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Chapter 6

Docosahexaenoic Acid Promotes Recovery of Motor Function by Neuroprotection and Neuroplasticity Mechanisms

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Abstract

The omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA), has been shown to promote recovery of motor function after spinal cord injury. This is likely to be at least partly due to neuroprotective effects of DHA. However, recent studies have shown that DHA also supports neuroplasticity after injury, such as promoting sprouting of spared corticospinal tract (CST) axons. In this chapter, we review the published studies showing that DHA promotes recovery of motor function in rodent models of spinal cord injury (SCI), and consider the available data on the underlying mechanisms. This includes effects on inflammation and on neuronal and oligodendrocyte survival at the injury site, and effects on spared CST axons and serotonergic axons. Current data support the hypothesis that DHA promotes recovery of motor function by both neuroprotection and neuroplasticity mechanisms. The significance of this, and the implications of combining DHA with rehabilitation strategies, will be discussed.

Keywords: thoracic spinal cord injury, cervical spinal cord injury, central pattern generator, V2a interneurons, DHA

1. Introduction

1.1. Polyunsaturated fatty acids and their role in neurology

Polyunsaturated fatty acids (PUFA) of the omega-3 and omega-6 series are lipids with major structural and signalling roles. The long-chain omega-3 PUFA docosahexaenoic acid (DHA) is present in significant concentrations in the central nervous system (CNS), where its synthesis

from the dietary precursor alpha-linolenic acid (LNA) occurs through desaturation, elongation and β-oxidation reactions. Its intermediate precursors are eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA). In humans, the conversion of LNA into DHA is below 5% [1, 2]. Therefore, an adequate dietary supply of DHA is required, especially because due to modern food manufacturing, there has been a significant decrease in the omega-3 intake and the omega-3 to omega-6 PUFA dietary ratio in the Western diet [3]. These changes have been linked to an increase in the incidence and the prevalence of diseases such as cancer, cardiovascular disease, rheumatoid arthritis, osteoporosis and asthma [4]. After absorption, the omega-3 PUFA can cross cellular membranes through specialized fatty acid transporters and interact with fatty acid-binding proteins (FABP). These proteins are a family of lipid chaperones involved in the extracellular and intracellular transport of fatty acids [5]. FABP bind long-chain fatty acids, with different ligand selectivity and binding affinity profiles. Changes in FABP levels have been reported following injury. For example, B-FABP is found in the brain and has very high affinity for DHA; it is detected in plasma as a high specificity and sensitivity biomarker of brain injury [6]. More recently, Figueroa et al. [7] reported increases in tissue levels of FABP-5 after spinal cord injury.

The principal omega-3 PUFA in the brain is DHA, representing 10–20% of the total fatty acid composition. Retinal tissue is also highly enriched in DHA. This fatty acid is a component of phospholipids, in particular phosphatidylethanolamine and phosphatidylserine; it is concentrated in cytoplasmic and synaptosomal membranes, growth cones, microsomal and mitochondrial membranes, and is also a component of myelin [8]. DHA-enriched phospholipids are present in the inner leaflet of the cytoplasmic membrane, and DHA chains are very flexible and transition rapidly between conformational states. This creates a unique microenvironment for the functioning of many proteins embedded in membranes, such as neurotransmitter receptors [9]. After release from membrane phospholipids by phospholipases, long-chain omega-3 PUFA such as DHA can be metabolized and produce a wide range of metabolites, designated with the generic term of "docosanoids", which include protectins, D-series resolvins and maresins. These DHA metabolites have intrinsic biological effects, which are mediated through specific receptors [10, 11]. Therefore, some of the effects induced by DHA may be due not to a direct action of the fatty acid but to specific metabolites which are derived from it, and which could attain significant levels, especially under conditions of chronic DHA supplementation [12].

DHA has an essential role in normal neurodevelopment, in particular for vision and cognition [13]. Supplementation with DHA has been shown to improve various aspects of learning and memory in children [14]. More than two decades ago, Martinez and collaborators [15] carried out seminal studies in children with peroxysomal disorders, which lead to a major DHA deficit, and showed that supplementation with DHA can lead to significant improvement in neurological function, and this is accompanied by improved myelination of the immature brain. At around the same time, studies initiated by Lazdunski and collaborators were providing evidence that omega-3 PUFA had significant neuroprotective potential under conditions which lead to CNS injury, such as seizures and ischaemia [16]. DHA has been increasingly linked to a variety of conditions, such as Alzheimer's disease and Parkinson's

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disease, and also schizophrenia, depression and attention deficit-hyperactivity disorder [17–19], and this has consolidated the idea that dietary supplementation with DHA could have therapeutic value across a spectrum of major disorders in neurology and psychiatry.

