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Dietary intervention with DHA and curcumin enhance spinal cord sensory motor learning

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Dietary intervention with DHA and curcumin enhance spinal cord sensory motor learning

A thesis submitted in partial satisfaction

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in Physiological Sciences

by

Michael Selvan Joseph

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ABSTRACT OF THE THESIS

Dietary intervention with DHA and curcumin enhance spinal cord sensory motor learning

by

Michael Selvan Joseph

Master of Science in Physiological Sciences

University of California, Los Angeles, 2012

Professor V. Reggie Edgerton, Chair

Given that the spinal cord is capable of learning sensorimotor tasks and that dietary interventions can influence learning involving supraspinal centers, we asked whether the presence of omega-3 fatty acid docosahexaenoic acid (DHA) and the curry spice curcumin (Cur) could affect spinal cord learning in adult spinal mice. Using Paw Withdrawal instrumental learning paradigm (PaWL) to assess spinal learning we observed that mice fed a diet containing DHA/Cur performed better in the spinal learning paradigm than mice fed a diet deficient in DHA/Cur. Previous studies in the cortex have demonstrated that both DHA and curcumin elevate the expression of brain derived neurotrophic factor (BDNF) and plays a major role in learning and neural plasticity. Here we examined the effect of DHA and curcumin on the Paw Withdrawal learning. The enhanced performance was accompanied by increases in mRNA of molecular markers of learning, i.e., BDNF, cyclic AMP response element-binding protein (CREB), Calcium/Calmodulin Kinase II (CaMKII), and syntaxin 3. Sequestering BDNF with TrkB

IgG in the lumbar spinal cord of DHA/Cur fed mice showed significant decrease in spinal learning, and levels of mRNA of BDNF, CaMKII, CREB and syntaxin 3. These results emphasize the capacity of select dietary factors to foster spinal cord learning and suggest the BDNF pathway may be a key mediator of spinal plasticity. Given the non-invasiveness and safety of the modulation of diet, this intervention should be considered in light of their potential to enhance relearning of sensorimotor tasks during rehabilitative training paradigms after spinal cord injury.

The thesis of Michael Selvan Joseph is approved.

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2012

DEDICATION

This thesis is dedicated to both my father and mother who have sacrificed their lives to provide me the opportunity to achieve my dreams. I would like to thank Dr. Niranjala JK Tillakaratne for her unwavering support for my purist of science and happiness.

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INTRODUCTION

The incident of spinal cord injury (SCI) in the United State is 25 to 59 new cases per million populations per year resulting in approximately 12,400 new cases in 2010 and a projected to reach 13600 cases by 2020 [1]. The ability of the spinal cord to learn new tasks or recover behavioral function in an activity dependent manner following SCI has clearly opened the possibility that this capacity can be used to promote functional recovery in spinal cord injured patients [2]. In SCI animals, locomotor training such as, training to step, stand, or avoid obstacles lead to improved performance of that task [3]. The mechanism involved in functional recovery may be through mechanisms such as reorganization of the surviving spinal neural circuitry, changes in neurotransmitter and receptors and learning related markers. Learning can be affected by dietary factors in addition to activity-driven plasticity. For example, dietary factors such as docosahexaenoic acid (DHA) and curcumin can improve learning in the brain by acting on molecular systems involved with synaptic plasticity [4,5]. DHA has also been shown to improve locomotor recovery after spinal cord injury.

Our overall hypothesis is *that dietary intervention with DHA and curcumin improves spinal cord learning through a brain derived neurotrophic factor (BDNF)-mediated mechanism*. We will use instrumental paw withdrawal learning (PaWL) paradigm in completely spinal cord transected adult mice to test this hypothesis addressing the following two specific aims:

Specific Aim1: Examine whether spinal learning is enhanced with dietary supplementation of DHA and curcumin (DHA/Cur).

Specific Aim2: Examine whether the enhancement of spinal learning is mediated through a BDNF- mediated pathway.

We will compare the performance and rate of paw withdrawal learning in mice fed with DHA/Cur to a control diet (CtrlDiet, no DHA and curcumin) (Specific Aim1). We will inject TrkB IgG stereotaxically into lumbar spinal cord to sequester BDNF protein and assess the changes

in performance of PaWL in DHA/Cur fed mice (Specific Aim2). We will measure and compare mRNA levels of BDNF and associated downstream molecular markers such as Calcium/Calmodulin Kinase II (CaMKII), cAMP response element-binding protein (CREB), and syntaxin 3, in lumbar spinal cords of DHA/Cur and CtrlDiet fed mice after PaWL (in both Specific aims) using RT-PCR method.

Showing dietary supplementation of DHA and curcumin can facilitate spinal learning via BDNF-related mechanism can be used as a therapeutic strategy in SCI patients.

BACKGROUND

Abbreviations: AMPK- activated protein kinase, ANOVA- Analysis of variance, BDNF –brain derived neruotrophic factor, CaMKII- Calcium/Calmodulin Kinase II, cAMP- cyclic adenosine monophosphate, COX-II- Cytochrome C Oxidase II, CtrlDiet- Control Diet, Cur- Curcumin DHA- docosahexaenoic acid, DRG- doral root ganglion, EPSP-Excitatory postsynaptic potential, FPI- fluid percussive injury, GAPDH- Glyceraldehyde 3-phosphate dehydrogenase, GRP40- G-protein receptor protein 40, LTP- Long-term potentiation, MAPK- Mitogen-activated protein Kinase, PaWL- Paw withdrawal learning, RD- Response Duration, RD_{bfl} - Response Duration of the best-fit-curve, RD_{bfl_max}-maximum RD of the best-fit-curve, RD_{bfl_min}-minimum RD of the best-fit-curve, RT-PCR- Reverse transcription Polymerase chain reaction, SOD- Superoxide dismutase, Sir2- Silent information regulator two, TBI- Traumatic brain injury, TrkB IgG- Tyrosine kinase B Immunoglobulin G, uMtCK- ubiquitous mitochondrial creatine kinase,

A. Sensory motor learning

Following SCI, the remaining circuitry is capable of acquiring complex motor tasks such as standing, stepping and adapt to perturbation while stepping on a treadmill [3,6,7]. Evidence suggests, training strategies (as body weight support treadmill training) can activate the circuitry to acquire or learn new tasks. Treadmill training provides sensory motor tasks that initiate and

reinforce the reflexive network in the injured spinal cord. The repetitive activation of this network results in improved stepping ability and molecular changes in the spinal cord [8]. For example, in low thoracic spinal cord transected cat, the locomotor recovery in the daily trained group displayed greater number of consecutive steps and more load or weight-bearing during treadmill stepping [9]. The electromyography recording at the soleus (ankle extensor) and tibialis anterior (ankle flexor) muscles in both treadmill trained and untrained, at the12 week time point showed significantly increased with improved coordinated muscle activity in the trained versus the untrained group. In a recent study when spinal cats trained to step on the treadmill were presented with a tripping perturbation, kinematic data showed the spinal cord was able to reprogram the stepping trajectory of the swing following the presentation of the perturbation [7]. EMG data suggested that the primary knee flexor semitendinosus contributed to this adaptive response. In human patients with American spinal injury association impairment scale (AIS) grade C and D, improved balance and walking following 20 sessions of body weight support locomotor treadmill training. These results support the intensive activity-based locomotor therapy can improve functional recovery in individuals with chronic incomplete SCI [10].

Although the mechanism is unclear, it remains that the activity dependent plasticity leads to locomotor recovery through the sensory motor adaptation or learning in the spinal cord. The common to all activity-dependent plasticity in locomotor recovery is the activation from cutaneous and proprioceptive (afferent) sensory input. Treadmill stepping in spinal transected animal involves activation of the cutaneous and muscle proprioceptive changes that initiate the changes in reflex pathways in the spinal cord. The stretch reflex circuitry begins with sensory receptors embedded in the muscle. The sensory afferents, Group Ia and Group II afferent receive sensory input from intrafusal muscle fibers as they transmit the limb dynamics or positioning in space to the spinal cord [11]. Cutaneous stimuli or electrical shock can activate the low threshold axon and activate the reflexive pathway. The efferent pathway consisting of alpha and gamma motoneurons activate muscle contraction resulting in limb correction. In

locomotor treadmill training in SCI animals is thought to activate this reflexive pathway and continued training results in improved locomotor recovery.

