The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

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	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Title page
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Main text Page 1
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Main text Page 2-3 Main text Page 3
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Main text Page 3
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Main text Page 4
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Suppl. M&M: Pages 2-3 Page 4 Pages 2-3 Main text Fig. 1
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Main text: Pages 4-8 Pages 4-8 Pages 4-8 Pages 4-8
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Main text: Page 4 Page 4

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	Main text: Page 4 Page 4 Page 4
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	Suppl. M&M: Pages 2-3 Page 5 Main text:
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	Suppl. M&M: Page 4
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Suppl. M&M: Page 4
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Main text: Pages15-16
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Main text: Pages 17-22 Figs. 1-4
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	Suppl. M&M: Pages 2-4 Suppl. M&M:
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Main text: Pages 17-25
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	Suppl. M&M: Page 4 N/A
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	Main text: Pages 26-30 Pages 29-30 N/A
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Main text: Pages 29-30
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Main text: Page 31

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.

Supplementary Materials

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

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1. Materials and methods:

1.1 *In vivo* study designs

Supplementary Table 1: Details of groups, animal numbers in treatment groups and primary outcomes for behavioural experiments

Experiment Description	Study type	Groups	Group size		Timeline of behavioural assessment	Primary behavioural outcomes
			Initial	After exclusion		
BBB	Acute DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/9/15/14/12	D1, D3, D5, D7, W2, W3, W4, W5, W6	Locomotor functional recovery
	Delayed DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/10/14/14/12	D1, D3, D5, D7, W2, W3, W4, W5, W6, W7	
At-level mechanical hypersensitivity	Acute DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/9/15/14/12	W2, W3, W4, W5, W6	Brainstem response (licking guarding, biting, vocalizing, jumping) in response to static mechanical stimulus applied at the level of the injury
	Delayed DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/10/14/14/12	W2, W3, W4, W5, W6, W7	
Below-level mechanical hypersensitivity (spinal reflex)	Acute DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/9/15/14/12	W2, W3, W4, W5, W6	Hind-paw withdrawal threshold in response to static mechanical stimulus applied on the later plantar surface of the foot
	Delayed DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/10/14/14/12	W2, W3, W4, W5, W6, W7	
Below-level mechanical hypersensitivity (brainstem response)	Acute DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/9/15/14/12	W2, W3, W4, W5, W6	Brainstem response (licking guarding, biting, vocalizing, jumping) in response to static mechanical stimulus applied at the lateral plantar surface of the foot
	Delayed DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/10/14/14/12	W2, W3, W4, W5, W6, W7	
Burrowing	Acute DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/9/15/14/12	W2, W3, W4, W5, W6	Grams of displaced gravel from the burrows
	Delayed DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/10/14/14/12	W2, W3, W4, W5, W6, W7	
PEAP	Acute DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/9/15/14/12	W6	Frequency of crossing to and time spent in the white zone
	Acute DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/10/14/14/12	W7	

Thigmotaxis	Delayed DHA regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/9/15/14/12	W6	Frequency of crossing and time spent in the virtual inner zone
	Delayed regimen	Naïve/ Sham/ DHA/ Vehicle/ Pregabalin	10/10/15/15/12	10/10/14/14/12	W7	

D – Days post-injury / W – Weeks post-injury.

Supplementary Table 2: Major domains of good laboratory practice to minimise the effects of experimental bias.

	Description of procedures
Sample Size Calculation	• Group size was determined by sample size estimation for each experiment using SigmaStat Version 3.5 (ANOVA sample size, desired power = 0.8, alpha = 0.05). • Effect sizes for estimation were derived from our previous studies (see below Table S3).
Inclusion and Exclusion Criteria	• In experiments for at-level brainstem response, rats that developed a decrease in the withdrawal threshold less than 70% from baselines were excluded. • For burrowing, rats that burrowed less than 500 g during training and at baselines were excluded. For PEAP, rats that spend less than 20% time in the white zone were excluded. • For locomotor recovery, rats which scored 2 or more points on the BBB scale at day 0 post-injury were excluded. Rats that did not reach a point of 9 on the BBB scale by day 14 were also excluded. All rats excluded from BBB assessment were also excluded for Horizontal ladder and Burrowing tests.
Randomization	• Animals were randomly assigned to sham, SCI vehicle-treatment, or SCI DHA-treatment groups using random allocation software [10].
Allocation Concealment	• The person performing SCI/sham surgeries and administering DHA/Pregabalin/vehicle was unaware of the allocation to treatment group*. • This was achieved by the blinding procedure described below.
Reporting of Animals Excluded from Analysis	• Rats showing marked behavioural changes, exudates around wound, or sensitivity to palpitation on handling attributable to surgery, drug, dosing procedure, infection from surgery were excluded. • Rats with significant surgical complications or whose general health deteriorates were excluded. • Any rat which showed a surgery-related weight of loss equal to more than 25% of the body weight pre-injury was excluded. • The details of the number of excluded animals and the reason for exclusion are stated in Supplementary Table 1.
Blinding procedures	• Codes were assigned to different treatments by an independent person and kept in a sealed envelope. The codes were not broken until the analysis had been completed. • The experimenter was 'blinded' to the treatments received and had no knowledge of the experimental group to which an animal was randomized.

Animal exclusion: In the acute DHA regimen study, the following animals were excluded/culled: 1x sham-operated due to fluid build-up under the surgical incision and 1x vehicle-treated rat due to non-injury/surgically related fluid build-up in the lungs; In the delayed DHA regimen study the following animals were excluded/culled: 1x DHA-treated rat due to non-injury/surgically related foot wound and

1x vehicle-treated rat due to non-injury/surgically related swollen and blocked gut. All conditions were reported to the animal facility vets and NACWO and registered with the Home Office.

Supplementary Table 3: Sample size estimation.

Sample size	15
Difference in means	19
Standard deviation	17.1
Number of groups	5
Power	0.80
Alpha	0.05

Based on Figure 7B (Baastrup *et al*., 2010, Pain 151:670-79)

1.2. Locomotor functional recovery

The recovery in the locomotor function of the rats following SCI was assessed by the commonly employed open field BBB scale [1]. Briefly, the BBB scoring scale used exclusively for the assessment of rat locomotor recovery, ranged between 0-21 points depending on the movements that the animal is able to perform, zero being complete paralysis and 21 for normal walking. A milestone in the rat's locomotor recovery is the score of 9 when it is able to support its body weight using its hind limbs, but still without having the ability to step. A score of 14 is another milestone in the functional recovery, when the rat is able to step in a coordinated manner.

Prior to surgeries the rats were acclimatized to a circular non-slip open field arena with 100 cm diameter and 60 cm height for three consecutive days and then baselines were taken. Each animal was allowed to

walk freely in the arena for 4 minutes, while two observers assessed the rat's joint movements and walking abilities, and took notes of the performance in pre-designed BBB scoring template sheets. Following SCI surgeries hind limb paralysis was confirmed for each animal after its recovery from anesthesia. BBB assessment was performed at days 1, 3, 5, 7 after injury and weekly thereafter until the end of the studies. Analysis of the recorded scores were performed blindly by another member of the laboratory group in reference to the BBB scale descriptions [1].

