervention with DHA reduces the number of white matter cells undergoing apoptosis after SCI. (A) Results from ImageJ-assisted co-localization analysis showed a significant reduction in the number of total DAPI/FragEL$^+$ and FragEL$^+$/NG2$^+$ cells in the WWM regions. Representative photomicrographs from white matter analyses show increased DNA fragmentation in control groups (B, C) when compared to DHA-pretreated animals at 7 dpi (D, E). Images from representative triple-labeled (DAPI, NG2, FragEL) sections show that vehicle groups had increased DNA fragmentation marker in NG2$^+$ cells (F) when compared to DHA-pretreated animals (G). Student's t-test analysis was carried out to determine the statistically significant differences between treatments. Error bars represent means ± SEM. **$P = 0.0043$, $n = 8$ in total WM analysis; *$P = 0.0320$, $n = 4$ in NG2$^+$ cell analysis. Scale bar: B-E, 50 μm; F, G, 10 μm. DAPI, blue; anti-NG2, red; FragEL, green; arrows = colocalization.

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DHA Pretreatment Increases NeuN$^+$, APC$^+$, and NG2$^+$ Cell Numbers

To determine the quantity of neurons, oligodendrocytes, oligodendrocyte lineage cells, and inflammatory cells in areas involved in the observed locomotor and electrophysiological responses, immunohistochemical analyses were performed at the critical apoptotic stage of 7 dpi. Detection of neuronal and oligodendrocyte phenotypes was assessed by anti-NeuN and anti-adenomatosis polyposis coli (APC) antibodies, respectively. Alternatively, the anti-NG2 antibody was used to determine the number of potential oligodendrocyte precursor cells (OPCs). Cell counts were made in relevant areas involved in rodent locomotion such as ventral gray matter (VGM), ventrolateral funiculus (VLF), and dorsal corticospinal tract (dCST; Fig. 5A). Our results revealed a significant increase in the number of NeuN$^+$ cells in the ventral gray matter of DHA-pretreated animals (* $P < 0.05$; vehicle, $n = 10$; DHA, $n = 9$). In addition, the relative number of oligodendrocytes and OPCs was doubled in the DHA-pretreated groups at 7 dpi (both * $P < 0.05$; vehicle, $n = 6$ rats; DHA, $n = 7$ rats). Representative images from sections labeled with anti-Neu N (Fig. 5C-D), anti-APC (Fig. 5E-F), and anti-NG2 (Fig. G-H) show increased numbers of neurons, oligodendrocytes, and oligodendrocyte precursor cells in the DHA-pretreated group at 7 dpi.
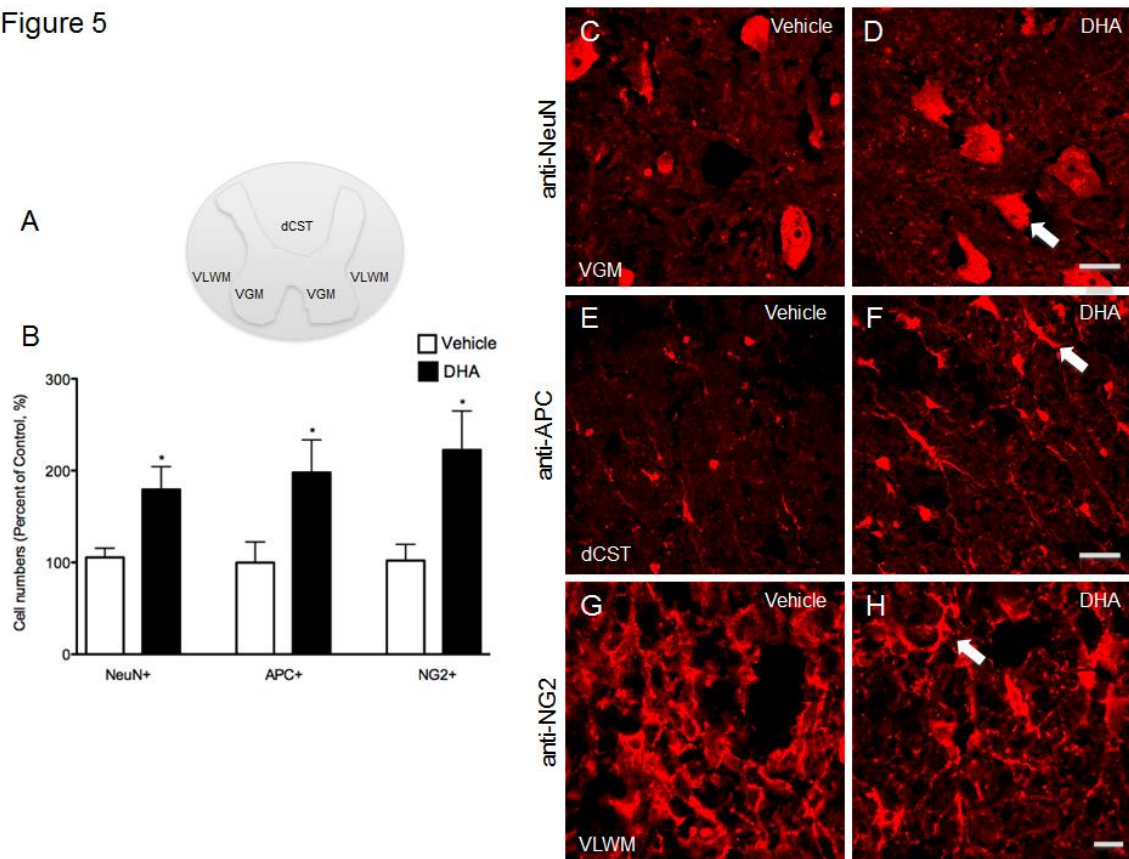
Figure 5. Pretreatment with DHA preserves neurons, oligodendrocytes, and oligodendrocyte precursor cells at one week after SCI. (A) Expression of cellular markers were quantified in the ventral gray matter (VGM), ventrolateral funiculi, and dorsal corticospinal tract (dCST). These regions were identified by superimposing a series of photomicrographs to a T10-T11 spinal cord diagram from the Watson, Paxinos, Kayalioglu spinal cord atlas. (B) Manual quantification of cell numbers and normalization to vehicle controls revealed increased numbers of NeuN$^+$, APC$^+$, and NG2$^+$ cells in the VGM, dCST, and VLF, respectively. Confocal images from sections immunoreacted against NeuN (C, D), APC (E, F), and NG2 (G, H) demonstrate increased immunoreaction to each marker in the DHA-pretreated animals when compared to controls. Student's t-test analysis was carried out to determine the statistical significance differences between treatments. Error bars represent means $\pm$ SEM. (NeuN, * $P < 0.05$, vehicle, $n = 10$, DHA, $n = 9$; both APC and NG2 * $P < 0.05$, vehicle, $n = 6$, DHA, $n = 7$. Scale bars: A-F, 50 μm; G-H, 25 μm.

Prophylactic Injection with DHA Did Not Result in Significant Anti-inflammatory

Effects at 1-week Following Compression Injury

Although the beneficial effects of inflammation in SCI remain controversial, it has been increasingly recognized that the recruitment and activation of inflammatory cells during the first week after trauma may play a critical role in modulating functional recovery after SCI [46]. To determine whether DHA pretreatment reduces the expression of these inflammatory markers in physiologically relevant areas, we quantified the number of immunoreactive particles using immunohistochemical analysis. Surprisingly, DHA pretreated animals showed no significant differences in the immunoreactivity to ED1 (monocytes/macrophages/microglia; Fig. 6A-E) and GFAP (astrocytes; Fig. 6F-J) when compared to vehicle controls ($P > 0.05$; n = at least 4 animals). To validate our ED1 results and determine whether our DHA prophylactic intervention reduced the activation of microglia, OX42 antibody was employed to examine the expression of CD11b/c in microglia in the spinal cord. No significant differences were observed between the studied groups (data not shown).
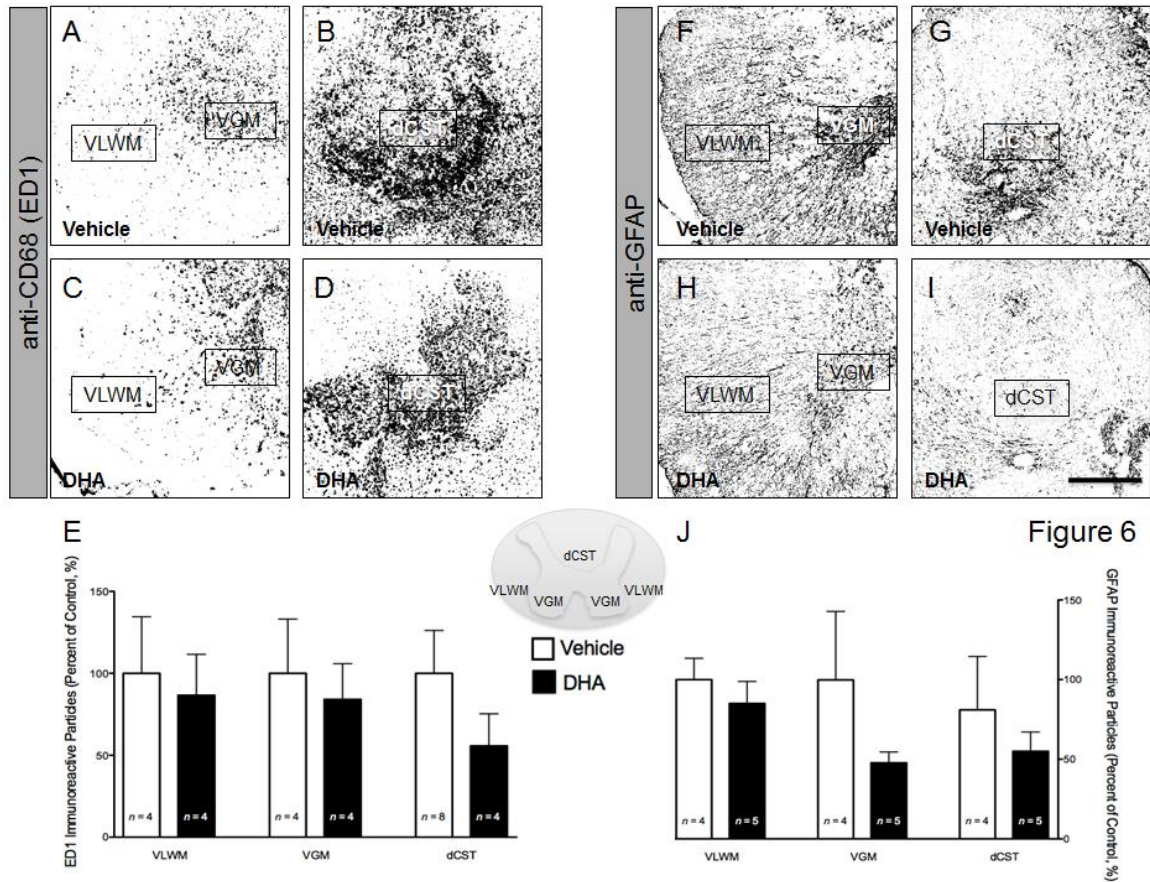
Figure 6. Pretreatment with DHA did not affect inflammatory markers after SCI. Photomicrographs from ED1 labeled monocytes/macrophages in animals injected with vehicle (A, B) or DHA (C, D). (E) Quantitative analysis showed no significant changes in the number of ED1 immunoreactive particles between treatment groups in any of the regions of interest (ROI) studied at 1 week post-injury. Representative images from GFAP labeling of astrocytes in animals injected with vehicle (F, G) or DHA (H, I). (J) Quantitative analysis at 1 week revealed no significant changes in the optical density of GFAP-immunoreactive particles between treatment groups. Error bars represent means ± SEM. *n* = at least 4 animals. VLWM, ventrolateral white matter; VGM, ventral gray matter; dCST, dorsal corticospinal tract. Scale bar: A-D and F-I, 200 μm.

DHA-pretreatment Induces the Up-regulation and Activation of Akt and CREB

After Contusive SCI

Akt and CREB have been associated with DHA protective effects on survival and myelin stability *in vitro* and *in vivo* [18-21]. We examined whether DHA-pretreatment increases the mRNA and protein phosphorylation levels of Akt and CREB after acute SCI. Real-time PCR analysis revealed that DHA pretreatment increased Akt and CREB levels 1.61- and 2.03-fold, respectively, at 7 dpi when compared to the vehicle controls (Fig. 7A; both $*P < 0.05$; $n = 4$ animals/group). Quantitative determination of Akt and CREB protein phosphorylation was performed by measuring the relative immunoreactivity to anti-pAkt and anti-pCREB antibodies. Gray-scale calibration of 0-255, representing white-to-black, permitted measurements of optical density values in immunoreactive sections. Quantitative analysis of pAkt and pCREB immunoreactivity in our DHA-pretreated group demonstrated a 1.59- and 1.70-fold increase, respectively, when compared to controls (Fig. 7B; both $*P < 0.05$; vehicle, $n = 4$ animals; DHA, $n = 5$ animals). Interestingly, DHA-pretreatment did not change the levels of pAkt and pCREB in the uninjured spinal cord (data not shown). Gray matter photomicrographs show pAkt (Fig. 7C-D) and pCREB (Fig. 7F-G) immunoreactivity in neuron like cells (arrows). These cells had an intensely positive pAkt and pCREB signal in nuclear and perinuclear regions (Fig. 7D and G). Antibody preabsorption with blocking peptides shows reduced immunoreactivity; thus confirming the specificity of each antibody (Fig. 7E and H).

We also examined whether DHA-pretreatment resulted in increased pAkt and pCREB levels in the different cellular phenotypes that were neuroprotected at 7 dpi. Gray matter double-labeling immunohistochemistry revealed increased neuron cell numbers

and immunoreactivity for pAkt (Fig. 8A-L) and pCREB (Fig. 9A-L). White matter

photomicrographs also demonstrated increased cell numbers with concomitant expression

of pAkt and pCREB in NG2$^+$ (Fig. 8M-R; Fig. 9M-R, respectively) and APC$^+$ cells (Fig.

8S-X; Fig. 9S-X, respectively).
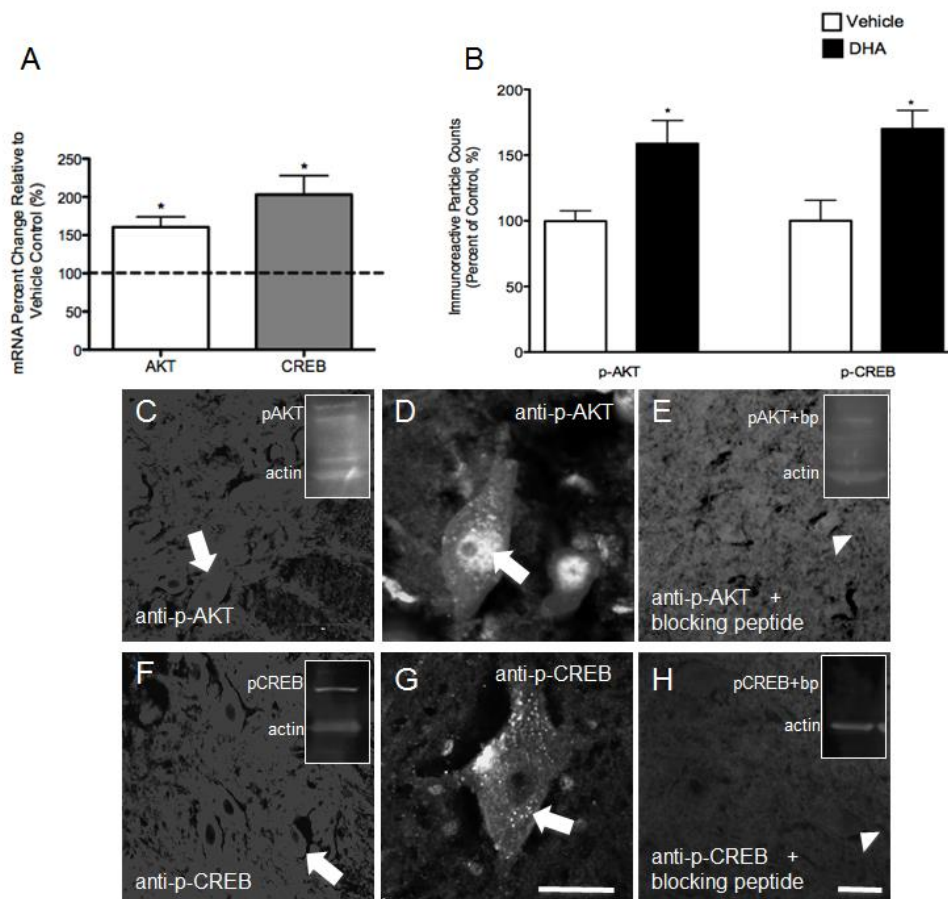
Figure 7. Intravenous administration of DHA before of SCI results in increased mRNA and phosphorylated protein levels of Akt and CREB at 7 days post-injury. (A) Quantitative analyses of real-time PCR crossing thresholds showed a significant increase in the mRNA levels of Akt and CREB in the DHA-pretreated group when compared to control animals. (B) Quantification of immunoreactive particle counts in ventral gray and white matter using ImageJ revealed increased levels of pAkt and pCREB in the DHA-pretreated group at 7 dpi. (C-H) Photomicrographs of immunostained section confirmed the specificity of pAkt and pCREB reactions. This immunoreaction was abolished after preincubating pAkt and pCREB antibodies with their respective blocking peptides (E, H). Antibody specificity was also demonstrated by immunoblot (C, E, F, H, insets). Student's t-test analysis was carried out to determine the statistical significance between treatments. Error bars represent means ± SEM. mRNA studies *$P < 0.05$; $n = 4$ per group; IMH studies *$P < 0.05$; $n = 4$, vehicle, $n = 5$, DHA. Arrows, immunoreactive cells in ventral gray matter. Arrowheads, no immunoreactive cells. Scale bars: D and G, 50 μm; C, E, F, and H, 50 μm.
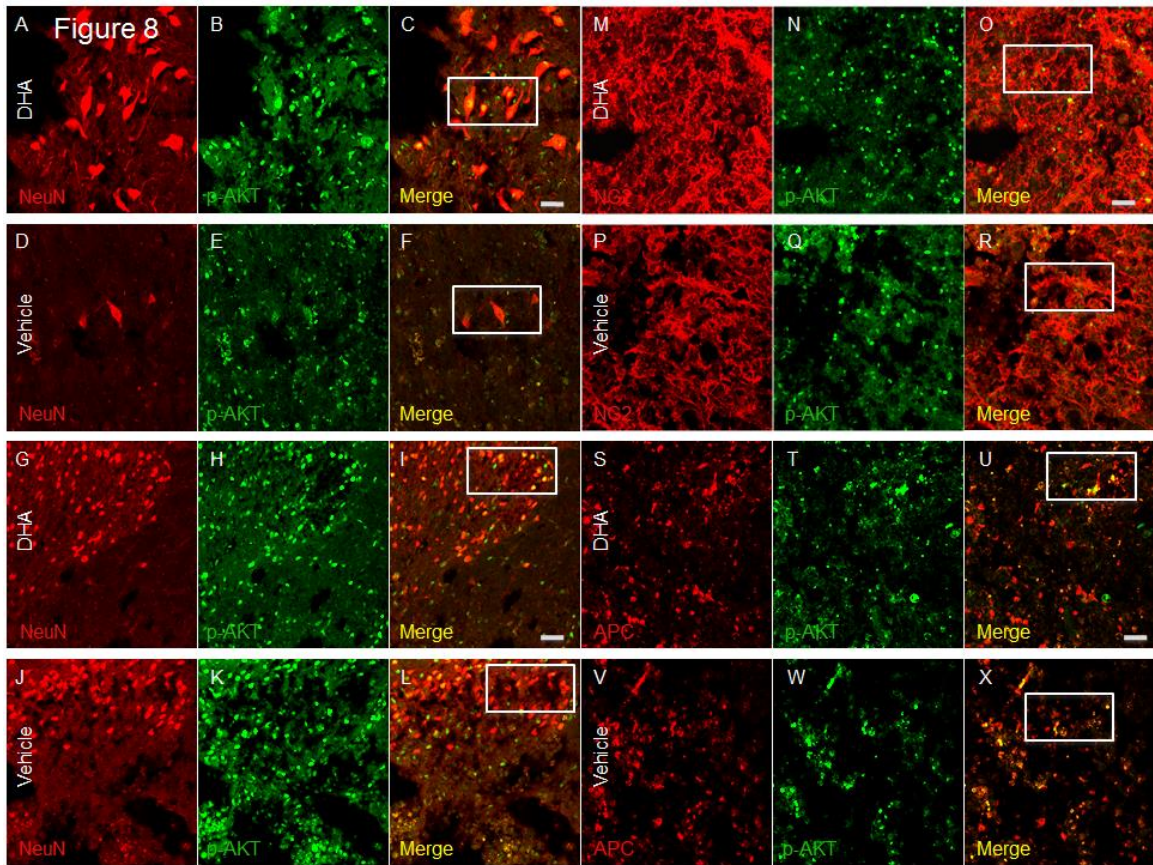
Figure 8. Double labeling and merge photomicrographs of NeuN, APC, and NG2 cells immureactive for pAkt in transverse sections at the ventral and dorsal gray matter, ventrolateral funiculus, and dorsal corticospinal tract of the T10-T11 spinal cord segment. pAkt expression and neuron cell numbers were higher in ventral gray motor neurons of DHA-pretreated (A-C) when compared to vehicle sections (D-F). pAkt was more abundantly expressed in dorsal gray motor neurons of DHA-pretreated animals (G-I) when compared to controls (J-L). Ventrolateral white matter photomicrographs show more pAkt and NG2^{+} cells in DHA-pretreated animals (M-O) when compared to vehicle controls (P-R). Images from representative dorsal columns/dorsal corticospinal tract in DHA-pretreated (S-U) and control groups (V-X). Scale bars: A-F, 50 μm; G-L, 50 μm; M-R, 50 μm; S-X, 50 μm.
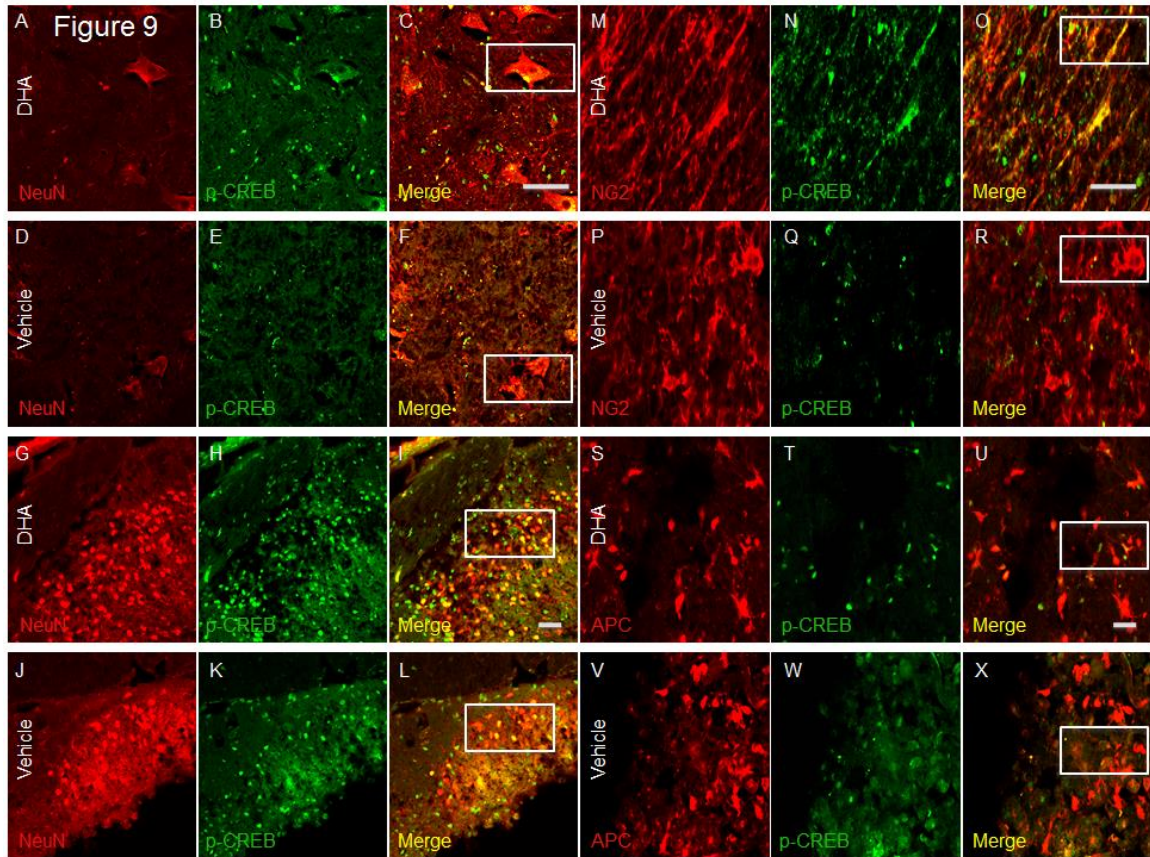
Figure 9. Double labeling and merge photomicrographs of NeuN, APC, and NG2 cells immureactive for pCREB in transverse sections at the ventral and dorsal gray matter, ventrolateral funiculus, and dorsal corticospinal tract of the T10-T11 spinal cord segment. Photomicrographs show increased pCREB expression and neuron cell numbers in ventral (A-F) and dorsal (G-L) gray motor neurons of DHA-pretreated (A-C, G-I) when compared to vehicle sections (D-F, J-L). pCREB was robustly colocalized with NG2 immunoreactivity in the ventrolateral white matter of DHA-pretreated animals (M-O) when compared to controls (P-R). Images from dorsal corticospinal tract revealed more pCREB in APC$^+$ cells of DHA-pretreated groups (S-U) when compared to controls (V-X). Scale bars: A-F, 50 μm; G-L, 50 μm; M-R, 50 μm; S-X, 50 μm.

Discussion

The results presented herein clearly demonstrate that docosahexaenoic acid (DHA), when injected in a prophylactic manner, resulted in amelioration of locomotor and conduction deficits observed during the acute phase of SCI. These acute beneficial effects were evidenced by (i) improved BBB scores, (ii) the emergence of motor evoked potentials, (iii) white matter tissue preservation, (iv) increased numbers of neurons, oligodendrocytes, and precursor cells, (v) activation of pro-survival and anti-apoptotic responses without reduction in inflammatory markers, and (vi) increased gene expression and activation of Akt and CREB. Altogether, these data suggest that DHA-mediated neuroprotection can occur even under significant inflammatory conditions and supports the prophylactic use of DHA in situations of anticipated high risk of damage to the spinal cord.

There are numerous clinical and occupational scenarios that are known to exhibit a significant risk for SCI. Example of these conditions include cerebral palsy, spina bifida, amyotrophic lateral sclerosis, vitamin B12 deficiency, herpes simplex viral infection, multiple sclerosis, atherosclerosis, iatrogenic ischemia, syringomyelia, spondylolysis, disc herniations, radiation toxicity, tumors, contact sports, and warfare [1, 4, 5]. Although the primary pathophysiology of non-traumatic SCI is disease-specific, the clinical signs, symptoms, and general therapeutic principles are similar to traumatic SCI. The clinical safety and neuroprotective properties of DHA make it an ideal prophylactic treatment in these situations because its ability to simultaneously inhibit multiple pathways involved in cell death and dysfunction such as apoptosis [47], excitotoxicity [48], ROS generation [49] and inflammation [50]. Interestingly, contrary to dietary DHA,

a single low dose DHA injection results in acute blood-brain-barrier penetration and incorporation in the brain [51] and is rapidly metabolized and accumulated in neurons and glial cells even at 7 days after the injection [43]. This is consistent with recent studies revealing that DHA injections are neuroprotective at 7 dpi when administered after injury to the spinal cord [27] whereas dietary supplementation with DHA did not confer neuroprotection [28]. It is reasonable that injection pretreatment with DHA could lead to a significant inhibition of very early damaging events and promote recovery after SCI.