B. Dietary intervention

i. DHA

Recent evidence indicates that select dietary factors such as docosahexaenoic acid (DHA) can improve learning in the brain by acting on molecular systems involved with synaptic plasticity [4,5]. DHA belongs to the omega-3 fatty acid family and has important roles in cell communication, synaptic plasticity, and hippocampal learning [12,13]. DHA has been shown to normalize levels of Brain-derived neurotrophic factor (BDNF), reduce oxidative damage, and counteract learning disabilities in animal models of brain trauma [12,14]. When various types of polyunsaturated fatty acids were added to dorsal root ganglion (DRG) cultures from prenatal, adult or aged mice, DHA added DRG cultures from aged mice showed the greatest amount of axonal and neurite outgrowth, suggesting that DHA can promote recovery and plasticity in neurons from adult and aged animals *[15].*

DHA is abundant in coldwater fishes such as salmon, making it easy to incorporate into an individual's diet. In Alzheimer's disease (AD), decreased levels of DHA in the diet are associated with increased risk. Dietary supplementation of DHA in transgenic mice expressing human gene known to cause AD, markedly reduced Abeta42 accumulation and oxidative damage, corrected many synaptic deficits and improved cognitive function [16]. In traumatic brain injury (TBI) model, rats fed with adequate DHA diet showed normalized levels of BDNF, Synapsin I, CREB, CaMKII in the hippocampus. In addition, DHA increased manganese superoxide dismutase (SOD) and Sir2 levels that is commonly reduced following TBI and is associated with promoting metabolic stress [17]. In prenatal mother's diet deficient in DHA resulted in diminished hippocampal neurite growth and synaptogenesis during prenatal development leading to neuronal and behavioral deficits in the progeny's adulthood. DHA

deficiency also resulted in diminished activated phosphorylated (p) learning markers such as pTrkB/TrkB, pBDNF and pCREB in the hypothalamus and hippocampus neurons in these animals [18]

Injection of DHA intravenously into adult rats 30min after a lateral hemi-transection injury was shown to improve functional recovery, when compared to a saline injected group, the DHA injected rats developed smaller lesion size, reduced cell death, and increased oligodendrocyte and neuronal survival. Functional assessment showed DHA treated rats significantly improved locomotor ability measured by open field walking, foot slip, and beam walk [19]. In a subsequent study, long term dietary intervention with DHA in rats with the same injury showed greater neuroprotection and improved functional recovery when compared to acute DHA injection alone. In another study, DHA was introduced via acute intravenous injection alone, or injection plus 6 weeks of dietary DHA into rats with a spinal compression injury. Providing DHA to these SCI rats acutely or chronically led to increased neuroprotection, white matter survival and improved function compared to rats that didn't receive DHA. Locomotor assessments showed as early as 4 weeks on animals on 6 week DHA diet showed significantly better locomotor capabilities as early as 4 weeks compared to acute DHA injected or no DHA given groups [20].

ii. Curcumin

The curry spice curcumin, used in India for many years for several medical purposes, has shown important effects in models of learning and plasticity in animals [14]. Curcumin is a low molecular weight with broad biological potent antioxidant, anti-inflammatory, and chemo-preventative effects. Curcumin supplementation has been shown to reduce cognitive [21,22] and locomotor deficits via normalizing levels of BDNF in rodents with brain trauma [23,24].

The Prevalence of Alzheimer's disease in India is 4.4 fold less than the US and curcumin is thought to be a major factor [25]. *In vitro* and *in vivo* studies have since established curcumin to be important in preventing neurodegenerative diseases such as Alzheimer's disease [26], cerebral ischemia [27] and treatment with curcumin can protect hippocampal neurons against

excitotoxicity. Curcumin supplementation has been shown to reduce cognitive [21,22] and locomotor deficits in rodents with brain trauma [23,24].

In a fluid percussion traumatic brain injury (FPI) model, dietary curcumin can counteract the energy loss due to excitotoxicity; decrease in levels of AMP-activated protein kinase (AMPK), ubiquitous mitochondrial creatine kinase (uMtCK) and Cytochrome C Oxidase II (COX-II) in rats [23]. Behavioral evidence suggests curcumin derivative fed to FIP rats can facilitate locomotor performance and normalize BDNF, CREB, synapsin-I, CaMKII, and oxidative stress related molecules such as SOD, Sir2 [24].

C. BDNF and Synaptic transmission

BDNF is a powerful modulator of neuronal excitability and synaptic transmission [28,29], and learning and memory in the hippocampus [30-32]. BDNF facilitates monosynaptic excitatory postsynaptic potentials (EPSPs) in motoneurons [33]. In addition, BDNF delivered to the injured spinal cord can induce hindlimb stepping [34]. BDNF mediated activation of CaMKII is a key intermediate in facilitation of early phase of long term potentiation (LTP) leading to a strengthening of synaptic efficacy [35,36]. CREB is one of the best described stimulus-induced transcription factors involved in gene transcription [37] and memory formation [30]. Syntaxin 3 protein is a presynaptic membrane bound protein that participates in vesicular docking that is up-regulated during synaptic plasticity [38-40].

D. The paw withdrawal-learning (PaWL) paradigm

PaWL paradigm was developed to examine mechanisms of plasticity involving a simple circuit that demonstrate instrumental spinal learning. The PaWL paradigm was based on a spinal learning technique developed by Horridge headless insects [41], later in rats [42] and mice [43].

The spinal cord is capable of learning was first demonstrated by Horridge. Electrodes were inserted into hindleg of headless insects with the first in a flexor muscle and the second at the distal end of the leg as the ground. The flexor muscle was stimulated to elicit a withdrawal response from the leg. Horridge set a vertical threshold above the insects' initial resting foot position and stimulated the leg with respect to new threshold and continued until the flexion response resulted in the acquisition of the new foot position. During 15-30 min of stimulation, the headless insect acquired a foot position above the threshold. To prove that the leg was elevated due to learning, a second insect setup was yoked to the first (master) insect. The leg of the yoked animal was shocked concurrently with the leg of the master animal regardless of its position. The yoked insects' foot position was not paired to its own shock, rather paired to the master insect (non-contingent shock) [41,44]. The leg receiving contingent shock in the master animal again showed persistent elevation while the legs of the yoked animals did not, demonstrating that the acquisition of new foot position is not an artifact of stimulation but due to learning.

Instrumental learning model in rats was later adapted from Horridge's insect model to show spinal learning in rat. In the rat instrumental learning model, tibilais anterior (TA) muscle was stimulated in rats whose spinal cord were severed completely at the thoracic level to eliminate supraspinal input to the spinal cord below the lesion. Similar to insect model, master and yoked pair is subjected to contingent and non-contingent shocks, respectively. Only the master rat learns to acquire a new threshold position set above the initial resting position to minimize the net exposure to the shocks [42].

The molecular characterization of spinal instrumental learning in the rat spinal cord showed modulation of learning related markers such as mitogen-activated protein kinase (MAPK), CaMKII, BDNF, CREB and Synapsin-1. The significance of these markers implore to the possibility that the same molecular mechanism of cortical learning and plasticity exists in the spinal cord [45].

MATERIALS AND METHODS

The experiments were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. The UCLA Chancellor's Animal Research Committee approved all procedures used in this study.

Diet

Adult male C57BL6 mice (Jackson Lab, Sacramento, CA), approximately 10 weeks of age, were housed in standard polyethylene cages in an environmentally controlled room (22–24°C) with a 12 h light/dark cycle. The mice were divided randomly into 4 groups (n=13/group): (1) Control Diet plus Sedentary (CtrlDiet); this group served as a control for all comparisons and (2) DHA plus curcumin (DHA/Cur Diet). After acclimatization for 1 week on standard mice chow, the mice were placed on diet, either a Ctrl or DHA/Cur (1.25% DHA; Nordic Naturals, Inc. Watsonville, CA + 500 ppm Curcumin: Sigma Aldrich) diet for 21 days. The diets were provided *ad libitum* and administered in powder form.

The two custom diets (CtrlDiet and DHA/Cur) used were based on the composition of the American Institute of Nutrition diet and prepared commercially (Dyets, Bethlehem, PA) as described previously [18] (Table 1). Both diets had the same macronutrients, vitamins, minerals, and basal fats (hydrogenated coconut and safflower oils). The only difference between the Ctrl and DHA/Cur diets was the higher amount of n-3 fatty acids (flaxseed oil, 0.48% and DHA, 1.2%). The dose of DHA and Curcumin used in this study were the same as used previously in rats [18] [21]

Surgical procedures

Spinal cord transection: The spinal cord of all mice was completely transected at the T7–T8 vertebral level as described previously [46]. Briefly, under 2% isoflurane anesthesia, a dorsal midline skin incision was made from T6 to T9 and the musculature covering the dorsal vertebral column was retracted to expose the spinal laminae. A partial laminectomy of the T7 and T8

vertebrae was performed to expose the spinal cord. The spinal cord, including the dura, was transected completely using microscissors. The completeness of the lesion was verified by separating the cut ends of the spinal cord with small cotton pellets and by passing a fine glass probe through the lesion site. The skin incision was closed using small surgical staples.

After surgery, the wound sites were treated with triple antibiotic ointment (Bacitracin) and the mice were given lactated Ringer's solution (1.5 ml/30 g body weight, s.c.). Because preventing leg extension during recovery has been shown to facilitate subsequent learning in rats [47], both hindlegs were bound with the knee and ankle joints fully flexed. The mice recovered in an incubator maintained at 37^0C until fully awake and then were returned to their home cages. The mice were allowed to recover for 24 hr before PaWL testing. To minimize bias, the surgeons and testers were blind to the diet and exercise conditions during surgery and PaWL testing. Thirty min after the completion of the PaWL test in Experiment 2, the spinal cord was quickly dissected, and fresh frozen on dry ice for mRNA measurements.

