Fatty acid suppression of glial activation prevents central neuropathic pain after spinal cord injury

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Abstract

About half of patients with spinal cord injury (SCI) develop debilitating central neuropathic pain (CNP), with no effective treatments. Thus, effective, safe, and novel therapies are needed urgently. Previously, docosahexaenoic acid (DHA) was reported to confer neuroprotection in preclinical SCI models. However, its therapeutic potential on SCI-CNP remains to be elucidated. Here, we demonstrated for the first time that intravenous DHA administrations with 3-day intervals (250 nmol/kg; starting 30 minutes after injury and maintained for 6 weeks) effectively prevented SCI-CNP development in a clinically relevant rat contusion model. SCI-CNP was assessed by a novel sensory profiling approach combining evoked pain measures and pain-related ethologically relevant rodent behaviours (burrowing, thigmotaxis, and place/escape avoidance) to mimic those for measuring human (sensory, affective, cognitive, and spontaneous) pain. Strikingly, already established SCI-CNP could be abolished partially by similar DHA administrations, starting from the beginning of week 4 after injury and maintained for 4 weeks. At spinal (epicenter and L5 dorsal horns) and supraspinal (anterior cingulate cortex) levels, both treatment regimens potently suppressed microglial and astrocyte activation, which underpins SCI-CNP pathogenesis. Spinal microgliosis, a known hallmark associated with neuropathic pain behaviours, was reduced by DHA treatments. Finally, we revealed novel potential roles of peroxisome proliferator–activated and retinoid X receptors and docosahexaenoyl ethanolamide (DHA’s metabolite) in mediating DHA’s effects on microglial activation. Our findings, coupled with the excellent long-term clinical safety of DHA even in surgical and critically ill patients, suggest that systemic DHA treatment is a translatable, effective, safe, and novel approach for preventing and managing SCI-CNP.

Keywords: Central neuropathic pain, Spinal cord injury, DHA, Burrowing, Thigmotaxis, PEAP

1. Introduction

Spinal cord injury (SCI) causes permanent impairment of motor and sensory functions. About 70% to 80% of patients with SCI suffer from different types of pain,\textsuperscript{21,52} among which central neuropathic pain (CNP) is present in about 50% of patients.\textsuperscript{7,21,52} At- and below-level SCI-CNP manifests as spontaneous, burning, stabbing, and/or electric shock-like sensation, stimulus-evoked allodynia and hyperalgesia, and often associates with paraesthesia and dysesthesia.\textsuperscript{20} SCI-CNP is difficult to treat and significantly impacts on daily functioning/rehabilitation, often leading to depression.\textsuperscript{57} No treatments in tolerable doses reliably produce satisfactory relief for a majority of patients with SCI-CNP\textsuperscript{20}; therefore, new therapeutic approaches are urgently needed.

Microglia and astrocytes contribute to the neuroinflammation underpinning SCI-CNP pathogenesis.\textsuperscript{25,33} They are activated chronically both at the injury site and in areas remote to injury, eg, the lumbar L5 spinal cord, and are responsible for the development and maintenance of SCI-CNP.\textsuperscript{25} They release glia transmitters that activate receptors and ion channels in the neuronal membrane\textsuperscript{25} and proinflammatory mediators including cytokines, chemokines, inducible nitric oxide synthase (iNOS), and prostaglandin E2 (PGE2) that induce CNP.\textsuperscript{17,28,40,60,61} Collectively, these substances initiate and maintain the activation of a positive feed-forward cycle between neurons and glia at spinal and supraspinal regions, enabling persistent neuronal hyperexcitability after SCI.\textsuperscript{25} Therefore, targeting the activation of these cells presents a potential therapeutic strategy for SCI-CNP.

Docosahexaenoic acid (DHA), an omega-3 fatty acid crucial for the development and functioning of the nervous system, inhibits proinflammatory cytokine production in microglial cell lines.\textsuperscript{17} When given as a single bolus intravenous (i.v.) injection combined with dietary supplementation, DHA conferred neuroprotection and improved locomotor function in rodent models of SCI.\textsuperscript{32,36} Other research showed that prophylactic dietary supplementation containing DHA and other fatty acids reduced thermal hyperalgesia in rats with SCI.\textsuperscript{19} However, the therapeutic potential of DHA to prevent and treat SCI-CNP has yet to be clarified.

Here, we hypothesized that systemic DHA treatment would prevent SCI-CNP development when given i.v. immediately after SCI and followed by repeated i.v. administrations (acute regimen),
and that it would abolish already developed CNP when given as repeated i.v. administrations at a chronic SCI (delayed regimen). By using a novel sensory profiling, including pain-related spontaneous and/or natural rodent behaviours (burrowing [affective], thigmotaxis [affective] and place escape/avoidance paradigm [PEAP] [cognitive]) to mimic clinical practice to estimate human SCI-CNP, we showed that the acute regimen prevented the development of spontaneous/affective and cognitive pain behaviours coupled with significantly attenuated at-level mechanical hypersensitivity in a clinically relevant rat contusion SCI model. We showed also that the delayed regimen partially abolished SCI-CNP. Using neurochemical and flow cytometry techniques, we showed that the mechanisms by which DHA prevents SCI-CNP include reduced microglial and astrocyte activations at both spinal and supraspinal regions. Finally, we used quantitative real-time polymerase chain reaction (qPCR) to reveal the potential roles of the peroxisome proliferator–activated receptor (PPAR), retinoid X receptor (RXR), and docosahexaenoyl ethanolamide (DHEA – DHA’s metabolite) in modulating microglial activation.

2. Materials and methods

2.1. Ethical statement

Experiments involving live animals were performed following UK Regulations (Animals Scientific Procedures Act, 1986) as well as IASP and National Institutes of Health guidelines for using laboratory animals. Animal studies were approved by the local Ethics Committee of the University of Aberdeen. The ARRIVA guidelines were followed, and an ARRIVA checklist was attached as a supplementary material (available at http://links.lww.com/PAIN/A853).

2.2. Study design

Male Wistar rats (180–200 g; Charles River, Tranent, United Kingdom) were housed (2–3 per cage) in a temperature/humidity/noise-controlled environment, kept on a 12:12-hour light–dark cycle, and allowed to acclimatize for 48 hours after delivery. Standard rat chow and tap water were available ad libitum. The numbers of experimental groups and timelines for behavioural tests are detailed in Supplementary Table 1 (available at http://links.lww.com/PAIN/A853). Good laboratory practice was followed to minimise bias (Supplementary Table 2, available at http://links.lww.com/PAIN/A853). Sample size estimation is described in Supplementary Table 3 (available at http://links.lww.com/PAIN/A853). Behavioural tests were performed during day phase. Batches of subset behavioural experiments (2–3 per group) were used due to limited laboratory capacity and to achieve comparable timings between test animals.

2.3. Contusion spinal cord injury surgery

An established rat contusion model of SCI-CNP was adopted. Two-percent isoflurane (Abbot Laboratories, Berkshire, United Kingdom) was used for general anaesthesia. Buprenorphine (0.3 mg/kg for postsurgery acute pain; Alstoe Animal Health, York, United Kingdom), carprofen (50 mg/kg for postsurgery inflammation; Pfizer, Tadworth, United Kingdom), and Baytril (5 mg/kg for postsurgery infection; Bayer Plc, Reading, United Kingdom) were administered subcutaneously (s.c.) before surgery. A T10 and partial T9 laminectomy was performed and followed by stabilising of the T8 and T11 spinous processes with a clamping apparatus and transferring the rat with the apparatus to the MASCIS impactor (rat model III; software 1.32.6; Rutgers University, New Brunswick, NJ). Next, a 10 g rod was released from a 12.5 mm height onto the exposed spinal cord and generated a vertical compression of the cord. Finally, muscle and skin layers were sutured, and rats were allowed to recover from anaesthesia before returning to home cages. Sham-operated animals underwent the same laminectomy procedure without contusion injury. Saline was given for 5 days (5 mL/day, s.c.) after surgery. Bladders were manually expressed twice daily until self-voiding was resumed. Baytril was given during the first 5 days to minimize urinary infection.

2.4. In vivo docosahexaenoic acid administration

Docosahexaenoic acid stocks (1 M; Sigma, Dorset, United Kingdom) were prepared and stored at −80°C. For in vivo treatment, stocks were diluted with sterile 0.9% saline to desired concentrations immediately before intravenous (i.v.) tail vein injection. For the acute regimen study, DHA (250 nmol/kg) was administered 30 minutes after surgery and thereafter every 3 days lasting for 6 weeks (Fig. 1A). The dosage choice was based on our previous studies. For the delayed regimen study, DHA (250 nmol/kg) was administered at the beginning of week 4 after surgery and thereafter every 3 days lasting for 4 weeks (Fig. 1B). For positive controls, pregabalin was administered intraperitoneally (30 mg/kg; Pfizer) 45 minutes before pain-related behavioural assessment at week 3 and week 6 for the acute regimen study and at week 6 for the delayed regimen study.