A striking finding of this study is that DHA pretreatment led to a significant reduction in locomotor deficits associated with contusive SCI, as evidenced by the improvements in BBB locomotor scores at different time points during the acute phase of SCI. Remarkably, DHA-pretreated animals exhibited extensive movements in all three joints and weight supported steps at 7 days post-injury (dpi). Our findings are supported by studies showing that pretreatment with polyunsaturated fatty acids (PUFAs) provide tolerance against ischemic-induced neurodegeneration [52], reduce disabilities after traumatic brain injury in rats [53], confer neuroprotection from brain hypoxia-ischemia [54], and increase resistance to PTZ-induced seizures [55].

Another important finding of our study is the emergence of transcranial magnetic motor evoked potential (tcMMEPs) electrophysiological responses at 7 dpi. The appearance of these responses provide a reproducible and objective assessment of axonal conduction of descending motor pathways in both the normal and injured spinal cord [32, 56, 57]. Moreover, this measurement is useful to evaluate the conduction capacity and integrity of descending myelinating pathways located in the medial ventrolateral funiculus (VLF) [37, 38]. Our results are the first demonstration of *in vivo*

electrophysiological improvements after acute bolus administration of DHA before SCI. This may represent preservation of myelinated fibers and return of function in spared reticulospinal fibers, which run mainly in the VLF and carry important motor information. Our anatomical and histological data support this idea by showing increased ventrolateral white matter tissue sparing and axonal integrity in DHA-pretreated animals when compared to vehicle controls at 7 dpi.

Both neurons and oligodendrocytes are extremely vulnerable to cell death following SCI [58]. Several lines of evidence demonstrate that during the first few days after injury, acute apoptotic and autophagic waves contribute to tissue damage and loss of function after SCI in animals [9, 10, 59-62], and humans [63]. Here, we demonstrated that bolus DHA injection given before SCI significantly increased neuronal and oligodendrocyte cell survival at 7 dpi. In support of this finding, DHA has been shown to be neuro- and oligoprotective when administered and supplemented after SCI [26-29]. Previous *in vitro* work from our laboratory is also in agreement with these neuroprotective actions after showing that preventive administration of DHA in advance of lipotoxic injury protects NGFD-PC12 cells from apoptotic cell death (Almaguel et al., 2009).

Proliferation of progenitor cells expressing NG2 proteoglycans may play a role in replenishing oligodendrocytes and may determine neurological outcomes after acute SCI [64-66]. It was recently proposed that these precursor cells might also have a broader relevance for gray matter physiology based on the observation that some of these cells can mature into cortical projection neurons [67]. Therefore, strategies targeting precursor cells with beneficial pro-survival agents may represent a viable mechanism for neuronal

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and oligodendrocyte replacement, spinal cord homeostasis, and functional recovery. Our results show, for the first time, not only that DHA pretreatment leads to increased numbers of NG2$^+$ cells, but also that DHA prevents apoptotic cell death in these cells after SCI. Apoptotic reduction may be partially responsible for the white matter sparing and improved functional behavior observed in our study [68, 69]. Future studies are needed to clarify the effects of DHA in precursor cell survival, proliferation, and differentiation after SCI.

Although DHA plays an important role in neuroprotection and functional recovery, we lack information regarding its underlying mechanisms after SCI. Previous reports suggest that therapeutical treatment with DHA after SCI may exert its neuroprotective effects, at least partially, through negative modulation of cycloxigenase-2 protein expression and by means of reducing peroxidation and oxidation products [28]. This same group demonstrated that DHA administration after SCI results in improved recovery associated with a significant reduction of inflammatory markers, such as ED1 [27, 28]. Contrary to this finding, this study showed no significant differences in the immunoreactivity levels of various inflammatory makers, including GFAP (astrocytes), CD68 (macrophages), and CD11b (microglia). This discrepancy suggests that DHA pretreatment may have different targets during the first few minutes after injury. DHA is rapidly and selectively incorporated into the central nervous system (CNS) when injected in rats [51] and it is also acutely released from cell membranes after SCI [70]. Thus, it is plausible that acute accretion of DHA in advance of SCI might promote neuroprotection by triggering the docosanoid pathway and DHA-derived messengers, including docosatrienes, resolvins, and neuroprotectins, such a neuroprotectin D1 (Bazan, 2006).

Also, while DHA multiple double bonds are excellent targets for lipid peroxidation and could potentiate neurotoxicity, DHA may also function as a free-radical scavenger to reduce the neuronal and glial susceptibility to oxidative stress after trauma. We cannot discard the possibility that DHA could reduce the effects of primary injury by modulating the biophysical properties of membranes and axons [71]. Future work is needed to study the acute targets of DHA when administered before injury to the spinal cord.

Dietary DHA increases the levels of activated Akt and CREB in rats [19]. These signaling pathways play a major role in DHA-mediated neuronal survival [72], provide resistance to CNS insults [73-75], and increase endogenous protection in the retina [76]. Our findings showing that Akt and CREB expression was up-regulated in DHA pretreated rats are in agreement with the involvement of these molecules in estrogen-mediated neuroprotection after SCI [77]. Current work in our lab is aimed at investigating whether Akt/CREB activation is required to exert DHA neuroprotective effects in a prophylactic manner (Descoberth et al., Unpublished observations).

In summary, our findings are the first to demonstrate that acute pretreatment with DHA renders neural tissue more resistant to SCI-induced secondary damage possibly by limiting the expansion of the apoptotic pathways (See summary in Table 1). This report substantiates the relevance of DHA as a preventive neuroprotective agent, which may confer a clear advantage in situations of anticipated neuronal damage, and deserves further consideration as an innovative approach to prevent neurodegeneration and injury expansion after SCI. Further studies testing the durability of these neuroprotective effects over longer post-SCI survival times are being tested.

Table 1. Summary.

Biological Level	Test	Observation
Physiological	BBB tcMMEP	Improved locomotor recovery Reduced conduction latencies
Anatomical and Histological	IMS	↑Luxol, ↑WMS, ↑CV
	IMH	↑NeuN, ↑APC, ↑NG2, ↓NG2/FRAGEL
Molecular	RT-PCR/IMH	↑AKT, ↑CREB

DHA pretreatment in advance of contusive SCI results in relevant physiological, anatomical, histological, and molecular changes. Our electrophysiological, immunohistochemical, and molecular biology assays indicate that two bolus injections of DHA given 1 week and 60 min before SCI reduces locomotor impairments and conduction latencies, increased neuroprotection, and results in increased mRNA and phosphorylated protein levels of the pro-survival proteins Akt and CREB.

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Author Disclosure Statement

No competing financial interests exist.

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CHAPTER THREE

DIETARY OMEGA-3 FATTY ACIDS PREVENT SPINAL CORD INJURY-INDUCED DHA DEFICIENCY, RESTORE INJURED NEUROLIPIDOME AND STIMULATE A ROBUST FUCNTIONAL RECOVERY

By

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Abstract

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) confer multiple health benefits and decrease the risk of neurological disorders. However, studies are needed to identify promising cellular targets and to assess their prophylactic value against neurodegeneration. The present study (1) examined the efficacy of a preventive diet enriched with ω-3 PUFAs to reduce dysfunction in a well-established spinal cord injury (SCI) animal model and (2) used a novel metabolomic analysis to identify potential mechanisms and neurolipidomic targets. Rats were fed with either control chow or chow enriched with ω-3 PUFAs (750 mg/kg/day) for 8 weeks before being subjected to a sham or a contusion SCI operation. We report new evidence showing that rats subjected to SCI after being pretreated with a diet enriched with ω-3 PUFAs exhibit significantly better functional outcomes. Pretreated animals exhibited lower sensory deficits, autonomic bladder recovery and early improvements in locomotion that persisted for at least 8 weeks after trauma. We found that SCI triggers a robust alteration in the cord PUFA neurolipidome, which was characterized by a marked DHA deficiency. This DHA deficiency was associated with dysfunction and corrected with the ω-3 PUFA-enriched diet. Multivariate data analyses revealed that the spinal cord of animals consuming the ω-3 PUFA-enriched diet had a fundamentally distinct neurolipidome, particularly increasing the levels of essential and long chain ω-3 fatty acids and lysolipids at the expense of ω-6 fatty acids and its metabolites. Altogether, dietary ω-3 PUFAs prophylaxis confers resiliency to SCI mediated, at least in part, by generating a neuroprotective and restorative neurolipidome.

Introduction

Spinal cord injury (SCI) leads to debilitating and long-lasting motor, sensory, and bladder dysfunction. Primary mechanical injury to the spinal cord initiates a complex cascade of secondary damaging events, including prominent metabolic alterations, axonal damage, inflammation, cell death, and demyelination.[1] Although current therapeutic strategies aimed at alleviating the secondary injury are promising,[2, 3] studies identifying and addressing potential determinants of vulnerability to neurological dysfunction in experimental models of injury are very limited. Accumulating evidence suggests that the susceptibility to damage that follows SCI depends on the intrinsic balance between neurorestorative and neurodestructive signals.[4]

Injury to the spinal cord results in a robust deregulation of membrane polyunsaturated fatty acid (PUFA) homeostasis; leading to membrane derangements and preferential release of arachidonic acid (AA; 20:4, ω-6 PUFA) and peroxidation of docosahexaenoic acid (DHA; 22:6, ω-3 PUFA).[5-11] These derangements can lead to ω-6 fatty acid metabolism and marked ω-3 PUFA deficiencies, altering PUFA nutritional requirements. Further, the essential role of these lipids in physiology and cell signaling is believed to be important in the neural responses to injury and could predispose nerve cells to dysfunction. For instance, AA and its potent pro-inflammatory mediators are implicated in secondary damage cascades and dysfunction after SCI.[12-16] In contrast, ω-3 PUFAs, such as DHA, are anti-inflammatory, confer neuroprotection, and play significant roles in facilitating functional recovery in various SCI animal models.[12, 17-22]

One important hypothesis arising from our work and from other published epidemiological evidence is that dietary ω-3 PUFAs can confer resiliency and facilitate recovery, even when administered before SCI. We recently reported that acute parenteral administration of DHA before injury leads to early improvements in conduction and locomotor function following SCI.[22] This finding is consistent with studies showing the efficacy of ω-3 PUFAs as a post-treatment modality to ameliorate secondary damage and dysfunction.[12, 17-24] However, there is still a significant gap in our knowledge concerning the therapeutic effects of prophylactic ω-3 PUFAs against neurotrama.

Here, we assessed whether SCI significantly alters ω-3 PUFA requirements in the cord, and whether pretreatment with dietary ω-3 PUFAs, without further supplementation, is sufficient to reduce dysfunction following SCI. Addressing this question is particularly important for populations where neurological injury is an unavoidable high risk (i.e., neurodegenerative conditions, contact sports, and military conflicts) and can have major implications for the identification of biomarkers of neural vulnerability to dysfunction.

Materials and Methods

Animals

Experimental procedures 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. A total of fifty-four female Sprague-Dawley rats weighing 182-212 g were received from Charles River Laboratories (Portage, MI). Animals were housed in individual cages with food and water *ad libitum*

and exposed to alternate light and dark periods of 12 h.

Study Design

The animals were allowed to acclimatize for one week after arriving to the animal facility and were randomly divided into two main groups: (Group A) animals received the control diet ($n = 27$) and (Group B) animals received the ω-3 PUFA-enriched diet ($n = 27$) (Fig. 1*A*). After 8 weeks on these diets, the rats were further divided into four groups based on their dietary and surgical interventions: Group 1 was the control diet, sham operated group ($n = 8$); Group 2 was the control diet, spinal cord injured group ($n = 19$); Group 3 was the ω-3 PUFA-enriched diet, sham operated group ($n = 8$); and Group 4 was the ω-3 PUFA-enriched diet, spinal cord injured group ($n = 19$). The respective dietary interventions were continued after surgery. Autonomic bladder, sensory and motor functions were assessed after surgery. The best attempts were made to perform behavioral tests in a blinded manner. Spinal cord tissue was collected for analyses at 8 weeks post-operation (wpo).

Diet Composition

Custom isocaloric AING-93-based diet formulations were designed with modifications to the fat composition (Bio-Serv, Frenchtown, NJ). The level of dietary fat was approximately 6% of dry weight supplied as either soybean oil (control chow) or menhaden fish oil (ω-3 PUFA-enriched chow). Both of the diets were stored refrigerated. Lab analyses using gas chromatography coupled with mass spectrometry demonstrated that the level of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in the

menhaden fish oil was 12.82-gm and 6.91-gm, respectively, per 100 gm of diet. The level

of cholesterol was 0.582-gm/100 gm. This amount was added to control diets to make the

levels consistent to that of the fish oil diet. The selection of soybean oil as control fat in

our diets was based on the following observations: (1) soybean oil is the principal source

of ω-3 fatty acids in the U.S., and (2) mammals have a limited capacity to convert DHA

from linolenic acid (LA: 18:3, ω-3).

Spinal Cord Injury Surgical and Post-Operative Procedures

After 8 weeks of receiving either dietary intervention, the animals were deeply

anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). The spinal

cord was injured at thoracic level 10 (T10) using the well-characterized NYU

Impactor.[25] Briefly, the skin and muscles overlying the spinal column were incised. A

laminectomy was performed at T9-T10, leaving the dura intact and the T8 and T12 spinal

processes were clamped to the Impactor. A 10-g rod was released from 12.5-mm onto the

exposed spinal cord to induce mild to moderate trauma. Analysis of the biomechanical

impact parameters revealed no significant differences between control and treated groups

(control diet group: 1.799 ± 0.051-mm, 0.478 ± 0.005 m/s, and 0.382 ± 0.007 m/s vs. ω-3

PUFA-enriched diet group: 1.748 ± 0.058-mm, 0.478 ± 0.008 m/s, and 0.385 ± 0.013 m/s

values for compression, impact velocity, and compression rate, respectively; average $\pm$

SEM). The sham animals received only a laminectomy to expose the spinal cord. During

surgery, the body temperature of the animals was maintained at 37°C using a

thermostatically controlled heating pad with a rectal thermometer (Physitemp TCAT-

2LV; Physitemp, Clifton, NJ). After surgery, the muscle layers were sutured and the skin

layers were closed with wound clips. 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. Two animals died because of

surgical complications during this study. All other animals were allowed to survive for 8

wpo.

Autonomic Function Testing

Autonomic Bladder Control Recovery

For all injured animals, the residual volume of urine expressed each morning was

measured during the first 11 days post-injury (dpi; period during which full recovery of

bladder function occurs after mild to moderate contusive injury in the rat). We also

recorded the day on which autonomic bladder control was restored (e.g., when the

maximal volume of urine collected in one session is consistently below 500 µL).

Motor Function Testing

Behavioral Evaluation of Spontaneous Locomotion.

Rats' spontaneous open-field locomotion was evaluated using the 22-point (0-21)

Basso-Beattie-Bresnahan scale.[26, 27] Briefly, animals were acclimatized to the open

field environment during 5 daily sessions one week before SCI. After the SCI, the open

field locomotion test was videotaped weekly. Subsequently, two observers assessed the

locomotive function, joint movement, paw placement and rotation, coordination, and tail

and trunk position and stability. For each hindpaw, the averaged consensus score was used for analyses. In this scale, a completely paralyzed rat scores 0, a rat with increasing joint movements but without weight support scores between 1 and 8 (recovery stage 1), a rat with abnormal locomotion but with weight supported steps scores between 9-13 (recovery stage 2), graded coordination patterns, paw position, and trunk stability scores between 14 and 20 (recovery stage 3), and a normal (and sham) rat scores 21.

Locomotor scores were transformed using a simple post hoc method that reduces error variance and improves the metric properties of the BBB, effect size, and power.[22, 28] This transformation produces a continuous distribution by pooling together BBB scores from 2–4 and 14-21 and thus avoids potentially suspect measures of performance from unlikely observations in the lower/higher end of the scale.

Sensory Function Testing

Habituation

Animals were habituated to sensory behavioral tests during 5 daily sessions held one week prior to baseline recordings.

Electronic von Frey Test

Sensory function was assessed by measuring the withdrawal threshold of the hindpaws in response to a mechanical stimulus using an electronic von Frey aesthesiometer (model 2391C; IITC Life Science, Woodland Hills, CA, USA). Each animal was placed in a Plexiglas chamber positioned on an elevated metallic grid floor, which provided access to the plantar surface of the hind paw. Animals were allowed to

acclimate to the environment for 30 min before testing. A rigid blunt tip attached to the

meter was applied to the plantar surface from under the floor. The withdrawal threshold

was defined as the average force (g) required for paw removal in five trials separated by a

1-min interval. The maximum and minimum threshold values were excluded from each

paw after each testing session. The data was normalized to the percent change from

baseline and sham animals. The normalized % change from baseline represents

[(withdrawal threshold $_{\text{injury @ baseline}}$ − withdrawal threshold $_{\text{injury @ time point X}}$) / withdrawal

threshold $_{\text{injury @ baseline}}$] X 100 − [(withdrawal threshold $_{\text{shams @ baseline}}$ − withdrawal

threshold $_{\text{shams @ time point X}}$) / withdrawal threshold $_{\text{shams @ baseline}}$] X 100.

Metabolomic Analyses

Metabolon's Sample Preparation and Metabolic Profiling

The unbiased metabolic profiling platform employed for this analysis combined

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). The

spinal cord samples were processed essentially as described previously.[29, 30] For each

sample, 100μL was used for analyses. Using an automated liquid handler (Hamilton

LabStar, Salt Lake City, UT), protein was precipitated from the plasma with methanol

that contained four standards to report on extraction efficiency. The resulting supernatant

was split into equal aliquots for analysis on the three platforms. Aliquots, dried under

nitrogen and vacuum-desiccated, were subsequently either reconstituted in 50μL 0.1%

formic acid in water (acidic conditions) or in 50μL 6.5mM ammonium bicarbonate in

water, pH 8 (basic conditions) for the two UHPLC/MS/MS^2 analyses or derivatized to a final volume of 50μL for GC/MS analysis using equal parts bistrimethyl-silyl-trifluoroacetamide and solvent mixture acetonitrile:dichloromethane:cyclohexane (5:4:1) with 5% triethylamine at 60°C for one hour. In addition, three types of controls were analyzed in concert with the experimental samples: 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. The experimental samples and controls were randomized across the platform run days.

For UHLC/MS/MS^2 analysis, aliquots were separated using a Waters Acquity UPLC (Waters, Millford, MA) and analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA), which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The MS instrument scanned 99-1000 *m/z* and alternated between MS and MS^2 scans using dynamic exclusion with approximately 6 scans per second. Derivatized samples for GC/MS were separated on a 5% phenyldimethyl silicone column with helium as the carrier gas and a temperature ramp from 60°C to 340°C and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific, Inc.) operated at unit mass resolving power with electron impact ionization and a 50-750 atomic mass unit scan range.

Metabolites were identified by automated comparison of the ion features in the experimental samples 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 metabolites were curated by visual inspection for quality control using software developed at Metabolon.[31]

RNA Isolation and Reverse Transcription

Animals were submitted to humane euthanasia with Fatal-Plus, and perfused transcardially with ice-cold 0.01 M PBS solution, pH 7.4 (Sigma-Aldrich). Spinal cord segments containing the injury areas adjacent to the injury site (5 mm each) were collected at 8 weeks post-operation (wpo). Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions and the RNA concentration was determined on a NanoDrop spectrophotometer (Thermo Scientific). cDNA was prepared with 800 ng of RNA by reverse transcription using SuperScript II and random primers (Invitrogen).

Real time PCR. PCR amplification and analyses were carried out on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The cDNA was amplified by PCR using pairs of primers specific for protein kinase B (Akt1; FWD: 5'-TAC CAT GAA CGA CGT AGC CA-3' and REV: 5'-AGG TGC CAT CAT TCT TGA GG-3'), and the cyclic AMP responsive element binding protein (CREB; FWD: 5'-CAT GGA CTC TGG AGC AGA CA-3' and REV: 5'-GGG CTA ATG TGG CAA TCT GT 3'). Each well contained 25 µL as the final PCR reaction volume containing cDNA, primers and the SYBR green PCR master mix (Applied Biosystems, Foster City, CA). All samples were run in triplicates. Thermal cycling conditions were as follow: 10 sec at 95°C for denaturation, and 30 sec at 60°C for annealing/extension. Relative mRNA quantities were compared between groups using the crossing threshold values.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; FWD: 5'-TGC CAC GAA GAC TGT GG-3' and REV: 5'-TTC AGC TCT GGG ATG ACC TT-3') was used as the internal control gene, and amplification specificity was checked using melting curve analyses. Mock reactions were performed as negative controls.

Immunodetection

Western Blot

Eight weeks after the operations, the spinal cord segments were processed for Western blot analysis as described previously.[32] Briefly, 5 mm spinal cord segments were dissected and immediately frozen. The tissue containing injured epicenter-to-caudal regions was then homogenized manually with a Dounce tissue grinder in 200 µL of 1% SDS in Tris–EDTA buffer with proteinase inhibitors (10 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 mM PMSF) and then sonicated. After centrifugation at 14,000 rpm, the supernatant was assayed for protein concentration using the method of Lowry[33] and bovine serum albumin as a standard. Thirty micrograms of protein were diluted in Laemmli's buffer, loaded and run in 4-12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked using Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE). The relative amounts of Akt and CREB proteins were detected using rabbit anti-Akt and anti-CREB antibodies (1:1000 each; Cell Signaling, Danvers, MA). After a 2 h incubation in primary antibodies, the membranes were washed three times for 10 min in Tris-buffered saline with 0.1% Tween-20 and incubated in donkey anti-rabbit IgG conjugated with IRDye® 800CW for 1 h (1:15,000; LI-COR Biosciences). Membranes were washed as above and the images were captured using an

Odyssey infrared imaging system (LI-COR Biosciences). Protein relative expression was standardized to β-actin levels, which were detected using mouse anti-β-actin antibodies (1:2000; Abcam, Cambridge, MA) and goat anti-mouse IRDye® 680RD secondary antibodies (1:20000, LI-COR Biosciences). Quantitative analyses were carried out using ImageJ 1.45I software (http://rsb.info.nih.gov/ij/ index.html).

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 (IBM: SPSS, Armonk, New York), Prism 5 software v5d (GraphPad Software Inc., San Diego, CA), MetaboAnalyst (www.metaboanalyst.ca), and the "R" program (http://cran.r-project.org/). Two-Way Analysis of Variance (ANOVA) followed by Bonferroni post-hoc comparisons were used to determine the effect of our dietary/surgical intervention and time on rat's pellet consumption, weights, residual urine volume, open-field locomotion scores, and sensory threshold changes. ANCOVA was also used to test multi-interactions between weight, pellet consumption and functional behavior at baseline and at different timepoints within and between the groups. All other data were assessed by one-way ANOVA, t-tests, or the Mann-Whitney *U* test. The Kolmogorov-Smirnov and Shapiro-Wilk normality tests were used to investigate outliers and spread. Outliers were also identified using the Grubbs' method, also known as ESD (extreme studentized deviate; www.graphpad.com). Only two animals were excluded from the study after using these exclusion methods in our behavioral data. To analyze neurometabolic alterations, multivariate data analysis (MVDA) -partial least squares projections to latent structures discriminant analysis (PLS-DA) - was performed using MetaboAnalyst software. Spearman's rank correlation tests

were used to explore associations between the top metabolic ratios contributing to model variability and the behavioral phenotypes. Data are presented as mean ± SEM. Statistical differences were considered significant at $p < 0.05$.

Results

The rationale behind the chosen ω-3 PUFAs dose, pretreatment schedule, and end-point measures was based upon (1) studies that indicate that animals that are fed diets enriched with 6% fish oil, such as the one used in this study, can take up to 8 weeks to significantly alter the lipid profile of the rat brain,[34] (2) studies that demonstrate that fatty acid incorporation occurs in a manner that is highly correlated with the amount consumed and with brain needs,[35, 36] (3) studies revealing that the beneficial effects of ω-3 PUFAs in the central nervous system (CNS) could be readily observed with dietary interventions lasting 6-8 weeks and at doses ranging from 10 mg/day to 400 mg/kg/day,[19, 37-39] and (4) evidence that supports that damage/repair processes take place following chronic SCI.[40-43] The study design is summarized in Fig. 1*A*.

Animals were housed in individual cages and the baseline food consumption was monitored daily. Food intake resulted in an average intake of 15 pellets per day (Fig. 1*B*). This amount resulted in approximately 500 mg docosahexaenoic acid (DHA) and 250 mg eicosapentaenoic acid (EPA) per kilogram of animal body weight (BW). It is known that rats and humans have similar dietary requirements for PUFAs (% of total energy). However, the relationship between body size and the rate of metabolic processes (and thus time) needs to be taken into account when dosing. For instance, the relationship between basal metabolic rate (BMR) and body weight in different size mammals is

described by the function $Y = aX^{0.75}$, where Y is basal metabolic rate (kJ/d), X is body weight (kg) and a is basal metabolic rate per $kg^{0.75}$ per day, which is 300 kJ/($kg^{0.75} \cdot$ d).

Thus, the BMR in different size species is proportional to the body weight raised to the 0.75 power, also known as the metabolic weight. Assuming that the BMR comprises 75% of the total metabolic rate, then the total metabolic rate can be described by the function $Y = 400\ X^{0.75}$. From this relationship, the total metabolic rate of a 70-kg human is $400 \times 70^{0.75} = 9680$ kJ or 138 kJ per kg body weight. Similarly, the total metabolic rate of a 200 g rat is $400 \times 0.200^{0.75} = 120$ or 600 kJ per kg body weight. Thus, the absolute energy expenditure expressed in kJ per kg body weight is greater in rats than in humans. Therefore, although the DHA dose used in this study (500 mg/kg) would be equivalent to approximately 35 mg of DHA/day in a 70-kg human, this amount would be far less when corrected for metabolic weight. Also, when using this correction, the 8-week feeding period used herein is analogous to approximately one year in humans.[44]

Two-way ANOVA analyses identified time as the only significant source of variation in food intake between groups [$F(8,1082) = 29.33$; $p < 0.0001$, $n = 8$ control groups, $n = 18$ experimental groups]. Post hoc analysis revealed a significant reduction in the amount of pellets ingested at one week post-operation (wpo) when compared to baseline consumption (~30 % reduction; $p < 0.05$). Normal feeding behavior was re-established by week 2 after surgery when compared to baseline values ($p > 0.05$).