Trk B IgG Injection: A separate cohort of mice were fed the DHA/Cur diet for 21 days and subdivided into two groups (n = 9/group): the spinal cord of one group was injected with physiological saline and the other group with TrkB IgG (R&D Research, rhTrkB/ Fc Chimera Cat. # 688). Immediately after the spinal cord transection surgery, the mouse was transferred to a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The spinal processes rostral and caudal to the injection site (L2-L3 spinal level) were secured using tissue clamps connected to the stereotaxic apparatus. Injections were made bilaterally at 0.4 mm from the midline and at a 0.32 mm depth [48,49]. A 10 µl Hamilton syringe fitted with a 100 µm (tip) glass pulled needle was used to inject 0.4 µl of 5 µg/µl of TrkB IgG in saline solution at both sites (total of 0.80 µl) or the same amount of saline as a vehicle control [45].

Following 24 hours after the spinal transection surgery and TrkB IgG / saline injection, the PaWL test was given. Again, the master only PaWL test was used to compare the spinal

learning in the two groups. Thirty min after the completion of the PaWL test in Experiment 3, the spinal cord was quickly dissected, and fresh frozen on dry ice for mRNA measurements.

PaWL testing

PaWL is a unique paradigm used to demonstrate that the spinal cord can learn a motor task. In this paradigm, a mouse that has undergone a mid-thoracic complete spinal cord transection learns to dorsiflex the paw above a predetermined threshold, when a mild electric shock is applied to the TA muscle. A learned response is determined by the amount of time the paw remains dorsiflexed above the threshold during a 30 min testing period. [43].

The PaWL test was conducted 24 h after the spinal cord transection surgery on mice that have received 3 weeks of DHA/Cur or CtrlDiet. The details of the PaWL testing for mice have been described previously [43]. Briefly, during testing the mice were restrained in a closed cloth harness with two slots cut at the end of the harness to allow for both hindlegs to hang freely. Two fine-wire hook electrodes were constructed by removing ~1 mm of insulation at the end of nylon-coated single strand stainless steel wires (California Fine Wire Co., Grover City, CA). Each wire was passed through a 32-gauge needle: one electrode was inserted intramuscularly into the left TA muscle and the second electrode was inserted subcutaneously at the base of the lateral malleolus on the same side to serve as a ground. The electrodes were attached to a Stimulator (S88, Grass Product Group; W.Warwick, RI), through a stimulus isolation unit (SIU5; Grass Product Group) and a constant current isolation unit (CCU1; Grass Product Group). A stimulus duration of 50 msec followed by a 10 msec delay between consecutive pulses was used throughout the PaWL test session as previously described [45].

The stimulation intensity is measured for each mouse, by determining the current:force curve relationship and then extrapolating the current required to produce 2/3 of the maximum TA force which was used for the PaWL testing. We previously have shown this to be the optimal force level needed to elicit instrumental paw withdrawal learning [43]. To do this, one end of a

silk thread was tied firmly around the distal end of the metatarsals just proximal to the metarsophalangeal joint, and the other end attached to a force transducer (Dual Mode Muscle Lever 300BLR, Aurora Scientific Inc., Aurora, Ontario, Canada). A sequence of stimuli from 0 to 1.0 mA, in increments of 0.1 mA, with 30 s delays between each stimulus, was administered and the force was recorded [43].

For the PaWL testing, foot is detached from the force transducer and positioned in front of a camera. The paw position was continuously tracked using a video-based point tracking system (CMUCam2; Carnegie Mellon University). The video information was converted to two-dimensional axial components [43]. To identify the resting position of the paw, 3 rapid priming stimuli were applied at the determined intensity.

A stringent vertical threshold for the paw was set at 1.5 mm above resting position. We initially tested vertical thresholds at 1, 1.5, and 2.0 mm in a pilot study using three mice in each of the CtrlDiet and DHA/Cur groups. At a 1 mm threshold both groups learned, indicating that this threshold was not discriminating. At 2 mm neither group learned, indicating that this threshold was too difficult. At the 1.5 mm threshold there was a group difference in the level of learning.

The duration of the PaWL test session was 30 min and the level of learning was assessed by the response duration. The response duration reflects the time that the paw is above the threshold during the PaWL test and incorporates the number of times the foot drops below the threshold resulting in a shock. The response duration is calculated as follows:

Response Duration= (time (sec) the paw is above the threshold)/(# of shocks + 1)

Data collected include the vertical and horizontal paw position, the threshold and the times when animals were shocked during all PaWL trials. Following the completion of the test, the data was post-processed using custom scripts written in MATLAB (The Math Works, Inc.,

Natick, MA, USA). Data are reported as the response duration for each min binned over the 30-min test (e.g., Fig. 2A) or as the sum of the response duration at each min of the 30-min test (total response duration) (e.g., Fig. 2C).

Rate of PaWL analysis

The master mice undergoes contingent stimulus paired to foot position while the yoked mice stimulation is not. To compare the spinal learning in this study, we used the master only model without the paired yoked group. PaWL testing between two diet groups using the mater only model result similar learning outcomes. Currently, we characterize the PaWL using the response duration curve. We set out to identify a mathematical model to better describe the performance and rate of PaWL. The plot of our response duration curve (Fig 2A) suggests a nonlinear regression model or a sigmoidal shaped curve. With the assistance of the Graph Pad software (GraphPad Prism 4.0), the best-fit curve was determined by taking the response duration curve and identified the best-fit curve with 95% confidence interval (GraphPad Prizim 4.0). Given that all the mice were tested under Master condition where the test contribution is the same, the curve fit analysis was used to characterize the quality and rate of PaWL in mice. From the sigmoidal curve fit data, the maximum response duration reached will be used as maximal learning performance (RD_{bfl_max}) and the rate of learning will be the time at which response duration has reached half the maximal response duration (V_{50}). In addition to response duration calculation this new criteria can help to show quality and rate of spinal learning.

Protocol to for the Sigmoidal best-fit curve analysis:

1. Response duration is first calculated;

   RD= (Time the paw flexed above threshold)/ (Number of shocks +1)

2. The mean response duration data for each test group was plotted and a best fit line with 95% confidence interval of our mean RD for each group.
3. Sigmoidal best-fit curve equation:

$$RD_{bfl} = RD_{bfl\_min} + (RD_{bfl\_max} - RD_{bfl\_min})/(1+ \exp((V_{50}\text{-Time}) / \text{Slope}))$$

   a. RD_{bfl} = Response Duration of the best-fit-line
   b. RD_{bfl_max} = maximum RD of the best-fit-line, represents the optimal learning performance.
   c. RD_{bfl_min} = minimum RD of the best-fit-line, represents the minimal learning performance.
   d. V_{50}= Is the time (min) at which the RD_{bfl} has reached half the maximal performance.

***RT-PCR* measurements**

Total RNA was isolated using the RNA STAT-60 kit (TEL-TEST, Inc., Friendswood, TX, USA) as per the manufacturer's protocol. Total RNA (100 ng) was converted to cDNA using iScript cDNA Synthesis kit (Bio-Rad). The SsoFast EvaGreen Supermix kit was used for qPCR and the cycling conditions were according to manufactory's protocol (Bio-Rad). The sequences of the primers were designed using the Integrated DNA Technologies (IDT) online software ("IDT SciTools RealTime PCR"). BDNF: forward (5′- TTACCTTCCTGCATCTGTTGG-3′); reverse (5′- AACATTGTGGCTTTGCTGTCCTGG -3′); syntaxin 3: forward (5′- GCTGGAAGA GATGTTGGAGAG -3′); reverse (5′- TGCTTGGAAATCTGGGAGTC -3′); CREB: forward (5′- ACAGATTGCCACATTAGCCC -3′); reverse (5′- GAGACTGGA TAACTGATGGCTG -3′); CaMKII: forward (5′- CTTTCAGCCAGAGATCACCAG -3′); reverse (5′- ACCAGTAACCAGAT CGAAGATAAG -3′). GAPDH: forward (5′- CTTTGTCA AGCTCATTTCCTGG -3′); reverse (5′- TCTTGCTCAGTGTCCTTGC -3′). The mRNAs for BDNF, syntaxin 3, CaMKII, and CREB were measured using the CFX96 Real-Time PCR Detection System (Bio-Rad). Glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) was used as an endogenous control to standardize the amount of sample loading. The amplification cycle at which the first significant increase of fluorescence occurred was designated as the threshold cycle (CT). The CT value of each sample then was compared with those of the internal standard. The resulting corrected values were used to make comparisons across the different experimental groups. The mean mRNA levels were computed for the four groups. To compare mRNA levels between all experimental groups, we expressed all values as a percent of CtrlDiet by dividing the mean mRNA level in each group by the mean of CtrlDiet mRNA level (Fig.2).