1.3. Greiss assay

In order to measure the release of nitrite in the medium of microglia cells activated with LPS or receiving LPS plus simultaneous or delayed DHA treatment, we performed a Greiss assay, as described previously [11]. Briefly, following treatments the medium in which the cells were incubated for the particular duration of time was collected in sterile Eppendorf tubes and stored at -80ºC. Greiss assay reagents were prepared as follows: Greiss reagent A (1% sulphanilamide (1mg; Sigma, UK) in 5% orthophosphoric acid (100 ml; Sigma, UK); Greiss reagent B (0.1% naphthylethylenediamine (100 mg; Sigma, UK) and 5% orthophosphoric acid (100 ml; Sigma, UK). Stock solution of 1 mM sodium nitrite ($Na\text{-}NO_2$, 69.00 g/mol) was prepared by dissolving 0.345 mg sodium nitrate (Sigma, UK) in 5 ml serum free DMEM media. Standard curve of sodium nitrite from 0-100 μM was prepared in base media. The media samples were defrosted. Standard curve and test sample were aliquoted in 100 μl triplicates into a 96-well plate. The Greiss reagent A and B were mixed in a 1:1 ration and 100 μl were added to each well. The samples were immediately quantified for change in absorbance at 544 nm using the Galaxy Fluorstar spectrophotometer (BMG Labtech, UK).

2. Supplementary results:

2.1. DHA treatment regimens result in improved locomotor functional recovery

The neuroprotective effects of DHA and its potential to improve locomotor functional recovery in different animal models of SCI, such as the static compression model, have been previously reported [4; 5; 8]. Thus, we used the BBB locomotor scale, as described in the literature [5; 7] to follow up the locomotor functional recovery of the rats with SCI. During the baseline measures, rats from different experimental groups obtained a score of 21 in accordance to the BBB scale (Supplementary Fig. 1a-b), reflecting on their unimpaired locomotor abilities. Following surgeries, rats with SCI developed significant locomotor disability in comparison to the naïve and sham operated animals, which was evident by marked reductions in the BBB scores in a range between 0 and 2 points in the first 3 days (Supplementary Fig. 1a-b). The locomotor deficits of SCI rats receiving vehicle treatment were continuous through the duration of both studies. Our analysis showed that there was a significant main effect of treatment on the BBB scores by the acute DHA treatment regimen ($F_{(1,27)}$=40.52, p=0.000012, two-way repeated-measures ANOVA), in which animals receiving DHA treatment significantly improved their BBB scores (i.e. more than 2 scores) when compared to those of SCI rats treated with vehicle, from week 2 post-surgery onwards (Supplementary Fig. 1a). We also found a significant main effect of time on the BBB scores ($F_{(8,216)}$=1196.44, p=0.0000001) and a significant treatment with time interaction ($F_{(8,216)}$=9.52, p=0.0011). In order to provide evidence that the improved locomotor recovery following SCI of the rats treated with DHA is a result of the neuroprotective properties of the compound, we collected spinal cord longitudinal sections from naïve, SCI vehicle-treated, and SCI DHA-treated rats and performed an immunostaining against GFAP and Laminin (an extracellular matrix marker), in order to assess the lesion cavity appearance. We demonstrated that the lesion cavities of rats that were given the acute DHA regimen appeared to be smaller than those of SCI rats treated with vehicle and the accumulation of laminin around the injury site also appeared to be reduced (Supplementary Fig. 7b, c).

We also assessed whether the delayed DHA treatment regimen would have an effect on the locomotor recovery of rats with SCI. Two-way repeated-measures ANOVA showed significant main effects of

treatment ($F_{(1,26)}$=13.75, p=0.001) and time ($F_{(3,78)}$=60.89, p=0.000057) on the BBB scores by the delayed DHA treatment regimen. Moreover, the analysis showed a significant treatment with time interaction ($F_{(3,78)}$=10.99, p=0.0017). We found that delayed DHA treatment led to significant improvement of locomotor functional recovery of SCI rats in weeks 6 and 7 post-surgery, when compared to that of vehicle-treated SCI rats (Supplementary Fig. 1b; p=0.0001, Tukey–Kramer *post hoc*). Our immunohistochemistry of GFAP and laminin staining did not show that there appeared to be any differences in the lesion cavity size of rats receiving delayed DHA treatment and rats receiving vehicle treatment following SCI surgeries (Supplementary Fig. 7d, e).

2.2. Effects of locomotor functions on complex pain-related outcome measures

We used BBB locomotor scale to monitor the hindlimb locomotor function on a weekly basis from week 2 post-surgery throughout the two DHA treatment regimen studies. We found that the locomotor functional recovery is in line with the ability of animals to enter the burrows. We observed that at week 2 post-surgery in the acute regimen study, vehicle-treated SCI rats already achieved occasional weight-supported plantar stepping, and continued to improve with frequent to consistent weight-supported plantar stepping from week 3 onwards; whilst DHA-treated SCI rats already achieved frequent to consistent weight-supported plantar stepping at week 2 (Supplementary Fig. 1). In the delayed regimen study, all SCI rats reached occasional weight-supported plantar stepping at week 2 and frequent to consistent weight-supported plantar stepping from week 3 onwards (Supplementary Fig. 1). Therefore, the locomotor recoveries from both regimen studies allowed all SCI rats from week 2 onwards to enter the burrows without difficulty and we found that SCI rats used both forepaws and hindpaws to displace gravels. For thigmotaxis and PEAP, all SCI rats achieved consistent weight-supported stepping at week 6 or week 7, therefore they can readily explore the open field arena and white/black zones.