1.2. Pathways controlling movement in the rat and possible strategies for promoting recovery of movement following spinal cord injury (SCI)

Locomotion in rodents is controlled by a central pattern generator (CPG) which generates the basic rhythm of limb flexion and extension and which, for the hindlimbs, is located in the lower thoracic and upper lumbar spinal cord. In recent years, great progress has been made in dissecting the organization of the CPG, by identifying subtypes of neurons based on their developmental expression of specific transcription factors (summarized in [20, 21]). Genetic ablation of specific transcription factors can then be used to probe the functional roles of different neuronal subtypes (see e.g. [22, 23]). **Figure 1** illustrates one possible model of the

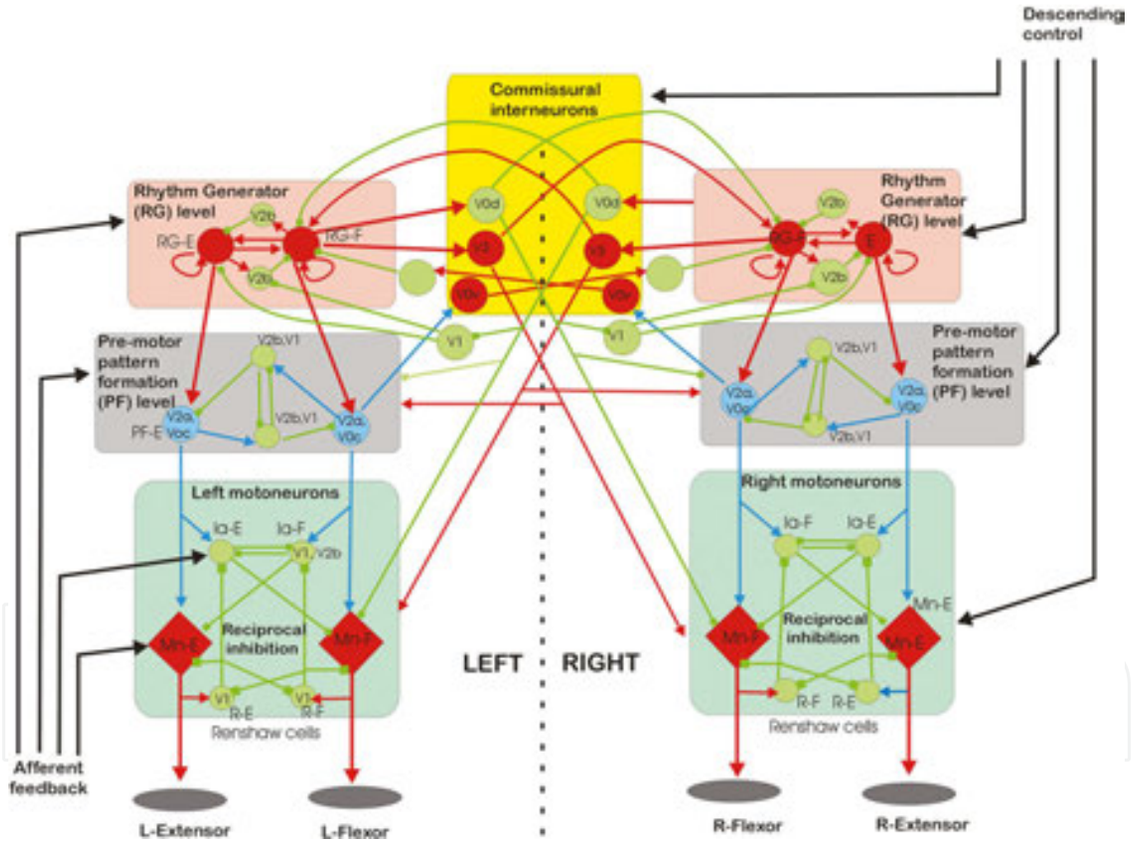

Figure 1. Schematic model of the rodent CPG controlling hind limb movement. Excitatory neurons are shown in red, and inhibitory neurons are shown in green. Rhythm generator (RG), pattern formation (PF) and motoneurons (Mn) are present for both flexion and extension on both sides of the spinal cord. V0d, V0v, V0c, V1, V2a, V2b and V3 are different types of interneurons which in some cases correspond to existing well-characterized neuronal types (e.g. V1 neurons include Renshaw cells). V2a neurons are shown in blue and have direct projections to motoneurons, plus projections to V2b/V1 PF inhibitory interneurons and to V0v excitatory commissural interneurons. Afferent feedback and descending control occur bilaterally, but for simplicity have only been shown ipsilaterally. The diagram is based on diagrams contained in Harris-Warrick [20] and Rybak and colleagues [21].