Statistics

Data are reported as the mean values ± standard error of the mean (SEM). For comparisons of learning between groups, across time (binned minute) during PaWL test and the interaction between group affect and over time was, a Two-way mixed analysis of variance (Mixed ANOVA) and Bonferroni post-hoc tests were used to determine differences, respectively. All comparisons were performed using the CtrlDiet or saline group as a control. Pearson product correlations were used to determine the relationships between response duration (learning) vs. all plasticity markers in the spinal cord. For comparisons including two groups (Experiment 3: BDNF blocking experiment), Mann Whitney T-tests were used. All analyses were performed using GraphPad 4.0 (GraphPad Software Inc. San Diego, CA). The level of significance was chosen as $P < 0.05$ for all comparisons.

RESEARCH DESIGN

Given the positive and complementary effects of DHA and curcumin on brain plasticity and learning, the purpose of Specific Aim1 and 2 was to determine the potential therapeutic role of a combination of DHA and Curcumin diet in improving spinal learning. We used the PaWL paradigm in mice to demonstrate instrumental spinal learning. Lastly, to show the dietary affect on spinal learning is mediated through the BDNF mediated pathway, we sequestered BDNF with TrkB IgG injected into the lumbar spinal cord before conducting PaWL. To evaluate the dietary and TrkB IgG effects on BDNF-mediated PaWL learning, we measured levels of BDNF, CaMKII, CREB, and Syntaxin3 mRNA in the lumbar spinal cord. The potential benefits of diet on spinal cord learning have a strong translational potential based on the high efficacy and low invasive profile.

Specific Aim1: Examine whether spinal learning is enhanced with dietary supplementation of DHA and curcumin (DHA/Cur). We will compare the performance and rate of paw withdrawal learning in mice fed with DHA/Cur to a control diet (CtrlDiet, no DHA and curcumin) (Specific Aim1, experiment 1 & 2).

Experiment 1

We first set out to show that the mice fed with DHA/Cur diet are capable spinal learning using the Master and Yoked PaWL paradigm. Following 21-days on the DHA/Cur diet (n=12), the mice were randomly assigned into the master or yoked groups. The master group received a shock contingent upon the paw position relative to a pre-set threshold position, while the yoked mouse received shock independent on its foot position. The PaWL testing was conducted in using a 1.5mm vertical threshold criteria and the response duration, the

performance and rate of learning were determined using Sigmoidal best-fit curve analysis as described in the materials and methods section.

Experiment 2

To test the potential therapeutic role of DHA and curcumin on spinal learning two groups of mice were placed on control diet (n=13) and DHA/Cur (n=13) diet for 21 days. At the end of the 21 days, the master only PaWL testing was conducted. Response duration, performance, and the rate of spinal learning were measured and compared between the groups (Specific Aim1). The fresh frozen lumbar spinal cord tissue was collected 30 min following PaWL testing to be processed for mRNA measurements of BDNF, CaMKII, CREB and syntaxin 3 using RT-PCR method (see Specific Aim2A below).

Specific Aim2: Examine whether the enhancement of spinal learning is mediated through a BDNF-mediated pathway. We will measure and compare mRNA levels of BDNF and associated downstream molecular markers such as Calcium/Calmodulin Kinase II (CaMKII), cAMP response element-binding protein (CREB), and syntaxin 3, in lumbar spinal cords of DHA/Cur and CtrlDiet fed mice after PaWL using RT-PCR method (Specific Aim 2A).

Experiment 3 (Specific Aim 2B).

We will inject TrkB IgG stereotaxically into lumbar spinal cord to sequester BDNF protein and assess the changes in performance of PaWL in DHA/Cur fed mice (Specific Aim 2B).

For this study we fed with DHA/Cur diet (n=16) for 21 days. The mice were then assigned into two groups; one group received TrkB IgG, while the second group received saline via stereotaxic injections into the lumbar spinal cord. Following 24 hours after the injections, PaWL was conducted to measure spinal learning. Response duration and the rate of spinal

learning was measured and compared between the groups. The fresh frozen lumbar spinal cord tissue collected following the PaWL test was then processed to measure BDNF, CaMKII, CREB and syntaxin 3 using RT-PCR protocol.

RESULTS

Experiment 1: Paw withdrawal performance in Master Vs. Yoked mice

Mice fed with 21 days of DHA/Cur showed that only the Master group learned to withdraw the paw. Response duration plotted over time and total response duration show that these measures are significantly higher in master mice than the yoked (Fig. 2A and 2B). Mixed ANOVA shows difference between master vs. yoke group ($F_{(1,29)}$=629.33, P<0.0001 n=6 per group), over the time $F_{(29,300)}$= 11.19, P<0.0001 n=6 and the interaction group x time is also significant $F_{(29,300)}$= 10.52, P<0.0001 n=6. Bonnferroni post hoc comparison between groups and time indicate at the 13th minute master achieved significance (p<0.001) difference over the yoked group. The yoked group did not show significant improvement in response duration for the 30-minute period. Paw withdrawal learning paradigm is unique method to demonstrate that the spinal cord can learn a proprioceptive task.

The performance of spinal learning is measured by the characteristics of sigmoidal best-fit curve (Fig.2C) characteristic show the RD_{bfl_max} = 58.55s with V_{50}= 13.4 min measured for the master group while the yoked group the RD_{bfl_max} = 1.09s with V_{50}= 11.82 min.

Experiment 2: The effect of DHA/Cur diet and exercise on spinal learning

PaWL paradigm was used to evaluate the spinal learning ability. Figure 3A showed the pattern of the paw withdrawal learning over 30 minute between DHA/Cur vs. CtrlDiet groups. Mixed ANOVA shows significant difference between DHA/Cur vs. CtrlDiet group ($F_{(1,29)}$=166.85, P<0.0001 n=13 per group), over the time $F_{(29,720)}$= 4.47, P<0.0001 n=13 and the interaction of

diet affect x learning over time is also significant $F_{(29,720)}$= 2.67, P<0.0001 n=13. Bonferroni post hoc comparison showed, at time 12^{th}-19^{th}, 23^{rd} and 26^{th} -30^{th} minute, the response duration for DHA/Cur is significantly greater (p<0.05) than CtrlDiet group (Fig.3A). The Mann Whitney T-test comparing the total response duration also showed that DHA/Cur group was significantly greater than the CtrlDiet group (Fig 3B). The Sigmoidal best-fit curve (Fig.4B) characteristic for the DHA/Cur curve with RD_{bfl_max} = 47.28s with V_{50}= 8.82 min while the CtrlDiet group with RD_{bfl_max} = 20.66 with V_{50}= 18.48 min. Dietary supplementation with DHA/Cur group performed greater and at faster rate over CtrlDiet group.

Molecular markers of learning and plasticity

We compared the relative expression of BDNF, CaMKII, CREB and syntaxin 3 mRNA in the lumbar spinal cord using the RT-PCR method. The results show that DHA/Cur group significantly increased BDNF (Fig. 5A), CaMKII (Fig. 5C), CREB (Fig. 6A) and syntaxin 3 (Fig. 6C) mRNA levels compared to the CtrlDiet group. Response duration, as a measure of spinal learning, across groups was positively and significantly correlated for BDNF (Fig. 5B), CaMKII (Fig. 5D), CREB (Fig. 6B) and syntaxin 3 mRNA levels (Fig. 6D). Taken together, the dietary combination of DHA/Cur increased the BDND-related learning markers in the spinal cord.

Experiment 3: Effects of sequestering BDNF in lumbar spinal cord (Specific Aim 2B)

A separate group of animals fed with DHA/Cur received TrkB IgG or vehicle (Saline) injection in the lumbar spinal cord prior to the PaWL test. Figure 7A shows the pattern of the paw withdrawal learning between the saline and TrkB IgG groups. PaWL learning in the group receiving TrkB IgG was compared to the saline injected group. Mixed ANOVA shows significant difference between TrkB IgG vs. saline injected group ($F_{(1,29)}$=179.08, P<0.0001 n=9 per group), over the time $F_{(29,480)}$= 3.40, P<0.0001 n=9 per group and the interaction of diet effect x learning over time is also significant $F_{(29,480)}$= 6.35, P<0.0001 n=9 per group). Bonferroni post hoc comparison showed, at time points 14^{th} -22^{nd} , 25^{th} and 26^{th} -30^{th} min, the response duration for

saline group is significantly greater ($p<0.05$) than TrkB IgG group (Fig. 7A). The Sigmoidal best-fit curve (Fig.7B) characteristic for the saline group with RD_{bfl_max} = 47.20s with V_{50}= 12.56 min while the Trk B IgG group RD_{bfl_max} 11.28s and V_{50} at 21.73 min. These results indicate that sequestering of BDNF protein with TrkB IgG diminished the total response duration when compared to the Saline treated group ($P<0.0001$; Fig 7B).