2.5. A novel sensory profiling approach to assess SCI-CNP

The approach consists of evoked mechanical hypersensitivity measurement at- and below-injury level (sensory component of pain). It also consists of pain-related spontaneous and/or natural rodent behaviours, ie, burrowing (affective component of pain), thigmotactic (affective component of pain), and PEAP (cognitive component of pain) behaviours, which were previously reported by us.

2.5.1. Spinal reflex–based below-level mechanical hypersensitivity

Below-level mechanical hypersensitivity was measured using an electronic von Frey device with 0.5 mm2 probe tip area (Somedic Sales AB, Norra Mellby, Sweden) as previously described. In 3 consecutive days, rats were acclimatized to the testing room for 45 minutes and then habituated to Plexiglas boxes for 60 minutes until exploratory behaviour ceased. The same Plexiglas box was used for the same rat throughout the duration of the study. Two consecutive baseline measurements were taken before surgery. The probe was applied to the midlateral plantar surface of the hind paw, delivering an increasing force (8–15 g/second) until active paw withdrawal. Both hind paws were tested 5 times with a 1-minute interval, and results were pooled. An average of data from 10 trials per rat was calculated as the withdrawal threshold in response to punctate static mechanical stimulation.

2.5.2. Brainstem response–based below- and at-level mechanical hypersensitivity

Brainstem responses (licking, guarding, vocalizing, jumping, or biting) to below- and at-level mechanical stimuli were measured as previously described. Acclimatization, habituation, and baseline assessments were performed as above. For at-level
brainstem responses, nylon von Frey filaments (Bioseb, FL) with increasing diameters exerting different forces—0.07, 0.16, 0.4, 0.6, 1.4, 2, 4, 6, and 8 g (cutoff) were applied 2 cm laterally to the SCI site, starting with 1.4 g filament. A total of 9 filament applications, 30 seconds apart, were performed per rat. For below-level brainstem responses, nylon von Frey filaments ranging from 1 to 26 g, ie 1, 1.4, 2, 4, 6, 8, 10, 15, 26 (cutoff) were used. The filaments, starting with 6 g, were applied to the midlateral plantar surface of both hind paws for 5 times each; a total of 10 filament applications, 30 seconds apart, were performed per hind paw, and bilateral results were pooled. An “up-down” method was used to calculate the 50% withdrawal threshold in response to at- and below-level mechanical stimulations.

2.5.3. Spontaneous and natural burrowing behaviour

Burrowing, an evolutionally conserved rodent behaviour, is easy to measure objectively by weighing the amount of gravel left in the burrow after testing. Plastic tubes were custom-made (32-cm-long and 10-cm-diameter) with one end sealed and the other opened as entrance (raised by 6 cm to prevent gravel [5 mm pea shingle; B&Q, Eastleigh, United Kingdom] loss). Before each burrowing training or assessment, rats were habituated to the

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Figure 1. Effects of systemic DHA treatments on below- and at-level mechanical hypersensitivity. (A and B) Study designs of acute and delayed DHA treatment regimens in vivo. Docosahexaenoic acid administered every 3 days, starting either 30 minutes (C) or week 4 (D) after SCI, significantly increased hind paw withdrawal thresholds to punctate mechanical stimuli, comparing with those of vehicle-treated rats ((C) weeks 2-6, \( P < 0.01 \) and (D) weeks 5-7, \( P < 0.001 \)). Both the acute and the delayed regimens significantly increased brainstem response thresholds to at-level mechanical stimulations, comparing with those of vehicle-treated rats (E) weeks 2-6, \( P < 0.05 \); (E) weeks 5-7, \( P < 0.05 \)). No differences in brainstem responses to hind paw mechanical stimulations were found in both regimen studies (G and H). \( N = 10 \) to 15 per group. Two-way repeated-measures ANOVA was used to determine the main effects of treatment (DHA vs vehicle) and time (weekly). Statistical significance of the differences between the groups was determined by 1-way ANOVA followed by the Tukey–Kramer post hoc test at each week point. *\( P < 0.05 \), **\( P < 0.01 \), or ***\( P < 0.001 \) vs vehicle. ANOVA, analysis of variance; DHA, docosahexaenoic acid; SCI, spinal cord injury.
testing room and cages (30 minutes each) with dim lighting (5 lux). Before baseline measurements, rats were trained to burrow in pairs in 1 cage with a tube containing 2500 g of gravel for 2 hours. The next day, rats that performed well were regrouped with those that performed less well in the first training, to facilitate peer learning. Two baseline assessments were conducted per rat in 2 consecutive days. Burrowing was assessed weekly after surgery starting from the second week, when plantar stepping was re-established [Supplementary Fig. 1, available at http://links.lww.com/PAIN/A853].

2.5.4. Spontaneous and natural thigmotactic behaviour

This is based on natural behaviours of rodents when they enter an open field to which they have not previously been exposed. Thus, rats were introduced for first time to an open field arena (100-cm-long and 100-cm-wide) in dim lighting (12 lux) at week 6 and week 7 after surgery for the 2 regimen studies respectively. Spontaneous movement of each rat in the arena was recorded for 15 minutes with a highly sensitive camera (Tracksys Ltd., Nottingham, United Kingdom), and movement tracks were analysed using EthoVision XT (v10; Noldus, Wageningen, The Netherlands). The duration spent in and frequency of entry to the virtual inner zone (40-cm-long and 40-cm-wide) were calculated.

2.5.5. Natural place escape/avoidance paradigm behaviour

This is based on natural behaviours of rodents in which they prefer hiding in the dark than exposing to bright conditions. The paradigm was custom-made with acrylic box (60-cm-long, 30-cm-wide, 30-cm-high; painted half white and half black; 12 lux lighting). Rats were allowed to acclimatize in the experiment room for 30 minutes before testing. Then each rat was introduced to the centre of the box and allowed a 5-minute habituation. A von Frey filament (2 g), a choice based on our previous studies, was next applied every 15 seconds either on the dorsal thorax (2 cm laterally to the injury site) when the rat was in the black zone or on the skull vertex when it was in the white zone, for 30 minutes during which free movement was allowed. Free movements of each rat were recorded using EthoVision, enabling the calculation of the cumulative time spent in and the number of crossings to the white zone.

2.6. Immunohistochemistry and quantitative analysis

At the end of the behavioural studies, animals (n = 5-6 per group) were perfused with 4% paraformaldehyde. Brains and spinal cords (L5 and injury epicenter) from SCI rats and naive/sham rats were postfixed, cryoprotected in 30% sucrose in phosphate-buffered saline (PBS), and dehydrated by graded 70%, 95%, and 100% ethanol, cleared through xylene, and mounted by Neo-mount (Sigma). To establish (Supplementary Fig. 1, available at http://links.lww.com/PAIN/A853), tissues were previously described.30,31 Iba-1 immunoreactive cells with “reactive” morphology (the process length is twice shorter than that of the soma diameter) were counted and expressed as the number per 50,000 μm². Double-immunoreactive (pp-38+/Iba-1+) cells were counted and expressed as a percentage of the total of Iba-1+ microglia. Astrocyte activation was determined by analysing the intensity of GFAP immunoreactivity; Images were converted into 8-bit using ImageJ, and a threshold was applied to render the staining as a binary image in black and empty spaces in white; then the area occupied by GFAP staining was calculated. Images from the ACC were also analysed for the numbers of Iba-1 immunoreactive cells and GFAP intensity. The person who conducted these analyses was blinded to treatment groups.

For the spinal cord histology, we used transverse sections from a 5-mm-long spinal cord segment which contained the lesion epicenter in the middle. Thus, a series of transverse sections with 14-μm thickness were cut from the rostral end of this segment towards caudal until the finish of sectioning. Owing to the cavity formation in the lesion epicenter which destroyed the dorsal horn, we selected transverse sections approximately 2000 μm both rostral and caudal to the middle of the segment. In these sections, despite still having small cavities, the superficial dorsal horns were largely intact thus allowing us to conduct imaging and image analysis for microglia and astrocytes. The right and left dorsal horn data were averaged per section. The rostral and caudal histological measurements were then averaged per rat.

2.7. Eriochrome cyanine staining and analysis

Eriochrome cyanine (EC) staining was used to analyze the amount of tissue sparing, i.e., spared white matter myelin from the spinal cord tissue of injured animals (n = 4-5 per group). Transverse 14-μm-thick cryostat sections approximately 2000 μm away from the epicenter in both rostral and caudal directions were selected for this purpose. Staining procedures were previously described.53 Briefly, sections were dried overnight at room temperature and then were placed in fresh acetone for 5 minutes, removed, and air dried for 20 minutes. Sections were incubated in EC solution (0.2 g EC R, 0.5 mL concentrated H₂SO₄, 96 mL ddH₂O, and 4 mL of 10% ferric ammonium sulfate solution, all from Sigma) for 30 minutes at room temperature and then washed in ddH₂O. The sections were then differentiated in 5% iron alum (Sigma) for around 15 minutes until the gray matter became visible. This was followed by differentiation in a borax-ferricyanide solution (1 g disodium tetraborate decahydrate, 1.25 g potassium ferricyanide, and 100 mL ddH₂O, all from Sigma) for 10 minutes. The slides were dehydrated by graded 70%, 95%, and 100% ethanol, cleared through xylene, and mounted by Neo-mount (Sigma). To calculate the area of spared myelin, bright-field images of 3 EC-stained sections per rat (rostral, epicenter, and caudal)
were taken by a Nikon DS-U3 camera. Tissue sparing was quantified by outlining areas with light and dark blue staining, analyzed by ImageJ, and calculated by dividing the spared white matter area by the spinal cord transverse cross-sectional area.