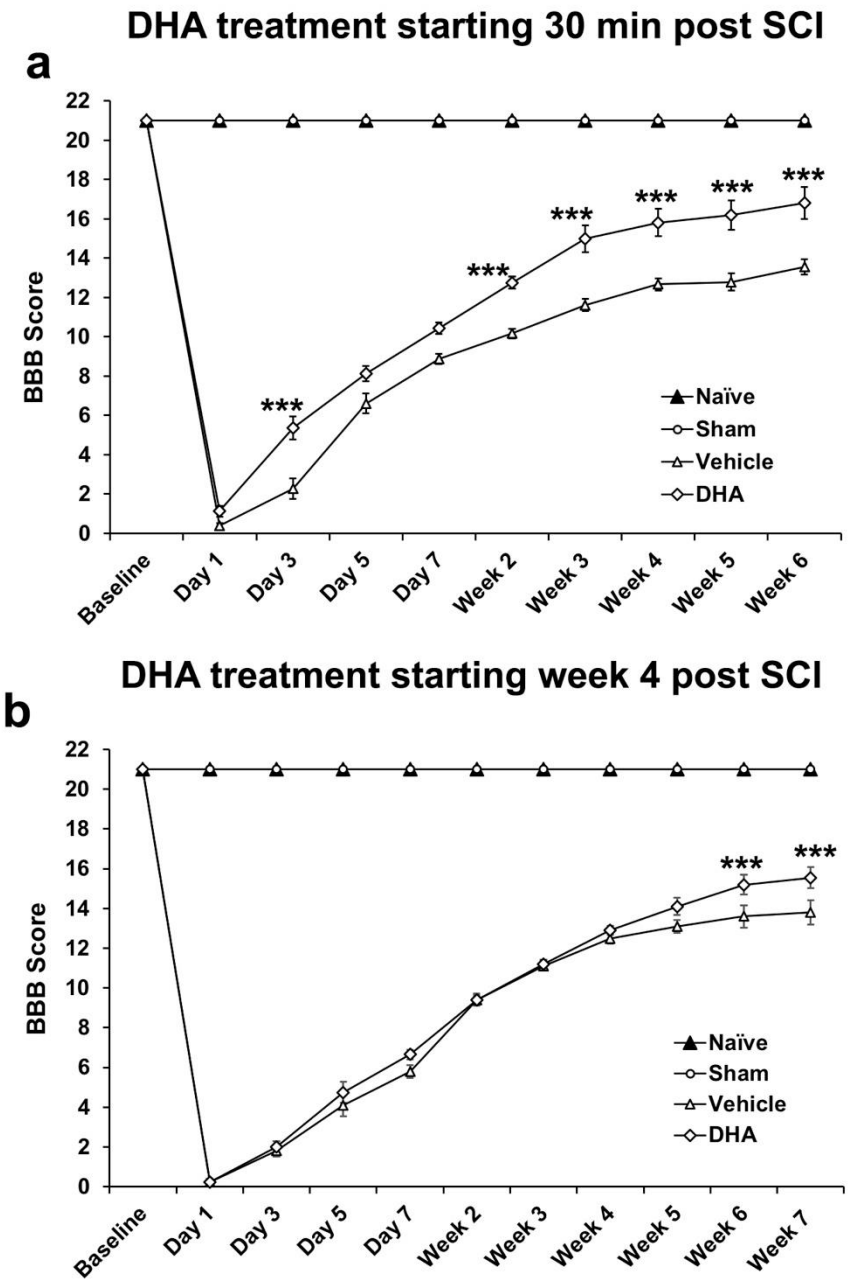
2.3. DHA significantly decreases the activation of astrocytes *in vitro* and *in vivo*

We also investigated the effects of DHA on astrocyte activation. DHA (0.8 μM) was found as the most efficacious in reducing GFAP-expression ($F_{(5,68)}$=44.25, p=0.00045, one-way ANOVA; Supplementary Fig. 4b) in cultured astrocytes co-treated with LPS and DHA for 4 hours; DHA also preserved non-reactive morphology (Supplementary Fig. 5). With qPCR, we showed that mRNA expression of iNOS, TNF-α, IL-1β, and IL-6 in cultured astrocytes was significantly reduced by DHA when compared to control (co-treatment with LPS/DHA: p=0.0009-0.0021; pre-activation with LPS followed by DHA treatment: p=0.0010-0.0054; t-tests in R; Supplementary Fig. 6).

With immunohistochemistry, we then elucidated that GFAP immunoreactivity in the dorsal horns and ACC was significantly reduced by systemic DHA treatments compared to vehicle-control [acute regimen: L5 (p=0.00017, Supplementary Fig. 7Ai-iii), lesion site (p=0.00036, Supplementary Fig. 7Bi-iii), ACC (p=0.00091, Supplementary Fig. 7Ci-iii); delayed regimen: L5 (p=0.0035, Fig. Supplementary Fig. 7Di-iii), lesion site (p=0.00019, Supplementary Fig. 7Ei-iii), ACC (p=0.0038, Supplementary Fig. 7Fi-iii); Mann–Whitney test].

Supplementary Figures:

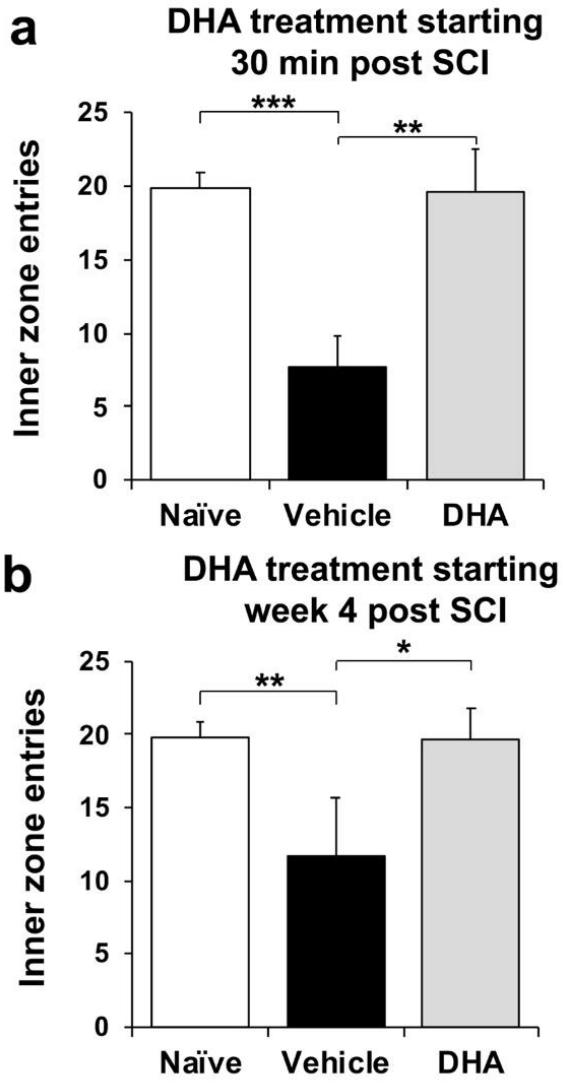
Supplementary Fig. 1



Supplementary Fig. 1. Effects of systemic DHA treatment on locomotor function recoveries. BBB locomotor assessment revealed that: (a) DHA treatment administered systemically every 3 days starting 30 minutes post-surgery (acute administration) significantly improved the locomotor functional recoveries of SCI rats between week 2 and week 6, when compared to those of the vehicle-treated group.

(b) DHA treatment administered systemically every 3 days starting 4 weeks post-surgery (chronic administration) improved the functional recoveries of SCI rats in week 6 and week 7, when compared to those of the vehicle-treated group. Each value is the mean ± SEM. N= 10-15 per treatment 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 one-way ANOVA followed by Tukey–Kramer *post hoc* test at each week point; ***$p<0.001$ *vs* vehicle.