Effects of sequestering BDNF in the spinal cord on BDNF-associated learning related markers

Following the PaWL test, we measured the levels of BDNF, CaMKII, CREB and syntaxin 3 mRNAs in lumbar segment by RT-PCR assays in mice injected with TrkB IgG or saline. Animals administered TrkB IgG showed a significant decrease in BDNF (79% of saline group, $P<0.01$; Fig. 8A), CaMKII (84% of Saline group, $P<0.05$; Fig 8B), and CREB mRNA (84% of saline group, $P<0.05$; Fig. 8C) while no significant changes were observed in levels of syntaxin 3 mRNA (Fig. 8D).

DISCUSSION

The principal findings were a diet supplemented with DHA/Cur enhances PaWL spinal learning and increases the levels of several learning-related cellular markers. Furthermore, we found that the spinal learning was mediated through the BDNF pathway as the selective blocking of BDNF signaling with TrkB IgG chimera resulted in decreased paWL learning and decreased mRNA levels of BDNF-related learning markers in the lumbar spinal cord.

Can diet modulate spinal learning?

The dietary effects on spinal learning were accompanied by significant increases in the levels of BDNF, CaMKII, CREB, and syntaxin 3 mRNAs. These results are consistent with previous studies in the brain showing the capacity of DHA [17,18,50-55] and curcumin [23,24,56,57] to counteract the effects of brain injury on hippocampal-dependent spatial learning performance [17,24,58-60]. DHA also has shown some beneficial effects when it was injected in the tail vein 30 min after a spinal cord injury, as indicated by increasing the survival of neurons and improving locomotor performance after a spinal cord hemisection or compression injury [19].

These results were associated with differential levels of DHA in the plasma membrane. DHA is critical for maintaining membrane fluidity, which is required for proper neuronal function and transmission of information [53,61]. A reduction in the levels of DHA can lower membrane fluidity leading to dysfunction of transmembrane receptors, potentially affecting the induction of LTP [54] and subsequent learning [55,62]. Membrane instability and loss of function is suggested by our results showing that the diet deficient in DHA was associated with a decrease in syntaxin 3, a pre-synaptic membrane vesicular transport marker that is regulated by DHA [63]. The levels of both DHA [64] and curcumin [14] can differentially regulate syntaxin 3 in the brain. A deficiency in DHA in the cerebral cortex has been shown to reduce phosphorylation of

the BDNF TrkB receptors [18] that, in turn, may reduce learning-related factors such as CREB [12,65]. DHA also may influence neurotrophic signaling by activating the PI3-K/Akt pathway that phosphorylates CREB [66]. The increased CREB activation then can influence the expression of other genes including BDNF [13,30,67]. In addition, DHA has been implicated as elevating neurogenesis and plasticity by binding the G-protein coupled receptor GRP40 [68]. The binding of DHA to GRP40 may provide a fast way to impact plasticity through second messenger pathways such as PLC, PIP3, PKC, and CREB and c-fos system [69-71].

Role of curcumin in spinal plasticity and learning

We combined curcumin with DHA based on its action in maintaining metabolic homeostasis that can be critical for supporting learning and memory [14,72]. For example, the beneficial action of curcumin on brain trauma is associated with stabilizing membrane homeostasis and reducing cognitive decay [14,23]. In addition, curcumin has been shown to reduce cell damage in models of toxicity in culture [73,74]. The combination of curcumin and DHA in the current study may be particularly effective for fostering plasticity as they act on similar molecular systems. For example, both DHA and curcumin can affect BDNF-related synaptic plasticity [17,18] and also may influence the metabolic actions of BDNF on glucose and molecules that are crucial for the production of ATP necessary for learning [75,76].

DHA is an essential fatty acid and a structural component of plasma membranes, synaptic vesicles, and membranes of other essential organelles important for brain function and, based on the present results, the spinal cord as well. In a recent study, deficiency of DHA during brain maturation was associated with reduced TrkB signaling in the brain as well as with increased risk for anxiety-like behavior during adulthood in rats [18]. Overall the evidence seems to indicate that some critical level of DHA and curcumin must play an important role in engaging mechanisms of synaptic plasticity and learning in the spinal cord.

CONCLUSIONS

Our results indicate that a dietary combination of DHA and curcumin facilitates spinal cord learning via a BDNF-related mechanism. The observation that the spinal learning was proportional to the levels of spinal cord DHA and the DHA-related synaptic marker syntaxin 3, suggest that the action of DHA on the plasma membrane is an important factor for spinal learning as it is in learning tasks involving supraspinal networks. Given that spinal learning can occur in the sensorimotor circuits of the spinal cord and that these circuits can be modulated by dietary modulation of DHA and curcumin, the potential of these interventions for improving recovery after a spinal cord injury should be examined.

Table 1: Dietary Content for CtrlDiet and DHA/Cur diets.		
Ingredients	**Amount (g/ 100g diet)**	
	CtrlDiet	**DHA/Cur**
Alacid 710, acid casein	20.0	20.0
Cornstarch	15.0	15.0
Sucrose	10.0	10.0
Dextrose	19.9	19.9
Maltose-dextrin	15.0	15.0
Cellulose	5.0	5.0
Salt-mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
TBHQ	0.002	0.002
Fat sources		
Hydrogenated coconut oil	8.1	7.45
Safflower oil	1.9	1.77
Flaxseed oil	none	0.48
DHA	none	1.20
Curcumin	none	Curcumin (500ppm)

.

Table 2: Sigmoidal best-fit curve data all PaWL				
	RD_{bfl_min}	RD_{bfl_max}	V_{50}	SLOPE
Master	1.14	58.55	13.4	3.22
Yoked	0.16	1.1	11.82	2.66
DHA/Cur	0.01	47.28	9.32	3.27
CtrlDiet	5.86	20.55	18.46	0.66
Saline	3.45	47.09	12.56	1.56
TrkB IgG	1.279	11.28	21.73	1.833

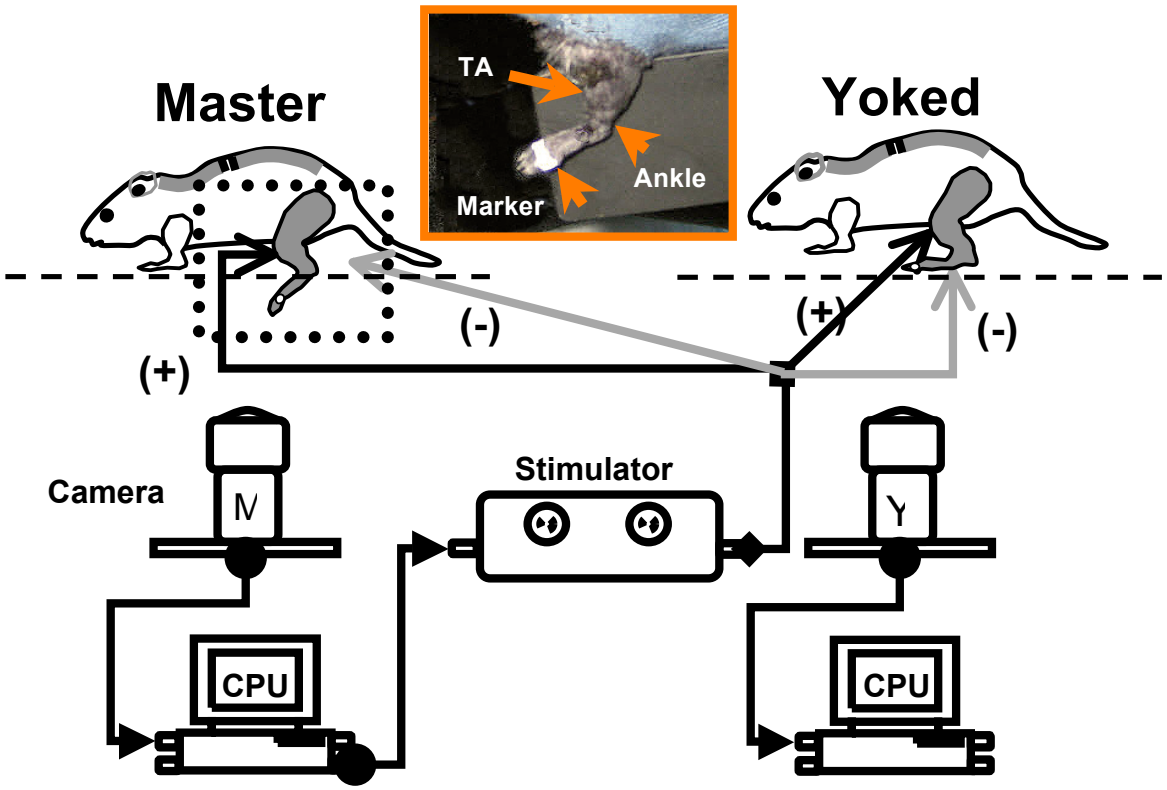

Figure 1: Schematic of PaWL instrumental learning in mice. If the paw position is below a predetermined threshold, fine-wire electrodes deliver a shock to the leg. Learning involves ankle dorsiflexion to hold the paw above the threshold. Two mice, master and yoked are placed in front of two cameras to monitor paw position. Inset box shows knee, and ankle joints, and metatarsal marker. Point tracking information from master mouse is collected by one computer and used to trigger let shock for both the master and yoked mice. A second computer monitors yoked mouse paw position and stimulation.