2.8. Glial cultures, in vitro docosahexaenoic acid treatment, and immunocytochemistry

Wistar rat pups (postnatal days 3-5) were used, and mixed microglial and astrocyte cultures were prepared as we previously described. Briefly, isolated cortical tissue was mechanically and chemically dissociated, and then, cells were seeded onto 60-mm poly-D-lysine (PDL; Sigma)–coated culture dishes in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 15% heat inactivated foetal bovine serum (Invitrogen, United Kingdom). After 4 days in culture, microglia were harvested and plated onto uncoated glass coverslips. The astrocyte layer was left to incubate for another 48 hours, and then, cells harvested by incubation with 0.25% Trypsin-EDTA solution (Sigma). Once astrocytes were detached, the cell suspension was centrifuged at 1500 rpm for 10 minutes, and then, cells were counted, resuspended, and plated onto PDL-coated coverslips.

Docosahexaenoic acid stock was diluted with 25 mL serum-free DMEM to obtain 200 μM stock solution for in vitro simultaneous treatment or delayed treatment (ST vs DT). For ST experiments, cultured cells were co-treated with DHA and lipopolysaccharides (LPS, 10 μg/mL; Sigma) for 4 hours. For DT experiments, cells were pre-activated by LPS for 4 hours, and then, DHA was added for another 4 hours. To determine the optimal DHA concentration, cultured cells were first treated with 0.8, 4, 8, or 32 μM DHA during the ST experiments. 0.8 μM was found as the optimal concentration and was used in subsequent ST/DT experiments. Cells were either fixed with 4% PFA for immunocytochemistry, lysed with RLT buffer (Qiagen, Manchester, United Kingdom) for qPCR, or processed for flow cytometry. Culture media were collected for the Greiss assay analysis (Supplementary Methods, available at http://links.lww.com/PAIN/A853).

For immunocytochemical analysis, cells were incubated with primary antibodies: rabbit anti-Iba-1 (1:1000), mouse anti-GFAP (1:1000), and mouse anti-FLAG/NOS type II (1:200; BD Bioscience, Berkshire, United Kingdom), followed by incubation with goat/donkey secondary antibodies: anti-rabbit Alexa Fluor488, anti-mouse Alexa Fluor568, or anti-mouse IgG2a Alexa Fluor555 (all 1:400; Fisher Scientific). Images from 5 random areas per coverslip were taken at ×20 objective magnification. Microglial and astrocyte morphologies were qualitatively assessed using bright-field images. Microglial/astrocyte culture purity was determined by calculating the percentage of Iba-1 or GFAP immunoreactive cells in the total of DAPI-stained cells. Coverslips with purities less than 95% were excluded from further analysis. The concentration of eluted RNA was quantified using a mixture of 4 Lc DNA L RNA with RNA-free water. cDNA synthesis was 4-5 per group. We also assessed microglial activation in vitro using flow cytometry. Cells from the ST/DT experiments were detached using StemPro Accutase Cell Dissociation Reagent (Fisher Scientific) and then fixed with 2% PFA for 10 minutes. Cell suspension was then centrifuged at 1500 rpm for 10 minutes. Cell pellet was washed with PBS, spun twice, and resuspended in 100 μL FACS buffer. Cells were incubated with CD11b/c-APC (clone-REA325) and CD86-PE (clone-24F) (Miltenyi Biotec) at 1:10 dilution (106 cells/50 μL) for 45 minutes at room temperature. Samples were then washed and resuspended in FACS buffer, followed by analysis using flow cytometry machine BD Fortessa (BD, Witternesh, United Kingdom) (5 × 105 events per sample) and FlowJo software (FLOWJO, LLC, Ashland, OR). Data were presented as numbers of ED9+/CD11b/c+ cells per mg of spinal cord tissue.

2.9. Flow cytometry analysis

2.9.1. In vivo flow cytometry

Fresh-frozen cord tissues were collected at the end of behavioural studies and assessed for microgliosis, known to be correlated with neuropathic pain behaviours, using flow cytometry as previously reported. Segments of L5 and epicenter cords (n = 4-5 per group) were immediately frozen on dry ice after harvesting and stored at −80°C. Samples were thawed on ice, weighed (milligram), and then enzymatically digested with collagenase type IV (1 mL; 0.5 mg/mL in PBS; Sigma) for 30 minutes at 37°C. The cell suspension was passed through a 40 μm cell strainer (BD Falcon, United Kingdom), followed by centrifuging at 400 rpm for 10 minutes and resuspending in 10% normal goat serum and 1% 2 μM EDTA for 15 minutes. Each sample was diluted in fluorescence-activated cell sorting (FACS) buffer to contain a minimum of 5 × 105 cells and incubated with CD172a-FITC (clone-ED9; microglial marker; Bio-Rad, Hertfordshire, United Kingdom), CD11b/c-APC (clone-REA325; microglial marker; Miltenyi Biotec, Bisley, United Kingdom), and CD45-VioBlue (clone-REA304; general macrophage marker; Miltenyi Biotec), all at 1:1 dilution for 45 minutes at room temperature. Samples were then washed and resuspended in FACS buffer, followed by analysis using flow cytometry machine BD Fortessa (BD, Witternesh, United Kingdom) (5 × 105 events per sample) and FlowJo software (FLOWJO, LLC, Ashland, OR). Data were presented as numbers of ED9+/CD11b/c+ cells per mg of spinal cord tissue.

2.10. Quantitative real-time polymerase chain reaction analysis

2.10.1. In vivo quantitative real-time polymerase chain reaction for spinal cord dorsal horn tissues

To assess relative mRNA expression levels of proinflammatory mediators iNOS, tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 in vivo, spinal cord dorsal horn tissues at L5 level and from lesion site segment containing the epicenter (n = 4-5 per group) were freshly dissected out using the open book method that we previously reported and processed for qPCR as we previously described. Tissues were dissected approximately 1.5 to 2 mm away both rostrally and caudally to the epicenter, where dorsal horns were relatively preserved. Left/right and rostral/caudal tissues from each animal were pooled. Tissues were immediately frozen on dry ice after harvesting and stored at −80°C until use. Frozen spinal cord tissue was homogenized in 1 mL Trizol (Fisher Scientific) and separated into phases by using 200 μL chloroform (Sigma). The aqueous phase was transferred in a fresh tube and cleaned up by using RNeasy Mini Kit (Qiagen), following the manufacturer’s instructions. The concentration of eluted RNA was quantified using Nano Drop 1000 spectrophotometer (Fisher Scientific). The cDNA SuperMix (WWR, Leicestershire, United Kingdom) was used to synthesise cDNA from RNA samples. A mixture of 4 μL cDNA SuperMix and 16 μL RNA samples was prepared for cDNA synthesis. RNA samples were diluted to a final concentration of 100 ng/μL RNA with RNA-free water. cDNA synthesis was performed by a thermal cycler according to the manufacturer’s instructions.
instructions, and the samples were stored at −20°C until further use. All used primers (Sigma) used are listed in Table 1. A total of 15 μL reaction solution, including 3 μL cDNA sample and 12 μL gene-specific master mix, was loaded in triplicates for each biological sample in a 384-well plate. cT values were obtained by Light Cycler 480 II thermal cycler and Cycler 480SW1.5.1 software (Roche, Hertfordshire, United Kingdom). The relative mRNA expression of each gene was calculated and expressed as fold change using the $2^{-\Delta\Delta Ct}$ method. Data were normalized against the reference gene YWHAZ.

2.10.2. In vitro quantitative real-time polymerase chain reaction
To assess relative mRNA expression levels of proinflammatory mediators iNOS, TNF-α, IL-1β, and IL-6 in microglia and astrocytes from the ST/DT experiments, qPCR was performed, as previously described. After a sterile PBS wash, a total of 500 ng RNA from individual samples was extracted using RNeasy Mini Kit (Qiagen), following manufacturer’s instructions. The RNA concentration was confirmed using Nano Drop 1000 spectrophotometer. cDNA synthesis, qPCR methods, and the relevant analysis were performed in the same manner, as described above.