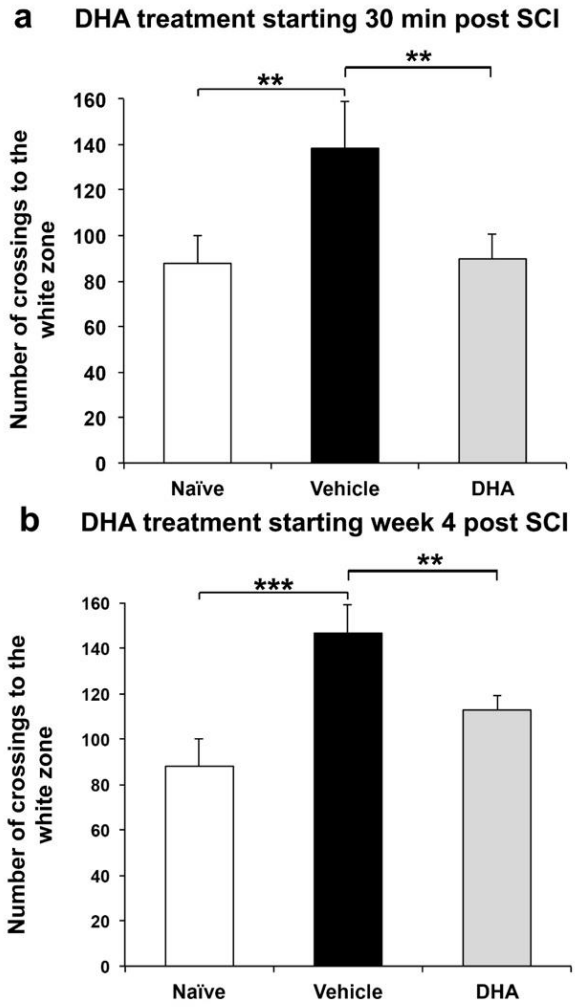
Supplementary Fig. 2



Supplementary Fig. 2. The effect of systemic DHA treatment on the frequency of entry in the virtual inner zone during the assessment of thigmotactic behaviour at week 6 and week 7. (a) The number of entries to the virtual inner zone of SCI rats at week 6 post-surgery was markedly decreased, when compared to that of the naïve group. DHA treatment in SCI rats commencing 30 minutes post-surgery and continued every 3 days thereafter for 6 weeks, significantly increased the frequency of crossing into the inner zone, when compared to that of vehicle-treated SCI rats. (b) SCI rats at week 7 post-injury crossed the virtual inner zone significantly fewer times than naïve rats. Delayed DHA

treatment in SCI rats administered 4 weeks post-surgery and its continued administration every 3 days thereafter for 4 weeks, significantly increased the frequency of entries into the inner zone, when compared to that of vehicle-treated SCI rats. Data are presented as the mean ± SEM. N=10-15 per treatment group. One-way ANOVA and Tukey–Kramer *post hoc* test were used for statistical analysis; *p<0.05, **p<0.01, or ***p<0.001 *vs* vehicle.

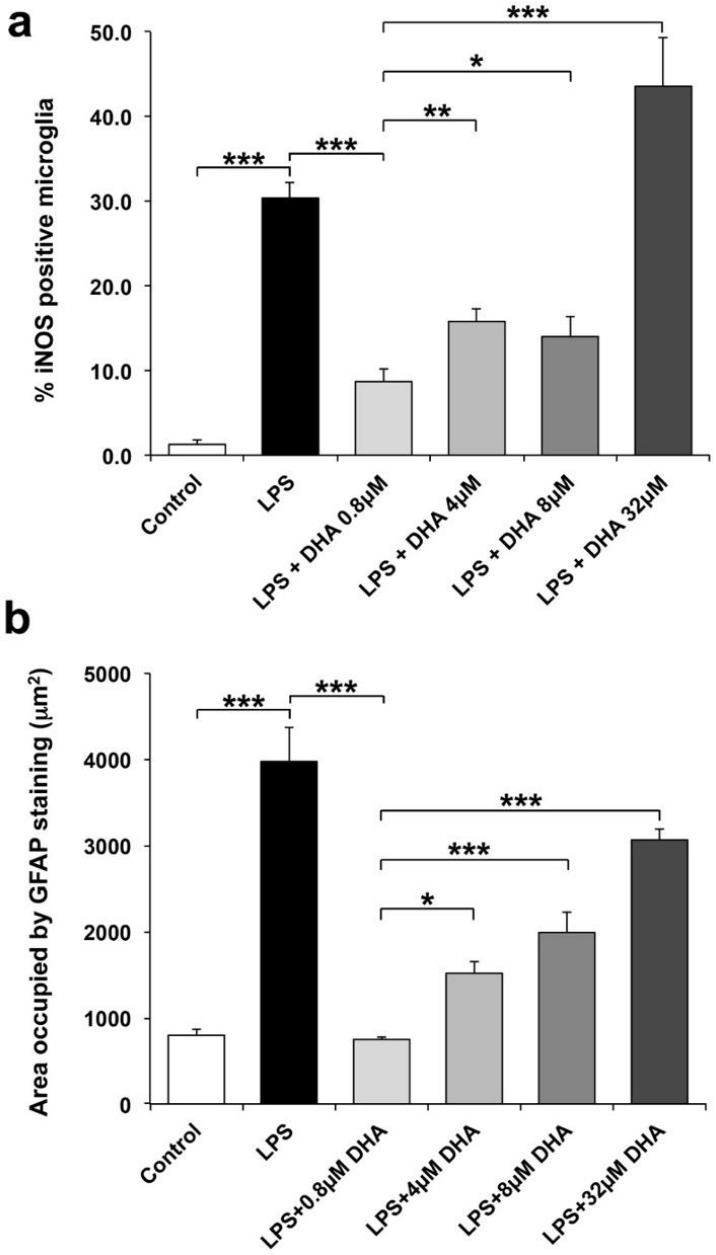
Supplementary Fig. 3



Supplementary Fig. 3. The effect of systemic DHA treatment on the number of crossing to the white zone of the PEAP box at week 6 and week 7. (a) The number of crossings to the white zone of the PEAP box of SCI rats at week 6 post-surgery was significantly increased, when compared to that of the naïve group. DHA treatment administered acutely post-surgery, significantly decreased the frequency of crossing into the white zone of the arena, when compared to that of vehicle-treated SCI rats. (b) The frequency of crossing of SCI rats into the white zone of the PEAP box at week 7 was notably increased when compared to that of naïve rats. Delayed systemic DHA treatment significantly decreased the number of entries of SCI-rats into the white zone, when compared to that of vehicle-treated SCI rats.

Data are presented as the mean ± SEM. N=10-15 per treatment group. One-way ANOVA Tukey–Kramer *post hoc* test were used for statistical analysis; *p<0.05, **p<0.01, ***p<0.001 *vs* vehicle.