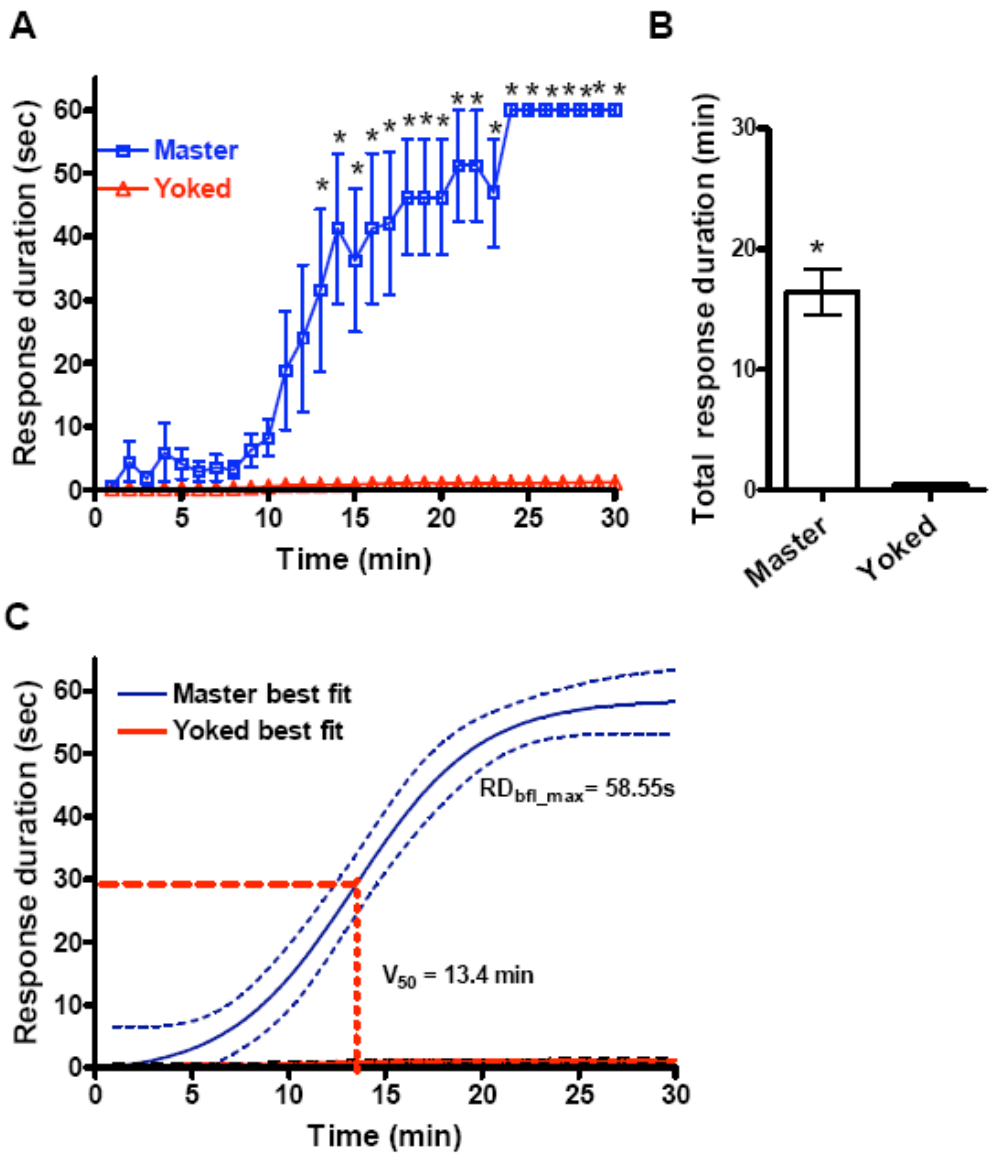

Figure 2: Spinal learning in DHA/Cur fed mice. A) PaWL learning in Master Vs Yoked comparison showed significant difference beginning at 13th to 30th min. B) Total response duration comparison shows, Master group shows significantly greater mean total response duration than Yoked group. C) Rate of learning confirmed by curve fit analysis suggests a sigmoidal shape with maximum performance at RD_{bfl_max} 58.55 for the master group with V_{50} at 13.4min. Yoked mice showed minimal change in response duration. Mixed ANOVA with Bonferroni posttests ($P<0.05$) and Mann-Whitney T-test, * significantly different from Master and Yoked groups $P<0.05$.

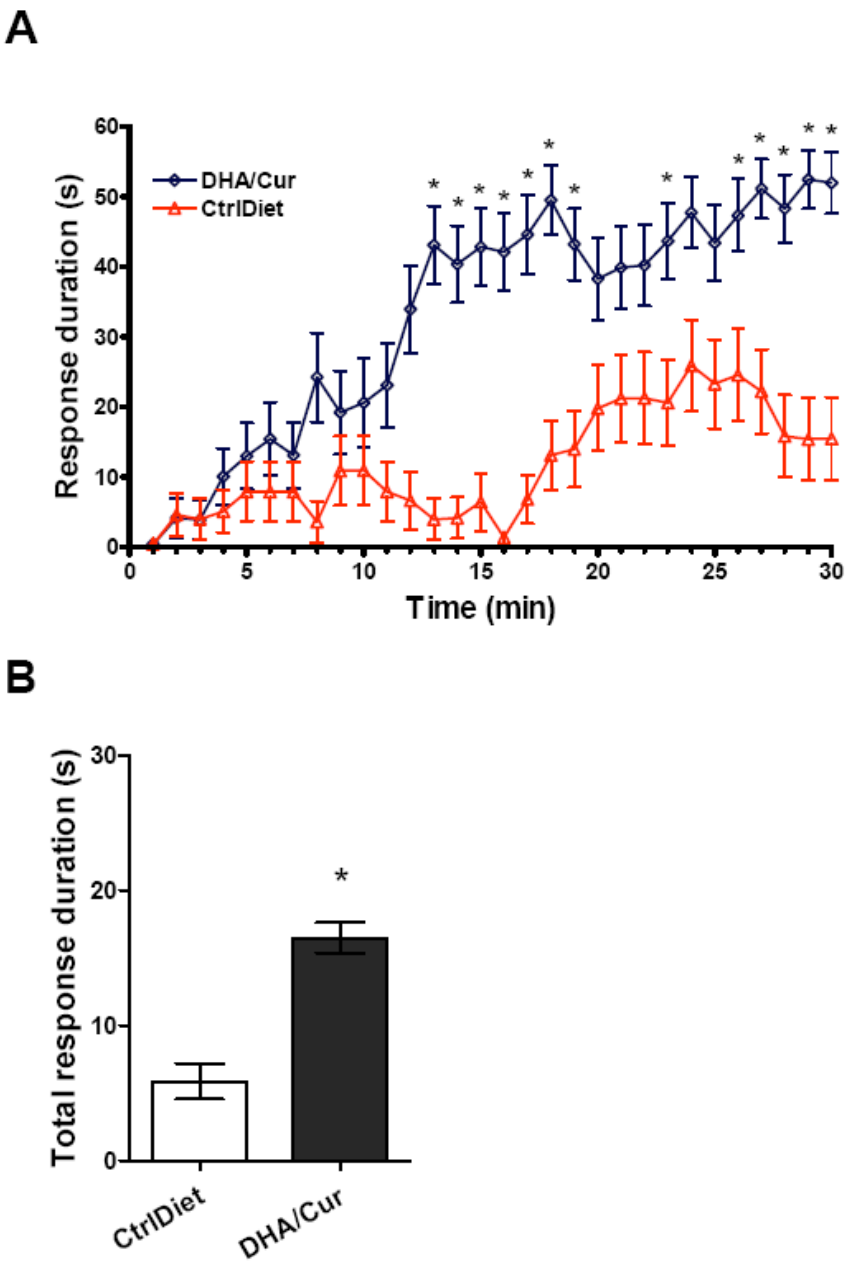

Figure 3: Spinal learning in DHA/Cur and CtrlDiet mice. (A) The groups receiving DHA/Cur had longer mean response durations than CtrlDiet groups. Mixed ANOVA show DHA/Cur is significantly different at time points 13-19, 23 and 26-30min. (B) Mean total response duration in the groups receiving DHA/Cur had longer total response durations than CtrlDiet groups. Mann-Whitney T-test comparing Ctrl vs. DHA/Cur diets. Values are mean ± SEM (n=13 /group).