2.11. Peroxisome proliferator–activated receptor and retinoid X receptor antagonist treatment of microglia in vitro
Docosahexaenoic acid is a well-known natural ligand of the PPAR and RXR receptors, which have been previously shown to have a regulatory role in various inflammatory- and immunity-related processes. To investigate the potential mechanisms through which DHA attenuates the activation of microglia in vitro, we treated the microglial cells with potent PPAR and RXR antagonists before simultaneous treatment with LPS and 0.8 μM DHA. PPARγ antagonist GW9662 (10 μM; Sigma) and RXR antagonist HX531 (2 μM; Tocris, Bristol, United Kingdom) initially reconstituted in dimethyl sulfoxide (Sigma) were prepared to final working concentration of 10 μM in serum-free DMEM medium, as described previously. After the cells were pretreated for 4 hours with either LPS and 0.8 μM DHA, the microglial cells were treated for 4 hours as described previously. At the end of the treatments, the cells were processed for the analysis of gene expression profiles of proinflammatory mediators, as described in the main methods section.

2.12. Docosahexaenoic acid ethanalamide treatment of microglia in vitro
To gain further insight into the mechanisms of DHA effects on microglial modulation, we investigated whether treatment with the DHA metabolite DHEA would also lead to a decrease in the activation of microglia in vitro. The rationale behind assessing the effects of DHEA are that first, the levels of this downstream substrate have been shown previously to be increased in the brain and the spinal cord after consumption or administration of omega-3 fatty acids, second, DHEA also possesses anti-inflammatory properties and enhances the beneficial effect of DHA, and finally, DHEA also binds to the PPAR, RXR, and CB1/2 cannabinoid receptors expressed by microglia. Docosahexaenoyl ethanolamide (Cayman Chemicals, Ann Arbor, MI) was diluted to stock concentration of 10 mM in the same way as described in the main text for DHA and further prepared in serum-free DMEM to a working concentration of 5 or 10 μM. The choice of these concentrations was based on previous studies. The microglial cells were treated for 4 hours with either LPS 10 μg/mL, a combination of LPS with 5 μM DHEA, or LPS and 10 μM DHEA, after which the cells were processed for the analysis of gene expression profiles of proinflammatory mediators, as described in the main methods section.

| Table 1 | Forward and reverse primers and their concentrations. |
|---------|------------------------------------------------------|
| Primer  | Forward primer 5′−3′                           | Reverse primer 3′−5′ | Working concentration (μM) |
| TNF-α   | AGCCCACTGTTGACAAAAACAC  | AGGTACACCCATGCGTGGCA | 0.5 |
| iNOS    | CACCTGGAAGTTCACCCAGT | ACCACTGTAATTGGGATGC | 0.5 |
| IL-6    | TCTTCCAACACCTGGAATGTC | TGGATGTCTGACCCAGGCA | 0.4 |
| IL-1β   | CACCTCCAAGCAGACAGACG | GGGTACATGTTGAGTACAC | 0.4 |
| YWHAZ   | GCTACTGGCTGAGGTGCT | TGCTGACTGCTCAGCAAT | 0.5 |

InOS, inducible nitric oxide synthetase; IL-6, interleukin 6; IL-1β, interleukin 1β; TNF-α, tumour necrosis factor-α.

2.13. Statistical analysis
Statistical analyses were performed using Prism7 Software (La Jolla) and SPSS Version 25 (IBM). All data were expressed as mean ± SEM. The primary outcomes for behavioural experiments are shown in Supplementary Table 1 (available at http://links.lww.com/PAIN/A853). For below- and at-level mechanical hypersensitivity, burrowing, and locomotor function assays, a two-way repeated-measures (between- and within-subject) analysis of variance (ANOVA) was used to determine the main effects of independent variables namely “treatment” (DHA vs vehicle) and “time” (weeks after injury). “Treatment” is a between-subjects variable, while “time” is a within-subject variable. Since naive animals and sham treatments did not receive DHA or vehicle treatment, we did not include the naive/sham groups in the two-way repeated-measures ANOVA. However, we used 1-way ANOVA for the naive, sham, vehicle, DHA, and pregabalin groups at each individual time (week), followed by the Tukey–Kramer post hoc multicomparison adjustment to calculate the significant levels. For thigmotaxis and PEAP, 1-way ANOVA followed by the Tukey–Kramer post hoc multicomparison adjustment was used to determine the main effect and significance levels. The box/Whisker plots for thigmotaxis/PEAP data were prepared using OriginPro 2018 (OriginLab, Northampton, MA).

Histological data were tested for normality first as we previously applied. Parametric (2-tailed unpaired Student t test) and nonparametric (Mann–Whitney test) tests were then used accordingly. For immunocytochemical, flow cytometry, and nitrite release analyses, the unpaired Student t test was used. One-way ANOVA was used to determine the effect of DHA concentration on cultured microglia and astrocytes. For qPCR, statistical
significance was calculated as we previously described, ie, by t tests in R (2-sided, Welch t test) on the ΔCT values, followed by adjusting the P values using the false discovery rate correction.43 We considered differences to be statistically significant when \( P < 0.05 \).

3. Results

Information for group sizes and primary outcome measures of the in vivo experiments is detailed in Supplementary Table 1 (available at http://links.lww.com/PAIN/A853). Animal exclusion from the in vivo experiments is detailed underneath Supplementary Table 2 (available at http://links.lww.com/PAIN/A853).

3.1. Systemic docosahexaenoic acid treatment attenuates spinal reflex–based below-level mechanical hypersensitivity

We measured the left and right hind paw withdrawal thresholds in response to mechanical stimuli, which were then averaged for each rat. Our analysis showed significant main effects of treatment on hind paw mechanical hypersensitivity in both studies (acute regimen: \( F_{(1,27)} = 75.735, P = 0.0001 \), Fig. 1C; delayed regimen: \( F_{(1,26)} = 57.221, P = 0.001 \), Fig. 1D; two-way repeated-measures ANOVA). We did not find significant main effects of time on hind paw mechanical hypersensitivity in both studies (acute regimen: \( F_{(4,108)} = 1.941, P = 0.109 \); delayed regimen: \( F_{(3,78)} = 1.157, P = 0.332 \)). However, there were significant treatment with time interactions in both studies (acute regimen: \( F_{(4,108)} = 5.323, P = 0.001 \); delayed regimen: \( F_{(3,78)} = 10.513, P = 0.0001 \)). Significant increases in withdrawal thresholds were observed in DHA-treated rats when compared with vehicle-treated rats (acute regimen/weeks 2-6: \( P < 0.0021 \); delayed regimen/weeks 5-7: \( P < 0.0062 \); Tukey–Kramer post hoc). However, the increased withdrawal thresholds of DHA-treated rats did not reach those of naïves (Figs. 1C and D).

3.2. Systemic docosahexaenoic acid treatment attenuates brainstem response–based mechanical hypersensitivity

Our analysis revealed significant main effects of treatment on brainstem response (licking, guarding, vocalizing, jumping, or biting)–based at-level mechanical hypersensitivity in both studies (acute regimen: \( F_{(1,27)} = 52.031, P = 0.00001 \), Fig. 1E; delayed regimen: \( F_{(1,26)} = 21.707, P = 0.0001 \), Fig. 1F; two-way repeated-measures ANOVA). We also found significant main effects of time on brainstem response in both studies (acute regimen: \( F_{(4,108)} = 2.406, P = 0.045 \); delayed regimen: \( F_{(3,78)} = 11.782, P = 0.001 \); two-way repeated-measures ANOVA). Moreover, there were significant treatment with time interactions in both studies (acute regimen: \( F_{(4,108)} = 6.385, P = 0.0001 \); delayed regimen: \( F_{(3,78)} = 17.28, P = 0.0001 \)). The mean at-level 50% pain thresholds were significantly higher in DHA-treated rats than vehicle-control rats (acute regimen/weeks 2-6: \( P < 0.022 \); delayed regimen/weeks 5-7: \( P < 0.028 \); Tukey–Kramer post hoc), but lower than naïves (Figs. 1E and F). Pregabalin-controls showed comparable (no statistical difference) brainstem responses to at-level mechanical stimulation to DHA-treated rats (Tables 2 and 3). The result showing no difference in brainstem responses to hind paw mechanical stimulation (Figs. 1G and H) was consistent with our previous findings.2

It is important to note that we assessed brainstem response–based at-level mechanical hypersensitivity 1 day before burrowing, thigmotaxis, or PEAP testing throughout the 2 regimen studies. We observed at-level mechanical hypersensitivity in all SCI rats (vs naïves, Fig. 1) before they were assessed next day for 1 of the 3 complex behaviours. However, at-level mechanical hypersensitivity was attenuated in rats receiving systemic DHA treatments (versus vehicle-controls; weeks 2-6; Fig. 1E and weeks 5-7; Fig. 1F) in the 2 regimen studies 1 day before testing for the complex behaviours.