CPG based on studies reported in [22, 23]. Flexor and extensor motoneurons are thought to have separate rhythm generators (RG) which provide input to a pattern formation (PF) network that controls flexor/extensor alternation. The RG network also connects to commissural interneurons which coordinate left/right hindlimb movement. As an example of the complexity of the system, we have highlighted the V2a class of excitatory interneuron in **Figure 1**. V2a neurons can be identified by their expression of the homeodomain protein Chx10 and receive input from the RG centres. They project to three different targets, namely (1) motoneurons, (2) V2b inhibitory interneurons that are part of the PF centre, and (3) V0v commissural interneurons [21]. They therefore can influence locomotion by direct monosynaptic inputs to motoneurons and by regulating the flexor/extensor and left/right coordinating centres. However, V2a interneurons are not only present in the lumbar CPG, they also occur in the cervical spinal cord and play a key role in skilled forelimb movement, by conveying an internal copy of the premotor signals. This is possible because of their dual innervation of cervical motoneurons and precerebellar interneurons [22].

The CPG can generate alternating hindlimb movements independently, but in the intact animal, the CPG is modulated by afferent feedback and by descending control. The effects of SCI depend on the spinal level of the injury. Rats subject to complete transection above the level of the lumbar locomotor centres show some spontaneous recovery of hindlimb movement [24], and this can be increased by a variety of treatments, including weight supported treadmill training [24], delivery of growth factors [25, 26] and implantation of various exogenous cells [27–29]. At least some of this recovery of function appears to be related to regeneration or sprouting of descending bulbospinal [30, 31] and corticospinal [25] pathways. In contrast, the lasting paraplegia that results from a SCI at lumbar level is thought to result from loss of spinal interneurons, possibly including components of the CPG [32]. An efficacious treatment for SCI might need to preserve and/or restore descending control pathways and repair damage to intrinsic networks such as the CPG.

2. The effects of PUFA treatment on recovery of movement following experimental SCI

2.1. Thoracic hemisection SCI

DHA promotes recovery of quadrupedal locomotion in rats and mice following thoracic hemisection [33], compression [34–36] and contusion [37] SCI, when acutely administered post-injury. Recovery is further improved if an acute injection of DHA is followed by a DHA-supplemented diet (**Figure 2**). The DHA treatment results in increased neuronal and oligodendrocyte survival [34] and decreased macrophage and microglial activation [36, 38] at the injury site. However, since the motoneuron pools that contribute to locomotion are not at thoracic level, it is likely that the effects on locomotion are due to preservation of descending axons passing through the injury site rather than preservation of thoracic neurons themselves. Analysis of axon numbers at the SCI site using generic axonal markers such as

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neurofilament and myelin basic protein (MBP) reveals that DHA preserves axons in white matter tracts [39] but, with the exception of serotonin (5-hydroxytryptamine, 5-HT), the origin and role of those axons have not been much explored. As outlined above, corticospinal and bulbospinal projections play an important role in motor control but mainly innervate motoneurons indirectly, via interneurons, and so are difficult to analyse (however, see Section 4 on pyramidotomy below). In contrast, 5-HT axons are derived from the medullary raphe nuclei, innervate many motoneurons monosynaptically and play key roles in modulating motoneuron excitability [40]. DHA treatment preserves the number of 5-HT axons at the level of a thoracic compression injury [39], including axons that appear to contact motoneurons (see **Figure 3**). It is therefore likely that at least some of the motor recovery is due to increased preservation of 5-HT axons that pass through the injury site and innervate motoneurons at lumbar level. 5-HT axons have a great capacity to sprout and make new connections [41]. Another possibility is therefore that some 5-HT axons are spared by the SCI but that DHA promotes sprouting and restoration of synaptic circuits by these axons. These possibilities have not been examined directly following thoracic SCI, but the effects of DHA on 5-HT axons have been studied in much more detail following cervical injury.