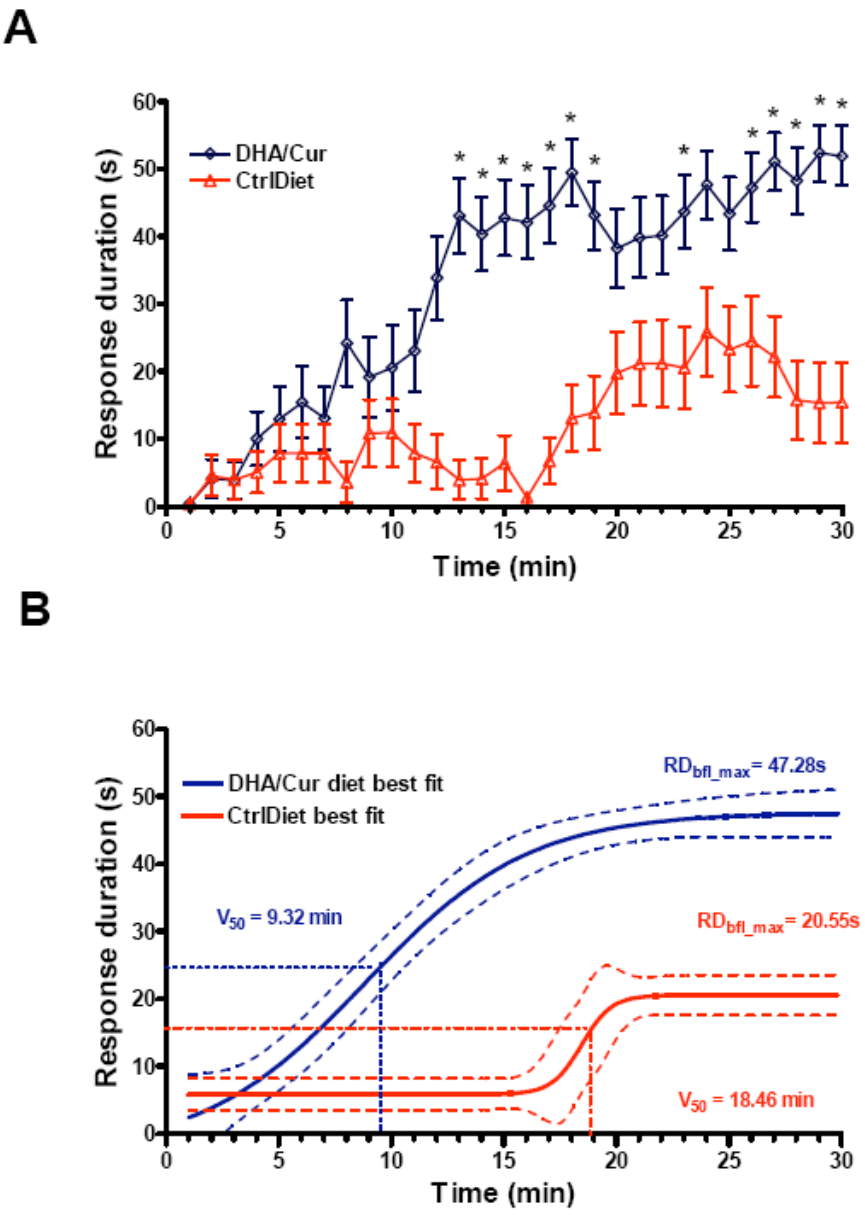

Figure 4: Graphical representation of PaWL and the rate of learning in DHA/Cur and Ctrl groups. A) PaWL learning represented by response duration plotted over time with DHA/Cur mice learning to dorsi-flex better than ctrl group. b) Sigmoidal curve fit analysis show a sigmoidal shape for both groups with maximum performance at RD_{bfl_max} 47.28s and V_{50} at 9.3 min for the DHA/Cur group (Blue) and RD_{bfl_max} 20.55s and V_{50} at 18.46min for the Ctrl group (red).

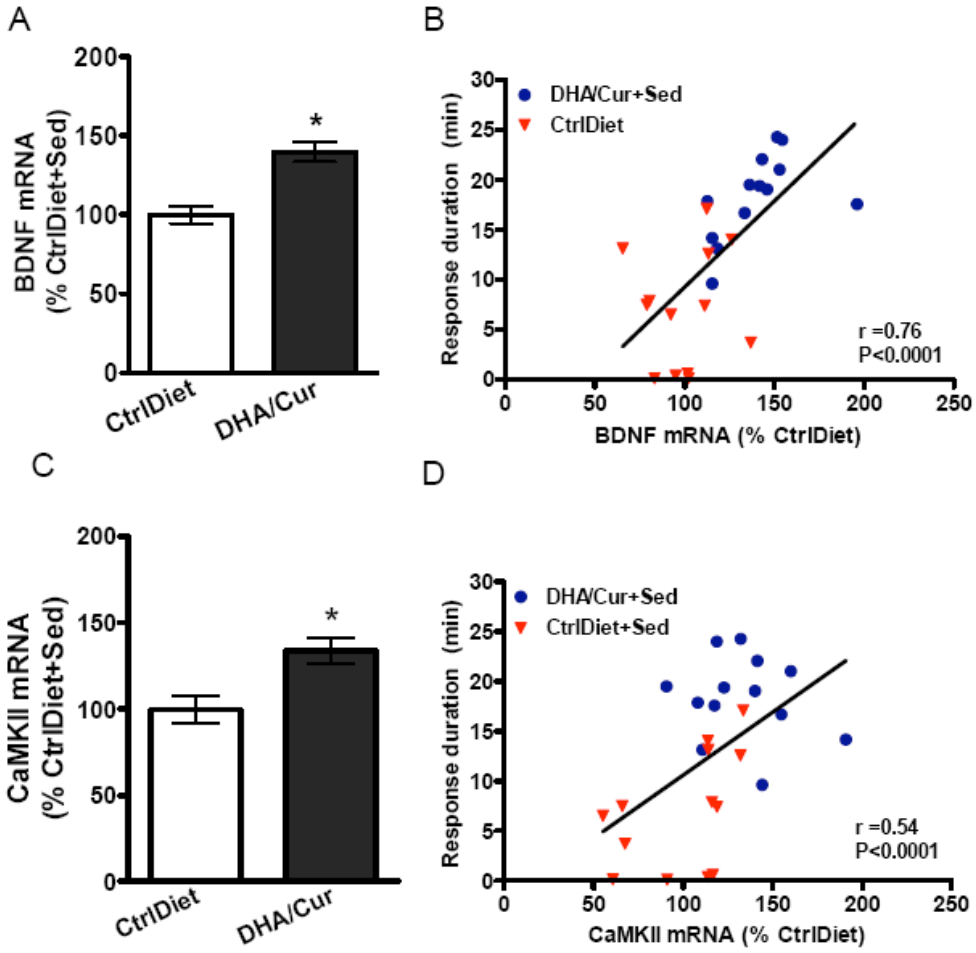

Figure 5: BDNF and CaMKII mRNA levels in the lumbar spinal cord after PaWL test. In DHA/Cur and CtrlDiet lumbar spinal cord A) BDNF and C) CaMKII mRNA levels are significantly greater in DHA/Cur group. A positive correlation between increase in mRNA levels and spinal learning for both B) BDNF (r= 0.76, P<0.0001) and D) CaMKII mRNA (r= 0.54, P<0.0001) to spinal learning. Mann-Whitney T-test, * significantly different from DHA/Cur and CtrlDiet group P< 0.05