Two-way ANOVA revealed that diet/surgical intervention [$F(3,7044) = 62.97$] and time [$F(8,3439) = 11.53$] had significant effects on animal weights ($p \leq 0.0001$ for both) (Fig. 1B). Post hoc analysis showed no significant differences in animal BW at baseline ($p > 0.05$). Although in both groups the injury resulted in a 8-10% reduction in

body weight, post hoc analysis demonstrated no significant differences in BW when comparing 1 wpi to baseline values in animals fed with the ω-3 PUFA-enriched diet ($p >$ 0.05). In contrast, significant BW loss was found in the control-diet fed animals at 1 week post-injury (wpi) ($p < 0.01$). In both diet groups, baseline BW was re-established at 3 wpi. It is noteworthy that although sham animals showed similar pellet consumption when compared to injured rats, no differences in weight were observed in the first few weeks after operations. In contrast, injured rats showed a marked metabolic disconnect, as evidenced by weight loss despite similar caloric intake to sham counterparts.
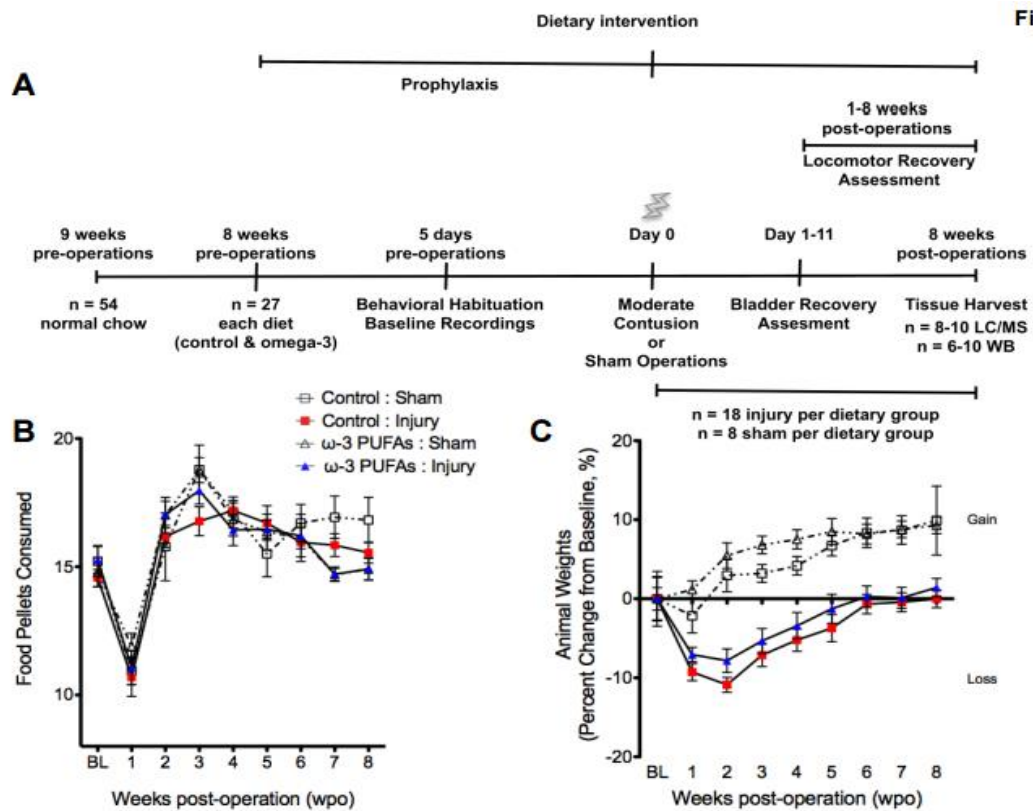
Figure 1. Dietary and weight monitoring. **(A)** Timeline outlining experimental design. **(B)** Baseline food resulted in an average intake of 15 pellets per day (500 mg docosahexaenoic acid and 250 mg eicosapentaenoic acid per kg of animal body weight per day). Surgery resulted in a significant reduction in food intake that returned to baseline levels at 2 weeks post-operation (wpo). No significant differences were found between groups ($p>0.05$, n=8–18 animals per group). **(C)** Dietary ω-3 polyunsaturated fatty acids (PUFAs) pre-treatment prevented significant weight losses when compared with injured rats receiving control diets at 1 week post-injury (wpi). Although dietary intake was similar, sham animals gained more weight than injured counterparts.

Dietary ω-3 PUFA Prophylaxis Accelerates Bladder Recovery, Improves Locomotor

Function, and Ameliorates Sensory Dysfunction

SCI results in a period of marked bladder dysfunction.[45] Because animals need assistance with bladder voiding during the first few days after SCI, we collected and quantified the residual urine volume to assess whether a dietary ω-3 PUFA intervention show efficacy in accelerating autonomic bladder recovery. Of special significance, ANOVA identified type of diet as a significant source of variance between groups in the course of 11 days post-injury (dpi) [$F_{(1,69.42)} = 5.88$; $p = 0.0208$, $n = 18$ per group] (Fig. 2*A*). Further, full autonomic recovery of bladder control (two or more days with a residual volume equal or less than 0.5 mL) occurred significantly earlier in animals receiving ω-3 PUFA-enriched diets (6.1 ± 0.6 dpi) when compared with controls (9.5 ± 0.4 dpi) (Unpaired t-test t = 4.97 df = 34; $p < 0.0001$) (Fig. 2*B*).

Figure 2
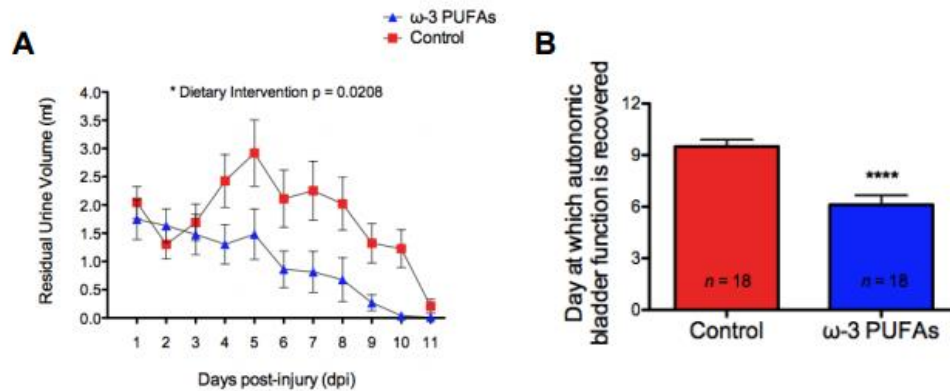
Figure 2. Beneficial effects of dietary ω-3 PUFAs prophylaxis on autonomic function after contusion injury. **(A)** Residual urine volumes (mL) differed significantly between control and ω-3 polyunsaturated fatty acids (PUFAs) pre-treated groups (analysis of variance, p=0.0208, n=18). Dashed line represents cutoff volume used to determine complete autonomic recovery. **(B)** For each animal, the number of days needed to attain full autonomic recovery was defined as residual volume of 0.5 mL for 2 or more consecutive days. Dietary ω-3 PUFAs prophylaxis resulted in fewer days to attain full bladder recovery (Mann-Whitney U test, p<0.0001, n=18

To characterize the effects of dietary ω-3 PUFA prophylaxis in motor recovery after SCI, we used the Basso Beattie, Bresnahan (BBB) locomotor behavior scale followed by score transformations.[28] Two-way ANOVA followed by Bonferroni's post hoc testing demonstrated significant differences in BBB locomotor scores between animals receiving ω-3 PUFA-enriched diets and control diets [Untransformed data: $F_{(3,8636)} = 460.1$; Transformed data: $F_{(3,1542)} = 117.7$; for both $p < 0.0001$] (Fig. 3*A* and *B*). A striking finding of this study is that locomotor recovery was significantly accelerated in the group of animals receiving ω-3 PUFA-enriched diets when compared to controls. This was evidenced by their ability to produce extensive joint movements and occasional weight supported steps at 1 wpi (transformed BBB scores, mean ± SEM: control diet = 1.53 ± 0.38, $n = 17$ vs. ω-3 PUFA-enriched diet = 5.47 ± 0.62, $n = 17$; $p < 0.0001$) (Fig. 3*B*). Interestingly, post hoc analysis showed that these differences in locomotor behavior persisted at least until 8 wpi ($p < 0.05$). End-point outcome measures showed that animals receiving control diets were able to step occasionally or frequently but with no signs of locomotive coordination at 8 wpi (transformed score: 8.2 ± 0.86). In contrast, rats receiving ω-3 PUFA-enriched diets displayed consistent weight-supported plantar steps (or frequent plantar stepping and occasional dorsal stepping), consistent coordination, and paw rotation during locomotion at 8 wpi (transformed score: 11.88 ± 0.08).

Figure 3
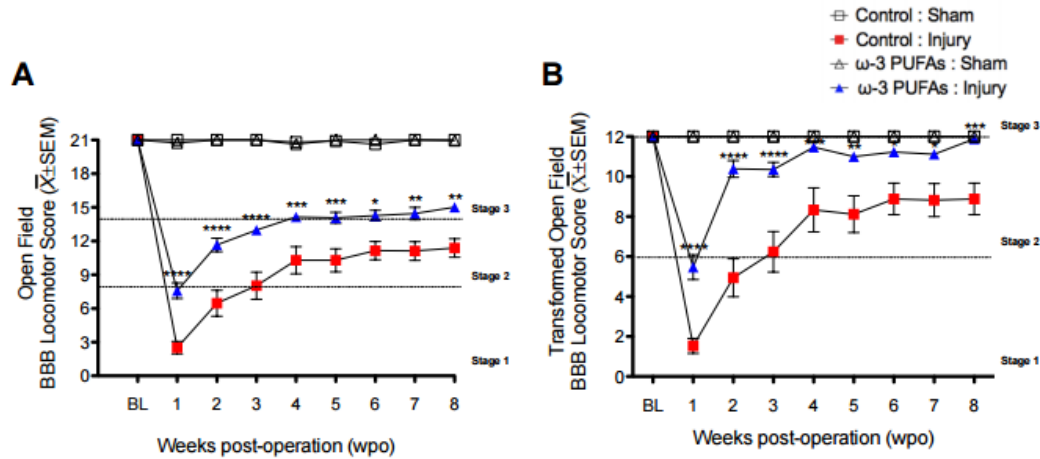
Figure 3. Dietary ω-3 polyunsaturated fatty acids (PUFAs) prophylaxis improves somatic function after contusion injury. **(A)** Dietary ω-3 PUFAs prophylaxis results in significant early and long-lasting functional milestones after SCI. **(B)** These results remained significant after pooling unusual observations and improving the metric properties using the Basso-Beattie-Bresnahan (BBB) score transformations. Interestingly, control animals reached a recovery plateau at 4 weeks (occasional weight supported plantar steps with no coordination: BBB score=10; transformed BBB score=8), whereas dietary prophylaxis resulted in prolonged recovery when compared with controls (displayed consistent weight-supported plantar steps and coordination at 8 weeks post-injury: BBB score=14; transformed BBB score: 12). Data are presented as mean±standard error of the mean (SEM); asterisks indicate significance level: Two-way analysis sof variance followed by Bonferroni *post hoc* *$p<0.05$, **$p<0.01$, ***$p<= 0.001$, *n*=8–18.

To examine the effect of dietary ω-3 PUFA on sensory function after SCI, the rats were subjected to electronic von Frey testing using a nonpunctate blunt probe. A remarkable finding of this study is that the ω-3 PUFA diet prevented major sensory deficits (mechanical hypoalgesia or loss of sensitivity) associated with chronic SCI in adult rats [43] [$F_{(1,14919)} = 8.592$; $p = 0.0039$] (Fig. 4A). Post hoc analyses showed significant differences in normalized hindpaw withdrawal thresholds between animals fed with control diets (≈ 66 % change from baseline) and ω-3 PUFA-fed groups (≈ 9 % change from baseline) at 8 wpi ($p < 0.01$) (Fig. 4A).

To determine whether a diet enriched in ω-3 PUFA reduces the likelihood of developing sensory dysfunction, we used the K-means clustering method to assign all rats to three groups according to their mechanical sensory thresholds changes at 8 wpi. Clustering resulted in a group of animals with statistically significant increases in hindpaw withdrawal thresholds at 8 wpi when compared to their respective baseline values (hypoalgesia cluster). The second cluster of animals did not show significant threshold changes between baseline and 8 wpo (normal cluster). The third cluster (hypoalgesia ++) also revealed hypoalgesic behaviors when compared to baseline (this cluster contained three animals fed with control diets). It is noteworthy that the average baseline value from this third cluster was also significantly lower when compared to the other two groups. Clustering validation results are summarized in Fig. 4B [hypoalgesic behavior (cluster): $F_{(2,634.2)} = 4.03$, $p = 0.022$; time: $F_{(1,2543)} = 32.33$, $p < 0.0001$]. Remarkably, we found that only 23% of the animals receiving ω-3 PUFA diets were clustered in the group of animals with significant sensory dysfunction (hypoalgesic clusters combined; Fig. 4C). In marked contrast, more than 75% of the control animals

showed significant sensory deficits. Fisher's exact test revealed that this difference was significant ($p = 0.02$; strength of association analysis demonstrated an odds ratio of 0.09 [95% CI = 0.01 to 0.60], with a sensitivity of 0.23 [95% CI = 0.05 to 0.54] and a specificity of 0.23 [95% CI = 0.05 to 0.54]).
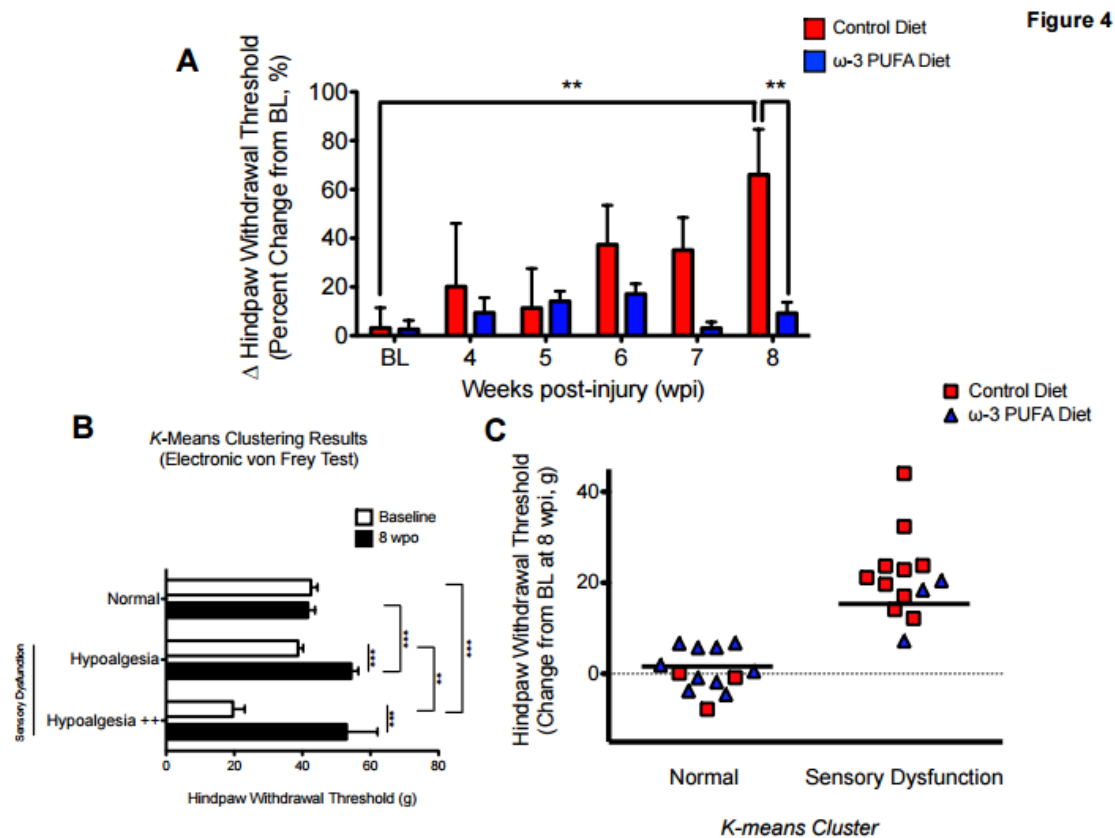
Figure 4. Dietary ω-3 polyunsasturated fatty acids (PUFA)s prevents sensory dysfunction after chronic spinal cord injury (SCI). **(A)** Electronic von Frey test shows that SCI results in increased withdrawal threshold (hypoalgesia). Remarkably, ω-3 PUFAs prophylaxis prevented alterations in paw sensitivity when compared with baseline (BL) ($p>0.01$). *Post hoc* revealed significant effects were observed between dietary groups at 8 wpi ($p<0.05$). Data are expressed as %Δ paw withdrawal threshold from BL (and normalized to %Δ in sham animals; mean±standard error of the mean [SEM], $n=13$). **(B)** K-means clustering divided mechanical sensory thresholds (g of force required to elicit hind paw withdrawal) into three groups: (1) normal (no differences between baseline and 8 weeks post-operation (wpo); two-way analysis of variance followed by Bonferroni's *post-hoc* test, $p>0.05$), (2) hypoalgesia (significant difference between baseline and 8 wpo; $p<0.05$), and (3) hypoalgesia++ (extremely significant difference between baseline and 8 wpo; $p<0.05$). Data are presented as mean±SEM. **(C)** Dietary ω-3 PUFAs prophylaxis significantly reduced the number of animals in which sensory dysfunction developed. K-means clustering was followed by the Fisher exact test to determine whether diet altered the predisposition of animals for development of sensory deficits. Analyses revealed that dietary ω-3 PUFAs reduced the probability of development of major sensory dysfunction (hypoalgesia) by 54% when compared with controls (Fisher exact test $p=0.02$, $n=26$).

Distinctive Neurolipidomic Signatures are Associated with Injury Operations and Dietary Interventions

To investigate the neurolipidome, we interrogated the levels of lipid metabolic features across 36 spinal cord epicenters using both LC/MS and GC/MS. Unsupervised hierarchical clustering was used to arrange the lipid metabolites on the basis of their relative levels across tissue samples and generate heat maps of the spinal cord neurolipidome (Fig. 5). Remarkably, we found distinctive metabolic lipid signatures in each studied group.
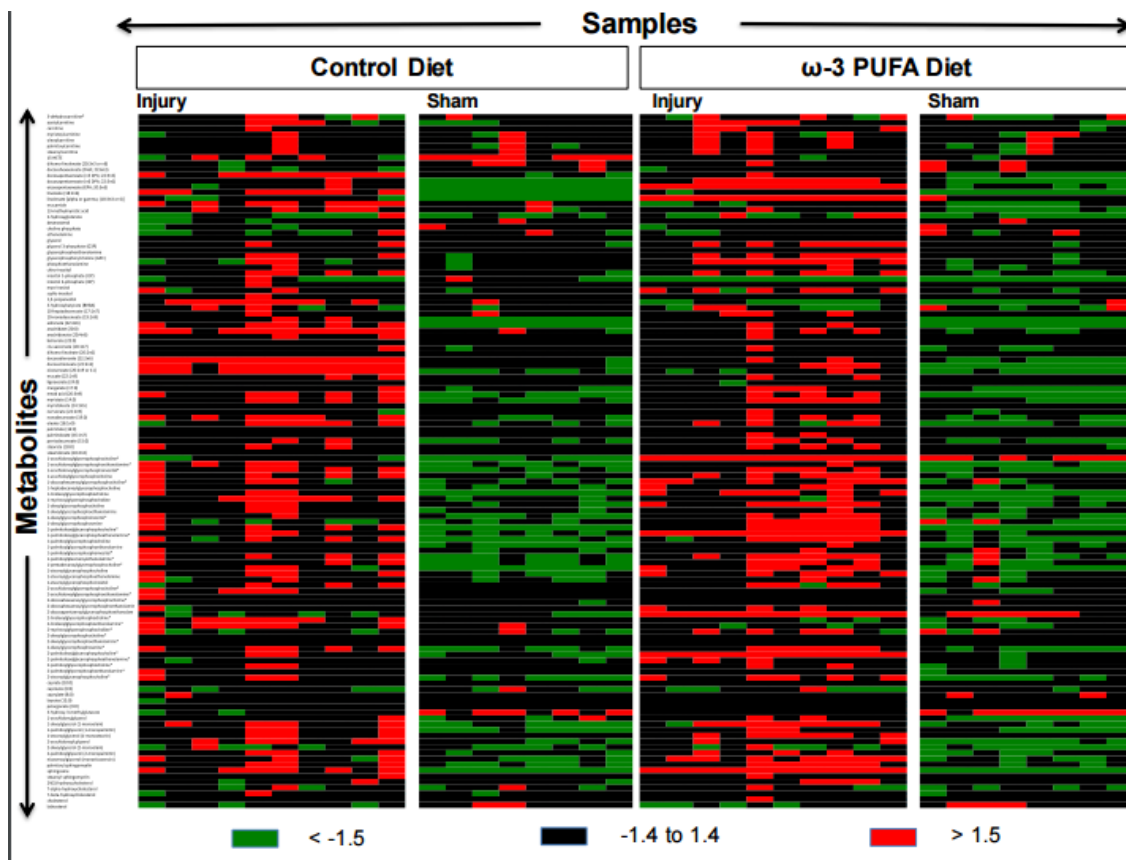
Figure 5. Heat map representation of unsupervised hierarchical clustering. Data are arranged in metabolites (rows) by sample type (columns). Metabolite family description: A, carnitine metabolism; B, eicosanoids; C, essential fatty acids and free fatty acids; D, glycerolipids; E, inositol metabolism; F, ketone bodies; G, long chain fatty acids; H, lysolipids; I, medium chain fatty acids; J, monoacylglycerols; K, sphingolipids; L, sterols and steroids. Green and red colors represent metabolite decreases and increases, respectively, relative to the median metabolite levels. See color scale. PUFA=polyunsaturated fatty acids; SCI=spinal cord injury.

Partial least squares projections to latent structures – discriminant analysis (PLS-DA) was performed to determine if the lipid features were capable of distinguishing group separations using a supervised projection technique (Fig. 6*B*). Of significance, our analyses revealed a projection in which the groups were significantly separated ($p < 0.01$ by permutation test). PLS-DA score plot was obtained using the variation scores of the first two principal components, PC1 (34.3%) and PC2 (18.1%) (Fig. 6*A*). Each plot mark corresponds to an animal subject and the variability in relative lipid metabolite levels detected for that animal. The first component, PC1, separated the sham animal spectra from injured animals. The second component, PC2, separated the spectra from control diets and animals receiving ω-3 PUFA-enriched diets. Hotelling's T^2 confidence ellipse, at a significance level of 0.05, revealed no outliers. Since score plots and loading plots are complementary, superimposing them depicts the impact of metabolites in the score plot model and show how the metabolites are correlated (most influential variables are further from zero on each loading; correlated variables are grouped together). As expected, the PLS-DA loading plot showed that the levels of ω-3 and ω-6 PUFAs had the strongest influence on the separation observed in our score plot model (Fig. 6*B*). Variable influence on projection (VIP) analyses, which reflects the impact of metabolites in the PLS-DA model, confirmed these findings by showing that PUFAs had a robust impact in both components of our PLS-DA model (VIP values higher than 1). Interestingly, DHA levels had only a slight effect in the projection model (low VIP values or low influence), which supports its fast metabolism and avid incorporation in neural membrane phospholipids. Figure 7 summarizes the main effects of chronic SCI and diet on the biosynthetic pathway of ω-3 and ω-6 PUFAs. We found that the ω-3 PUFA-enriched diet

significantly skewed the PUFA metabolism towards increased ω-3 at the expense of reduced ω-6 PUFA levels in both sham and injured animals.

Figure 6
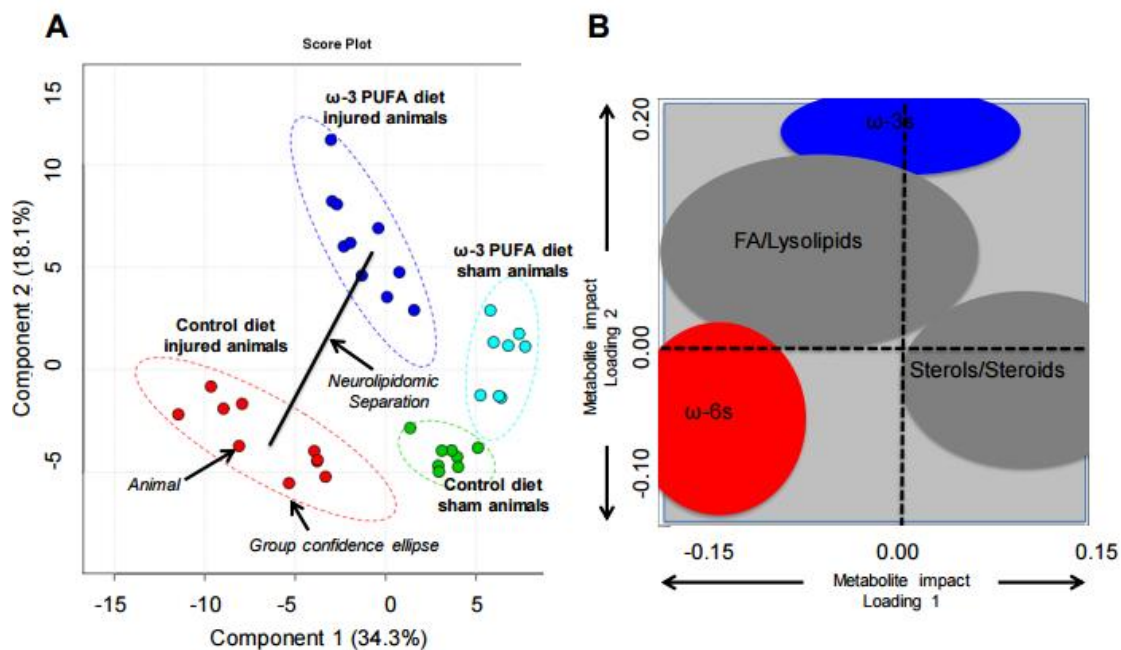
Figure 6. Multivariate data analyses. **(A)** PLS-DA score plot containing two first components. Each plot mark corresponds to an observation (individual rat spinal cord sample). The confidence ellipses are based on the Hotelling T^2 and illustrate the 95% confidence regions. Principal component 1 (PC1; *x*-axis) describes differences in the spectra between sham and injury operations. Principal component 2 (PC2; *y*-axis) shows a marked separation between control and ω-3 diets. Score plot analyses revealed statistically significant separations between groups ($p<0.01$ by permutation test). **(B)** Loading plot between PC1 and PC2. Loading 1 explains differences between sham and injured animals, whereas features in loading 2 explain differences between dietary interventions. Groups with stronger impact are further away from the plot origin (represented by dashed lines). Data demonstrate that ω-3 polyunsaturated fatty acids (PUFAs) contributed significantly to lipidomic differences between injured animals (location in loading 2 is far from zero). In contrast, analyses revealed that ω-6 PUFAs present a major contribution to both model loadings (loading 1: surgery effects and loading 2: diet effects), validating their role in chronic spinal cord injury and modulation by dietary ω-3 PUFAs. Correlated metabolites were grouped together to facilitate data presentation.
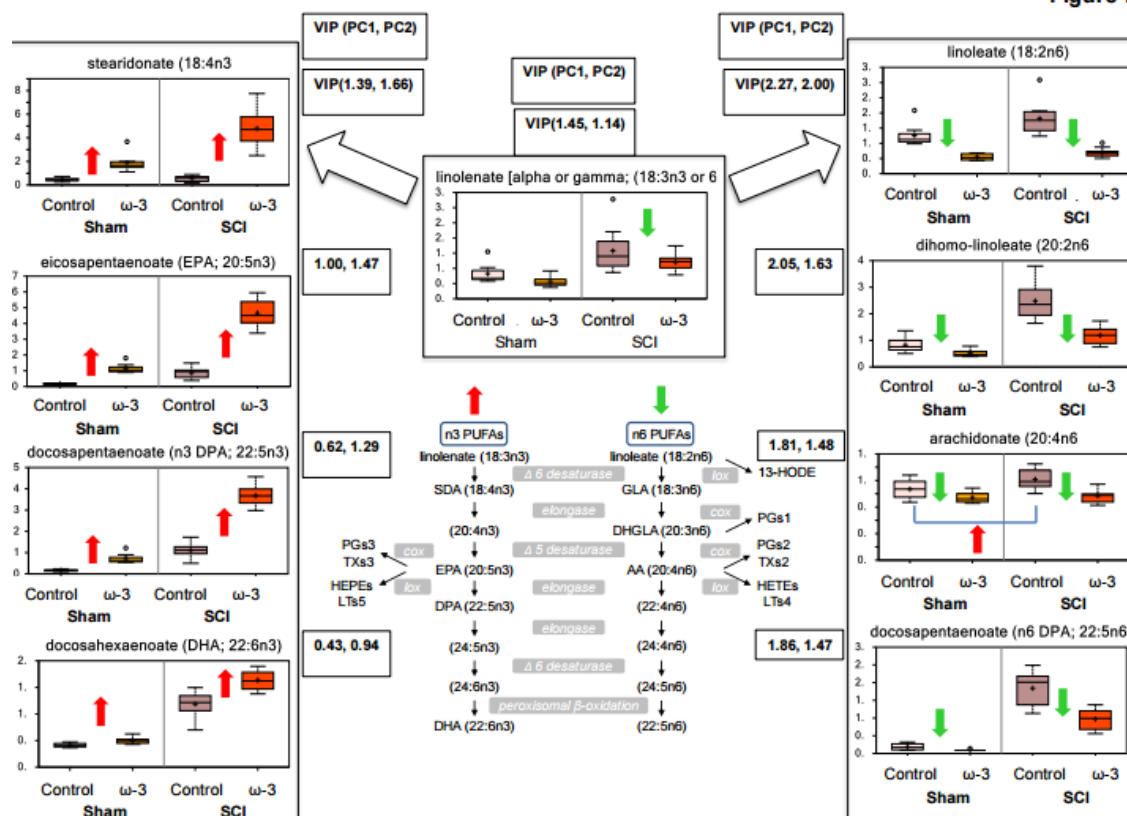
Figure 7. Chronic spinal cord injury (SCI) results in marked deregulation of ω-3 **(A)** and ω-6 **(B)** Polyunsaturted fatty acid (PUFA) metabolic pathways corrected by ω-3 PUFA-enriched diet. Green (decrease) and red (increase) arrows represent statistically significant alterations when compared with controls (two-way analysis of variance followed by *post-hoc* testing was performed. Differences were significance at $p<0.05$). **(C)** Box and whiskers legend depict metabolite relative levels relative to the median metabolite levels in each group. For each ω-3 and ω-6 PUFA metabolite, variable influence on projection (VIP) values were included for projections to latent structures discriminant analysis principal component (PC) 1 and 2. For each component, VIP scores higher than 1 are considered significant contributors to the group separations. **(D)** Summary of PUFA biosynthetic pathways. AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; GLA, γ-linolenic acid; DPA, docosapentaenoic acid; SDA, stearidonic acid; PG, prostaglandins; LT, leukotrienes; TX, thromboxanes; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid.