3.3. Systemic docosahexaenoic acid treatment prevents deficit in pain-related spontaneous burrowing behaviour

Burrowing is an ethologically relevant pain-related behaviour, reproducible in different laboratory settings.1,30,49,56 The behavioural construct is that pain would interfere with a rat’s motivation to burrow, which can be measured by the amount of gravel displaced from a tube. At baseline, no difference was measured in gravel displacement in the 2 regimen studies (Figs. 2A and B). We first demonstrated a significant main effect of treatment on burrowing performances by the acute regimen (weeks 2-6; \( F_{(1,27)} = 109.338, P = 0.000001 \), two-way repeated-measures ANOVA, Fig. 2A). There was also a significant main effect of time on burrowing performances by the acute regimen (\( F_{(4,108)} = 5.214, P = 0.001 \)). However, there was no significant treatment with time interaction (\( F_{(4,108)} = 0.957, P = 0.434 \)). Compared with naïves, vehicle-treated rats developed significant burrowing deficits from week 2 which were maintained up to week 6 after surgery (Fig. 2A). By contrast, DHA-treated rats displaced significantly more gravel than vehicle-treated rats (eg, week 4: 1696.2 ± 5.8 vs 840.6 ± 7.8 g, \( P = 0.00044 \), Tukey–Kramer post hoc, Fig. 2A). Strikingly, DHA-treated rats and naïves in the acute regimen study demonstrated comparable burrowing performances (\( P = 0.18-0.99 \), Tukey–Kramer post hoc). Next, our analysis revealed a significant main effect of treatment on burrowing performances by the delayed regimen (weeks 4-7; \( F_{(1,26)} = 455.212, P = 0.0001 \), two-way repeated-measures ANOVA, Fig. 2B). However, there were no significant main effects of time on burrowing performance (\( F_{(3,78)} = 2.492, P = 0.066 \)) nor any significant treatment with time interaction (\( F_{(3,78)} = 0.467, P = 0.706 \)). Vehicle-controls maintained significant burrowing deficits during this period when compared with naïves. Delayed DHA treatment significantly attenuated the reduced burrowing performances developed before treatment when compared with vehicle-control (eg, week 6: 1366.8 ± 5.4 g vs 891.3 ± 6.1 g, \( P = 0.032 \), Tukey–Kramer post hoc, Fig. 2B), but such attenuated performances were still lower than naïves (\( P = 0.026-0.0042 \), Tukey–Kramer post hoc). Pregabalin-controls produced comparable burrowing performances in both studies to DHA-treated rats (Tables 2 and 3). These data establish that systemic DHA treatment prevents the development of affective/motivational aspects of SCI-CNP shown by burrowing behaviour.

3.4. Systemic docosahexaenoic acid treatment prevents pain-related spontaneous thigmotactic behaviour

We next tested thigmotaxis—another ethologically relevant pain-related rodent behaviour.5,30,31,42 As prey animals, rats prefer to adhere to the perimeter of a novel arena, conflicting with their drive to explore arena centre.50 The behavioural construct is that if a rat is in pain, it would be in its survival interest to decrease its exposure to predators. We assessed thigmotaxis by analyzing spontaneous free movements of rats in an open-field arena. Since this is a novel test that could be affected by repeated testing,11 rats were only tested once at week 6 and week 7 for the 2 studies, respectively. In the acute regimen study, we found...
significant treatment effects on inner zone cumulative time and entry frequency (time: $F_{(2,36)} = 39.8, P = 0.0075$, Figs. 3A–D; frequency: $F_{(2,36)} = 11.8, P = 0.0050$, Supplementary Fig. 2a; 1-way ANOVA, available at http://links.lww.com/PAIN/A853). Vehicle-control rats had significantly reduced cumulative time and entry frequency at week 6 after surgery vs naïves (time: $P = 0.00,042$, Supplementary Fig. 2a, available at http://links.lww.com/PAIN/A853; Tukey–Kramer post hoc). Docosahexaenoic acid treatment starting immediately after surgery caused significant increases in cumulative time and entry frequency at week 6 when compared with vehicle-treated rats (time: $37.2 \pm 7.2$ seconds vs $11.2 \pm 3.4$ seconds, $P = 0.0025$, Figs. 3B–D; frequency: $P = 0.003$, Supplementary Fig. 2a, available at http://links.lww.com/PAIN/A853; Tukey–Kramer post hoc); cumulative time by DHA treatment was markedly similar to naïves ($P = 0.199$, Figs. 3A, C, and D, Tukey–Kramer post hoc). We also found significant treatment effects on thigmotactic behaviour by the

### Table 2

**Behavioural outcome measures comparing pregabalin-treated animals with other experimental groups: Part A—at-level mechanical hypersensitivity, below-level mechanical hypersensitivity, and burrowing outcome measures.**

| Behavioural outcome measures | Treatment regimen | Behavioural assessment time points |
|-----------------------------|-------------------|-----------------------------------|
|                             |                   | Week 3 after surgery (mean ± SE)  | Week 6 after surgery (mean ± SE)  |
|                             |                   | Naive | DHA | Vehicle | Pregabalin | Naive | DHA | Vehicle | Pregabalin |
| At-level mechanical         | Acute             | 7.8 ± 0.2⁹ | 2.5 ± 0.2 | 1.3 ± 0.1† | 3.2 ± 0.1 | 7.5 ± 0.5* | 3.9 ± 0.6 | 1.1 ± 0.1† | 5.1 ± 0.5 |
| hypersensitivity            | Delayed           | n/a   |    |         |           | 7.9 ± 0.1* | 2.2 ± 0.3 | 1.0 ± 0.2† | 4.2 ± 0.4 |
| Below-level mechanical      | Acute             | 63.5 ± 1.3* | 55.5 ± 2.1 | 48.2 ± 1.8† | 58.0 ± 2.1 | 66.8 ± 1.5* | 57.2 ± 1.3 | 41.1 ± 1.6† | 56.6 ± 2.5 |
| hypersensitivity            | Delayed           | n/a   |    |         |           | 68.9 ± 1.9* | 47.9 ± 1.6 | 33.4 ± 1.4 | 53.0 ± 3.0 |
| Burrowing behaviour         | Acute             | 1980.7 ± 199.5§ | 1524.7 ± 72.1 | 797.9 ± 135.4† | 1379.5 ± 128.2 | 2173.0 ± 122.8 | 1816.8 ± 85.5 | 876.3 ± 74.8† | 1548.2 ± 61.3 |
|                             | Delayed           | n/a   |    |         |           | 1888.2 ± 184.2 | 1371.3 ± 97.4 | 897.4 ± 43.9† | 1600.5 ± 66.8 |

Units are 50% response threshold (g), paw withdrawal threshold (g), and gravel displaced (g) for at-level mechanical hypersensitivity, below-level mechanical hypersensitivity, and burrowing outcome measures.

One-way ANOVA followed by Tukey–Kramer post hoc; multicomparison adjustment was used for statistical analysis.

* $P < 0.05$ vs DHA, vehicle, and pregabalin.
† $P < 0.05$ vs DHA.
‡ $P < 0.05$ vs vehicle.
§ $P < 0.05$ vs naïve. Sham data are not included, as they are similar to naïve.

### Table 3

**Behavioural outcome measures comparing pregabalin-treated animals with other experimental groups: Part B—thigmotaxis and place escape/avoidance paradigm outcome measures.**

| Behavioural outcome measures | Treatment regimen | Behavioural assessment time points |
|-----------------------------|-------------------|-----------------------------------|
|                             |                   | Week 6 (acute) or week 7 (delayed) after surgery (mean ± SE) |
|                             |                   | Naive | DHA | Vehicle | Pregabalin |
| Thigmotaxis                 |                   | Time in inner zone | Acute | 42.8 ± 2.8* | 36.4 ± 3.5 | 10.2 ± 1.8† | 22.8 ± 3.8 |
|                             |                   | Delayed | 38.3 ± 2.8† | 26.6 ± 1.7 | 15.4 ± 2.2† | 26.0 ± 2.5 |
|                             |                   | Frequency into inner zone | Acute | 19.7 ± 1.1 | 19.5 ± 2.9 | 7.6 ± 2.1 | 22.0 ± 3.3 |
|                             |                   | Delayed | 19.8 ± 1.1 | 19.7 ± 2.1 | 11.7 ± 3.9† | 20.8 ± 2.7 |
|                             |                   | PEAP | Time in white zone | Acute | 529.6 ± 79.9 | 537.4 ± 46.2 | 1048.0 ± 47.4† | 608.0 ± 59.9 |
|                             |                   | Delayed | 542.0 ± 64.2§ | 762.6 ± 31.2 | 1102.3 ± 41.6† | 590.9 ± 60.7 |
|                             |                   | Frequency into white zone | Acute | 87.9 ± 12.0 | 89.7 ± 11.1 | 138.7 ± 20.3† | 89.8 ± 5.3 |
|                             |                   | Delayed | 88.4 ± 12.1§ | 113.5 ± 6.5 | 146.9 ± 12.0† | 86.0 ± 5.3 |

Units are seconds for thigmotaxis—time in inner zone and PEAP—time in white zone and entry numbers for thigmotaxis—frequency into inner zone and PEAP—frequency into white zone. One-way ANOVA followed by Tukey–Kramer post hoc; multicomparison adjustment was used for statistical analysis.