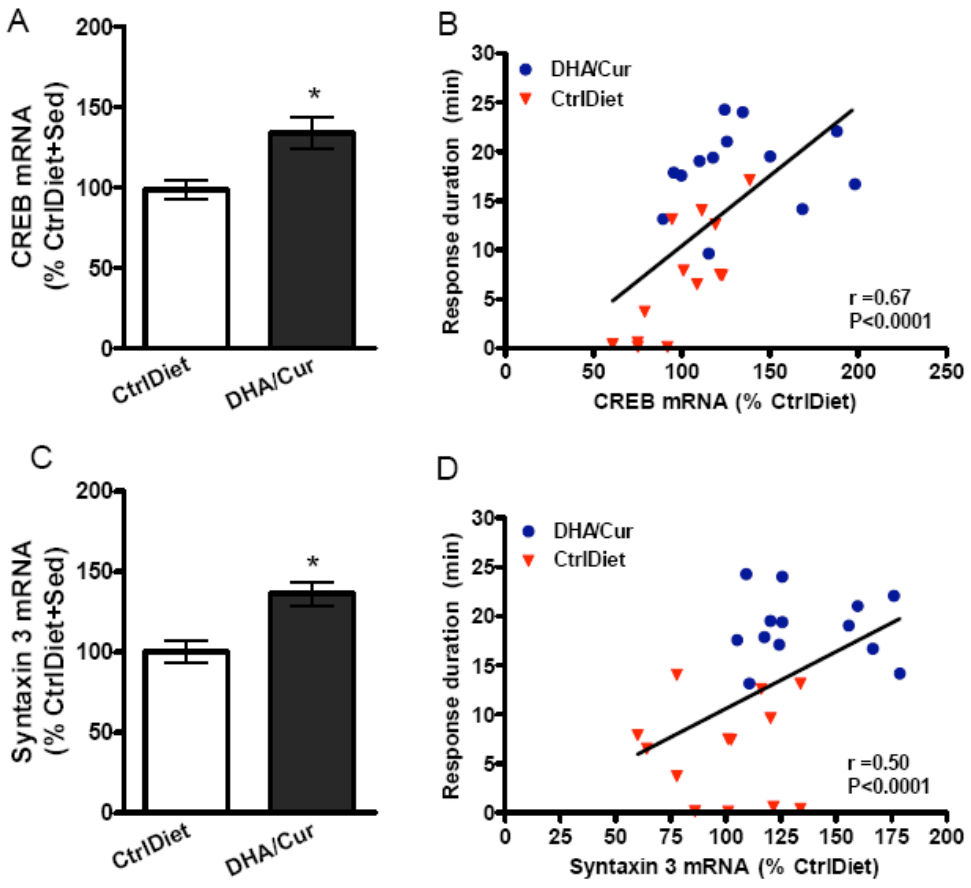

Figure 6: CREB and Syntaxin 3 mRNA levels in the lumbar spinal cord after PaWL test. Comparison between DHA/Cur and CtrlDiet shows A) CREB and C) Syntaxin 3 mRNA levels are significantly greater in DHA/Cur group. A positive correlation between increase in mRNA level and spinal learning for both B) CREB (r= 0.67, P<0.0001) and D) Syntaxin 3 mRNA (r= 0.50 P<0.0001) to spinal learning. Mann-Whitney T-test, * significantly different from DHA/Cur and CtrlDiet group P< 0.05

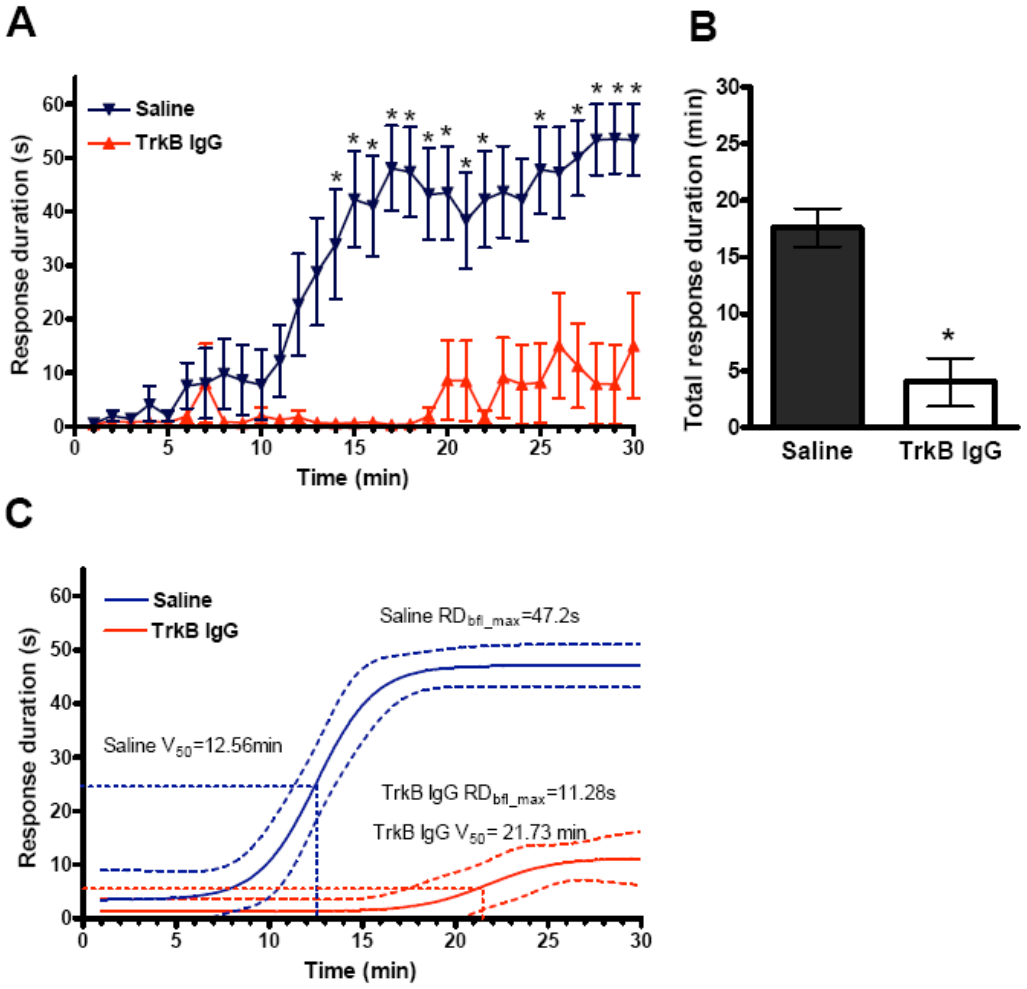

Figure 7: PaWL in DHA/Cur fed mice injected with TrkB IgG or Saline into the lumbar spinal cord. (A) PaWL learning in the group receiving TrkB IgG compared to the saline injected group. At time point 14-22, 25 and 26-30min saline group significantly different from TrkB IgG group. B) Mean total response duration was lower in theTrkB IgG group. C) Sigmoidal curve fit analysis for the saline injected mice (blue), RD_{bfl_max} 47.09s and V_{50} at 12.56 min and for the TrkB IgG group RD_{bfl_max} 11.28s and V_{50} at 21.73 min (red). Values are mean ± SEM (n= 9/group). Mixed ANOVA with Bonferroni posttests ($P<0.05$) and Mann-Whitney T-test where * shows significantly different from TrkB IgG and Saline injected group $P< 0.05$.

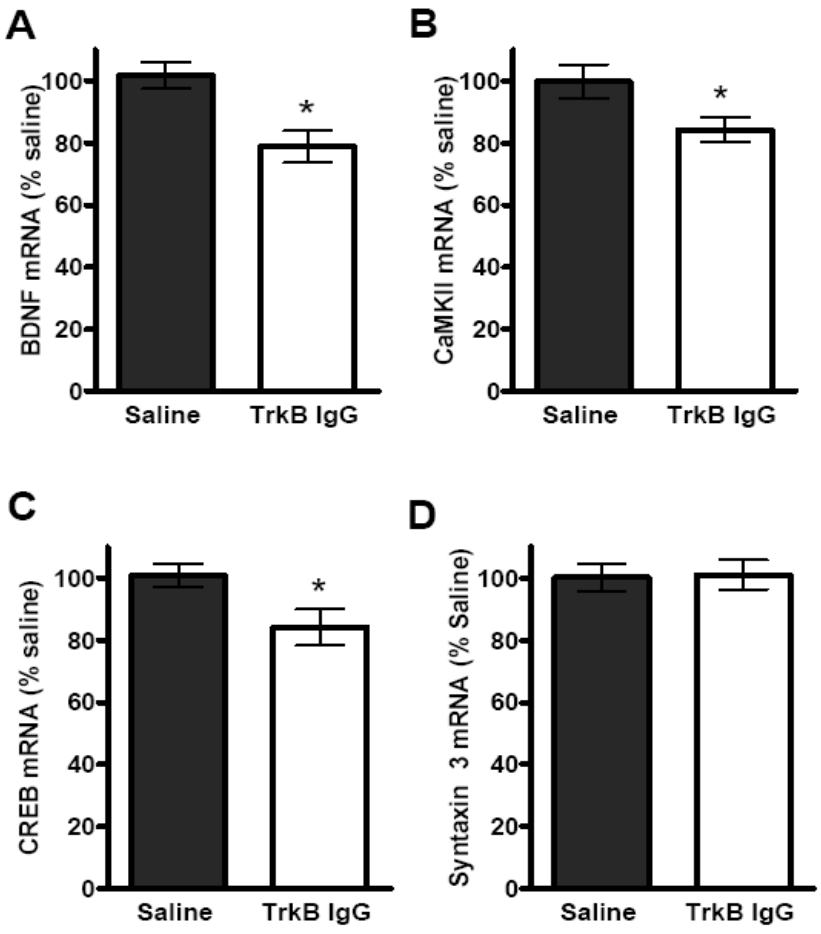

Figure 8: Levels of molecular markers of learning in the lumbar spinal cord. RT-PCR measure and comparison between the groups show (A) BDNF, (B) CaMKII, (C) CREB and (D), but not syntaxin 3 mRNA levels were lower in the group receiving TrkB IgG compared to the saline injected group. Values are mean ± SEM (n= 9/group). Mann Whitney T-test, showing * is $P< 0.05$, significantly different from Saline.

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