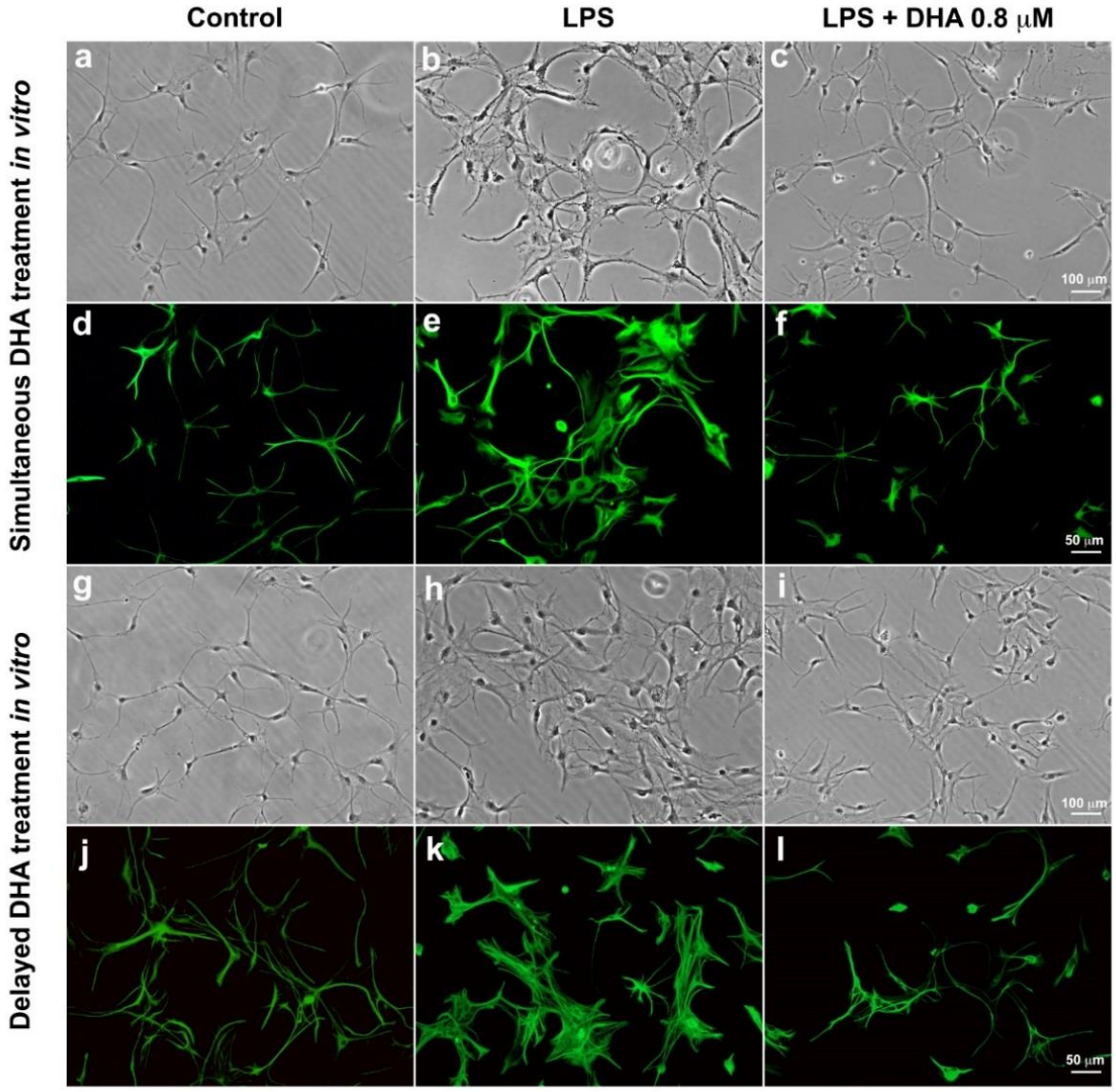
Supplementary Fig. 4



Supplementary Fig. 4. Analysis of the optimal concentration of DHA for modulation of LPS-induced microglial and astrocyte activation *in vitro*. (a) Microglial cell cultures treated with 10 μg/ml LPS over a period of 4 hours had a significantly higher percentage of microglia expressing iNOS compared to that of control cultures. Simultaneous treatment with DHA at four different concentrations revealed that 0.8 μM DHA had the highest efficacy in reducing the number of iNOS-positive microglia.

(b) Cultures treated with 10 μg/ml LPS for 4 hours had a significantly higher number GFAP immunoreactive astrocytes, when compared to control astrocyte cultures. Treatment with DHA at concentration of 0.8 μM applied simultaneously during the 4 hours of LPS exposure, significantly reduced the GFAP immunoreactivity of the astrocytes, when compared to that of LPS treated only astrocyte cultures. Data are presented as the mean ± SEM. N=3 biological replicates/n=9 technical replicates per treatment group. One-way ANOVA and Tukey–Kramer *post hoc* test were used for statistical analysis; *p<0.05, **p<0.01, ***p<0.001 *vs* LPS + 0.8 μM DHA. ***p<0.001of LPS *vs* control or LPS + 0.8 μM DHA.