Chronic Spinal Cord Injury Dysfunction is Linked to DHA Deficiency and Corrected

by a Preventive ω-3 PUFA Dietary Intervention

A number of studies have shown that feeding animals increased amounts of fish oil ω-3 PUFAs has a profound effect on PUFA biosynthetic pathways, particularly increasing tissue levels of DHA (22:6, ω-3), DPA (22:5, ω-3) and EPA (20:5, ω-3) at the expense of AA (20:4, ω-6).[46] Our results validate these findings and demonstrate that chronic SCI results in dramatic alterations in PUFA metabolism that can be corrected and prevented by dietary prophylaxis with ω-3 PUFAs. Previous evidence supports that dietary supplementation with ω-3 PUFAs results in improved CNS function.[47] On the other hand, deficiencies in ω-3 PUFAs have been associated with numerous neurological impairments.[48-52] DHA deficiency is accompanied by concomitant increases in docosapentaenoic acid (DPA; 22:5, ω-6) levels, thus the ratio of ω-6 DPA to DHA has been suggested as a biochemical marker of low ω-3 PUFA status.[53, 54] Using liquid and gas chromatography coupled with mass spectrometry (LC/MS and GC/MS), we measured the relative levels of ω-6 DPA and DHA in the spinal cord lesion/laminectomy epicenter area. We used the ω-6 DPA to DHA ratio to determine: (1) whether chronic SCI results in a DHA deficiency, (2) whether our dietary intervention altered this ratio, and (3) whether this deficiency has functional consequences in SCI. One-way ANOVA showed very significant differences in ω-6 DPA to DHA ratios between groups $[F(3,10.66) = 67.53, p < 0.0001]$. Post hoc analyses demonstrated that SCI resulted in a significant ω-6 DPA to DHA ratio increase (DHA deficiency) when compared to sham animals at 8 wpo ($p < 0.0001$ and $p < 0.01$, respectively) (Fig. 8*A*), showing that DHA nutritional requirements may be enhanced after SCI. Remarkably, the dietary ω-3 PUFA

diet significantly reduced this DHA deficiency when compared to the animals fed with control chow during chronic SCI ($p < 0.0001$; $n = 10$). Although DHA levels were increased in the animals receiving diets enriched with ω-3 PUFAs, no significant differences were observed when comparing the ω-6 DPA to DHA ratio in sham animals from both dietary groups ($p > 0.05$, $n = 8$ rats per sham group). This supports the proposed idea that PUFAs are tightly regulated under physiological conditions and shows that the control diet itself did not generate the DHA deficiency. It is also noteworthy that the observed effects are not likely due to a deficiency in ω-6 fatty acids in the animals fed with ω-3 PUFA. This is supported by the finding that diet type had no significant effect on the levels of mead acid (20:3, ω-9), a biomarker of linoleic acid (18:2, ω-6) deficiency (data not shown).

To investigate the potential pathophysiological consequences of DHA deficiency in recovery after SCI, we performed Spearman's correlation analyses to determine the relationship between the ω-6 DPA to DHA ratio and functional behavior. Because it has been proposed that molecular determinants of early autonomic recovery may also influence locomotor function (and vice versa),[55] we also investigated the correlation between chronic DHA deficiency and bladder recovery. Notably, increases in the ω-6 DPA to DHA ratio were positively associated with the number of days needed to full bladder recovery in animals receiving control diets (r value = 0.80, $p < 0.001$; $n = 17$ pairs) (Fig. 8*B*). The ω-6 DPA to DHA ratio showed a robust negative correlation with BBB locomotor scores (r = -0.82, $p < 0.0001$) (Fig. 8*C*). Further, this ratio was positively correlated with mechanical hypoalgesia (r = 0.74, $p < 0.01$) (Fig. 8*D*). Together, these

findings show for the first time that DHA deficiency after SCI may increase susceptibility

for dysfunction and/or hinder functional recovery.

Figure 8
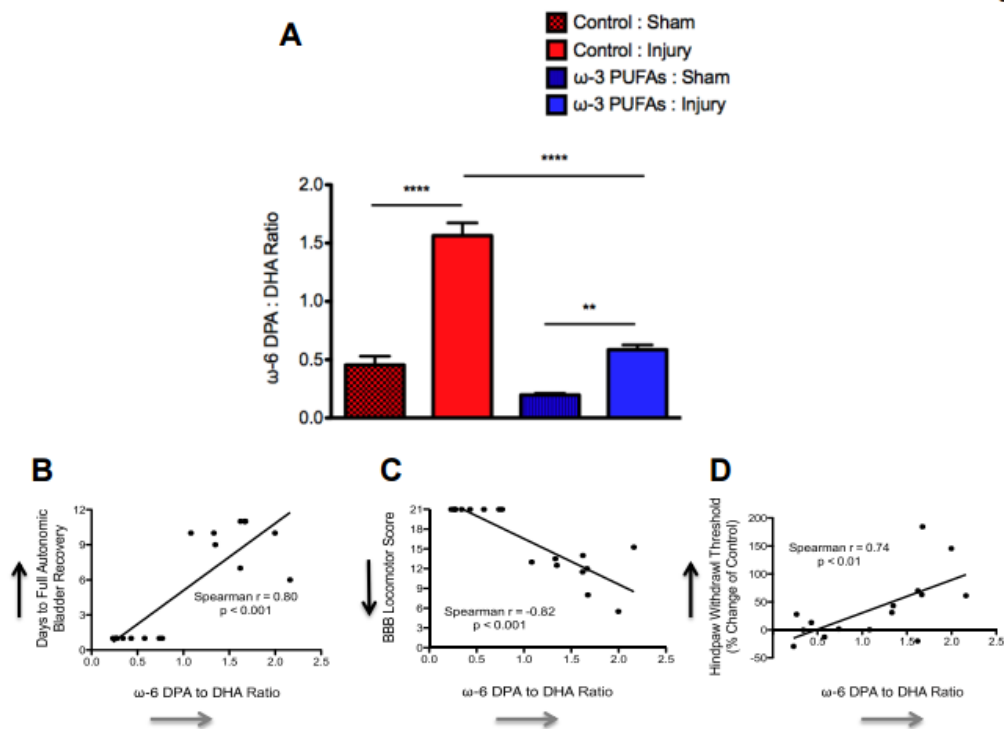
Figure 8. Chronic spinal cord injury results in significant dicosahexaenoic acid (DHA) deficiency that is corrected by a ω-3 polyunsaturated fatty acid (PUFA)-enriched diet. **(A)** Deficiency of the dietary-essential DHA (22:6, ω-3) was measured by assessing the levels of docosapentaenoic acid (DPA) (22:5, ω-6). After injury, both dietary groups presented significant increases in the ω-6 DPA to DHA ratio. Notably, injured animals consuming ω-3 PUFAs showed a significant reduction in the ω-6 DPA to DHA ratio when compared with injured animals receiving control diets at 8 weeks post-injury (wpi). This reduction was comparable to the baseline levels observed in the sham-operated animals receiving the control diet. The ω-6 DPA to DHA ratio was similar in both sham groups. Spearman rank correlation showed that the ω-6 DPA to DHA ratio is strongly associated with bladder **(B),** locomotor **(C),** and sensory function **(D).** Data were generated by correlating the levels of the ω-6 DPA to DHA with the number of days needed for full bladder recovery (1–11), the Basso-Beattie-Bresnahan (BBB) locomotor scores (0–21), and mechanical threshold changes from baseline at 8 wpi (% change from baseline). This was repeated for each animal receiving control diets (n=15–17 pairs for each behavioral test). R and p values are included for each graph. Line depicts the regression line. Black arrows in Y-axes represent direction of increased dysfunction in each behavioral test. Grey arrows in X-axes represent direction of increased DHA deficiency.

Dietary ω-3 PUFAs Increase Protein Kinase B/Akt and CREB mRNA Levels, Even in

Sham-operated Animals

Protein kinase B/Akt (Akt) and the cyclic AMP-response-element-binding protein

(CREB) have been implicated in regulating neural plasticity[56-58] and pro-survival and

regenerative responses in SCI.[22, 59-61] Prior studies from our lab and others have

shown that DHA-mediated beneficial effects may be mediated, at least partially, through

activation of Akt and/or CREB signaling pathways.[22, 62] To determine whether a diet

rich in ω-3 PUFAs results in modulation of these neuroreparative molecules after chronic

SCI, we examined Akt and CREB mRNA levels at 8 wpi. ANOVA followed by

Bonferroni's multiple comparison test revealed no significant changes in Akt mRNA

levels when comparing both injury groups [$F_{(3,0.26)} = 0.91$, $t = 0.40$, CI(-0.76 to 0.56), p

> 0.05] (Fig. 9*A*). In contrast, our analyses revealed a 1.9-fold increase in CREB mRNA

levels in animals that were fed with ω-3 PUFAs when compared to controls at 8 wpi

[$F_{(3,3.73)} = 9.03$, $t = 3.44$, CI(-1.72 to -0.09), $p < 0.05$, $n \geq 4$) (Fig. 9*A*). Western blot

analyses demonstrated that n ω-3 PUFA-enriched diet resulted in a 2.6-fold increase in

Akt protein levels when compared to controls at 8 wpi (Mann Whitney U (4.0), two-

tailed, sum (25,53), $p = 0.026$, $n = 6$) (Fig. 9*B*). Further, when compared to controls,

dietary ω-3 PUFAs induced a significant 4.3-fold increase in the protein levels of CREB

at 8 wpi (Mann Whitney U (2.0), two-tailed, sum (17,49), $p = 0.017$, $n = 6$) (Fig. 9*B*).
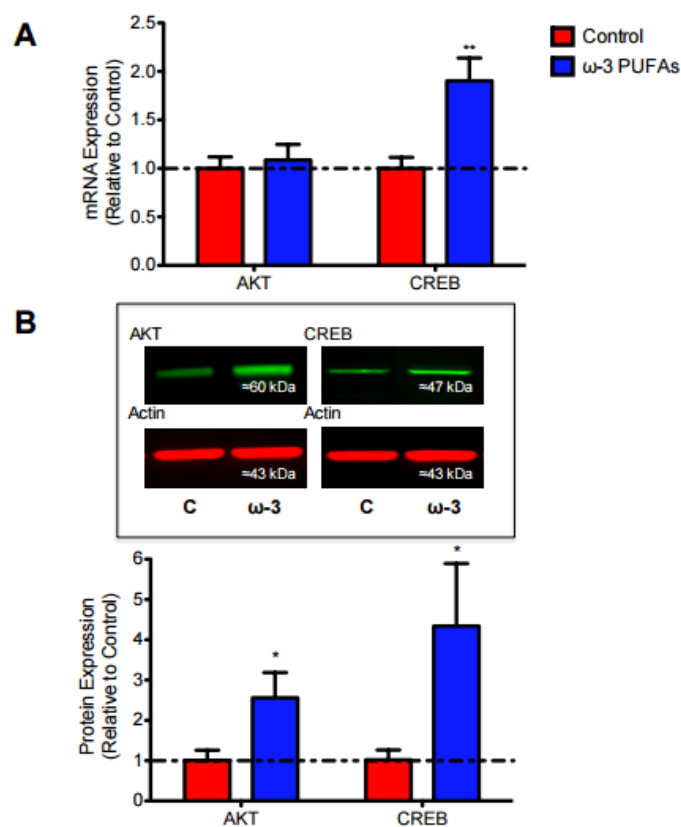
Figure 9. Dietary ω-3 polyunsaturated fatty acids (PUFAs) prophylaxis results in increased expression of pro-restorative signaling molecules. **(A)**Quantitative analyses of real-time polymerase chain reaction crossing thresholds showed a significant increase in cyclic AMP responsive element binding (CREB) protein mRNA levels in the ω-3 PUFA-pre-treated group when compared with control animals at 8 weeks post-injury (analysis of variance followed by Bonferroni *post hoc*, $p<0.05$, $n≥4$–5). **(B)** Immunoblot of spinal cord samples showing Akt (60 kDa) and CREB (approximately 47 kDa) protein upregulation after dietary pre-treatment with ω-3 PUFAs. Analyses show that dietary ω-3 PUFA prophylaxis resulted in increased Akt and CREB protein levels when normalized to actin levels (Mann-Whitney *U* test, $p<0.05$, $n=6$). Error bars represent standard error of the mean.

Discussion

The present study shows that a diet enriched in ω-3 polyunsaturated fatty acids (PUFAs) prophylaxis is sufficient to ameliorate autonomic, motor, and sensory function after experimental contusion SCI in rats. These improvements showed a significant association with the cord lipidome, particularly an ω-3 PUFAs accumulation at the expense of ω-6 PUFAs. Chronic SCI resulted in distinct lipidomic signatures, including a marked DHA deficiency, which was accompanied by dysfunction and a limited capacity to support functional recovery. Notably, a diet enriched with ω-3 PUFAs was effective in reducing this deficiency and resulted in the up-regulation of the pro-survival and repair proteins, Akt and CREB. A proposed mechanism for ω-3 PUFA-mediated prophylaxis against SCI is presented in Fig. 10.
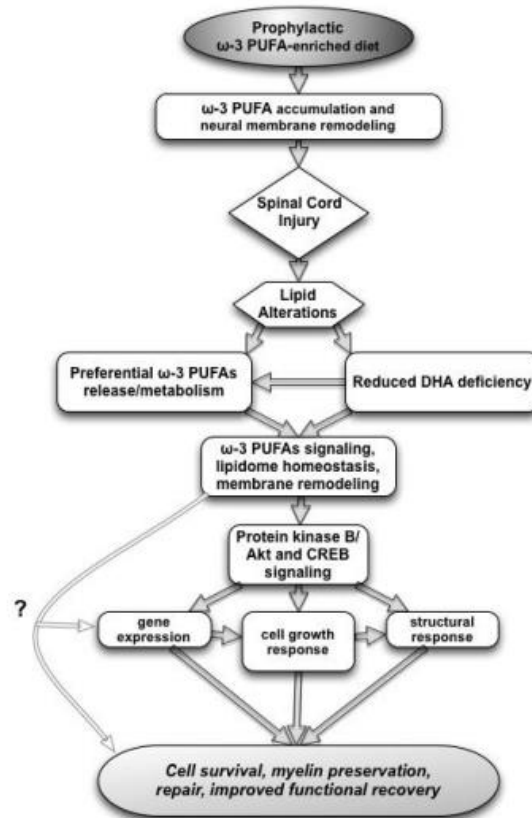
Figure 10. Putative mechanisms underlying the beneficial effects of dietary ω-3 polyunsaturated fatty acids (PUFAs) prophylaxis in spinal cord injury (SCI). A preventive nutritional therapy with ω-3 PUFAs results in accumulation of ω-3 PUFAs in the spinal cord neural membranes, which may provide extended neuroprotection and repair substrates after SCI. Lipid metabolism alterations under this dietary regime leads to reduced ω-3 deficiencies, particularly dicosahexaenoic acid (DHA), and preferentially activates ω-3 PUFA metabolism. This response may result in regaining PUFA homeostasis and enhanced pro-restorative signaling, such as protein kinase B/Akt and cyclic AMP responsive element binding (CREB) protein and/or additional uncharacterized mechanisms. Omega-3 PUFAs may provide important substrates implicated in stress resistance, such as induction of antioxidant gene expression, cell growth, and membrane homeostasis and remodeling. Together, this response should result in improved tissue sparing, plasticity, and repair.

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Pretreatment with a ω-3 PUFA-enriched Diet Improves Somatic and Autonomic

Recovery after Contusion SCI

The spinal cord is at high risk of injury during surgeries, high-impact sports, military conflicts, and in several congenital and degenerative CNS disorders.[63-65] Of significance, for patients undergoing SCI, the recovery of autonomic bladder function is a high priority for improving their quality of life.[66] Following incomplete SCI, there is initial disruption of bladder control followed by partial recovery, which correlates negatively with injury severity and damage.[67] Full autonomic bladder function recovery is a consequence of adaptive plasticity, particularly sprouting and reconnection of spinospinal pathways.[68] Here, we found that a ω-3 PUFA-enriched diet not only results in amelioration of autonomic bladder function but also accelerates its complete recovery, suggesting reduced damage and/or activation of repair responses.

Recent published reports have shown that supplementation with ω-3 PUFAs or with interventions that increase DHA levels promote locomotor recovery when administered after SCI.[12, 17-24] These findings led us to examine the efficacy of a preventive diet enriched with ω-3 PUFAs to reduce dysfunction after injury. Our results validate the role of fatty acids in functional recovery and show for the first time that dietary enrichment with ω-3 PUFA alone, without additional DHA supplementation or AA blockade, is sufficient to significantly improve hindlimb function in a clinically relevant contusion SCI. This was evidenced by weight-supported steps during the first week after injury in the group of animals receiving ω-3 PUFA prophylaxis, which represents an important finding because voluntary locomotion with full weight support requires supraspinal control.[69, 70] More importantly, our results show that these

beneficial effects are long lasting because BBB scores continued to improve in relation to controls for at least 8 weeks. These rapid and prolonged beneficial effects suggest that dietary ω-3 PUFA prophylaxis may be attributable to a combination of early (i.e., neuroprotection, plasticity, and remyelination) and late (i.e., sprouting and regeneration) protective/repair mechanisms. Although previous findings demonstrate that a combined intravenous and dietary DHA regime has the ability to sustain functional recovery, the efficacy of dietary DHA had limited positive effects when administered alone after injury.[18] Our findings suggest that the lack of dietary DHA efficacy reported by these investigators may have resulted as consequence of reduced food intake and altered lipid metabolism during the first week post injury, which may lead to suboptimal DHA levels. Likewise, administration of fenretinide (a retinoic acid analog shown to reduce AA and increase DHA), after 24 hours post-injury failed to sustain locomotor recovery in mice,[71] suggesting the relevance of the PUFA balance in the acute stages of SCI and supporting a role for endogenous ω-3 PUFAs in prophylaxis against neurotrauma. The novel experimental paradigm used in this study, which fed the animals with an ω-3 PUFA-enriched diet before SCI, underscores the value of the pretreatment. Further, these data clearly demonstrate a potential therapeutic window for ω-3 PUFAs, which may be dependent on the ω-3 source and dosage, and warrants further investigation.

Sensory deficits and pain are common clinical problems after chronic SCI.[72] We show that a diet enriched with ω-3 PUFA prevents the development of major sensory dysfunction (hypoalgesia), as evidenced by no significant changes in the force required to elicit hindlimb withdrawal when compared to controls. Although the pathophysiological mechanism responsible for the loss or presence of sensory function after injury are only

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partly understood, evidence demonstrates a paradoxical combination of sensory loss within the area where chronic pain is felt suggesting that both neurodegenerative and pro-inflammatory responses may play a role [73-74] Because we used the spinal cords for lipidomic profiling and RNA/protein extractions, we could not morphologically determine the extent of injury in this study. However, sensory function has been correlated with the amount of white matter spared.[75] Further, assessment of recovery using the BBB locomotor scale and bladder function provide an indirect measure of injury magnitude and spared tissue.[27, 67] Along with the results of others, we have shown that this ω-3 PUFA-mediated positive functional outcomes could be explained, at least in part, by the ability of DHA and EPA to dampen various secondary damage events, activate neuroprotective mechanisms, increase white matter sparing, and reduce axonal pathology in SCI.[12, 17-22]

ω-3 PUFA-enriched Diet Reduces DHA Deficiency and Results in Distinctive Lipid Signatures Associated with Functional Recovery After SCI

The precise mechanisms underlying the beneficial effects of dietary ω-3 PUFAs prophylaxis in the CNS are only partly understood, and our findings showing the important nutritional contributions and implications in functional behavior after trauma reveal various novel features. First, although the CNS is highly resistant to dietary PUFAs deficiencies,[76, 77] our data show that SCI itself leads to DHA nutritional deficiencies, which were associated with functional impairments. This data is supported by rodent studies showing that SCI results in an acute reduction in DHA plasma levels[71] and accelerates DHA metabolism.[78] Second, both chronic SCI and dietary

supplementation with ω-3 PUFAs had a profound impact on the spinal cord neurolipidome.

Notably, dietary enrichment with ω-3 PUFAs prevented a chronic DHA deficiency and led to across-the-board increases in short and long chain ω-3 PUFAs, which were accompanied by similar decreases in ω-6 species. Retroconversion of DHA to shorter chain PUFAs is known to occur and could account, in part, for the increased levels of other ω-3 PUFAs.[79, 80] On the other hand, the corresponding decrease in ω-6 PUFAs supports an ω-3 PUFA role in modulating genes and enzymes involved in the transport, synthesis, esterification, storage, and degradation of fatty acids.[81] Moreover, the PUFA balance can impair the interconversion of EPA and DHA by modulating the activity of Δ6-desaturase *in vivo*.[82] Although little is known about the particular mechanisms involved in the regulation of lipid metabolism after SCI, it is now clear that an ω-3 PUFA-enriched diet causes a global shift towards ω-3 lipids, which may have an important impact on resiliency to damage.

Despite its therapeutic and prognostic potential, the literature is scarce with regard to reports discussing endogenous determinants of vulnerability to neurological dysfunction in experimental models of injury. A very likely post-traumatic endogenous site is the cell membrane, which undergoes marked structural and functional alterations. After SCI, membrane phospholipids are converted into potent signaling molecules through the action of multiple phospholipases, including the calcium-dependent phospholipase A_2 that preferentially hydrolyzes AA from the *sn*-2 position of phospholipids [83]. Therefore, altering the membrane phospholipid acyl-chain composition by administration of ω-3 PUFAs has the potential to change phospholipid-

mediated cell signaling by three important processes: (1) altering the availability of AA, (2) changing the suitability of the phospholipids to serve as substrates for an array of phospholipases, and (3) mediating broad scale changes to membrane lipid composition which, in turn, may affect fluidity, signaling, and function. Although the involvement of membrane remodeling in the response to SCI has not been studied extensively, abnormal remodeling following injury has been implicated in membrane protein and channel dysfunction.[84, 85]

A third and final notable feature of this study is that dietary supplementation with ω-3 PUFAs results in increased levels of protein kinase B/Akt and CREB. Interestingly, several studies, including ours, have shown that Akt and CREB may play a role in DHA-mediated neural resiliency, neuroprotection, and plasticity.[22, 52, 62, 86] Our finding supports the idea that ω-3 PUFAs may modulate cellular switches involved in controlling vulnerability to damage after SCI. Future studies incorporating inhibitors of Akt/CREB could provide further insight on whether this mechanism is essential for the prophylactic efficacy of dietary ω-3 PUFAs in SCI.

In summary, we show that SCI leads to chronic DHA deficiency associated with dysfunction. We present the first evidence that demonstrates that dietary ω-3 PUFA prophylaxis results in distinctive neurolipidomic alterations that may reduce cellular vulnerability and facilitate functional recovery after SCI. Our findings have significant implications for the current Western diet. For instance, recovery after neurotrauma may be hindered by our modern diet. Thus, based on clinical and epidemiological evidence for the beneficial effects of ω-3 PUFAs,[87] together with evidence of safety and tolerability,[88, 89] dietary ω-3 PUFAs prophylaxis against neurotrauma deserve

consideration.

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CHAPTER FOUR

METABOLOMICS UNCOVERS DIETARY OMEGA-3 FATTY ACID-DERIVED

METABOLITES IMPLICATED IN ANTI-NOCICEPTIVE RESPONSES AFTER

EXPERIMENTAL SPINAL CORD INJURY

By

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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.

Key words: DHA; EPA; dietary fatty acids; endocannabinoid metabolome; spinal cord injury; chronic pain

Abbreviations: Spinal cord injury (SCI); docosahexaenoic acid (DHA); omega-3 polyunsaturated fatty acids (O3PUFAs); endocannabinoids (eCBs); N-acyl ethanolamines (NAEs); docosahexaenoyl ethanolamine/synaptamide (DHEA); docosapentaenoyl ethanolamine (DPEA); eicosapentaenoyl ethanolamine (EPEA); thermal hyperalgesia (TH); hindpaw withdrawal latency (HWL); ultrahigh performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS^2); gas chromatography/mass spectrometry (GC/MS); growth-associated protein 43 (GAP43); calcitonin gene-related peptide (CGRP); partial least square-discriminant analysis (PLS-DA); glycerophospho-containing N-acyl ethanolamines (GP-NAEs); Basso, Beattie and Bresnahan (BBB); linoleyl ethanolamine (LEA); arachidonoyl ethanolamine (AEA); eicosenoyl glycerol (EG); 2-arachidonoyl glycerol (2-AG); 2-palmitoyl glycerol (2-PG); 1-oleoyl glycerol (1-OG); palmitoyl ethanolamine (PEA); phospholipase D (PLD); phospholipase A/B or α-β-hydrolase 4 (Abh4); glycerophosphodiesterase (GDE1); N-acyl phosphatidyl ethanolamine (NAPE); glycerol-3-phosphate (G3P); lyso-phosphatidic acid (LPA); phosphatidic acid (PA).

Introduction

Chronic neuropathic pain is one of the most important determinants in the perceived quality of life of spinal cord injury (SCI) patients [1]. 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 [2, 3]. Neuroinflammation is regarded as a hallmark mechanism underlying injury progression and pain processing [4, 5], and thus represents an attractive target for therapeutic strategies [6, 7].

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 [8]. 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 [9]. Recent studies have shown that O3PUFAs and their derivatives can exert strong antinociceptive effects against thermal and chemical stimulation in various animal models [10-12]. 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

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dysfunction [13]. 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 [13, 14]. 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 [15-19]. 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 [20-22], 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 [23-26], 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.