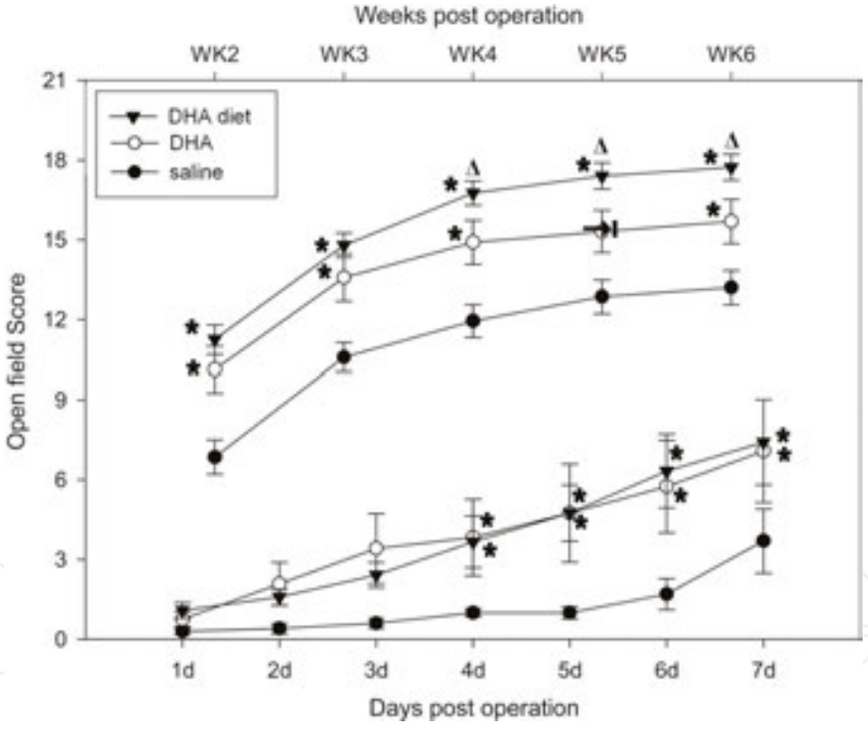

Figure 2. Locomotor performance of thoracic compression SCI rats injected with saline or DHA and of animals injected with DHA and then fed on a DHA-enriched diet. Results for days 1–7 are shown in the bottom half of the graph, plotted against the bottom X-axis. Results for 2–6 weeks are shown in the top half of the graph, plotted against the top X-axis. Both DHA- and saline-treated animals showed similar low levels of locomotor function 1 day after surgery, as assessed on the BBB open field task. During the first week, DHA-treated animals had improved motor function recovery compared with saline-treated animals (days 5, 6 and 7; $*p < 0.05$). Between week 2 and week 6, DHA-treated animals continued to perform better than saline-treated animals ($*p < 0.05$). In addition, from the 4th week onwards, animals injected with DHA followed by maintenance on the DHA-enriched diet, performed better than animals only injected with DHA (weeks 4, 5 and 6; $^{\Delta}p < 0.05$). Error bars represent SEMs. Reproduced from [34] with permission.