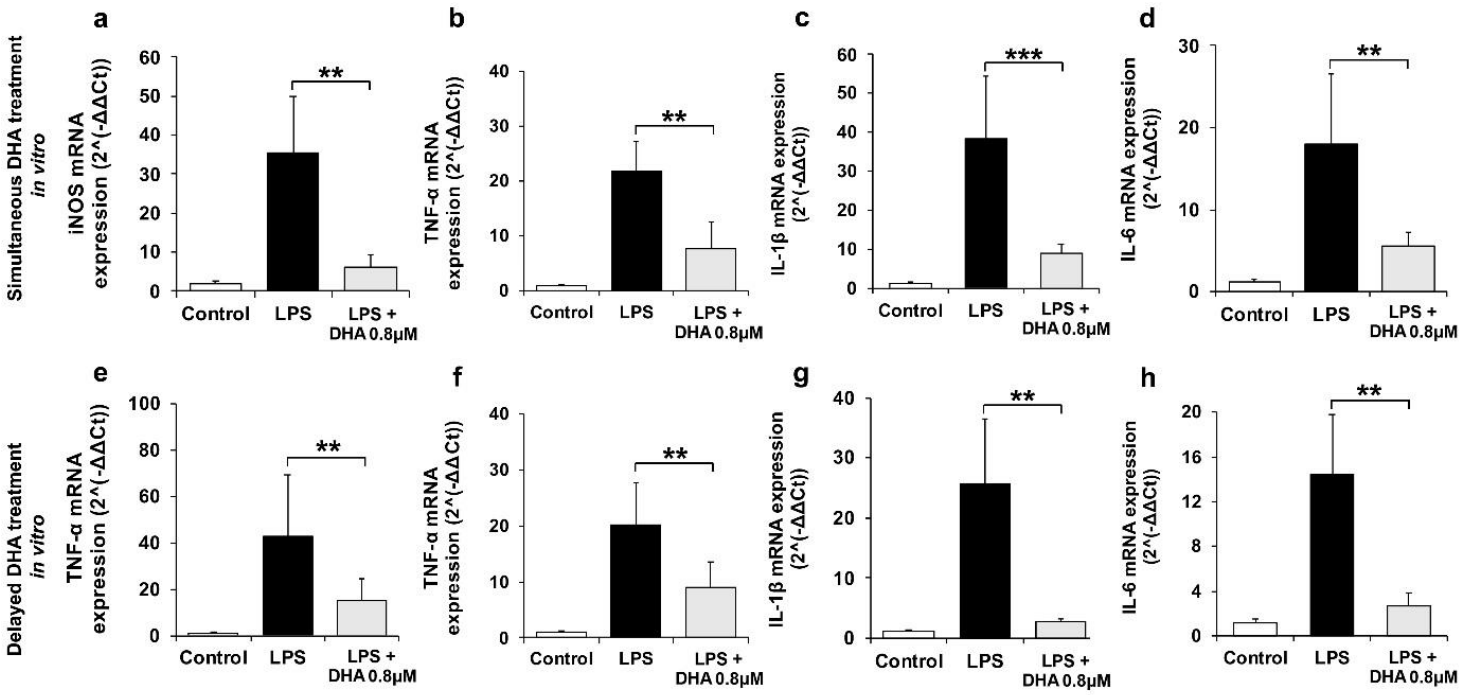
Supplementary Fig. 5



Supplementary Fig. 5. DHA attenuates LPS-induced astrocytes activation *in vitro*. Phase-contrast live images of (a) control astrocytes, (b) LPS-treated astrocytes, and (c) astrocytes treated with 0.8 μM DHA during simultaneous exposure to LPS for 4 hours revealed that DHA treatment led to the preservation of the non-reactive morphology of astrocytes manifested in the control cultures, characterized by slender cell processes and small cell bodies. Fluorescent images of (d) control, (e) LPS-treated, and (f) astrocytes treated with 0.8 μM DHA simultaneously during 4 hours of LPS stimulation, showed that DHA treatment reduced the high GFAP staining intensity of astrocytes (indicator of their

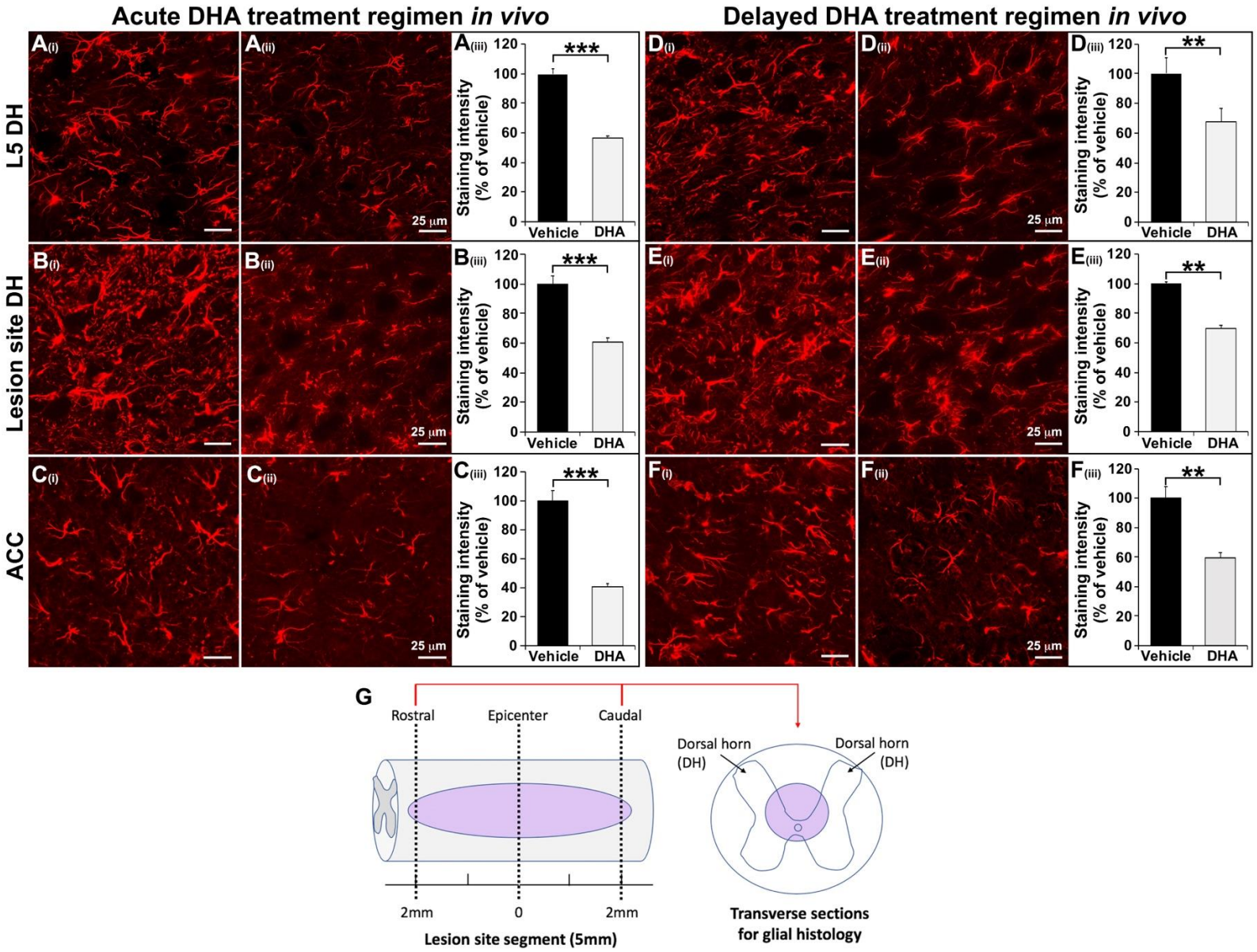
activation) seen in LPS-treated astrocyte cultures. Bright field live images of (g) control, (h) LPS-treated, and (i) pre-activated for 4 hours with LPS astrocytes, which received a delayed DHA treatment at the concentration of 0.8 μM for 4 additional hours post-activation, demonstrated that DHA markedly reversed the LPS-induced activated morphology of astrocytes nearly to that of control cells. Fluorescent images of (j) control, (k) LPS-treated, and (l) DHA-treated pre-exposed to LPS astrocytes revealed that 0.8 μM DHA treatment reduced the high GFAP staining intensity seen in the LPS-treated astrocyte cultures.