Materials and Methods

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 [13].

Diet Composition

Custom AIN-93-based diets were prepared with modifications to the fat composition as described previously [13]. 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.

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 [27]. 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 [28]. 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.

Nociceptive Testing

Thermal hyperalgesia (TH) was assessed using the well-established Hargreaves withdrawal test to thermal noxious stimulus [29]. 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 [30-33]. 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 [34-37]. Furthermore, the repetitive nature of the Hargreaves test makes it very susceptible to learning phenomena [38]. 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 [39-43]. 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 [13]. Briefly, the nociceptive

phenotype was determined by the following equation:

EQ.1 HWL$_\%$ = [(HWL$_{ib}$-HWL$_{ix}$) / HWL$_{ib}$] - [(HWL$_{sb}$-HWL$_{sx}$) / HWL$_{sb}$] (x100)

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*.

Metabolomic Profiling

Unbiased metabolic profiling was performed as previously described [13].

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 [44]. Archived mass spectrometry data from our previously reported study, which was curated for only identified 'named' compounds in Metabolon's chemical reference library [13], 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 [45, 46], SCI-mediated chronic pain [47], and recently as a marker of SCI progression [48]. 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).

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 [49, 50]. 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.

Immunodetection

Immunofluorescence methods has been described previously [14]. 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 [14]. 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.

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 [51, 52]. 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 [53-55]. 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.

Results

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 [13]. 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.

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 $\pm$ 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 $F(3,49253) = 40.22$, $p = 0.0001$, $n = 8\text{-}18$ 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

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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 $p = 0.0008$; $n = 18$).
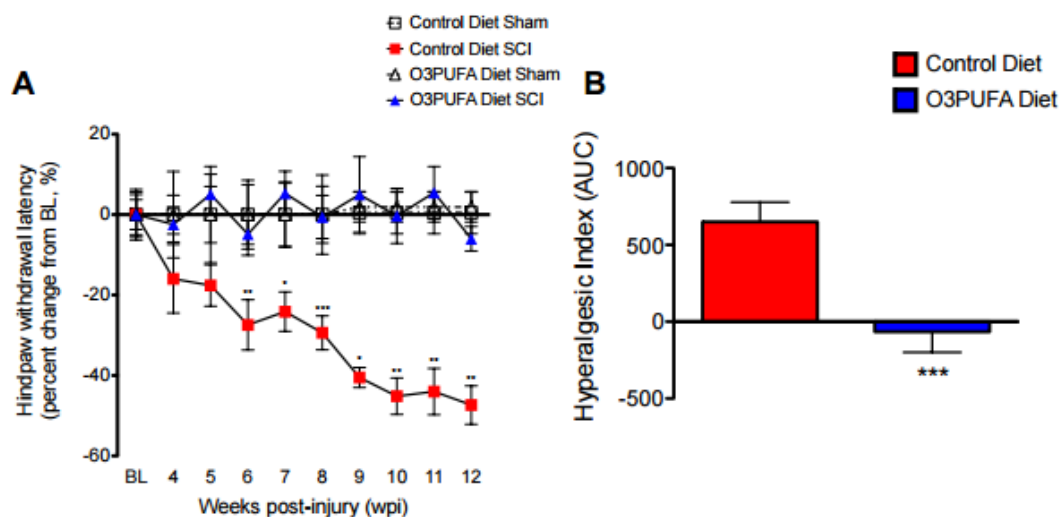
Figure 1. Responsiveness to thermal stimulation in animals receiving control and O3PUFA-enriched diets. (A) Thoracic contusion to the spinal cord leads to below-level thermal hyperalgesia in animals receiving control diets. Hindpaw withdrawal latencies (averaged percent change from baseline) are plotted 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; $n = 18$.

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Metabolomic Profiling Reveals Distinctive Endocannabinoid Signatures Associated

with Chronic SCI and Dietary O3PUFAs

We used an untargeted metabolomics approach [13] 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.
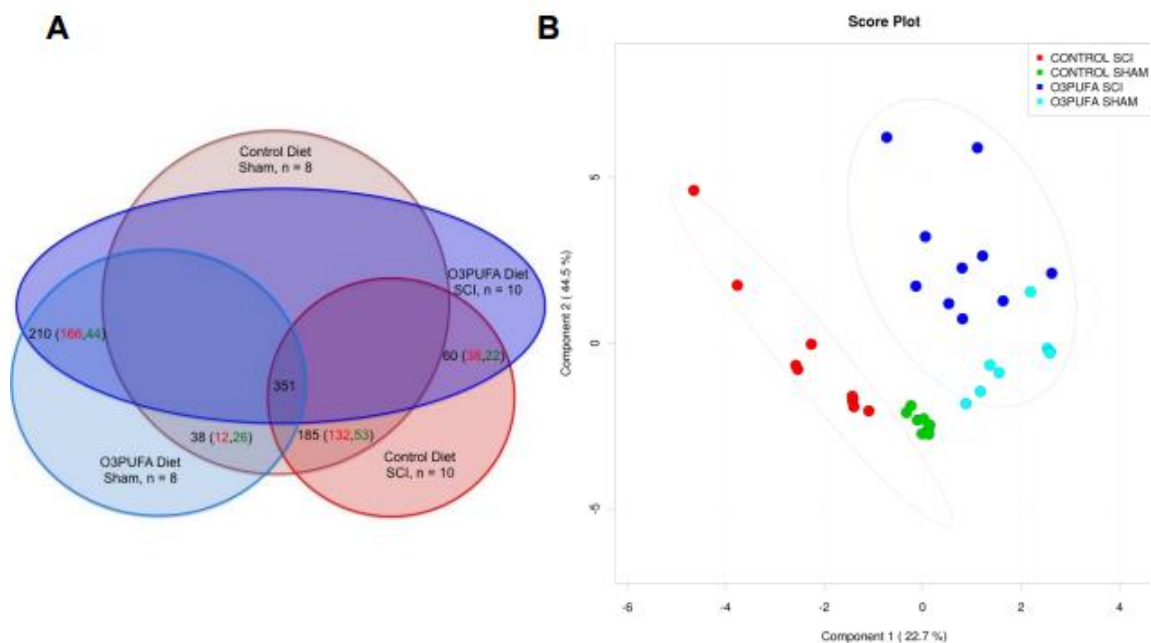
Figure 2. SCI and dietary O3PUFAs modulate the endocannabinoid-related neurometabolome. (A) Venn diagram depicting the numerical interactions among data sets. ANOVA contrasts analyses were used to evaluate the regulation pattern differences between groups. The total number of features detected across 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.

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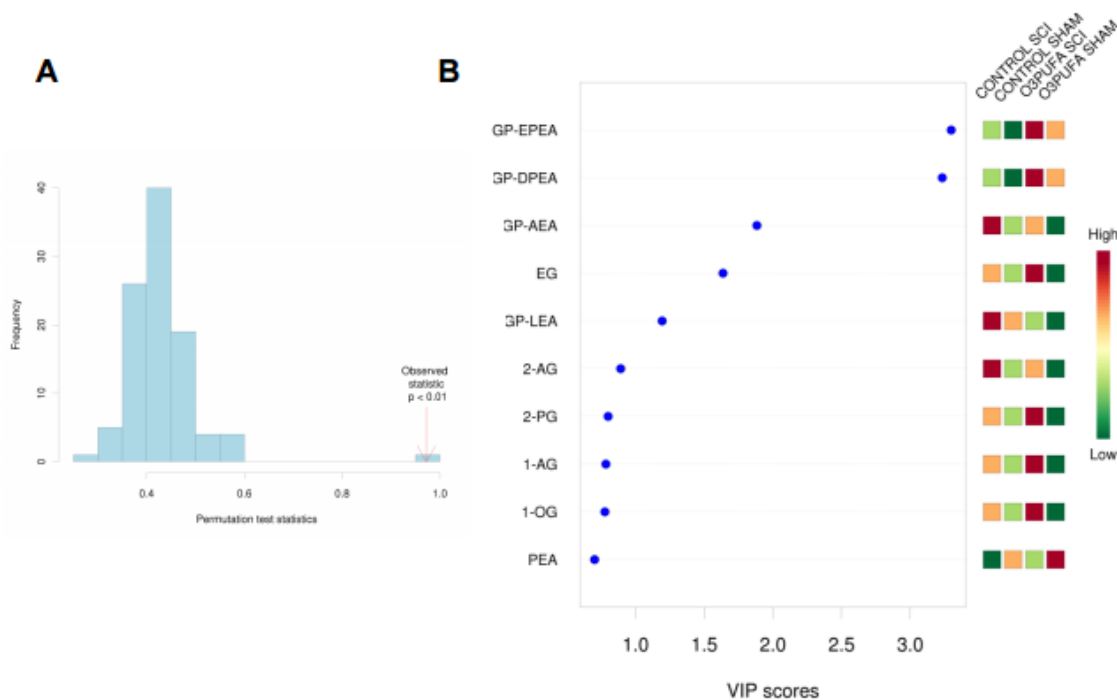
Figure 3. PLS-DA model validation and metabolite impact. (A) Prediction accuracy training permutation test validate the PLS-DA model by showing a significant observed statistic ($p < 0.01$). (B) The variable influence on projection (VIP) analyses, which reflect the importance of metabolites showed the 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.

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[56], 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 [57, 58]. 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.
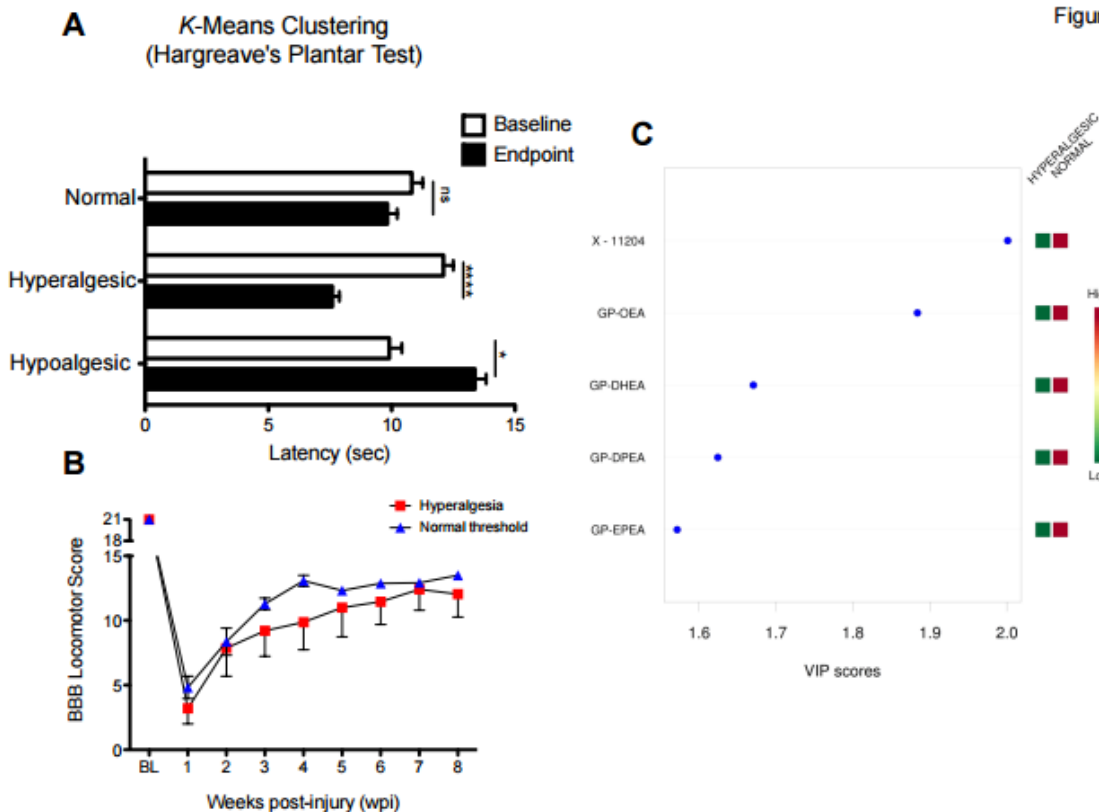
Figure 4. Chronic O3PUFAs consumption leads to a robust accumulation of diet-derived glycerophospho ethanolamines in the spinal cord. (A) Box and whiskers graphs illustrate a marked increase in the levels of O3PUFA-dervied GP-NAEs (relative to the median metabolite levels in each group). (B) Potential metabolic pathways for the biosynthesis of NAEs. This illustration is adapted and modified from previous reports [57, 58]. 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.

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.

Table 1

	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, Metabolites, 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
	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
	eicosapentaenoyl glycerophosphoethanolamine (GP-EPEA)	1.83	1.8	10.27	10.15
	1-palmitoylplasmenylethanolamine	2.28	1.78	1.48	1.15
	1-palmitoy lglycerol (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

LEGEND

Green: indicates significant difference ($p\leq0.05$) 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 ($p\leq0.05$) 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

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 X 10^{-15}; DPEA = 2.40 X 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 [59, 60]. Together, our findings suggest that these diet-derived metabolites may play significant roles in antinociception.
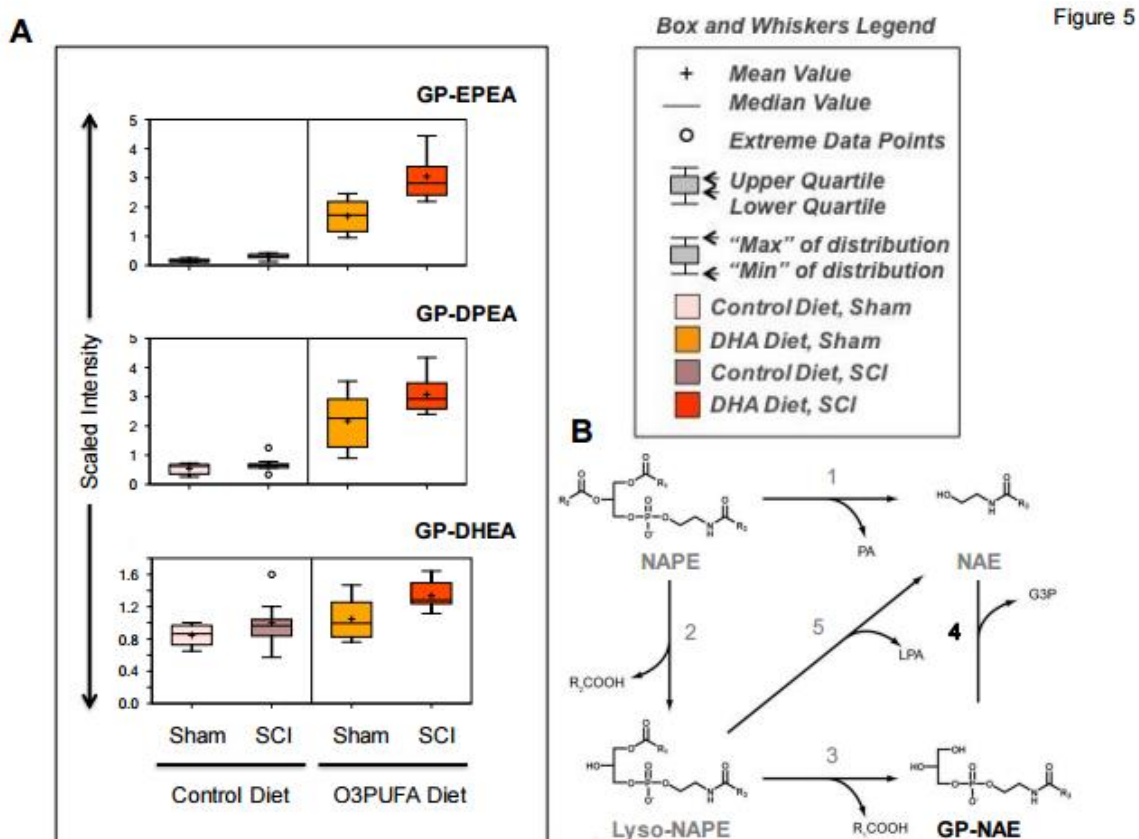
Figure 5. Metabolic features correlated with pain-like phenotypes. Scatter plot shows the significant relationship between the levels of (A) ethanolamines containing 22-carbon N-acyl chains, (B) GP-DHEA, (C) GP-DPEA, (D) PEA and the hindpaw thermal withdrawal latency change from baseline. For every correlation, the Spearman r was higher than 0.50, with a $p < 0.05$, XY = 20 pairs.

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 [61, 62]. 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 [63]. 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.
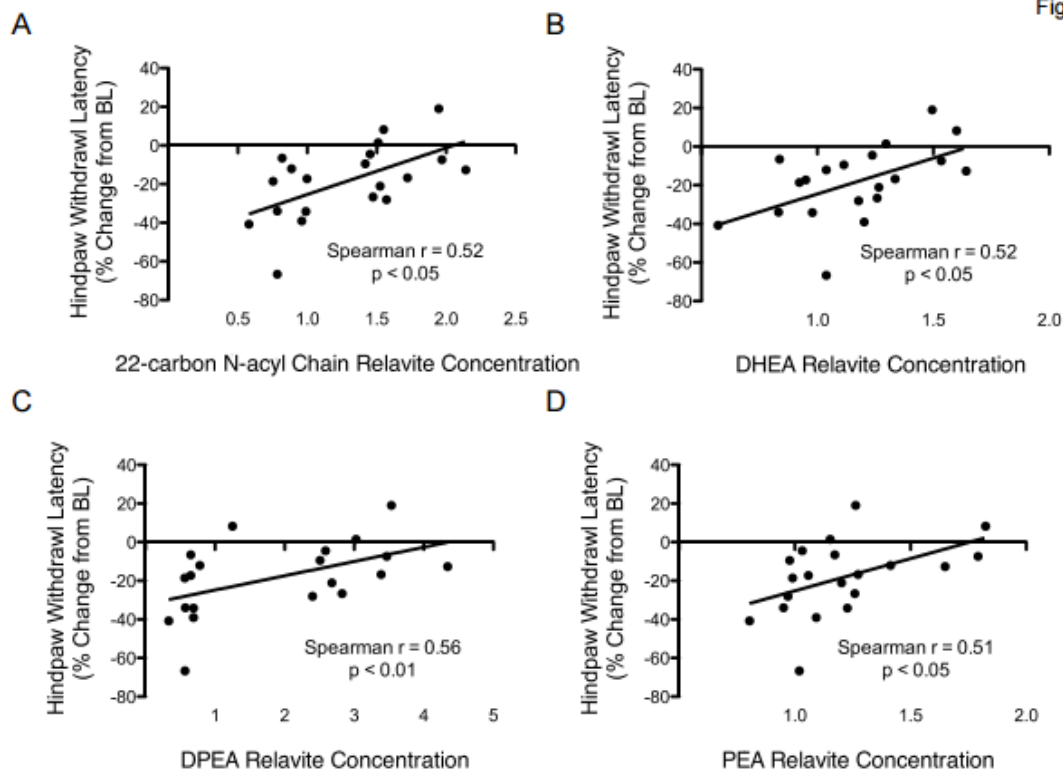
Figure 6

Figure 6. (A) *K*-means clustering divided animal based on their nociceptive behavior (Δ latency = latency$_{endpoint}$ - latency$_{baseline}$). 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.

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 [64].

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 [65, 66]. 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).

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 [67-69]. 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).
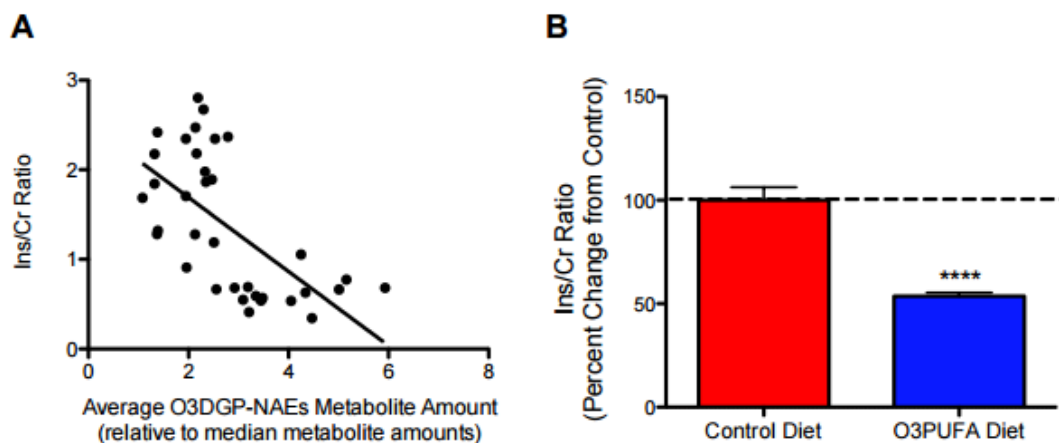
Figure 7

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.
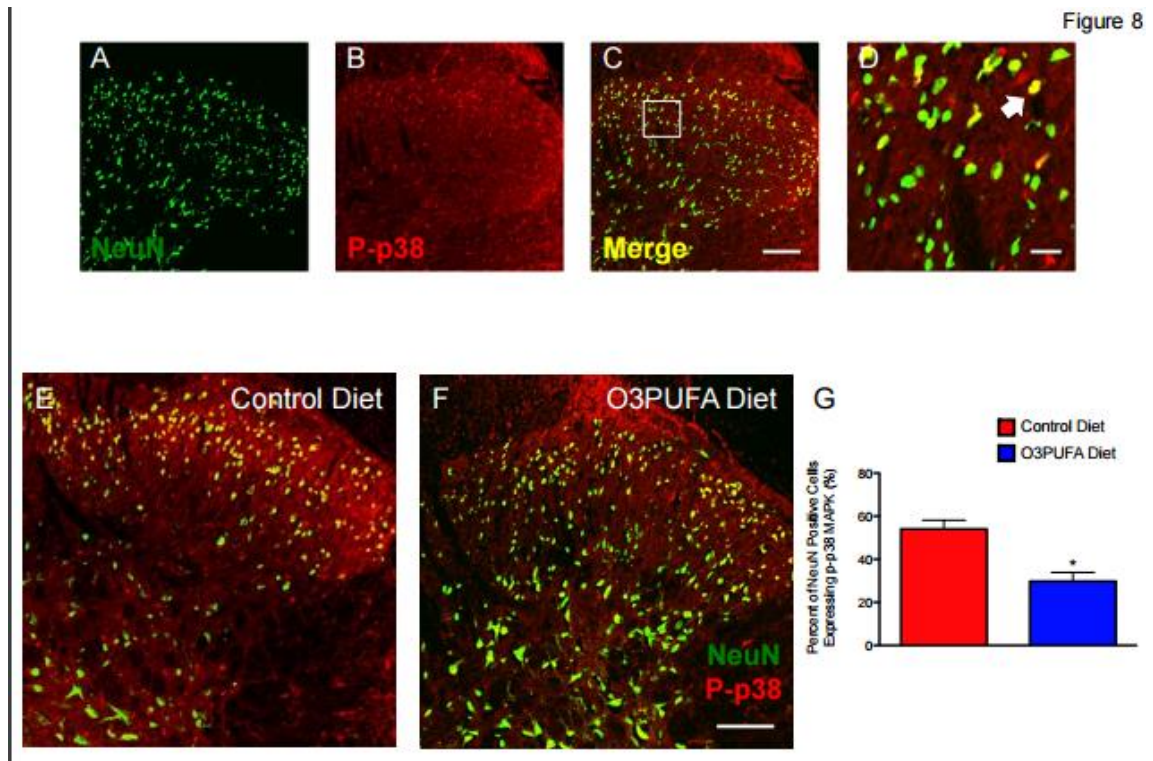
Figure 8. Preventive dietary O3PUFAs reduce the expression of phosphorylated p38 in below-level dorsal horn neurons. At 12 weeks post-injury, laser confocal microscopic evaluation revealed dorsal NeuN-positive neurons (A) containing the phosphorylated p38 MAPK (B). Merged photomicrographs (C) and inset (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.
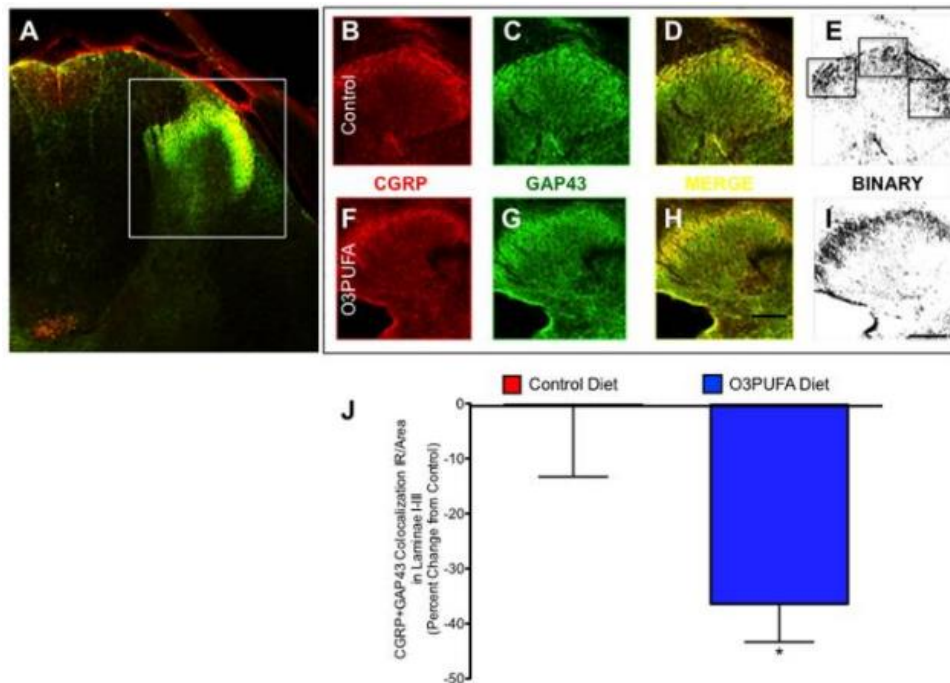
Figure 9. Dietary O3PUFA-pretreatment reduces nociceptive fiber sprouting following chronic SCI. (A) Double labeled spinal cord section showing calcitonin gene-related peptide (CGRP) and growth-associated protein 43 (GAP43) immunoreactivity (IR) in spinal cord. Confocal photomicrographs showing CGRP (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.

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 [65, 66, 70]. 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 [71], anti-inflammatory molecules [72], or by prophylactic cell transplantation strategies [73, 74]. 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 [75, 76].

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 [13]. Further, when the spinal cord is enriched with O3PUFAs before SCI, these lipids mediate robust neuroprotection, recovery, and activate pro-restorative responses [13, 14, 77]. Several recent studies have shown the importance of PUFAs in nociception, supporting the hypothesis that dietary lipids are key elements of the nociceptive pathways [9-12, 78]. 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 [13]. 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 [79-80].