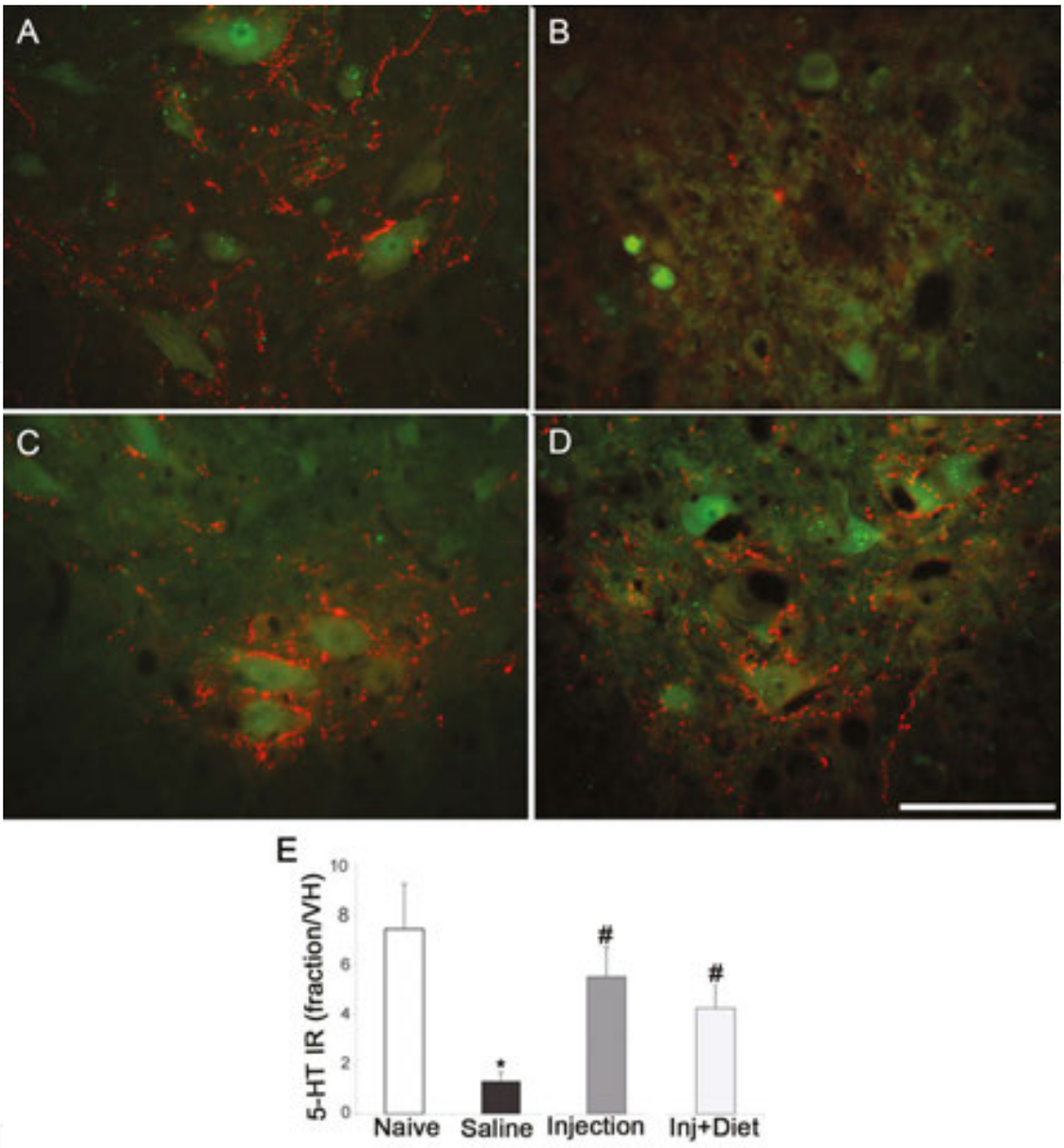

Figure 3. Immunofluorescence micrographs showing thin, 5-HT immunoreactive (IR) axons (red) that innervate NeuN labelled neurons (green) in the ventral horn (VH). In naïve animals (A), a dense network of beaded 5-HT-positive fibres can be seen surrounding large NeuN neurons. At 6 weeks post-thoracic compression injury, a marked loss of 5-HT-positive fibres was seen in saline-treated animals (B) compared with the groups receiving DHA injection alone (C), or in combination with a DHA-enriched diet (D). Quantitative analysis (E) at 6 weeks post-injury revealed a significant loss of 5-HT IR in the VH of saline-treated animals following injury. A significant amelioration in the loss of 5-HT IR was observed in the VH in both DHA-treatment groups (significantly different from *naïve or #saline-treated animals at $p < 0.05$; scale bar = 100 μm). Reproduced from [39] with permission.

2.2. Experimental cervical SCI

The majority of experimental studies have focused on thoracic injury, but the most common clinical injury is at cervical level (57% of SCI). Recently, we have therefore examined the effects