Supplementary Fig. 6



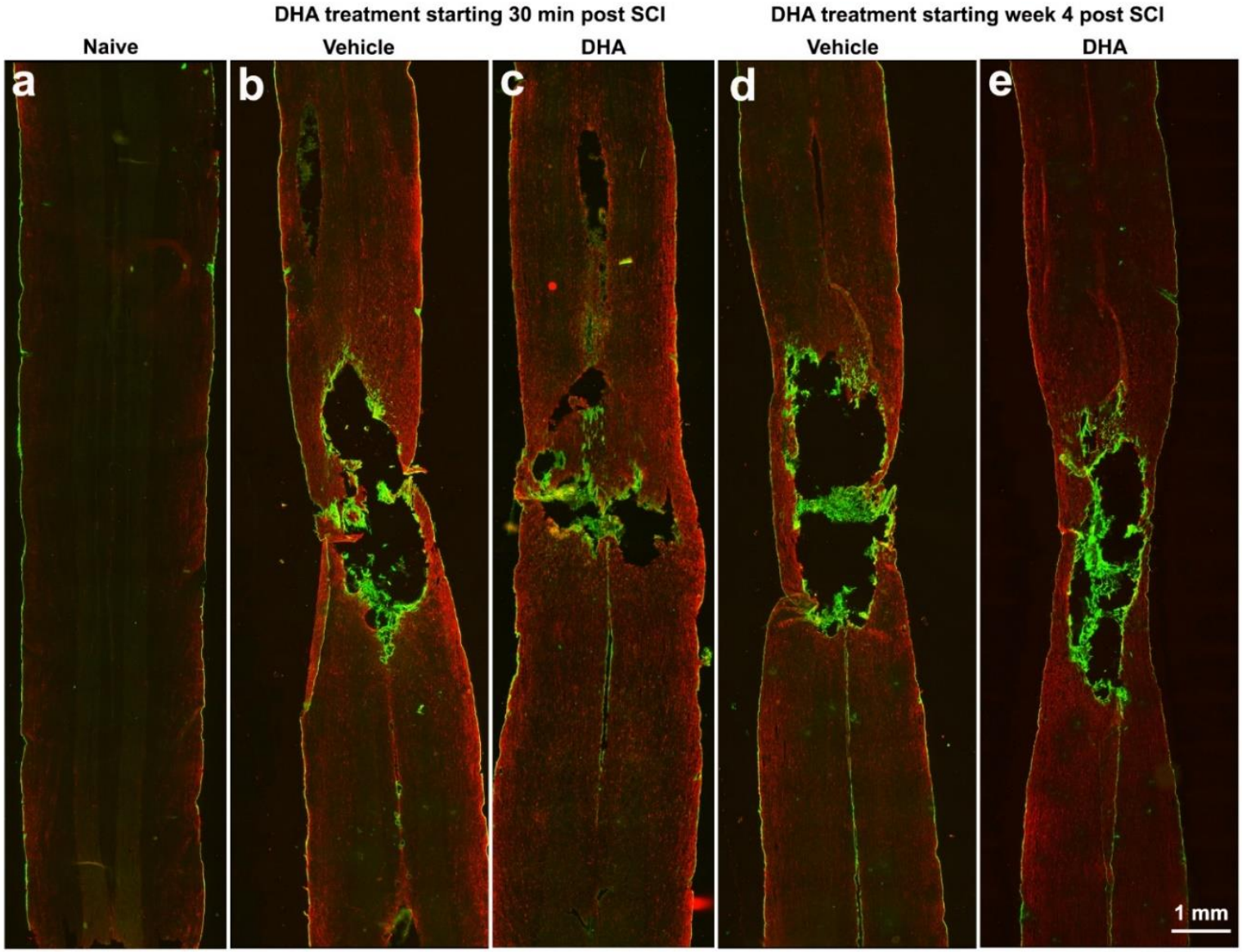
Supplementary Fig. 6. ***In vitro*** **DHA treatment of LPS-activated astrocytes significantly decreased the relative mRNA expressions of proinflammatory mediators.** Analysis of the qRT-PCR results for iNOS, TNF-α, IL-1β and IL-6 mRNA expression levels demonstrated that: (a-d) simultaneous treatment of the astrocytes with 0.8 μM DHA while exposed to LPS for 4 hours led to a significant reduction in the relative expressions of these proinflammatory genes, when compared to their expressions in astrocyte cultures stimulated with LPS only; (e-h) delayed treatment of astrocytes with 0.8 μM DHA following 4 hours of pre-treatment with LPS also significantly lowered the relative expressions of proinflammatory genes, when compared to astrocyte cultures stimulated with LPS only.

Supplementary Fig. 7



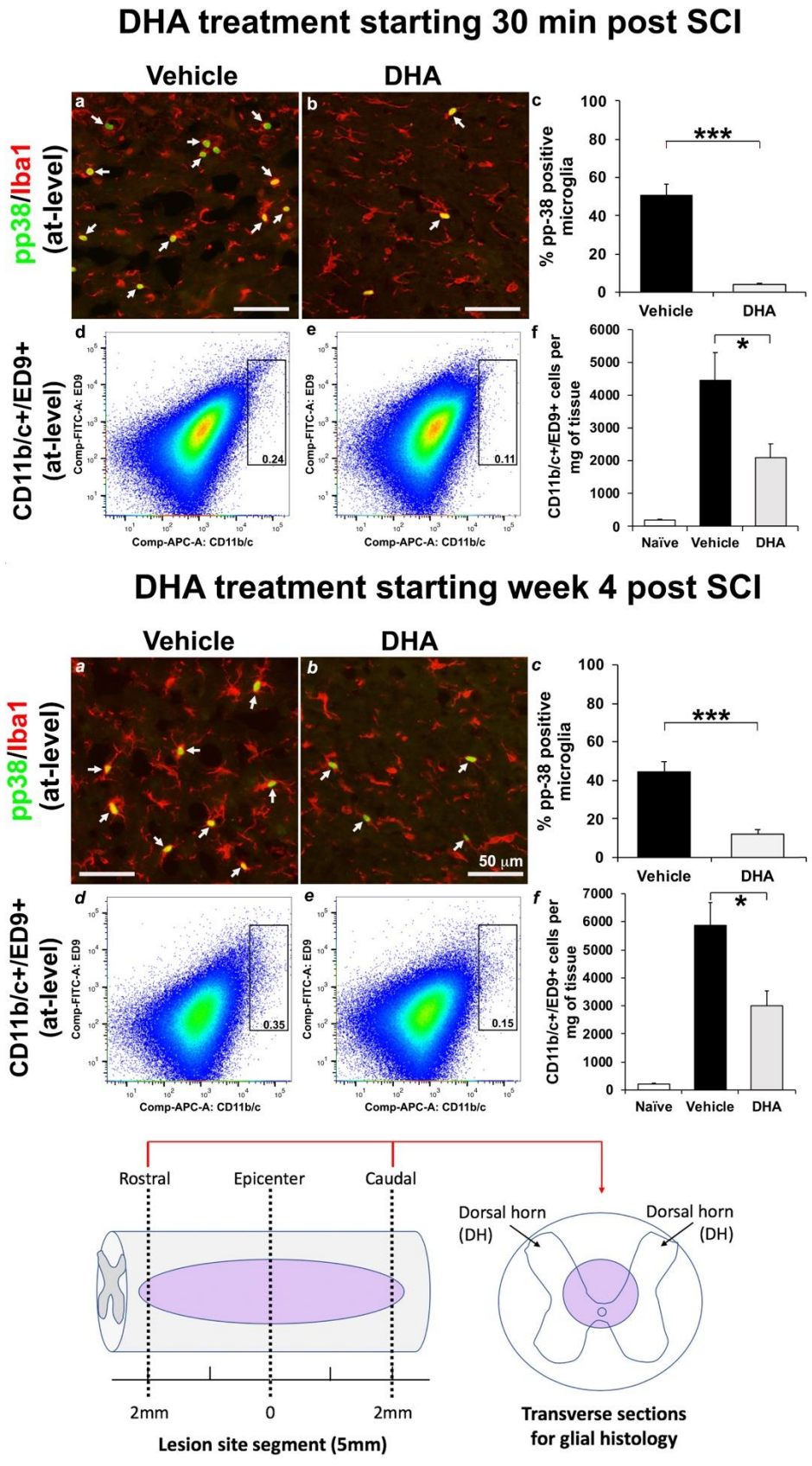
Supplementary Fig. 7: The effects of systemic DHA treatments on astrocyte activation. The acute regimen significantly decreased astrocyte activation (morphology/GFAP intensity) in dorsal horns (DH) of L5 (**A**$_{(i\text{-}iii)}$) and epicenter (**B**$_{(i\text{-}iii)}$) levels and the ACC (**C**$_{(i\text{-}iii)}$), comparing to vehicle treatment. The delayed regimen also reduced astrocyte activation in L5 dorsal horns (**D**$_{(i\text{-}iii)}$), lesion site dorsal horns (**E**$_{(i\text{-}iii)}$), and ACC (**F**$_{(i\text{-}iii)}$), in contrast to that of vehicle-treated rats. N=5-6 per group. Mann–Whitney test was used for statistical analysis. (G) is a schematic drawing of the lesion site segment from where lesion site DHs were analyzed for GFAP staining. *p<0.05, **p<0.01, ***p<0.001 *vs* vehicle.