* $P < 0.05$ vs vehicle.
† $P < 0.05$ vs naïve, DHA, and pregabalin.
§ $P < 0.05$ vs DHA and pregabalin.
§ $P < 0.05$ vs naïve. Sham data are not included, as they are similar to naïve.

ANOVA, analysis of variance; DHA, docosahexaenoic acid; PEAP, place escape/avoidance paradigm.
behaviour to DHA-treated rats (Tables 2 and 3). These data suggest that systemic DHA treatment can prevent SCI-CNP at affective/decision-making levels shown by thigmotactic behaviour.

3.5. Systemic docosahexaenoic acid treatment prevents pain-related place escape/avoidance paradigm behaviour

We previously assessed cortical processing of SCI-CNP using PEAP, where rats naturally prefer to hide in the dark (naive) and avoid exposure to bright conditions (aversive). The behavioural construct is that if a rat feels pain (e.g., due to painful stimulation received at the injured area) in a dark arena, it would rather choose to enter a bright arena to avoid that stimulation. As a novel test, PEAP behaviour was only assessed at week 6 and week 7 for the 2 studies respectively, by analysing free movements of rats between the white/black zones of PEAP. We found significant treatment effects on white zone cumulative time and crossing number by the acute regimen (time: $F_{(2,38)} = 30.61, P < 0.0026$, Figs. 4A-D; number: $F_{(2,38)} = 5.78, P = 0.0083$, Supplementary Fig. 3a, available at http://links.lww.com/PAIN/A853; 1-way ANOVA). Compared with naïves, cumulative time and crossing number at week 6 after surgery were significantly increased in vehicle-treated rats (time: $P = 0.0017$, Figs. 4A, B, and D; number: $P = 0.0010$, Supplementary Fig. 3a, available at http://links.lww.com/PAIN/A853; Tukey–Kramer post hoc). Strikingly, rats receiving DHA treatment starting immediately after surgery significantly reduced their cumulative time (~47%) and crossing number (~35%) when compared with vehicle-control (time: $P = 0.0028$, Figs. 4B-D; number: $P = 0.0059$, Supplementary Fig. 3a, available at http://links.lww.com/PAIN/A853; 1-way ANOVA). As expected, we observed significantly affected PEAP behaviour in vehicle-control vs naïves at week 7 after surgery (time: $P = 0.00019$, Figs. 4E, F, and H; number: $P = 0.0010$, Supplementary Fig. 3b, available at http://links.lww.com/PAIN/A853; Tukey–Kramer post hoc). Rats receiving delayed DHA treatment significantly improved cumulative time (~26%) and crossing number (~23%) compared with vehicle-control (time: $P = 0.0011$, Figs. 4F-H; number: $P = 0.0080$, Supplementary Fig. 3b, available at http://links.lww.com/PAIN/A853; Tukey–Kramer post hoc), but their cumulative time was more than naïves ($P = 0.030$, Tukey–Kramer post hoc). Pregabalin-controls in both studies showed comparable PEAP behaviour with DHA-treated rats (Tables 2 and 3). These data indicate that systemic DHA treatment can prevent SCI-CNP at cognitive level shown by PEAP behavior.

3.6. Docosahexaenoic acid significantly reduces microglial activation in vivo

With immunohistochemistry, we revealed the effect of systemic DHA treatment on activated microglial morphology in L5 dorsal horns vs vehicle-control in the acute regimen study ($P = 0.0003$, Fig. 5A(i), (ii), (iii), Mann–Whitney test). We next measured the expression of pp-38, a hallmark for SCI-CNP. Compared with vehicle-control, pp-38+/−/Iba-1+ microglial numbers in the dorsal horns were significantly reduced by DHA treatment in the acute regimen study (L5 $P = 0.00019$, Fig. 5B(i), (ii), (iii); lesion site $P = 0.00041$, Supplementary Fig. 3a-c, available at http://links.lww.com/PAIN/A853; Mann–Whitney test). Microgliosis, a key process in neuropathic pain pathogenesis, was significantly inhibited by DHA treatment vs vehicle as shown in the numbers of ED9+/-CD11b/c+ microglia per mg cord tissue by flow cytometry in the acute regimen study (L5 $P = 0.0048$, Fig. 5C(i), (ii), (iii); lesion site $P = 0.038$, Supplementary Fig. 9d-f, available at http://links.lww.com/PAIN/A853; Student t test). In the lesion site dorsal horns, we also found significant effect of the acute DHA regimen on activated microglial morphology ($P = 0.00041$, Fig. 5D(i), (ii), (iii), Mann–Whitney test). We also saw a significant

Figure 2. Effects of systemic DHA treatments on burrowing behaviour. In the acute regimen study, DHA-treated rats had significantly increased burrowing performances in contrast to vehicle-treated rats (A) weeks 2-6, $P < 0.01$. Moreover, there were no significant differences in burrowing performances between DHA-treated and naïve rats (A) weeks 2-6, $P > 0.05$. In the delayed regimen study, burrowing performances of SCI rats receiving DHA treatment were significantly attenuated in comparison with those observed in vehicle-treated rats (B) weeks 4-7, $P < 0.05$, but still significantly lower than those of the naïve rats (B) weeks 4-7, $P < 0.05$. N = 10 to 15 per group. Two-way repeated-measures ANOVA was used to determine the main effects of treatment (DHA vs vehicle) and time (weekly). Statistical significance of the differences between the groups was determined by 1-way ANOVA followed by the Tukey–Kramer post hoc test at each week point. *$P < 0.05$, **$P < 0.01$, or ***$P < 0.001$ vs vehicle. ANOVA, analysis of variance; DHA, docosahexaenoic acid; SCI, spinal cord injury.

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reduction in Iba1-immunoreactive microglial numbers in the ACC by the acute DHA regimen compared with vehicle-control \((P = 0.0006, \text{Fig. 5E(i), (ii), (iii)}; \text{Mann–Whitney test})\). In the delayed DHA regimen study, we found that in the dorsal horns, activated microglial morphology \((L5 \ [P = 0.0005, \text{Fig. 5F(i), (ii), (iii)}]; \text{Mann–Whitney test})\) and pp-38/Iba-1 microglial numbers \((L5 \ [P = 0.00065, \text{Fig. 5G(i), (ii), (iii)}]; \text{lesion site} \ [P = 0.00048, \text{Supplementary Fig. 9a–c, available at http://links.lww.com/PAIN/A853}; \text{Mann–Whitney test})\) were significantly reduced by DHA treatment compared with vehicle-control. We also found significantly reduced numbers of ED9/CD11b/c microglia per mg cord tissue \((L5 \ [P = 0.037, \text{Fig. 5H(i), (ii), (iii)}]; \text{Mann–Whitney test})\) and pp-38+/Iba-1+ microglial numbers \((L5 \ [P = 0.00065, \text{Fig. 5G(i), (ii), (iii)}]; \text{lesion site} \ [P = 0.00048, \text{Supplementary Fig. 9a–c, available at http://links.lww.com/PAIN/A853}; \text{Mann–Whitney test})\) were significantly reduced by DHA treatment compared with vehicle-control. We also found significantly reduced numbers of ED9+/CD11b/c+ microglia per mg cord tissue \((L5 \ [P = 0.037, \text{Fig. 5H(i), (ii), (iii)}]; \text{Mann–Whitney test})\). We noted that the proportion of pp-38+/Iba-1+ microglia in rats receiving DHA treatment in the acute regimen study was much lower than that seen in the delayed regimen study: ~5% \((\text{Fig. 5B(iii)}\) vs 20% \((\text{Fig. 5G(iii)}\). These data establish that systemic DHA treatment reduces spinal and supraspinal microglial activation.

3.7. Docosahexaenoic acid significantly spares white matter in vivo

To assess the severity of the contusion SCI used in our studies and also to investigate the possible effects of systemic DHA treatments on tissue sparing that might underlie changes observed in pain-related behaviours, transverse cryostat sections from the lesion site segments were stained for myelin and white matter sparing was quantified at rostral, epicenter, and caudal locations of the segments \((\text{Figs. 6A–R})\). In the acute regimen study, large percentages of white matter were lost at the rostral, epicenter, and caudal sections of vehicle-treated animals when compared with naive \((\text{Figs. 6A–I})\). However, no difference in white matter sparing at 3 locations was observed between vehicle- and DHA-treated animals in the delayed regimen study \((\text{Figs. 6J–O})\). Strikingly, systemic DHA treatments starting immediately after surgery significantly increased the percentages of white matter at 3 locations when compared with vehicle-control \((P = 0.0000015-0.0000048, \text{Fig. 6Q}; \text{Mann–Whitney test})\) at week 6 after SCI.
3.8. Docosahexaenoic acid significantly reduces proinflammatory mediators in vivo

To further verify the effects of DHA treatment on proinflammatory and algogenic mediators iNOS, TNF-α, IL-1β, and IL-6 in the spinal cord dorsal horns at lesion site and L5 levels, we performed qPCR analysis of relative mRNA expression of these mediators. We found that at week 6 after SCI, the acute DHA treatment regimen significantly reduced mRNA expression levels of iNOS, TNF-α, IL-1β, and IL-6 compared with vehicle-control (*P = 0.0002-0.032; t tests in R, Figs. 7A–H). Moreover, we also found that delayed DHA treatment regimen starting week 4 after SCI significantly reduced mRNA expression levels of these mediators at both dorsal horn levels at week 7 when compared with vehicle-control (*P = 0.048, t tests in R, Figs. 7I–P).