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of DHA in a cervical (C5 level) hemisection SCI model [42]. The cervical level of injury also has the advantage of allowing us to examine the effects of DHA treatment on a skilled motor task, namely retrieval of food using the forepaws. Rats are trained to retrieve food by extending a forelimb through a gap in a perspex box and grasping food pellets which are on a staircase [43]. Following SCI at C5 level, rats lose the ability to recover the food pellets, but recover partial function if treated acutely with DHA (**Figure 4**). Following cervical hemisection, 5-HT axons show a remarkable degree of plasticity. Rostral to the hemisection, there is an increase in 5-HT immunoreactivity which is maintained up to 3 weeks. Caudal to the hemisection, there is a transient loss of 5-HT immunoreactivity at 1 week, but levels are restored by 3 weeks (**Figure 5B**). DHA treatment results in an even greater number of 5-HT immunoreactive axons caudal to the injury (**Figure 5B**), and increased density of 5-HT axons contacting motoneurons (**Figure 5C–E**). However, 5-HT axons are not the only ones to sprout in response to DHA treatment. Corticospinal tract (CST) axons on the intact side of the spinal cord were identified by anterograde labelling with the tracer biotinylated dextran amine (BDA). Following hemisection, a small number of these axons sprout across the midline to innervate the denervated side of the spinal cord. However, after DHA treatment, this number doubles [42]. It is therefore possible that the recovery of skilled movement is due to sprouting of 5-HT and CST axons and formation of novel circuits. However, since the increase in axons occurs at the same level as the hemisection, it is possible that the increase in 5-HT and CST axons is due to neuroprotection rather than neuroplasticity. We have therefore examined the effect of DHA treatment in a third model of SCI, namely unilateral pyramidotomy in the mouse [42].

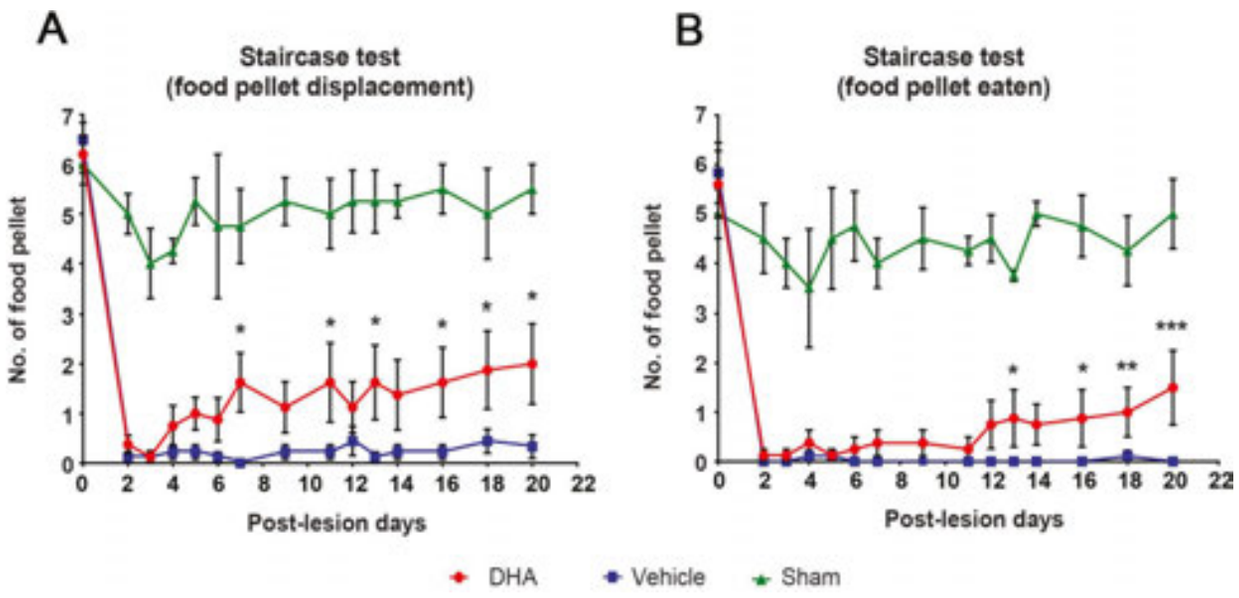

Figure 4. DHA enhances functional recovery following cervical lateral hemisection in the rat. A, B, In the Montoya staircase test, all injured animals lost the ability to displace (gross motor function) or eat (fine motor function) the food pellet after cervical lateral hemisection 2 d after injury. The animals treated with vehicle did not recover any food retrieval ability (blue squares), but DHA-treated animals gradually recovered food retrieval ability (red circles) from around 2 weeks onwards, compared with uninjured sham operated animals (green triangles). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to vehicle-treated animals. Results represent mean ± SEM. Reproduced from [42] with permission.