Several lines of evidence demonstrate that the level of spinal cord damage play significant roles in the development and maintenance of pain-like behaviors [61, 62, 81], 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 [13, 14]. 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 [82] and antinociceptive [20-22] responses. Notably, it has been shown that SCI rats exhibit profound alterations in the metabolic pathways associated with these lipids [83]. 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 [56, 84-88]. The findings reported in the present study support

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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 [59, 60, 89] and reduces

pain-like behaviors in experimental models of neuropathic pain [53, 90]. 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 [91] and may contribute to the beneficial effects mediated

by dietary O3PFAs [92-94]. 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 [95]. 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 [96]. 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 [65, 66, 97]. 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 [98]. More recent studies showed that DHA also impairs p38 MAPK signaling in microglial cultures [99]. 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 [100] and to the expression of calcitonin gene-related peptide in rats [101]. 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 [28, 30-33], 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 [102]. 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 [103-105], futures studies should employ integrated pain-associated behaviors to facilitate data interpretation and to gain better understanding of the antinociceptive targets of dietary O3PUFAs. 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 [106], 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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CHAPTER FIVE

EFFECTS OF DIETARY VITAMIN E SUPPLEMENTATION IN BLADDER

FUNCTION AND PLASTICITY DURING SPINAL CORD INJURY

By

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Abstract

The pathology associated with Spinal Cord Injury (SCI) generates debilitating autonomic dysfunctions, paralysis and significant sensorimotor impairments. These outcomes may be influenced an increased levels of free radical part of the oxidative stress observed after the trauma. The present study investigates the impact of an eight weeks dietary enrichment with vitamin E (alpha-tocopherol) on key functional outcomes prevalent during the acute phase of SCI. Female adult Sprague-Dawley rats were fed either with a normal diet or a regiment supplemented with Vitamin E for eight weeks. Following the treatment animals were exposed either to a contusion SCI or Sham and evaluated using standard functional behavior analysis. We report that dietary vitamin E promoted locomotor recovery and a reduction in conduction deficits. Rats consuming the Vitamin E enriched diet showed a significant improvement in the BBB locomotor scores and a significant accelerated bladder recovery. The dietary intervention did not seem to preserve neurons in the ventral horn of injured rats, but it significantly increased the numbers of oligodendrocytes. Further analysis showed, that dietary Vitamin E inhibited H-reflex depression and increased the levels of supraspinal serotonin immunoreactivity. Our findings support the complementary use of vitamin E to ameliorate sensory and autonomic dysfunctions observed following SCI such as bladder dysfunction.

Introduction

Primary mechanical injury to the spinal cord sets in motion a complex cascade of secondary harmful events that result in serious neurological dysfunction and paralysis. Following the initial lesion, events associated with a secondary injury can last several days and weeks after injury. Disorders associated with the secondary injury include dramatic metabolic alterations [1- 9], a general increase in oxidative stress and inflammation [10-20], demyelination [20-25], and apoptosis [26-29]. During the first week after injury there is an extensive loss of neurons and oligodendrocyte [23,28-33], lipid peroxidation [10,34], and axon degeneration [14]. The production of free radicals during this period is believed to contribute to these detrimental outcomes by disrupting cell membranes, causing organelle dysfunction, and disturbing calcium homeostasis [34]. The free radical production peaks at 12 h after the initial injury and it remains elevated for at least 1 week after injury. Examples of elevated free radicals after injury are hydrogen peroxide, hydroxyl radical, and peroxynitrite radical [14,35]. Previous studies have shown a reduced antioxidant defense after SCI [36] and antioxidant agents such as tempol have shown to be neuroprotective in the context of SCI [37].

Previous reports from our laboratory showed that dietary prophylaxis with omega-3 lipids is protective during SCI. For instance, dietary omega-3 polyunsaturated fatty acids (O3PUFAs) modulated multiple pathways that contribute to secondary acute and chronic damages following SCI [38-39]. Administration of O3PUFAs restored the spinal cord lipid homeostasis, confers neuroprotection, prevents sensorimotor dysfunction and neuropathic pain, and facilitates locomotor recovery following acute and chronic SCI when administered before and during SCI [38,39,40]. Another nutrient with demonstrated

antioxidant capability that could be important in stimulating recovery following SCI injury is vitamin E. Previous studies using dietary vitamin E supplementation for 5 days prior to the SCI in cats found a reduction of arachidonate and prostanoids [41] associated with less ischemia [42] and enhanced locomotion recovery [43]. Further, dietary vitamin E for 8 weeks before SCI decreased reactive oxygen species (ROS) while improved locomotion, blood flow and spinal cord evoked potentials, and decreased ROS in rats [44]. While these studies did not use the standardized scale to measure locomotion after SCI, the Basso, Beattie, and Bresnahan (BBB) scale, their findings provided support for a promising neuroprotective role of vitamin E. Further, dietary vitamin E supplementation for 14 days after injury was shown to improve BBB scores [45-46] potentially involving the inhibition of lipid peroxidation products such as thiobarbituric acid reactive substances and malondialdehyde [44,47-48].

Thus, prior studies suggest a neuroprotective role of vitamin E during SCI, but little is known about potential targets, primary cellular processes involved and whether it can affect other primary functions affected by SCI. For instance, it is unknown whether dietary prophylaxis with vitamin E can specifically address specific impairments associated with SCI such as bladder dysfunction and spasticity. These processes can be especially sensitive to oxidative stress, serotonin levels or adequate number of oligodendrocyte. The present study evaluates the effects of two months dietary exposure to vitamin E supplementation on selected physiological outcomes and examined potential mechanisms. The underlying hypothesis of the current study is that a balanced antioxidant dietary regimen containing adequate levels of vitamin E may enhance the ability of the spinal cord to response to traumatic injury. The data suggest that dietary

vitamin E supplementation significantly improved BBB locomotor scores following SCI. Further, we also report for the first time that prophylactic vitamin E administration stimulates bladder recovery, inhibited H-reflex depression, supraspinal serotonin levels and preserved oligodendrocytes survival.

Materials and Methods

Experimental procedures were compliant with the Loma Linda University School of Medicine regulations and institutional guidelines consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Rats.

Animals

Experimental procedures were compliant with the Loma Linda University School of Medicine regulations and institutional guidelines consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Rats. All animal work reported in this manuscript was performed under the approval the Loma Linda University Health Institutional Animal Care and Use Committee approval number 8170021. A total of 60 female Sprague-Dawley rats weighing 182–212 g were acquired from Charles River Laboratories (Portage, MI, USA). The female rats were housed in individual cages with food and water ad libitum with alternated exposure of light and dark periods of 12 h.

Study Design

The female rats were acclimatized for 1 week after arrival at the animal care facility and were randomly divided into two main groups: (Group 1) rats on the control

175

diet ($n = 30$) and (Group 2) rats on the vitamin E-enriched diet ($n = 30$) (<u>Figure 1</u>). After

8 weeks, the rats were further categorized based on their dietary and surgical

interventions: Group 1a was the control diet/sham operated group ($n = 15$); Group 1b was

the control diet/spinal cord injured group ($n = 15$); Group 2a was the vitamin E-enriched

diet/sham operated group ($n = 15$); and Group 2b was the vitamin E-enriched diet/spinal

cord injured group ($n = 15$). Dietary interventions were continued after surgery for 1

week post-operation (wpo). During the first week after operation functional recovery and

behavioral analysis was performed in a blinded manner. Spinal cord tissue was collected

for analyses at 1 wpo. We opted for not including a group with a vitamin-E enriched diet

after injury only because it has been shown the number of pellets eaten the week

following spinal cord injury, the acute phase, substantially decreases [39,40]. In a setting

where the sub-acute and/or chronic effects of a vitamin-E enriched diet post injury were

being studied, such group would definitely contribute to the study, but not in our acute

phase, study.

Diets

AIN-93G-based custom isocaloric diet formulations were prepared with modified

fat compositions (Bio-Serv, Frenchtown, NJ). The amount of dietary fat was supplied as

either soybean oil (control diet) or vitamin E-enriched diet and it was approximately 6%

of dry weight. They were both stored in a refrigerated area. Gas chromatography and

mass spectrometry analysis showed principal nutrients in the diet as follow: (i) the

amount of Vitamin E in the control diet was .0816 IU/gram and the Vitamin E-enriched

diet had 51 IU/gram; (ii) total saturated fat was 1.13 g/100gram and 1.00 g/100gram, total

monosaturated fat was 1.61 g/100gram and 1.49 g/100gram, total polysaturated fat was

4.09 g/gram and 4.34 g/gram for control diet and Vitamin-E enriched diet, respectively;

(iii) the percentage for kcal carbohydrates was 64.7 and 60.5, the percentage for kcal

protein was 18.8 and 21.1, and the percentage for kcal fat was 16.5 and 18.4 for control

diet and Vitamin-E enriched diet, respectively. The selection of soybean oil as the control

fat in our diets was based on our previous studies [1-3]

Surgical and Post-Operative Procedures

The female rats were on dietary pretreatment for eight weeks. After that they

were anesthetized with a combination of ketamine/xylazine (80 mg/kg and 10 mg/kg,

respectively). The New York University (NYU) Impactor was used to generate the spinal

cord lesions [4]. This device causes the necessary trauma to induce bladder dysfunction,

which suggests this model is appropriate for evaluating the therapeutic potential of our

intervention ([5, 6]). In order to expose the spinal cord to generate the contusion, the skin

and the muscles overlying the spinal column were removed. Laminectomy was

performed at the T9-T10 level and the T8 and T12 spinal processes were clamped to the

Impactor exposing the dorsal surface of the spinal cord. The dorsal surface was then

subjected to weight drop impact with a 10-g rod released from a height of 12.5-mm.

Sham rats were not subjected to weight drop impact and only received a laminectomy.

The female rats body temperature was maintained at 37°C during the whole procedure.

After laminectomy or weight drop impact, muscle layers were carefully sutured and skin

layers closed. Crede's maneuver was performed three times per day to express the

bladders of the injured rats until voiding reflexes were restored. All rats were inyected

twice per day with Cefazolin (from Bristol Myers Squibb, New York, NY; 25 mg/kg, s.q.) for 5 consecutive days and Buprenex® (also known as buprenorphine; from Reckett and Colman Pharmaceuticals, Inc. Richmond, VA; 0.05 mg/kg, s.c.) for 3 consecutive days. Rats were sacrificed 1 week post-operation and the spinal cord tissue was dissected and collected for immunohistochemical analysis.

Behavioral Evaluation of Spontaneous Locomotion

The Basso-Beattie-Bresnahan (BBB) scale is a 22-point (0–21) measurement scale that evaluate the rat's spontaneous open-field locomotion [55]. First, the rats were acclimatized in an empty-plastic black pool simulating an open field environment for five daily sessions 1 week before SCI. After SCI, rats were videotaped weekly in the open field locomotion test. In a blind manner, two observers independently assessed: (1) locomotive function, (2) joint movement, (3) paw placement and rotation, (4) paw coordination, (5) tail position, and (6) trunk position and stability. The average score from both blind observers for each hind paw was used for analyses. According to this measurement scale, a score of 0 is given to a completely paralyzed rat, scores between 1 and 8 are given to rats with increasing joint movements without weight support (recovery stage 1), scores between 9 and 13 are given to rats with abnormal locomotion that are able to produce weight supported steps (recovery stage 2), scores between 14 and 20 are given to rats that have reached graded coordination patterns, paw position, and trunk stability (recovery stage 3), and a score of 21 is given to normal (and sham) rats with no dysfunction in locomotion.

H-reflex Recording

The rats were placed on a heated (approximately 37 °C) metal platform. The

Hoffmann's reflex (H-reflex) was recorded from the plantar muscles of the hind paw,

with the active needle electrode (30-gauge) inserted between the fourth and fifth

metatarsals, and the reference electrode inserted in the skin of the fifth digit. To elicit the

H-reflex and study its rate sensitivity, the tibial nerve at the ankle was stimulated for 0.1

ms at 0.1, 0.3, 1, 2, 3 and 5 Hz using the TECA Sapphire 4ME EMG unit. The cathode

needle was inserted subcutaneously at the ankle, just above the heel, and the anode needle

was inserted subcutaneously at the plantar surface of the heel. The intensity of the

stimulus was adjusted to elicit the maximal consistent H-wave amplitude. The recorded

signal was passed to a differential amplifier and bandpass filtered at 0.1 Hz and 10 kHz.

The analog signal was then sent to an A/D converter and the digital waveform (recorded

at 30 kHz) and stored. Sixteen consecutive waveforms were collected at each frequency.

The differences in amplitudes of M- and H-waves as determined by the peak-to-peak

values of each waveform were used to calculate the H-reflex depression. The investigator

was blinded to the experimental groups during data recording and analyses.

Autonomic Bladder Control Recovery

Crede's maneuver was used to express the bladders of the injured rats. We used

this technique to express the bladder three times a day until bladder function was

restored. Each morning we counted how many rats in each diet regained bladder control

before 7 dpi and after 7 dpi. Bladder function is restored when the maximum amount of

collected volume is 500 μL or less for at least two consecutive days.

Immunohistochemistry Studies and Microscopy

Spinal cord coronal sections were dried at room temperature for 10–15 min,
washed with Phosphate Buffer Saline (PBS) for 5 min, and post-fixed with 4% PFA for
10 min. The sections were blocked with 20% Bovine Serum Albumin (BSA) for 2 h at
room temperature, and incubated at 4 °C in antibody solutions containing either mouse
anti-Neuronal Nuclei (NeuN) monoclonal antibody (clone A60, 1:100; from Millipore,
Billerica, MA, USA), mouse anti-adenomatous polyposis coli (APC)-7 monoclonal
antibody (clone CC-1, 1:200; from Calbiochem, San Diego, CA, USA), and rabbit
polyclonal anti-5HT antibody (1:500; from Abcam, Cambridge, MA, USA) to examine
the immunoreactivity (IR) and cell numbers of neurons, mature oligodendrocytes, and IR
of serotonin. On the next day, the sections were incubated with Alexa Fluor®488 or 594-
conjugated donkey anti-mouse (1:500; from Invitrogen, Carlsbad, CA, USA). Control
slides were incubated without primary antibodies to further confirm the specificity of the
IR. The slides were examined with a BIOREVO BZ-9000 fluorescent microscope
(Keyence, Itasca, IL, USA)

Statistical Analyses

Data are presented as mean +/– SEM. One-way analysis of variance (ANOVA),
followed by Bonferroni post-hoc comparisons, was used to determine the effect of spinal
cord injury and vitamin E supplementation on open-field locomotion scores, H-reflex
depression, NeuN+ cell counts, APC+ cell counts, and 5HT IR. Unpaired *t*-test was used
to analyze the difference of APC+ cell counts between uninjured and injured vitamin E
groups. Fisher's Exact Test was used to determine the effect of vitamin E in bladder

recovery during the first week post-SCI. Statistical analyses were performed using Prism

6 Software (GraphPad Software Inc., San Diego, CA, USA). Outliers were identified

using the Grubbs' method, also known as ESD (extreme studentized deviate). Only one

rat was excluded from the study after using these exclusion methods. Statistical

differences were considered significant at $p < 0.05$.

Results

Dietary Vitamin E Improves Locomotor Recovery after SCI

One week after behavioral habituation period, female Sprague-Dawley rats were

provided ad libitum access to one of two diets: control diet or vitamin E-enriched diet

(See Table 1 for detailed diet composition). The rats remained on their assigned diets for

a total of 8 weeks before injury and 1-week post-SCI. Functional recovery was assessed

during the first week after injury. At the end of the study, the spinal cords were harvested

and tissues were used for immunohistochemical studies (Figure 1 summarizes the study

timeline). The BBB locomotor grading scale was used to evaluate the effects of vitamin E

pre-administration on the functional recovery of injured rats. A video recording of each

subject's performance in the open field was obtained at 1 and 7 days post-injury (dpi).

We found significant improvements in locomotor behavior in rats from dietary vitamin E

prophylaxis group ($n = 12$) compared to rats fed with control diet at 7 dpi ($n = 14$)

(Figure 2A) (F (3, 4190) = 367.2, CTL INJ, $n = 14$ versus VIT E INJ, $n = 12$ *** $p <$

0.001). Still images from representative rats fed control diets shows limited movement,

dragging of hindlegs, and slight movement of hindlimb joints (Figure 2B) at 7 dpi.

Interestingly, rats fed with the vitamin E enriched diet exhibited signs of intermediate

locomotor recovery, as indicated by their ability to generate extensive movements of all

three joints with dorsal-stepping patterns (Figure 2).

Table 1. Detailed diet composition for Control and Vitamin E – Enriched Diet

Ingredient	AIN-93G Control diet (%)	AIN-93G Vitamin E
Soybean Oil	7	7
Vitamin E, IU kg	.0816	51
Total Saturated Fat	1.13 g / 100 g	1.00 g / 100g
Total Monosaturated Fat	1.61 g / 100 g	1.49 g / 100 g
Total Polysaturated Fat	4.09 g / 100 g	4.34 g / 100 g
Percentage kcal Carbohydrates	64.7	60.5
Percentage kcal Protein	18.8	21.1
Percentage kcal Fat	16.5	18.4
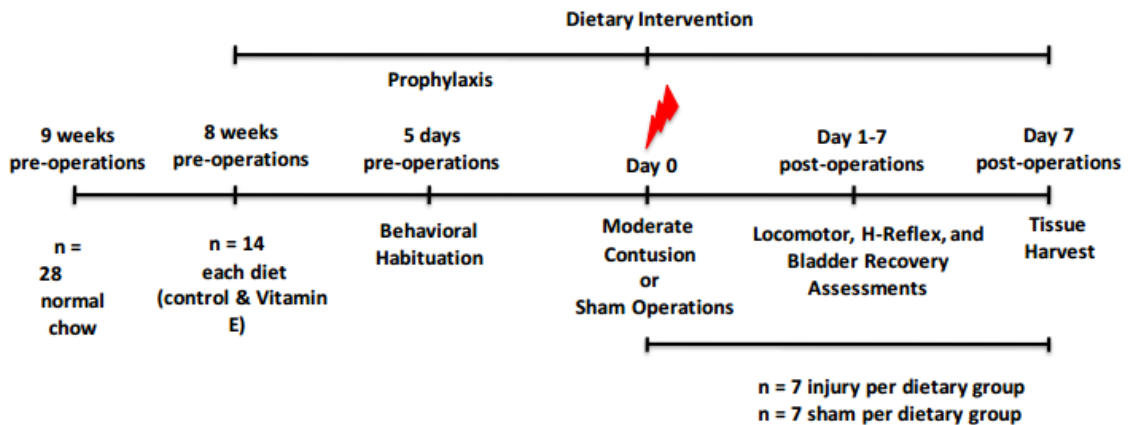
Figure 1. Timeline showing the vitamin E diet supplementation schedule and the time points of behavioral assays, surgical procedures, and tissue sample collection.
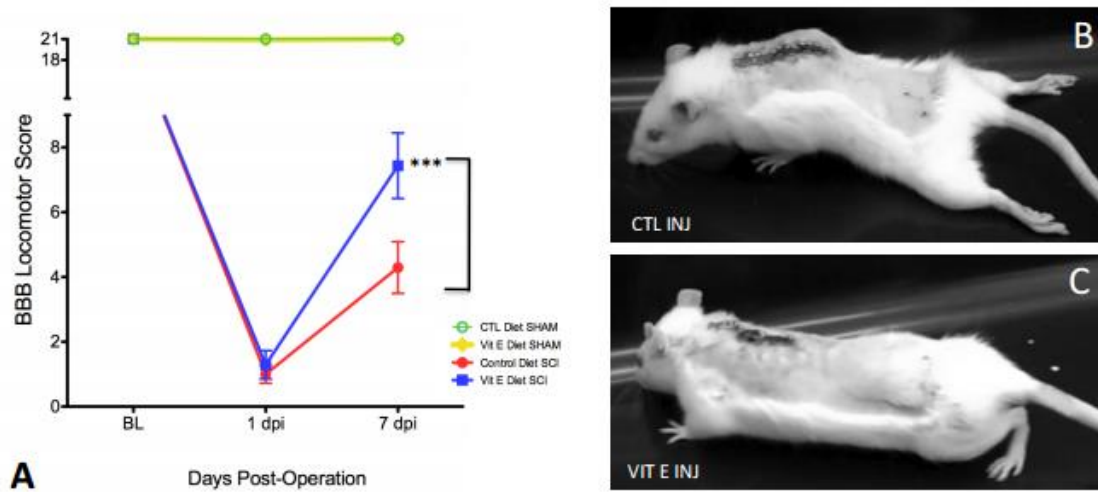
Figure 2. Beneficial effects of dietary vitamin E prophylaxis on the hindlimb neurological function of rats after a moderate injury, as assessed by the Basso-Beattie-Bresnahan (BBB) locomotor rating scale. (**A**) Effect of dietary vitamin E prophylaxis on locomotor function. Time (in days) following pretreatment and spinal cord compression (injury) is shown on the horizontal axis, BBB locomotor rating scores are shown on the vertical axis. Injured rats in the dietary vitamin E prophylaxis group had higher BBB scores of at least 7 when compared to controls. Open-field locomotion still images from control diets- (**B**) and dietary vitamin E prophylaxis rats (**C**) at 7 days post-injury (dpi). Mean dietary vitamin E prophylaxis scores revealed that most rats exhibited extensive movements of three joints with dorsal-stepping patterns at 7dpi. Bonferroni test analysis was carried out to determine the statistically significant differences between diet treatments. Error bars represent means±standard error of the mean (CTL INJ versus VIT E INJ ***$p<0.001$; CTL INJ, n=14; VIT E INJ , n=12).

Dietary Vitamin E Prophylaxis Restores H-reflex Depression at 7dpi at 5Hz

Hyperreflexia and spasticity are common complications in SCI with limited availability for safe and effective treatment. A central mechanism in spasticity is hyperexcitability of the spinal stretch reflex. This reflex presents symptomatically as a velocity-dependent increase in tonic stretch reflexes and exaggerated tendon jerks, resulting in functional deficits, pain, and musculoskeletal deformities. Given that the Hoffmann's reflex (H-reflex) can be evoked in rats, we chose to study the efficacy of our dietary intervention to restore this reflex. As expected, we found reduced H-reflex depression as the stimulation frequency was increased in SCI rats. Notably, the rats that consumed the vitamin E-supplemented diet showed improved H-reflex depression, suggesting less SCI-induced hyperreflexia. Sham-operated rats exhibited normal H-reflex depression in both dietary groups (control diet and vitamin E prophylaxis) (Figure 3A).

Vertical bars in Figure 3B represent the size of the difference between the M-wave and the H-wave at 5 Hz compared to low frequency stimulation (0.1 Hz). A lower vertical bar on the *y*-axis indicates a bigger difference between the M-wave and H-wave (the latter being smaller), thus showing more H-reflex depression. A higher vertical bar on the *y*-axis value indicates a smaller difference between the M-wave and the H-wave at 5 Hz compared to 0.1 Hz thus less H-reflex depression.

In uninjured control rats and uninjured rats that received and vitamin E diet, the H-wave steadily decreased with increasing stimulus frequency with the maximum frequency being 5 Hz. However, in injured rats that received a control diet before SCI, the H-wave depression was inhibited showing how the H-reflex was less sensitive to increased stimulus frequency at 5 Hz. As shown in Figure 3, injured rats that received a

vitamin E diet before SCI showed a decrease in H-wave depression similarly to uninjured

control rats and uninjured vitamin E rats. This finding is indicated by a lower H-wave

amplitude at 5 Hz compared to injured rats from a control diet (Figure 3) $(F (3, 20,287) =$

8.231, (** $p < 0.01$ CTL SHAM vs. CTL INJ and ** $p < 0.01$ CTL INJ vs. VIT E INJ

and * $p < 0.05$ VIT E SHAM vs. CTL INJ; CTL SHAM $n = 6$, CTL INJ $n = 6$, VIT E

SHAM $n = 6$, VIT E INJ $n = 7$). In summary, H-reflex depression became abnormal after

SCI only in injured rats that received a control diet before SCI but not in injured rats that

received a vitamin E diet before SCI.
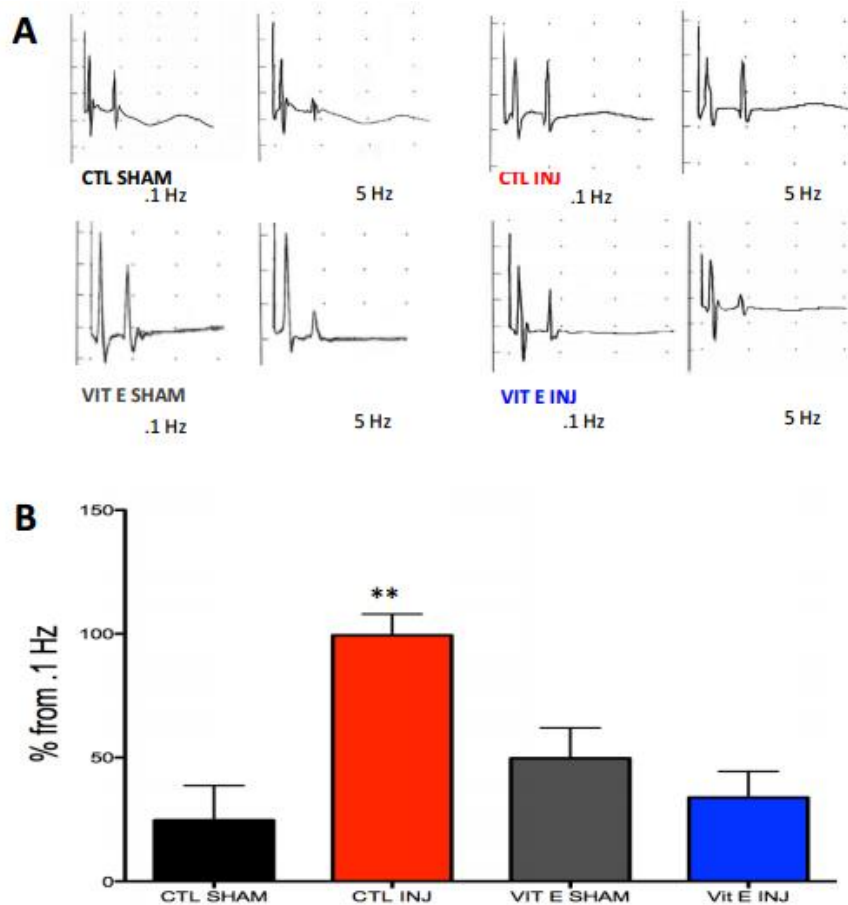
Figure 3. Dietary vitamin E prophylaxis restores H-reflex depression at 7dpi at 5Hz. Increased amplitudes on the y axis at 5Hz indicate less H-reflex rate depression, whereas decreased amplitudes indicate more rate depression. The H-reflex depression after increased frequency (i.e. 5 Hz) was abnormal in rats on a control diet after SCI but it was restored in rats on dietary vitamin E prophylaxis after SCI at 7dpi (**A**). Higher percent changes from .1Hz at 5 Hz indicate abnormal H-reflex rate depression after SCI in rats on a control diet but not in rats on dietary vitamin E prophylaxis at 7dpi where there was a lower percent change from .1Hz (**B**). (**P < 0.01 CTL SHAM vs CTL INJ and **P < 0.01 CTL INJ vs Vit E INJ and *P < 0.05 VIT E SHAM vs CTL INJ; CTL SHAM n=6, CTL INJ n=6, VIT E SHAM n=6, VIT E INJ n=7)

Beneficial Effects of Dietary Vitamin E Prophylaxis on Autonomic Function After SCI

SCI results in a period of distinctive bladder dysfunction[7-14]. Manual collection and quantification of the residual urine volume was done to assess whether dietary vitamin E prophylaxis show efficacy in accelerating autonomic bladder recovery (Fig. 4). For each rat, the number of days needed to attain full autonomic recovery was defined as residual volume of 0.5 mL for 2 or more consecutive days. *A* key finding is that Fisher's Test analysis of contingency tables found a significant decrease on the number of days (<7) required for bladder control recovery in the rats on the vitamin E prophylactic group. (*P < 0.05 CTL INJ vs VIT E INJ; CTL INJ n=19, VIT E INJ n=13).

	Bladder Recovery	
	< 7 dpi	> 7dpi
Control Injury	34%, n=10	66%, n=19
Vitamin E Injury	69%, n=9	31%, n=4

Figure 4. Beneficial effects of dietary vitamin E prophylaxis on autonomic function after contusion injury. **(A)** Residual urine volumes (mL) differed significantly between control and dietary vitamin E prophylaxis pre-treated groups at 7dpi. For each rat, the number of days needed to attain full autonomic recovery was defined as residual volume of 0.5 mL for 2 or more consecutive days. Dietary vitamin E prophylaxis resulted in fewer days (<7) to attain full bladder recovery (*P < 0.05 CTL INJ vs VIT E INJ; CTL INJ n=19, VIT E INJ n=13).