3.9. Docosahexaenoic acid significantly reduces microglial activation in vitro

We also investigated the effects of DHA on modulating microglial activation in vitro. In experiments where cells were cotreated with activation agent LPS and DHA (0.8, 4, 8 or 32 μM) for 4 hours, we found 0.8 μM as the optimal concentration to reduce iNOS.
immunoreactivity ($F_{(5,50)} = 76.15, P = 0.00024$, 1-way ANOVA, Supplementary Fig. 4a, available at http://links.lww.com/PAIN/A853) (Figs. 8A(i), (ii), (iii), and B(i), (ii), (iii)). With qPCR, we found that DHA significantly reduced microglial mRNA expression of algogenic mediators iNOS, TNF-α, IL-1β, and IL-6 compared with control ($P = 0.0091, 0.0006, 0.024$, and 0.026; t tests in R, Fig. 8C(i), (ii), (iii), (iv)). Using the more sensitive flow cytometry, we confirmed the ability of DHA to reduce microglial activation, ie, number of proinflammatory CD11b/c+/ED9+ microglia in L5 spinal cords, comparing with vehicle treatments; C(i–ii) and H(i–ii) = examples of flow cytometry data from vehicle- and DHA-treated rats. Finally, both DHA regimens (E(i–ii) and J(i–ii)) led to marked reductions in Iba-1 immunoreactive microglia in the ACC, in contrast to vehicle treatments. N = 5 to 6 per group. The Mann–Whitney test and unpaired Student t test were used where appropriate. K is a schematic drawing of the lesion site segment from where lesion site DHs were analyzed for microglial Iba-1 staining. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs vehicle. ACC, anterior cingulate cortex; DHA, docosahexaenoic acid.

Figure 5. Effects of systemic DHA treatments on microglial activation in vivo. Both regimen studies showed significantly reduced activated microglial morphology per 50,000 μm$^2$ by DHA treatments in L5 (A(i–iii) and F(i–iii)) and lesion site (D(i–iii) and I(i–iii)) dorsal horns (DH), comparing with vehicle treatments. Both DHA regimens led to significant reductions of microglial pp38 immunoreactivity in L5 dorsal horns, comparing with vehicle treatments (B(i–iii) and G(i–iii); white arrows = Iba-1/pp38 double-immunoreactive microglia). Flow cytometry further showed that both DHA regimens (C(i–iii) and H(i–iii)) significantly reduced microgliosis, ie, the numbers of proinflammatory CD11b/c+/ED9+ microglia in L5 spinal cords, comparing with vehicle treatments; C(i–ii) and H(i–ii) = examples of flow cytometry data from vehicle- and DHA-treated rats. Finally, both DHA regimens (E(i–ii) and J(i–ii)) led to marked reductions in Iba-1 immunoreactive microglia in the ACC, in contrast to vehicle treatments. N = 5 to 6 per group. The Mann–Whitney test and unpaired Student t test were used where appropriate. K is a schematic drawing of the lesion site segment from where lesion site DHs were analyzed for microglial Iba-1 staining. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs vehicle. ACC, anterior cingulate cortex; DHA, docosahexaenoic acid.
significantly reduced numbers of iNOS+/Iba1+ microglia (Fig. 8E) and nitrite release by microglia (Fig. 8F) when cells were cotreated with LPS and DHA. Further experiments where cells were preactivated by LPS for 4 hours and followed by DHA (0.8 μM) treatment for another 4 hours showed similar effects on iNOS expression (Figs. 8G(i), (ii), (iii), and H(i), (ii), (iii)), mRNA expression of iNOS, TNF-α, IL-1β, and IL-6 (Fig. 8I(i), (ii), (iii), (iv)), and CD86+/CD11b/c+ number (Fig. 8J(i), (ii), (iii), (iv)), further validating the ability of DHA to modulate microglial activation.

3.10. In vitro evidence of potential mechanisms of docosahexaenoic acid on microglial activation

Microglia express PPARγ and RXR receptors, which are known to involve in inflammation and can be bound by DHA. Therefore, we used antagonists to preblock PPARγ or RXR or both for 4 hours in cultured microglia, which were then cotreated with LPS and DHA (0.8 μM) for 4 hours. With qPCR, we found that preantagonism of each receptor alone caused significantly higher mRNA expression of iNOS, TNF-α, IL-1β, and IL-6 (P = 0.0012-0.0020; t tests in R; Figs. 9A, B, and D). These data suggest that DHA could act through these receptors to manifest its effects on reducing microglial activation. We finally investigated DHEA, a known DHA metabolite that can bind to PPAR and cannabinoid receptors on microglia, by cotreating cultured microglia with LPS and DHEA for 4 hours. We saw a significant decrease in microglial mRNA expression of iNOS, TNF-α, IL-1β, and IL-6 by DHEA (5 μM) when compared with control (P = 0.0001-0.026; t tests in R; Figs. 9E–H), suggesting that DHEA could partially mediate the effects of DHA on microglial activation.

4. Discussion

SCI-CNP is a debilitating complication without effective treatments. SCI-CNP are needed urgently. Here, we reported that systemic administrations of the natural compound DHA, starting immediately after injury and then maintained for 6 weeks, successfully prevented the development of pain-related spontaneous/affective and cognitive behaviours coupled with significantly attenuated at-level mechanical hypersensitivity in a clinically relevant rat model of SCI-CNP. Similar DHA treatment, given at a chronic SCI stage when CNP had already been established, partially abolishes pain-related spontaneous/affective and cognitive behaviours coupled with significantly attenuated at-level mechanical hypersensitivity in a clinically relevant rat model of SCI-CNP.
cognitive behaviours. Because our data were obtained using a novel sensory profiling approach combining evoked pain measures and non-evoked pain-related spontaneous and/or natural rodent behaviours to mimic the clinical practice, they have significant implications for clinical translation.

4.1. Docosahexaenoic acid reduces neuroinflammation that underpins SCI-CNP pathogenesis

Neuroinflammation plays a crucial role in SCI-CNP. Microglia become chronically activated in the lesion site and L5 dorsal horns as well as supraspinal areas, subsequently activating ERK1/2, p-38 mitogen-activated protein kinase (MAPK), and CAMII pathways that are known to be important for SCI-CNP development. The activation of p-38 MAPK, a hallmark for SCI-CNP, causes the release of proinflammatory mediators, including iNOS, TNF-α, IL-1β, and IL-6, which are significantly reduced by DHA treatment.

Figure 7. Effects of systemic DHA treatments on the production of proinflammatory mediators in the dorsal horns of lesion site and L5 spinal cords. Quantitative real-time polymerase chain reaction analysis showed that iNOS, TNF-α, IL-1β, and IL-6 mRNA expression levels were significantly reduced at week 6 after the acute DHA treatment regimen (A–H) and at week 7 after the delayed DHA treatment regimen (I–P), when compared with the vehicle groups. The relative mRNA expression levels of these proinflammatory mediators were similar between DHA-treated and naive animals. N = 4 to 5 per group. Statistical analysis was performed by t-tests in R. *P < 0.05, **P < 0.01, ***P < 0.001 vs vehicle-treated animals. DHA, docosahexaenoic acid; iNOS, inducible nitric oxide synthase; TNF-α, tumour necrosis factor-α.