Supplementary Fig. 8



Supplementary Fig. 8. Lesion cavities at week 6 and week 7 following SCI *in vivo*. Representative fluorescent images of 15 mm spinal cord segments containing the lesion cavity stained with GFAP (red) and laminin (green) revealed that: (a) Naïve spinal cord is lacking laminin staining due to the absence of lesion cavity and the staining intensity of GFAP is very low due to the absence of astrocyte activation in the healthy cord. (b) The lesion cavity at week 6 of SCI rats receiving vehicle treatment is evident from the pronounced accumulation of astrocytes (GFAP) and extracellular matrix (laminin) around the injury site. (c) Intravenous DHA treatment administered 30 minutes following SCI surgery and every 3 days thereafter until the end of the study, appeared to reduce the cavity size after the injury and the accumulation of astrocytes and extracellular matrix around the site of impact. (d) The lesion cavity at week 7 of SCI rats receiving vehicle treatment seemed similar to that of vehicle-treated SCI rat at week 6. (e) Chronic administration of DHA treatment did not appear to reduce the cavity size as compared to that of the vehicle-treated group. N=2 per treatment group.

Supplementary Fig. 9



Supplementary Fig. 9. The effects of systemic DHA treatment on SCI-induced microgliosis at the lesion site of SCI rats at week 6 and week 7 post-surgery. DHA treatment administered 30 minutes following SCI and every 3 days thereafter significantly reduced the expression of pp-38 by microglial cells (b&c) and significantly decreased the number of CD11b/c and ED9 double-positive microglial cells per mg spinal cord tissue (e&f), when compared to those of vehicle-treated SCI rat (a&c and d&f). Similarly, DHA treatment administered 4 weeks following SCI and every 3 days thereafter, significantly reduced the expression of pp-38 by microglial cells (*b&c*) and significantly decreased the number of CD11b/c and ED9 double-positive microglial cells (*e&f*), when compared to those of vehicle-treated SCI rats (*a&c* and *d&f*). Data are presented as the mean ± SEM; N=4-5 per treatment group. Wilcoxon-Mann-Whitney and unpaired student's *t*-test were used for statistical analysis. The bottom panel is a schematic drawing of the lesion site segment from where lesion site DHs were analyzed for GFAP staining. *p<0.05, **p<0.01, ***p<0.001 *vs* vehicle.

Supplementary Discussion

The intravenous dose of FFA DHA used in the current studies is 250 nmol/kg in a volume of 5 ml/kg. If assuming the average body weight of young adult rats is 250 g, then the concentration of DHA in the injected bolus solution is 62.5 μM. We do not know how this injected FFA DHA concentration would be translated into the plasma FFA DHA concentration and how long this plasma DHA concentration would last. A recent study conducted in humans showed that repeated weekly 4-hour intravenous infusion of omega-3 fatty acids (fish oil formulation) including DHA led to significantly increased FFA DHA levels in the plasma (7-fold post-infusion verse pre-infusion) after the first infusion, which was highly repeatable across each of the later infusions up to 24 weeks [3]. We expect that the plasma FFA DHA concentration in our studies would have significantly increased after the first intravenous injection, and this increase would have been maintained across each of the later injections (every 3 days). Future studies are needed to investigate the pharmacokinetic studies for DHA, which is beyond the scope of the current studies.

We injected a bolus of FFA DHA solution at 62.5 μM as stated above. We do not know the exact percentage of this 62.5 μM DHA that would have reached the brain and spinal cord; that would require further investigations that are beyond the scope of the current studies. It has been demonstrated in the literature that <1% of radiolabelled omega-6 arachidonic acid, when given intravenously, reached rat brains (with an intact blood-brain barrier) [6]. It has also been shown that about 1% of radiolabelled DHA was found to be incorporated in rat brain lipids after an intravenous infusion [9]. Therefore, by analogy, it is likely that in our studies a small proportion of the injected FFA DHA entered the CNS and was incorporated rapidly in phospholipids (may be higher than 1%, considering the blood-brain barrier is compromised following spinal cord injury). If assuming 1% of injected FFA DHA reached the CNS, the

concentration of incorporated FFA DHA in the CNS would be around 0.625 μM. The FFA DHA in brain has been reported to be as low as 1.3 μM [2]. Therefore, the assumed 0.625 μM concentration of DHA is likely to remain in a physiological range and should be also compatible with the affinity of this fatty acid for targets such as ion channels and RXR receptors, which may be activated by FFA DHA [7]. In our *in vitro* experiments, we examined 0.8, 4, and 8 μM FFA DHA concentrations, and found that DHA at all three concentrations was able to reduce microglial and astrocyte activation (Supplementary Fig. 4), with 0.8 μM being found as the optimal concentration. Therefore, the *in vivo* FFA DHA concentration in the CNS as assumed above would be in line with the *in vitro* concentrations used in the current studies.

Based on the findings from the human study involving repeated weekly intravenous infusion of omega-3 fatty acids including DHA as discussed above [3], we would expect that following the initial bolus FFA DHA injection in our studies, the FFA DHA levels in the plasma would have increased more than 7-fold, which was seen with the human study. This is because the human study used emulsion form of omega-3 fatty acids which would require an extra step of hydrolysis to release FFA DHA. We would also expect that a peak plasma concentration would have been reached very quickly after each intravenous injection, as previous evidence showed that about 1% of the dose of radiolabelled DHA injected intravenously was found in the rat brain 15 minutes after the injection. Future studies would need to establish the concentration range of DHA in the plasma including the peak plasma concentration following one DHA injection and before the next injection. In our studies, we used a repeated intravenous administration regime, i.e. every 3-day intervals, which would be a favourable regime in patients in the acute and subacute periods after SCI. We would anticipate that our regime would have maintained a constant plasma supply of FFA DHA to be incorporated into the CNS at a physiological range as discussed above. It is important to note that only FFA DHA in the plasma would be able to cross the blood-brain barrier

and reach the CNS, so that CNS cells such as neurons, microglia and astrocytes can take up FFA DHA using general FFA uptake mechanisms or via receptor binding [3].

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