Dietary Vitamin E Does Not Preserve Neurons at 1 Week After SCI

To determine the number of motor neurons in the ventral gray matter of the spinal cord, immunohistochemical analyses were performed during the first week following SCI. This represents a critical period in apoptotic cell death in SCI models [15-19]. Consistent with previous findings, we found a significant decrease in the number of neuronal nuclei positive (NeuN+) cells in the ventral gray matter of injured rats when compared to uninjured sham rats at 1-week post-SCI. Representative images from sections labeled anti-NeuN (Fig 5A) did not show significant differences in motor neuron cell counts when comparing vitamin E-fed rats with controls at 7 dpi ($p > 0.05$) [F (3, 13, 256) = 13.19, **P < 0.01, CTL SHAM, n=6 vs CTL INJ, n=6; ***P < 0.001 CTL SHAM, n=6 vs VIT E INJ, n=7; P > 0.05, CTL INJ, n=6 vs VIT E INJ, n=7)]
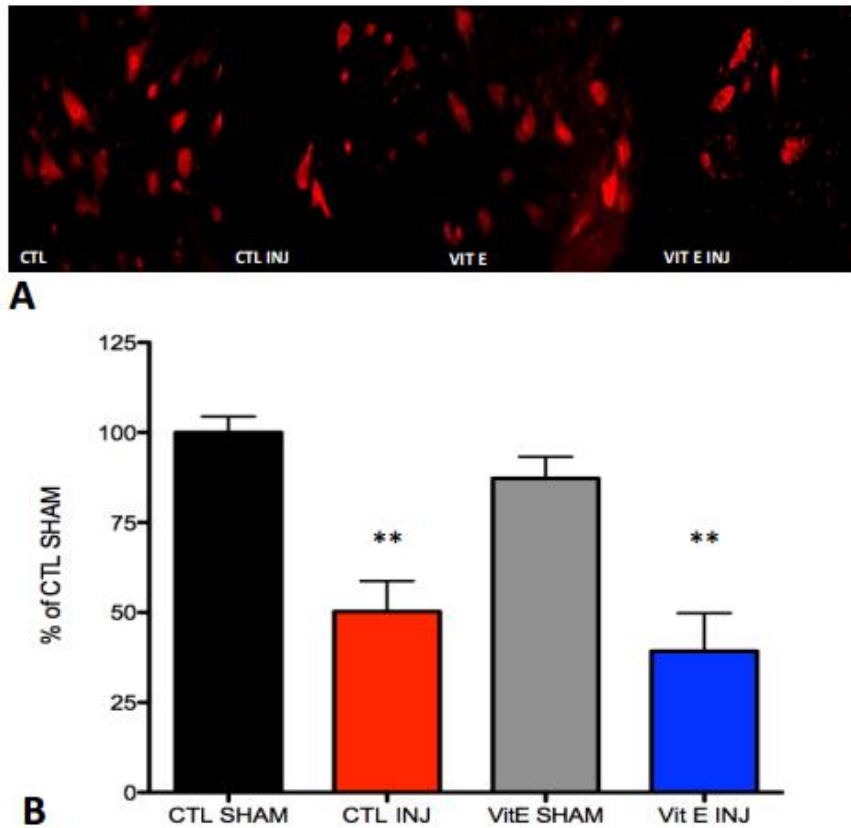
Figure 5. Vitamin E prophylaxis doesn't preserve neurons at 1 week after spinal cord injury (SCI). (**A**) Expression of neuronal markers was quantified in the ventral gray matter (VGM). (**B**) Manual quantification of cell numbers and normalization to controls shams revealed decreased numbers of NeuN^{+} cells in the VGM in injured control rats and injured vitamin E fed rats. The number of NeuN+ cells in the ventral gray matter of the spinal cord from injured control rats was not significantly different from injured vitamin E fed rats. Bonferroni test analysis was carried out to determine the statistically significant differences between diet treatments. Error bars represent means±standard error of the mean (**P < 0.01, CTL SHAM, n=6 vs CTL INJ, n=6; ***P < 0.001 CTL SHAM, n=6 vs VIT E INJ, n=7; P > 0.05, CTL INJ, n=6 vs VIT E INJ, n=7).

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Dietary Vitamin E Preserves Oligodendrocytes Following SCI

We performed immunohistological analyses to determine the quantity of

oligodendrocytes in the white matter at 7 dpi. Oligodendrocytes were immunodetected

using the anti-adenomatosis polyposis coli (APC) antibody. Our results confirmed

previous studies showing a significant decrease in the number of oligodendrocytes at one-

week post-SCI. Representative images from injured sections labeled with the APC

antibody show increased number of APC positive cells in the spinal cord of rats fed the

vitamin E diet when compared to controls. Additionally, there was an increased number

of APC positive cells in the spinal cord of injured rats that were fed the Vitamin E diet

compared to control injured rats. There was no significant difference between APC

positive cells between uninjured rats in the control diet and Vitamin E diet. ($p < 0.05$)

(Fig. 6) [F (3, 26.619) = 18.05, ***P < 0.001, CTL SHAM, n=6 vs CTL INJ, n=6);

**P<0.01, VIT E INJ, n=7 vs CTL INJ, n=6); P>0.05, CTL SHAM, n=6 vs VIT E

SHAM, n=6]. There was a significant difference in APC positive cells between

uninjured rats in the Vitamin E diet and injured Vitamin E diet when they were analyzed

using an unpaired t-test (P=0.0059).
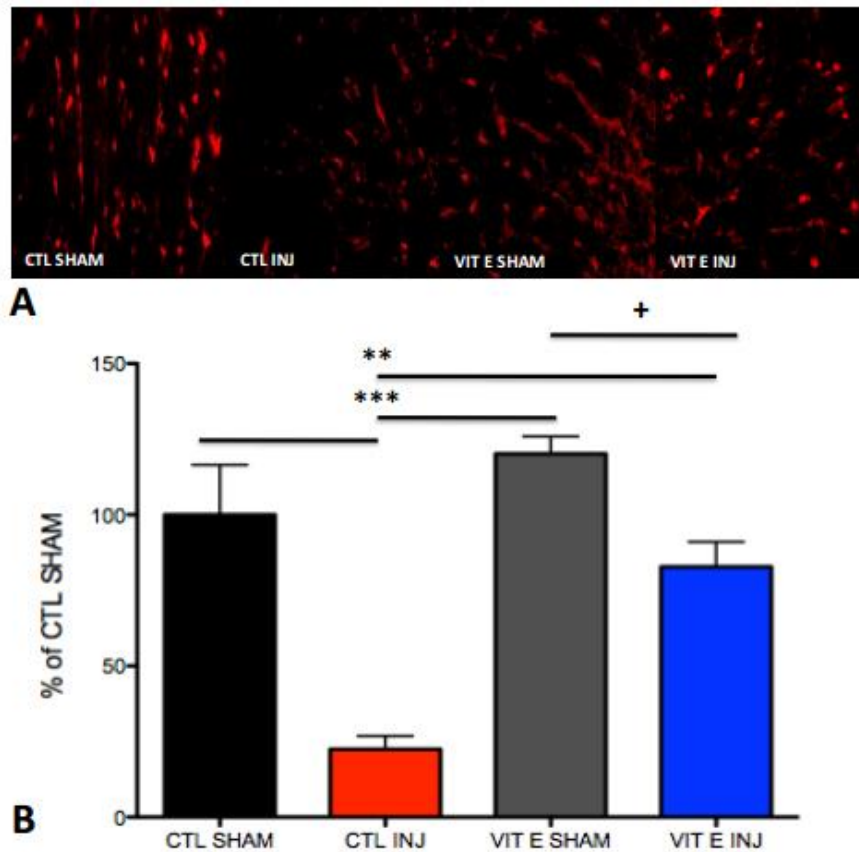
Figure 6. Vitamin E prophylaxis preserves oligodendrocytes at 1 week after spinal cord injury. (**A**)Expression of oligodendrocyte markers was quantified in the white matter of the spinal cord. (**B**) Manual quantification of cell numbers and normalization to control shams revealed decreased numbers of APC+ in the white matter of injured control rats, but not in injured vitamin E fed rats. There was a statistically significant increase of APC+ cells in vitamin E fed rats compared to injured control rats. Bonferroni test analysis was carried out to determine the statistically significant differences between diets. There was a significant difference in APC positive cells between uninjured rats in the Vitamin E diet and injured Vitamin E diet when they were analyzed using an unpaired t-test *(P=0.0059). Error bars represent means±standard error of the mean (****P < 0.0001, CTL SHAM, n=6 vs CTL INJ, n=6; P > 0.05 CTL SHAM, n=6 vs VIT E INJ, n=7; ***P<0.001, VIT E INJ, n=7 vs CTL INJ, n=6).

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Dietary Vitamin E Upregulates Serotonin Immunoreactivity Following SCI

Previous studies have shown decreased serotonin levels after moderate-contusive SCI[20]. Serotonin inhibits afferent transmission and spinal reflexes and plays crucial roles in motor control and recovery[21-27]. Serotonergic signaling is a key mechanism underlying neuronal hyperexcitability after SCI, which has been demonstrated to underlie the pathogenesis of spasticity after SCI [28-32]. Here, we investigated the effects of this dietary intervention on serotonin levels in the spinal cord. We found a significant increase in the levels of serotonin in rats fed the vitamin E- prophylactic diet compared to the control diet in uninjured (*$P < 0.05$, CTL SHAM, n=5 vs VIT E SHAM, n=5) and injured rats (*$P < 0.05$, CTL INJ, n=5 vs VIT E INJ, n=5) (Fig. 7b). Interestingly, we found no significant difference between sham and injured rats ($P > 0.05$, p = at least 5 rats).
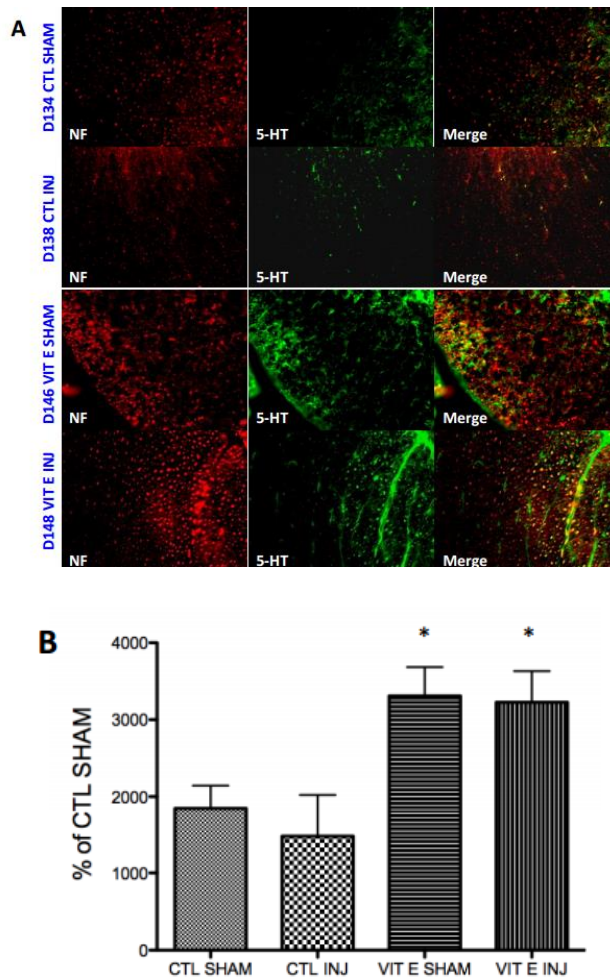
Figure 7. Vitamin E prophylaxis upregulate subspinal serotonin immunoreactivity in the white matter at 7dpi. Subspinal serotonin IR was quantified in the white matter at 7dpi in all four groups (Fig. 7a). There was a diet-dependent upregulation of 5-HT-IR in uninjured rats (*P < 0.05 , CTL SHAM, n=5 vs VIT E SHAM, n=5) and injured rats (*P < 0.05 , CTL INJ, n=5 vs VIT E INJ, n=5) (Fig. 7b). There was no difference between uninjured and injured rats in the control diet (P > 0.05, CTL SHAM, n=5 vs CTL INJ, n=5) and between uninjured and injured rats in the vitamin E diet (P > 0.05, VIT E SHAM, n=5 vs VIT E INJ, n=5).

Discussion

The present study reports novel findings showing that a two-month chronic dietary supplementation with vitamin E (alpha-tocopherol) improves recovery during the acute phase of SCI and identify potential targets. The significant prophylactic effects of vitamin E supplementation included improved functional locomotor outcomes, accelerated bladder recovery measured in urinary retention time, and reduced hyperreflexia after SCI. Further, the dietary intervention increased numbers of oligodendrocytes and increased supraspinal serotonin IR, indicating potential targets underlying the restorative potential of vitamin E in SCI.

Spinal cord injury has a devastating effect on affected individuals. Patients experience serious secondary complications such as urinary retention, a sign of autonomic bladder dysfunction, that has serious practical physiological and psychological consequences [77,78]. Thus, the search of alternative complementary interventions with the potential to lessen the effect of this condition is needed. Although there have been several studies considering the effects of vitamin E during spinal cord injury, little progress has been made in developing appropriate evidence-based complementary therapies. Questions needed to be address for further assessment include appropriate dosage, timing of administration and proper use of dietary prophylaxis. Also, while several studies have shown the effects of vitamin E on improvement locomotion and other parameters, these effects on locomotion has not been quantified using the BBB scores and it is unknown whether this complementary treatment is beneficial in addressing urinary retention time, or the H-reflex. To properly evaluate a potential vitamin E effect on these parameters it is important to ensure that the animal is exposed

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to the proper levels of vitamin E. The current study uses dietary prophylaxis with vitamin E by exposing the animals to a chronic dietary intervention for two months before the animal were subject to the traumatic contusion injury on the cord. A chronic dietary intervention was deemed to be a better approach to avoid potential hurdles associated with vitamin E stability. This approach allowed us to assess with confidence key functional outcomes and determine whether vitamin E had a significant impact. We also proceeded to assess selective functional parameters and quantifying vitamin E effects on locomotion using for the first time the BBB scores.

The well documented antioxidant actions of vitamin E would be beneficial in physiological process that, while affected by the injury, still have functional connections. In this context, we evaluate whether bladder dysfunction was a good target for this treatment. Current interventions used to reduce bladder dysfunction after SCI include cholinergic muscarinic receptor antagonists [79,80,81,82,83,84,85], chemical blockade of C-fiber afferent neurotransmission with capsaicin or resiniferatoxin [86,87,88,89,90,91,92] and alpha1- acetylcholine receptor (AR) receptor antagonists [93,94,95,96,97,98,99]. Additional interventions include suppression of motorneuron or interneuron excitation in the spinal cord by glycine, GABA agonists, and baclofan is being used to treat dysfunctional contraction of the external urethral sphincter [100,101,102,103,104,105,106]. Botulin toxin, a presynaptic neuromuscular blocker, is now FDA-approved to treat bladder hyperreflexia by inducing reversible muscle weakness [107,108,109,110,111,112].

Various studies support the use of antioxidant therapy to address bladder function following SCI. For instance, treatment with quercetin was shown to improve bladder

contractility, while decreasing reactive oxygen species, plasma cytokines, and caspase 3, and prevented depletion of free radical scavengers after SCI in rats [113]. Additional treatment with antioxidant cranberry extract supplements for at least 6 months showed a decrease in urinary tract infections [114]. These approaches to address bladder recovery following SCI suggest that complementary therapies that can improve nerve conduction may be useful as part of comprehensive treatment. In this context, vitamin E could specifically decrease urinary retention through the improvement of nerve conduction. For instance, spinal cord evoked potentials after injury showed greater recovery of both amplitude and latency in a vitamin E supplemented group compared to control [44,48]). Also, in the present study we found that vitamin E supplementation significantly inhibits the H-reflex depression.

These findings are consistent with a role of vitamin E in enhancing nerve conductivity in the injured cord which is may also explain the significant increase in the BBB locomotion scores seen in these animals fed with the enriched vitamin E diet reported here. These data expand previous work showing vitamin E improving locomotion when administered in a prophylactic manner [43,44,48] or after injury [45,46]. When compared to vitamin C supplementation after injury, vitamin E was shown to be more effective for locomotion recovery [46]. Proven actions of vitamin E in reducing lipid peroxidation products such as thiobarbituric acid reactive substances [44,48] and malondialdehyde [47] may be responsible at least in part for these effects [36]. Further, vitamin E improves recovery by decreasing ischemia [42] and downregulating arachidonate and prostanoids [41].

The improved outcomes exhibited by the rats that consumed the vitamin E-enriched diet were associated with an increased preservation of oligodendrocytes of the injured cord. Interestingly, this increase of oligodendrocytes survival was not accompany with an increased survival of neurons. This finding is consistent with reports showing the high vulnerability of oligodendrocytes to oxidative stress. For instance, oligodendrocytes and pre-oligodendrocytes have been shown to be highly sensitive to abnormal high levels of intracellular free radicals that follow cysteine deprivation and treatment with vitamin E and other ROS scavengers (ascorbate, idebenone, and *N*-tert-butyl-alpha-phenylnitrone) promote their survival [115,116]. Vitamin E also protects murine oligodendrocytes in culture from ROS generation and apoptosis caused by cytotoxic oxysterols [117,118,119], and from lipid peroxidation in combination with ascorbate acid [120]. Although this was not directly addressed in the present study, a significant increase in oligodendrocytes in the rats that consumed the vitamin E diet may be implicated in the improvement seen on H-reflex depression, bladder reflex recovery, and locomotion in these rats because of myelin and axonal preservation [25,121,122,123,124,125,126].

Therefore, vitamin E may be able to exert its beneficial effects in the event of spinal cord injury even in the absence of neuronal preservation due to increased survival of oligodendrocytes. Future clinical study may be necessary to assess the value of a similar strategy to treat bladder dysfunction and improve nerve conductivity following SCI.

Our results showing a significant upregulation in serotonin levels in rats fed a vitamin E prophylactic diet further strengthens vitamin E as a potential therapeutic agent for SCI complications. This is supported by several studies showing an improved voiding

efficiency after SCI following increased catecholaminergic and serotonergic axonal growth [127,128,129,130]. This is consistent with findings showing a direct correlation between low serotonin and abnormal H-reflex depression [64]. Serotonin depletion has also been found to positively correlate with the degree of paralysis and disease severity in rat model for multiple sclerosis [131,132]. In models of SCI regeneration of spinal serotonergic neurons is associated with functional recovery [133,134].

In summary, we propose increased survival of oligodendrocytes and upregulation of serotonin levels as potential mechanisms through which dietary vitamin E prophylaxis improves locomotion, H- reflex depression, and bladder recovery in the context of SCI.

The findings reported herein are consistent with previous studies from our lab showing that administration of antioxidants such as omega-3 fatty acids stimulate recovery following contusion injury to the spinal cord [38,39,40]. To date, several studies have shown the beneficial effects of antioxidants to restore function after trauma. However, studies investigating the effects of dietary vitamin E in neural repair are limited. Our findings suggest the potential of this nutritional-based intervention to ameliorate functional impairments and cell survival following SCI. Furthermore, our study supports the importance of nutrition to ameliorate the augmented state of cellular oxidative stress observed during SCI.

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CHAPTER SIX

SPATIOTEMPORAL EXPRESSION OF FAT/CD36 AFTER CENTRAL NERVOUS SYSTEM TRAUMA AND ITS POTENTIAL IMPLICATIONS FOR ALTERED LIPID TRANSPORT IN SPINAL CORD INJURY

Abstract

Traumatic injury to the central nervous system (CNS) is a devastating life event for which no current effective treatments are available. A growing body of evidence shows that complementary approaches including administration of Vitamin E and omega-3 polyunsaturated fatty acids (ω3PUFAs) are capable of attenuating CNS damage, while promoting functional recovery after spinal cord injury (SCI). However, the precise molecular mechanisms coupling ω3PUFAs and Vitamin E to pro-restorative targets, is not well understood. Due to the hydrophobicity of ω3PUFAs and Vitamin E, membrane transport may be required to facilitate their mobilization and meet the increased metabolic demand at the injury site. The fatty acid translocase/membrane cluster of differentiation 36 (FAT/CD36) is a B class scavenger receptor, which has been implicated in the uptake and signaling of hydrophobic molecules, including ω3PUFAs and Vitamin E. This study investigated the 1) FAT/CD36 mRNA/protein expression 2) cellular profile of FAT/CD36 3) potential modulation of FAT/CD36 by ω3PUFAs and Vitamin E in neurons and oligodendrocytes following thoracic contusion SCI (T10) in adult rats. We found that FAT/CD36 mRNA levels are increased at 7 days post-injury while its protein levels remained constant despite marked neuronal and oligodendrocyte loss. Immunofluorescence microscopy showed low FAT/CD36 immunoreactivity (IR) in pro-inflammatory astrocytes expressing the glial fibrillary acidic protein (GFAP) and in

CD11b-positive microglia. We did not observe FAT/CD36 in NG2+ oligodendrocytes precursor cells. Surprisingly, we observed the highest IR in motor neurons and interneurons of the ventral gray matter and mature oligodendrocytes expressing the adenomatous polyposis coli protein (APC). We found increased neuronal FAT/CD36 expression in the rats that consumed Vitamin E after SCI. Altogether, our data shows a differential expression pattern for FAT/CD36 in SCI, which seems to amenable to dietary Vitamin E. Our results suggests that FAT/CD36 may be an important player in the promotion of cellular uptake, transport, and/or metabolism of fatty acids in neurons following SCI. Given the beneficial roles of ω3PUFAs and Vitamin E in ameliorating functional recovery, FAT/CD36 may be a contributor to basic protection mechanisms in the injured spinal cord. Future pharmacological studies will confirm the role of FAT/CD36 in cell survival after SCI.

Introduction

Spinal cord injury (SCI) is a major cause of disability in the US, devastating the lives of more than 12,000 young adults annually. Although SCI may prove difficult to prevent in most instances, there is a need to further investigate factors affecting susceptibility to functional dysfunction in the event of injury. In ongoing studies, we have shown that the metabolism of fatty acids is markedly deregulated during the first few days after SCI. In agreement with others, our studies show that these alterations may determine the extent of functional recovery after such insult. We demonstrated that omega-3 polyunsaturated fatty acids (O3PUFAs) and vitamin E are major modulators of

repair mechanisms following SCI in rodents. However, the molecular mediators coupling these beneficial fatty acids to functional recovery after SCI remain largely understudied.

The fatty acid translocase membrane cluster of differentiation 36 (FAT/CD36) is a B class scavenger receptor highly expressed in models of neurological and cardiovascular injury and implicated in the uptake and signaling of fatty acids and vitamin E [1-4]. FAT/CD36 have been shown to be both beneficial and detrimental after injury [5-10]. The objectives of this study are to investigate 1) the spatiotemporal FAT/CD36 expression and 2) whether FAT/CD36 can be targeted by ω3PUFAs and Vitamin E following thoracic contusion SCI (T10) in adult rats. We hypothesize that FAT/CD36 is acutely upregulated following SCI and it is modulated by ω3PUFAs and Vitamin E. This hypothesis is based on: 1) FAT/CD36 ability of transporting hydrophobic molecules such as O3PUFAs and Vitamin E and 2) our studies showing that O3PUFAs are neuroprotective after SCI. Our rationale is that, knowledge of how O3PUFAs and vitamin E modulate neuroprotection after SCI will provide the foundation for the development of interventions aimed at restoring function. Here, we characterized the expression and putative targets and roles of FAT/CD36 in experimental SCI. We anticipate that this study will provide new evidence for the molecular mechanisms involved in the beneficial effects of nutritional fatty acids in neurological injury, which is expected to lead to new therapeutic targets for SCI.

Understanding how dietary omega-3 polyunsaturated fatty acids and vitamin E mediate neuroprotection and nervous system repair will require examining specific molecular targets regulated by these lipids. These interactions include, but are not limited to transport, internalization, and metabolism. The fatty acid translocase/cluster

differentiation receptor FAT/CD36 facilitates DHA metabolism and functions as a ROS scavenger receptor [4, 11], making this protein an ideal coupling molecule between diet and function. This study focused on the spatiotemporal expression and roles of FAT/CD36 and its potential role in nutrient-mediated repair following SCI in rats.

The most innovative feature of this study is the identification of lipid transport proteins as potential therapeutic targets for traumatic neurological injury (Figure 1). This idea is in agreement with findings showing that genetic deletion of a major membrane lipid transport protein, FAT/CD36, exacerbates traumatic brain injury (TBI) in neonatal mice [10]. In marked contrast, FAT/CD36 appears to mediate detrimental effects after CNS injury in the adult. This is evidenced by findings showing that FAT/CD36 deletion or blockade ameliorates recovery after TBI, SCI, and cardiac ischemia [6, 8, 9, 12, 13]. Together, this evidence suggests that the roles of this lipid transporter are context dependent. It is also reasonable to assume differences in fatty acid levels between the neonatal and adult brain may play a role in determining the role of this lipid transporter [14-18].

Our Preliminary studies have shown that SCI triggers a robust lipid deregulation, characterized by docosahexaenoic acid (DHA) deficiency. This DHA deficiency was correlated with neurological dysfunction such as poor locomotion, chronic neuropathic pain, and delayed bladder reflex recovery. We found that consumption of a diet rich in O3PUFA corrects this DHA deficiency and ameliorates neurological dysfunction in SCI rats [19]. Studies have shown that Vitamin E also improves recovery after SCI in rodents [20-23]. However, the mechanisms implicated in these beneficial responses remain unknown. Vitamin E decreases DHA peroxidation, suggesting a potential mechanism

controlling to repair and protection[24, 25]. Further, both vitamin E and DHA bind to the

FAT/CD36 receptor protein[1, 2, 4, 11, 26]. The experimental data obtained is expected

to help lay foundation for future interventions that target lipid transport in order to

prevent secondary damage and neurodegeneration.
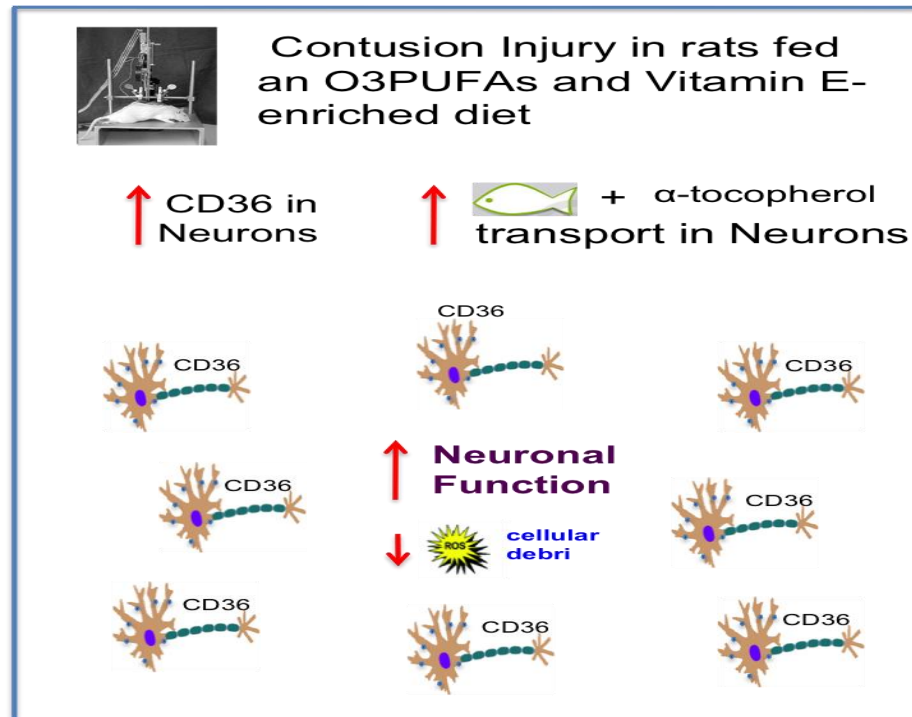
Figure 1. Suggested FAT/CD36 role in SCI in the context of O3PUFAs and Vitamin E transport.