4.2. Docosahexaenoic acid’s effects on dorsal horn neuronal hyperexcitability

Electrophysiological studies show that SCI results in neuronal hyperexcitability in the dorsal horns at and below the injury level,
which mediates enhanced nociceptive processing in pathological pain states. Particularly, pp-38 MAPK plays a pivotal role underlying dorsal horn neuronal hyperexcitability as discussed above. Thus, increased microglial pp-38 MARK levels correlate with dorsal horn neuronal hyperresponsiveness and CNP after contusion SCI in adult rats. Moreover, minocycline and pp-38 inhibitor SB203580 significantly reduce dorsal horn microglial pp-38 MAPK, which results in attenuated dorsal horn neuronal

Figure 8. Docosahexaenoic acid modulates microglial activation in vitro. Microglia, simultaneously treated (ST) with DHA and LPS, preserved resting bipolar morphology (A(iii)) seen with the controls (A(i)), contrasting to activated morphology seen with LPS only (A(ii)). Docosahexaenoic acid in ST experiments reduced iNOS expression, mRNA expression levels of iNOS, IL-6, TNF-α, and IL-1β, and nitrite releases by microglia, comparing with LPS only (B(ii–iii), C(i–iv), E–F). Microglia with delayed DHA treatment (DT) after 4 hours of LPS pre-exposure (G(iii)) reversed activated morphology seen with LPS only (G(ii)). DHA in DT experiments also reduced iNOS expression, mRNA expression levels of iNOS, IL-6, TNF-α, and IL-1β, and nitrite releases by microglia, comparing with LPS only (H(ii–iii), I(i–iv), K–L). Flow cytometry further verified that the numbers of proinflammatory CD11b/c^+CD86^+ microglia were significantly reduced by DHA in ST and DT experiments, comparing with LPS only (J(i–iv), K(i–iv)). N = 3 biological replicates/n = 9 technical replicates per group. The unpaired Student t test and t tests in R were used where appropriate. *P < 0.05, **P < 0.01, ***P < 0.001 vs LPS-treated microglia. DHA, docosahexaenoic acid; DT, delayed treatment; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharides; TNF-α, tumour necrosis factor-α.
hyperexcitability. The downstream pathways activated by microglial pp-38 MAPK lead to the release of numerous proinflammatory mediators that can bind to and activate dorsal horn neuronal membrane receptors and/or ion channels, triggering neuronal pp-38 MAPK-CREB-ERK pathway that causes persistent neuronal hyperexcitability. Among those mediators, TNF-α, IL-1β,
and IL-6 produced by microglia after SCI are known to produce hyperactivity of dorsal horn neurons and contribute to CNP development and maintenance.17,40 Our results demonstrated that DHA significantly reduced microglial pp-38 MAPK immuno-reactivity in lesion site/L5 dorsal horns, which is mirrored by a significant reduction of mRNA levels of iNOS, TNF-α, IL-1β, and IL-6 in lesion site/L5 dorsal horns. These data strongly suggest that DHA might have stopped the positive feedback cycle between microglia and dorsal horn neurons that maintains persistent neuronal hyperexcitability, leading to the prevention of CNP seen in the acute study and partial reversal of CNP seen in the delayed study. Here, we did not assess neuronal/astrocyte pp-38 MAPK expressions, which also contribute to dorsal horn neuronal hyperexcitability after SCI.10,25 Because DHA could interact with receptors and ion channels on dorsal horn neurons and astrocytes as discussed below, we suggest that DHA treatments may have also reduced neuronal/astrocyte pp-38 MAPK levels in lesion site/L5 dorsal horns.

4.3. Mechanisms of docosahexaenoic acid’s action in reducing neuroinflammation

Recent evidence shows that dietary DHA supplementation for 60 days leads to increased DHA levels in the retina, cortex, hypothalamus, and hippocampus in adult rats.46 Other evidence shows the incorporation of intravenously injected radiolabeled DHA into the rat brain.45 Therefore, we are confident that DHA in our studies would have reached the spinal cord of SCI rats. Docosahexaenoic acid’s effects on attenuating microglial/astrocyte activation are likely mediated by DHA binding to and activating PPAR and RXR receptors as their natural ligand,12–14 since these receptors are expressed by microglia18,51 and astrocytes.18,51 This is supported by our evidence demonstrating that preinhibiting these receptors reduced DHA’s ability to decrease mRNA levels of pain-producing proinflammatory mediators in cultured microglia coexposed to LPS. It is possible that DHA’s effects on microglial activation could be through its metabolite DHEA,19 an endocannabinoid, which can bind to PPAR and cannabinoid receptors4,6,19,41 on microglia. This notion is supported by our data showing that DHEA significantly reduced mRNA levels of proinflammatory mediators in cultured microglia coexposed to LPS. Future studies are needed to measure DHA/ DHEA levels in the spinal cord and explore the full role of DHEA on microglial activation which is beyond the scope of current studies.

4.4. Docosahexaenoic acid’s unique potential to target multiple mechanisms underlying SCI-CNP

We suggest that DHA’s effects on SCI-CNP could be attributed to its neuroprotection of neurons and oligodendrocytes,32 which was supported by improved locomotor function in both regimen studies and increased white matter sparing in the acute regimen study. Thus, DHA may interact with glutamate receptors32 and voltage-gated sodium/calcium channels32 on dorsal horn neurons, which are known to play roles in neuronal hyperexcitability. This makes DHA a promising treatment for SCI-CNP, as it can target multiple mechanisms underlying SCI-CNP.

4.5. A novel sensory profiling approach mimics clinical practice

Our work is timely in addressing a recent call for the use of sensory profiling in animal models.46 The preclinical literature on SCI-CNP mainly relies on spinal reflex-based withdrawal paradigms, which hardly reflect the affective, cognitive, and spontaneous aspects of pain and have minimal clinical validity. Our previous and current work showed that below-level SCI-CNP in rats, measured by mechanically evoked hind paw withdrawal thresholds, did not involve brainstem responses, which is likely due to the presence of spastic syndrome.32 Here, we combined at-level mechanical hypersensitivity basing on brainstem responses with pain-related spontaneous and/or natural rodent behaviours addressing the motivational, affective, and cognitive components of pain. These pain-related behaviours included burrowing, thigmotaxis, and PEAP, which are ethologically relevant and reproducible in different laboratory settings. Most importantly, they mimic those used for measuring the impact of pain and spontaneous component of pain in clinical settings. Such an approach would significantly add translational benefit for new drug development for SCI-CNP.

Consistent with our previous reports,2,3 we observed that all rats with the injury severity adopted here developed brainstem response-based at-level mechanical hypersensitivity. Therefore, our SCI-CNP model does not reflect SCI-CNP prevalence seen in human patients. It is known that different injury severities and injury devices used can influence SCI-CNP prevalence in animals.33 Similar situations also exist in common preclinical models of peripheral nerve trauma-induced neuropathic pain and drug-induced peripheral neuropathies, all of which are instrumental in understanding the pathophysiological mechanisms and developing novel analgesics for neuropathic pain.

4.6. Docosahexaenoic acid’s translational potential for SCI-CNP management

We suggest that the 2 DHA regimens could be readily translated to the clinic because DHA’s clinical safety and tolerability is well documented. Docosahexaenoic acid–containing fish oil capsules are available for public consumption. Other DHA-containing preparations are already used in clinical settings involving surgical and critically ill patients.8 A recent 14-month trial with consumption of DHA-containing capsules in patients with chronic SCI did not find neuroprotective effects, but proved DHA’s long-term patient safety.34 In our studies, burrowing behaviour, which is conserved in rodents and reflects their general well-being, was significantly improved in DHA-treated rats, further demonstrating the safety and translational potential of DHA for SCI-CNP management in the clinic. We suggest that future studies should investigate the pharmacokinetics of DHA, the optimal dose, best administration route (eg, i.v. vs dietary supplementation), and formulation (eg, free fatty acid format used here vs lipid emulsions) for systemic DHA treatment for SCI-CNP and to establish whether the effects on SCI-CNP by the 2 DHA regimens would continue if treatments stop, which are beyond the current scope. Further discussion on DHA’s preclinical translation addressing pharmacokinetics and relevance of concentrations to target pharmacology can be found in “Supplementary Discussion” section.

In summary, our studies using clinically relevant animal modelling revealed huge potential of systemic DHA treatments for not only preventing SCI-CNP but remarkably also for treating already established SCI-CNP. Considering the unmet clinical need for SCI-CNP, our strategies involving systemic DHA treatments represent one of the most promising approaches available for SCI-CNP management. These strategies also could benefit millions of patients who suffer from CNP after other CNS injuries and diseases including brain trauma, stroke, and multiple sclerosis.
Conflict of interest statement

N.B. Finnerup has received honoraria for serving on advisory boards or speaker panels from Teva, Novartis, Astellas, Grünenthal, Mitsubishi Tanabe, Novartis, and Teva Pharmaceuticals outside the submitted work. The remaining authors have no conflicts of interest to declare.

Part of the work was presented as abstracts for World Congress on Pain (Yokohama, Japan, 26-30th September, 2016), International Congress on Neuropathic Pain (Gothenburg, Sweden, 15-18th June, 2017), and SNF Annual Meeting (San Diego, CA, 3-7th November, 2018).

Acknowledgments

The authors thank Ms Leilani Barry and Ms Hristina Stefanova for their assistance with immunocytochemistry and qPCR work. The authors thank Dr Franziska Denk for her kind advice on in vivo tissue qPCR. We are grateful of Prof Colin McCaig for his critical and constructive review of the manuscript. We thank the Development Trust of the University of Aberdeen and the Scottish Rugby Union for their financial support. Part of the revision work was supported by a Science Initiatives Panel Award by the Institute of Medical Sciences of the University of Aberdeen.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A853.

Supplemental video content

A video associated with this article can be found at available online at http://links.lww.com/PAIN/A854.

Article history:

Received 25 February 2019
Received in revised form 17 June 2019
Accepted 9 July 2019
Available online 29 July 2019

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