Materials and Methods

Animal Subjects, Diet Intervention, and Spinal Cord Injury

Young adult female-Sprague Dawley rats were allowed to acclimatize for 1 week

after arriving at the animal facility. Rats were placed on four different groups according

to their diets: control diet, O3PUFA diet, vitamin E diet, and O3PUFA/Vit E diet. The

spinal cord was injured at thoracic level 10 (T10) using the well-characterized NYU

Impactor. Spinal cord tissue was collected for analyses at 7 days after injury. For more detailed information about these procedures see Figueroa et al., 2013 and Cordero et al., 2016.

Real Time-PCR

RNA was extracted from epicenter sections of Sprague- Dawley female rat spinal cords subjected to control diet injury and control diet sham treatments. To observe changes in mRNA expression of CD36, the extracted RNA from each of the samples was subsequently used to make cDNA. Using the Bio-Rad C100 Thermal Cycler, the sequence of interest was amplified through Real-Time RT-PCR for forty cycles. The data for each of the samples, normalized to GAPDH as the house-keeping gene, was evaluated using the Bio-Rad CFX Manager.

Immunoblotting

Rabbit anti-FAT/CD36 polyclonal antibody was used to detect the protein levels of FAT/CD36 from spinal cord tissue homogenates following previously published protocols from our laboratory.

Immunofluorescence

Double immunofluorescent labeling experiments were conducted with antibodies for different cellular markers and FAT/CD36 to study the spatial distribution of FAT/CD36 in the spinal cord and the effect of SCI in FAT/CD36 expression. The sections for both injured animals and SHAM animals were washed, blocked and

incubated in rabbit FAT/CD36 and either mouse anti-neuronal nuclei (NeuN), anti-cluster differentiation 11b (CD11b), anti-glial fibrillary acidic protein (GFAP), anti-adenomatous polyposis poli (APC), anti- NG2 Chondroitin Sulfate Proteoglycan (NG2), to examine immunoreactivity (IR) of FAT/CD36 in neurons, microglia, astrocytes, mature oligodendrocytes, and immature oligodendrocytes respectively.

Statistical Analyses

Data are presented as mean +/– SEM. Unpaired t-test was used to analyze the difference of FAT/CD36 mRNA expression through RT-PCR, FAT/CD36 overall protein expression through immunoblotting, and CD36+ ventral horn neurons and CD36+ mature oligodendrocyte counts through immunofluorescence between uninjured and injured groups. One-way analysis of variance (ANOVA), followed by Bonferroni post-hoc comparisons was used to determine the effect of spinal cord injury and O3PUFA/Vitamin E supplementation in CD36+ ventral horn and CD36+ mature oligodendrocyte counts through immunofluoresence. Statistical analyses were performed using Prism 5 Software (GraphPad Software Inc., San Diego, CA). Outliers were identified using the Grubbs' method, also known as ESD (extreme studentized deviate). Only one rat was excluded from the study after using these exclusion methods. Statistical differences were considered significant at $p < 0.05$.

Results

FAT/CD36 mRNA Levels are Upregulated and the Protein Levels Remained

Constant at 7dpi

FAT/CD36 mRNA was upregulated at 7 dpi (Fig. 2A). There was a trend for

increased FAT/CD36 protein levels, but we did not find a significant change in the spinal

FAT/CD36 protein levels at 7dpi (Fig. 2B). This finding is important because this time

point is characterized by a wave of neuronal apoptosis and inflammation. It is reasonable

to propose that maintaining the levels of this protein in a pro-inflammatory and

degenerative condition may be an important mechanism for neuronal survival. There are

several possibilities that may explain this phenomenon. First, it has been shown that

steady-state transcript abundances only partially predict protein abundances, suggesting

that after experimental errors have been eliminated, other modes of regulation must be

invoked to explain how the levels of proteins are set within cells. For example, it has

been implied that ~40% of the variation in protein concentration can be explained by

knowing mRNA abundances. Several mechanisms have been proposed to play a role in

this discrepancy. Regulatory proteins may have to be produced and degraded very rapidly

to react to a stimulus, whereas structural or housekeeping proteins would be much longer-

lived. This is consistent with a very unstable FAT/CD36 protein prone to degradation

since it is a regulatory protein that is strongly induced after injury.

Another mechanism that may explain the partial prediction of protein abundances

by mRNA transcripts is explained by evidence showing that the abundance of an mRNA

is often an excellent proxy for the presence of a protein: that is, for whether or not that

protein is detectable within the cells and not necessarily for protein abundance. This is

also consistent with our FAT/CD36 levels at 7dpi, which were not upregulated like the mRNA transcript, but were not downregulated either. Lastly it has been found that mRNA transcripts exhibiting shorter 3′UTRs with fewer micro-RNA (miRNA)-binding sites showed decreased miRNA-mediated translation repression. Thus, longer 3′UTRs will produce less protein abundance due to increased miRNA-mediated translation repression[27]. Second it is possible that FAT/CD36 is upregulated in the spinal cord after 7 dpi but only in specific areas. This phenomenon was seen and explained by Cruz-Orengo et al., 2007 where they only found upregulation of EphA4 receptor in the white matter and not in the gray matter after finding an upregulation of the EphA4 receptor mRNA transcript [28]. This may be due to massive cell death in the gray matter after SCI, therefore changes in FAT/CD36 protein levels could be missed due to dilution of the protein content in our samples.
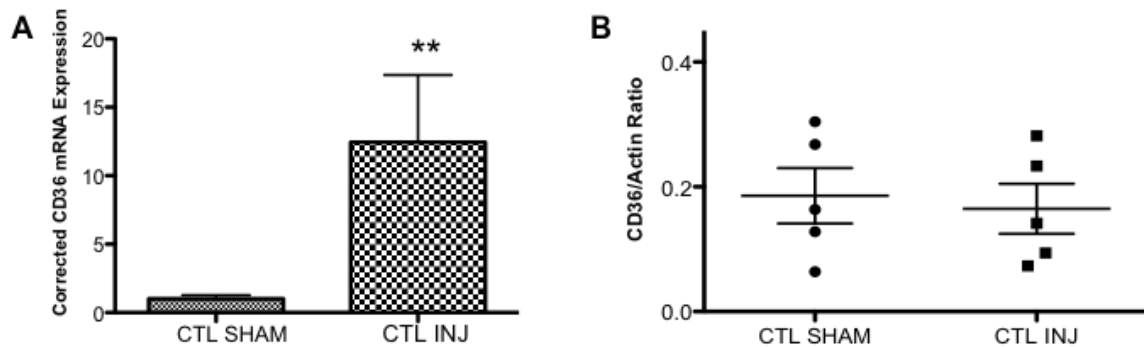

Figure 2. CD36 mRNA Levels are Upregulated and the Protein Levels Remain Constant at 7dpi.

FAT/CD36 Spatial Expression in the Spinal Cord

Despite the fact the vast majority of the literature has evaluated the roles of FAT/CD36 in immune cells, its expression and functions in neurons remains unknown.

Preliminary findings supporting this study show that FAT/CD36 is highly expressed in NeuN-positive cells (neurons) of the spinal cord (Fig 3, panel A-C) and mature oligodendrocytes (Fig 3, panel M-O). We found evidence demonstrating low FAT/CD36 expression in microglial cells (Fig 3, panel D-F), and immature oligodendrocytes (Fig 3, panel P-R), confirming other studies. FAT/CD36 was not found in GFAP-positive cells (astrocytes). (Fig 3, panel G-L).
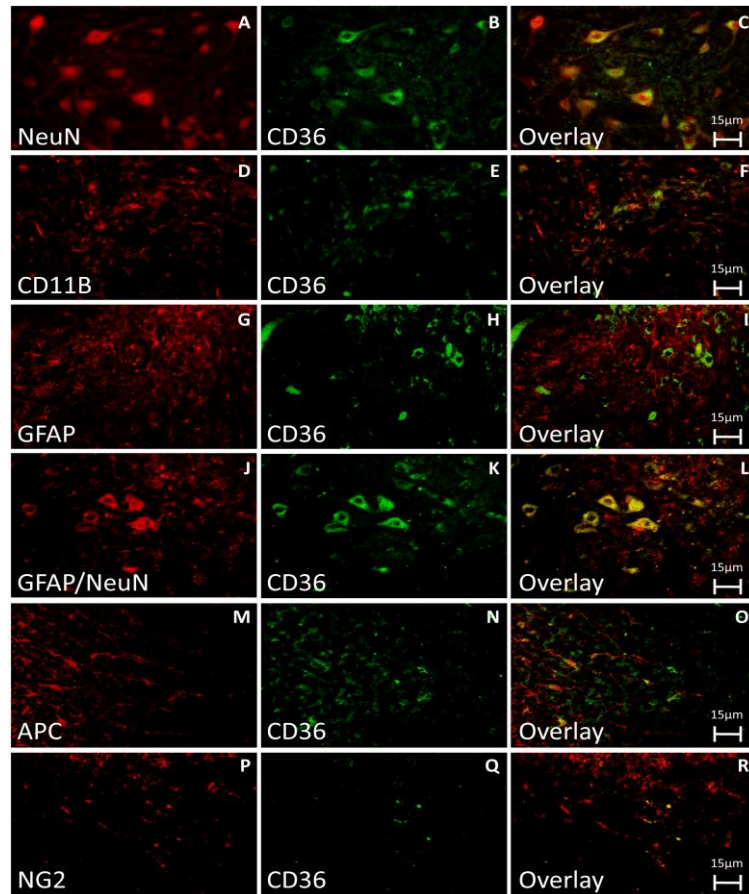

Figure 3. CD36 Spatial Expression in the Spinal Cord

FAT/CD36 Levels are Upregulated at 7dpi in Ventral Horn Neurons

The overall FAT/CD36 protein levels, measured through immunoblotting, were not upregulated at 7 dpi. Despite this, we did find an upregulation of FAT/CD36 protein levels, measured through immunofluorescence, in ventral gray matter neurons. This is an example of the phenomenon explained above where even though the overall protein expression did not change, its upregulation is seen in specific areas only, i.e. in a specific cell population such as ventral gray matter neurons. An upregulation of FAT/CD36 in the surviving neurons may indicate a potential role for this protein in ventral horn neurons after injury.
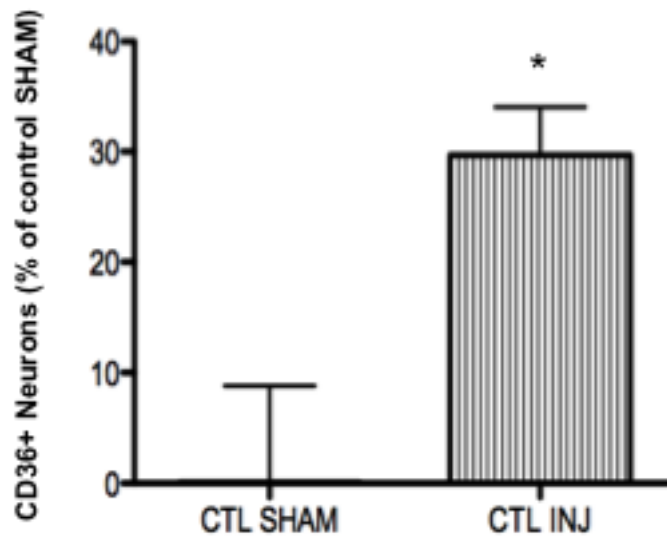

Figure 4. CD36 levels are upregulated at 7dpi in ventral horn neurons.

FAT/CD36 Levels Remained Constant at 7dpi in Oligodendrocytes

Similar to the total FAT/CD36 protein levels in the spinal cord, immunofluorescence revealed FAT/CD36 expression in oligodendrocytes at 7 dpi (Fig.

5). It is of importance to mention we counted the amount of FAT/CD36+

oligodendrocytes and not total amount of CD36 expression levels. This indicates most of

the oligodendrocytes that survived after SCI were expressing FAT/CD36, which

potentially indicates a role for this protein in oligodendrocytes after injury.
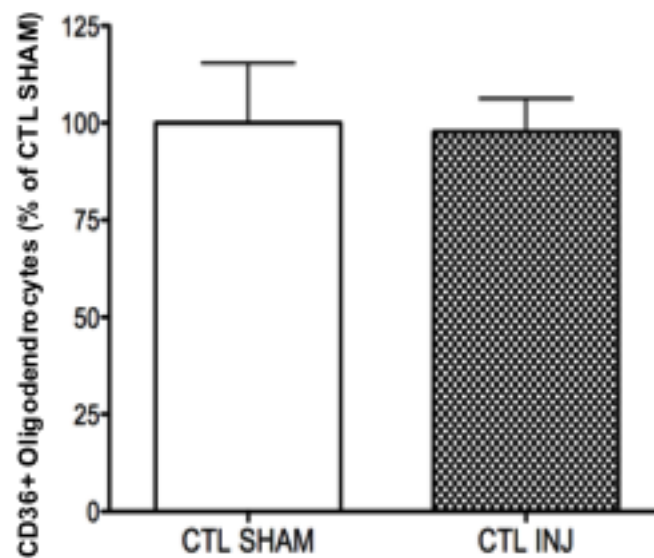
Figure 5. CD36 levels remained constant at 7dpi in oligodendrocytes

NeuN/CD36 Ratio was Upregulated in Injured Rats by a Vitamin E-enriched Diet Only

A Vitamin E-enriched diet increased FAT/CD36 expression in neurons after 7

days post-spinal cord injury. Interestingly, the ω3PUFAs -enriched diet did not increase

the FAT/CD36 expression (Fig 6). These results are suggestive of FAT/CD36 playing a

role in the improved recovery after SCI observed in rats fed a Vitamin E-enriched diet.
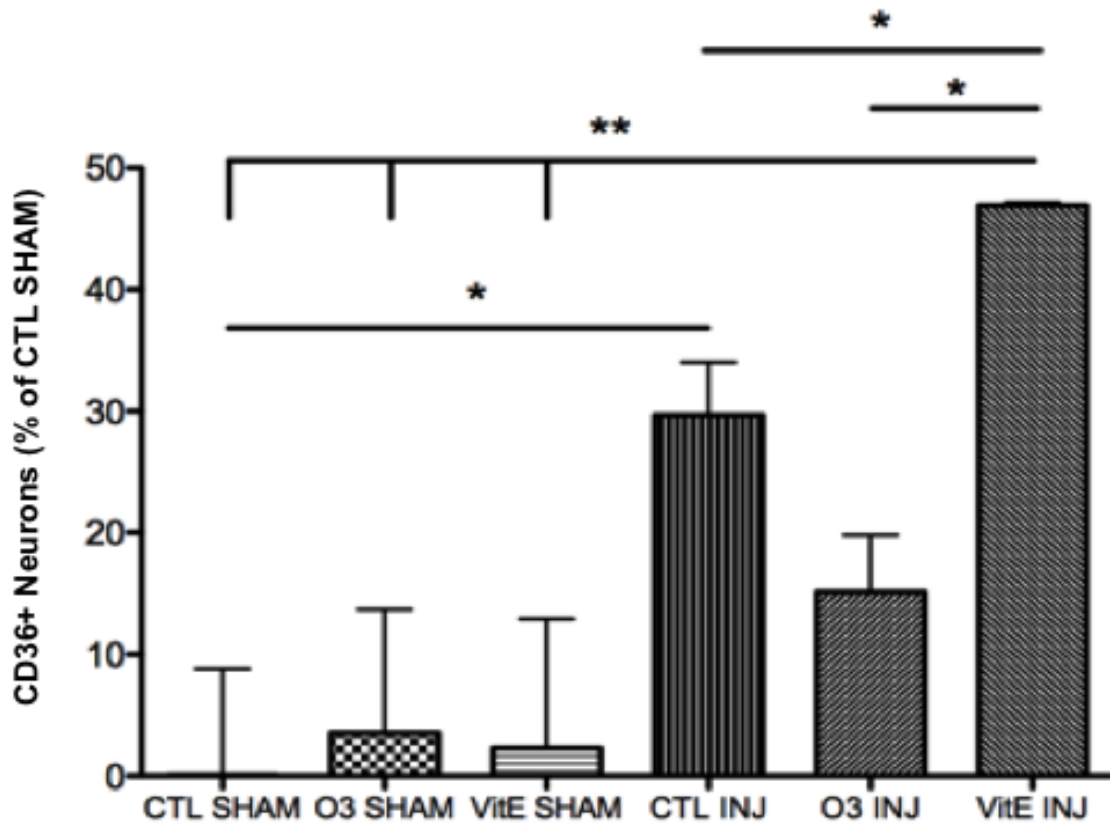
Figure 6. NeuN/CD36 Ratio was Upregulated by a Vitamin E-enriched Diet Only at 7dpi

APC/CD36 Ratio was Upregulated in SHAM Rats by an ω3PUFAs -

enriched Diet Only

An ω3PUFAs-enriched diet increased FAT/CD36 expression in oligodendrocytes in SHAM operated rats but not in SCI at 7dpi. Interestingly, the Vitamin E-enriched diet did not increase the FAT/CD36 expression in either group (Fig 7). These results are suggestive of increased transport of ω3PUFAs by FAT/CD36 in uninjured oligodendrocytes but not in surviving oligodendrocytes after SCI.
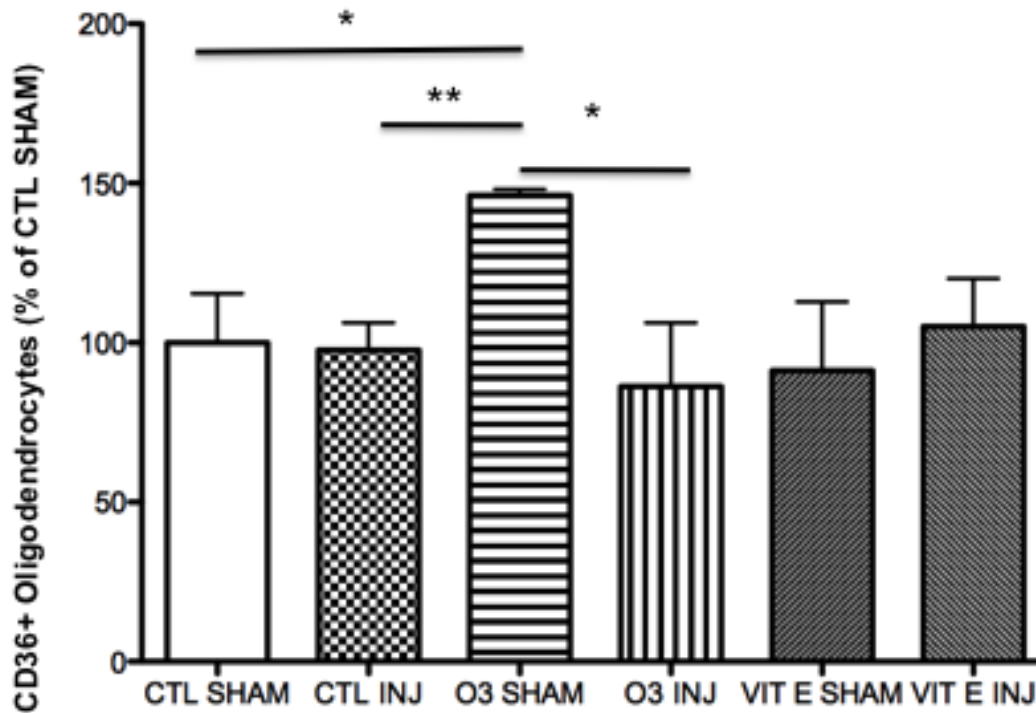
Figure 7. APC/CD36 Ratio was increased by ω3PUFAs Diet Only

Discussion

Our study investigated FAT/CD36 as a potential therapeutic target of Vitamin-E and ω3PUFAs. Our results indicate that Vitamin-E and ω3PUFAs modulate FAT/CD36 in ventral horn neurons and mature oligodendrocytes in the white matter of the spinal cord. We found that FAT/CD36 was mainly expressed in these two cell populations in the naïve spinal cord. While FAT/CD36 protein levels remained constant, we found that mRNA levels were upregulated at 7 dpi. FAT/CD36 was upregulated in ventral horn neurons and remained constant in mature oligodendrocytes at 7 dpi. We found that consumption of a Vitamin-E enriched diet increases FAT/CD36 expression in ventral

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horn neurons in SCI rats at 7dpi. Interestingly, we found increased FAT/CD36 in the SHAM rats that consumed the ω3PUFAs-enriched diet upregulated at 7 dpo.

FAT/CD36 has been shown to have a double-edge role in various pathological processes including Alzheimer's disease [29-35], cerebral ischemia, cerebral hemorrhage [36-38], and cerebral malaria [39-42]. In re-myelination after nerve crush injury it has been shown to have a beneficial effect [43] and in Parkinson's disease and Prion disease the studies show a detrimental effect [44, 45]. These studies suggest FAT/CD36 role in the nervous system is complex and possibly depends on many factors. In agreement, this study suggests that the lipid microenvironment previous to SCI may contribute to the functions of FAT/CD36.

FAT/CD36 has been shown to have a deleterious effect in SCI contributing to inflammation and microvascular dysfunction [46, 47]. In these studies, mice were not fed a prophylactic diet, thus it is possible that the lipid microenvironment before and after injury was very different to lesion microenvironment in our study. Our findings suggest a potential role of FAT/CD36 in the uptake of Vitamin E and ω3PUFAs in ventral horn neurons and oligodendrocytes, which can promote improved functional recovery. This is consistent with the increased overall mRNA expression and constant overall protein expression of FAT/CD36 in the spinal cord at 7dpi. Furthermore at the cellular level there was constant expression of FAT/CD36 in oligodendrocytes and upregulated expression in ventral horn neurons at in the spinal cord at 7 dpi without any diet intervention or injury (naïve spinal cord). Additionally in rats with prophylactic diets, FAT/CD36 was upregulated in ventral horn neurons by Vitamin E diet only after injury and upregulated in oligodendrocytes in ω3PUFAs diet in SHAM rats only. Based on these results we

postulate that the regulation of FAT/CD36 expression in the spinal cord is dependent on whether the lipid microenvironment is enriched with Vitamin E or with ω3PUFAs.

In conclusion, FAT/CD36 is highly expressed in ventral horn neurons and oligodendrocytes in the context of Vitamin E and ω3PUFAs enriched lipid microenvironment suggesting a role in the functional recovery conferred by these prophylactic diets in SCI.

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CHAPTER SEVEN

CONCLUSION

The Effects of DHA Injections in SCI at 7 Dpi in SCI

One of the main accomplishments from our studies was providing evidence for prophylaxis interventions that can strengthen the spinal cord in order to ameliorate functional dysfunction after SCI. DHA injections before injury limited apoptosis of key cells in the nervous system and downregulated molecules involved in apoptotic pathways. In the acute setting, DHA was proven to be neuroprotective (Table 1).

Table 1. Effects of DHA Injections in SCI at 7 dpi

Level	Test	Result
Behavioral	BBB and tcMMEP	Improved locomotor recovery, decreased conduction latencies
Histological	Immunostaining Immunohistochemistry	↑Luxol,↑WMS,↑CV ↑NeuN,↑APC,↑NG2,↓NG2/FRAGEL
Molecular	RT-PCR/IMH	↑AKT,↑CREB

Effects of an O3PUFAs-enriched Diet at 8 and 12 wpi

A prophylactic diet enriched with O3PUFAs vs regular diets showed chronic DHA deficiency correlated with decreased neurological recovery (Table 2). We were first to provide evidence prophylaxis with O3PUFAs-enriched diets alter the neurolipidome in the spinal cord resulting in decreased cellular vulnerability potentially explaining the improvement in neurological recovery after SCI. Specifically we found an O3PUFAs-

enriched diet resulted in an altered neurometabolome that correlated with decreased

nociceptive behaviors (Table 3).

Table 2. Effects of an O3PUFAs-enriched diet at 8 wpi.

Level	Test	Result
Behavioral	Residual Urine Volume, BBB, Hindpaw Withdrawal Treshold	Accelerated bladder recovery, improved locomotion, and decreased sensory dysfucntion
Molecular	ω-6 DPA: DHA ratio	Increased DHA Content

It is important to mention the implications of our prophylactic studies. Our

western diets if low in O3PUFAs will result in chronic DHA deficiency, which means

our current diet has the potential of hindering neurological recovery after neurotrauma.

On the other hand, individuals may have the ability of decrease functional dysfunction in

the event of neurotrauma with a safe and tolerable approach that is easily available to the

general population. In addition to functional recovery our prophylactic diet with

O3PUFAs attenuated chronic pain after SCI and modulated lipid mediators that have

been implicated in pain. It is possible our approach can be part of a multifactorial strategy

to target chronic pain in SCI.

Table 3. Effects of an O3PUFAs-enriched diet at 8 and 12 wpi

Level	Test	Result
Behavioral	Hindpaw Withdrawal Latency	No thermal hyperalgesia developed in animals with a O3PUFA-enriched diet
Molecular	Metabolomic analysis	↑N-acyl ethanolamine precursor correlating with decreased thermal hyperalgesia, altered endocannabinoid metabolone,
Histological	Immunocytochemistry	↑p38, decreased sprouting of CGRP-containing fibers (pain fibers)

Effects of a Vitamin E-enriched Diet at 7 dpi

We provided evidence that a two-month chronic dietary supplementation with vitamin E (alpha-tocopherol) also aids in the functional recovery of SCI during the acute phase and increased survival of motor neurons and oligodendrocytes (Table 4). We found that supraspinal serotonin IR is a potential target for the underlying mechanisms of vitamin E example on the effects of our diet in the prevention of neurotrauma.and its prophylactic effects in this acute phase. Vitamin E prophylaxis is yet another

Table 4. Effects of a Vitamin E-enriched diet at 7 dpi

Level	Test	Result
Behavioral	Residual Urine Volume, BBB, H-reflex	Improved locomotor recovery, accelerated bladder recovery, restored H-reflex depression
Histological	Immunohistochemistry	↑NeuN,↑APC,↑5-HT in Neurofilaments

FAT/CD36 Expression After SCI and its Modulation by an O3PUFAs and

Vitamin E-enriched Diet

Our last step was to study the transport of both O3PUFAs and Vitamin E by
FAT/CD36, a scavenger receptor and lipid transporter. First we found on the acute setting
that it was highly expressed in ventral horn neurons and oligodendrocytes (Table 5).

Table 5. CD36 expression at 7 dpi

	Total mRNA	**Total Protein**	**In NeuN+ cells**	**In APC+ cells**
CD36	↑	↔	↑	↔

Next FAT/CD36 levels were upregulated in oligodendrocytes by O3PUFAs and
in neurons by Vitamin E. These results suggest O3PUFAs and Vitamin E lipid-rich
microenvironments may play a role in the functional recovery conferred by these
prophylactic diets in SCI (Table 6).

Table 6. CD36 modulation by O3PUFAs and Vitamin E-enriched diets at 7 dpi

	NeuN+	**APC+**
O3PUFAs	↔	↑ in SHAM rats
Vitamin E	↑ in INJ rats	↔

In conclusion, while there are many studies that aim to treat the neurological dysfunction in SCI there is still room for improvements in the treatments for SCI. Our studies provided evidence for the value of studying preventive approaches such as O3PUFAs and Vitamin E prophylaxis for the group of people at a higher risk of suffering from SCI.