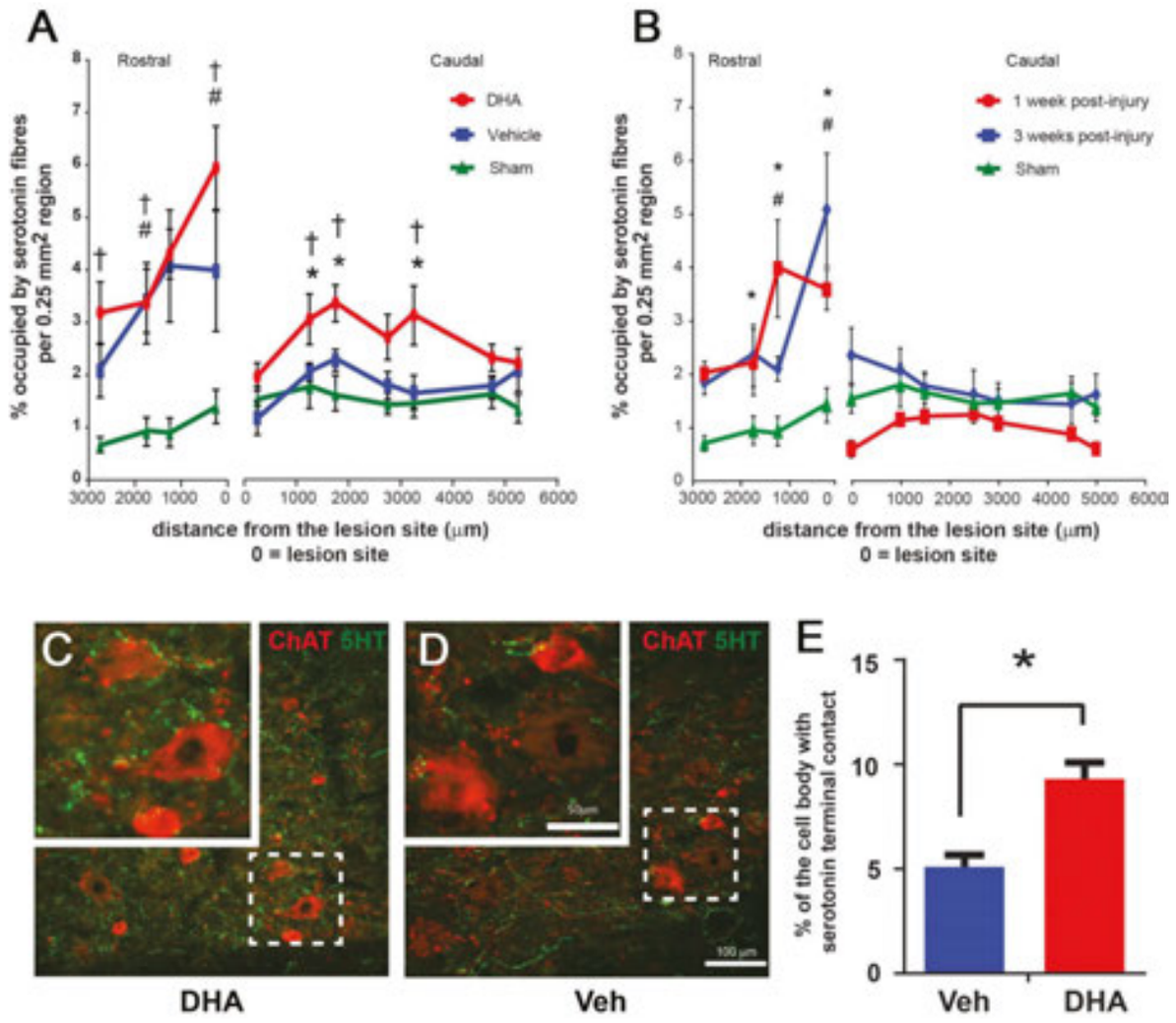

Figure 5. DHA enhances 5-HT (serotonin) fibre regrowth following cervical lateral hemisection in the rat. (***A***) Serotonin fibres in the rostral part of the lesion site were significantly increased in both treatment groups at 3 weeks post-injury, but only the DHA-treated group (red circles), not the vehicle group (blue squares), had significantly increased serotonin in the caudal region compared with the sham operated animals (green triangles). (***B***) Serotonin fibres in the rostral part of the lesion site were significantly increased at 1 week (red square) and 3 weeks (blue circle) compared with the sham operated group after lateral cervical hemisection. However, in the caudal region, 1 and 3 weeks after injury were similar to the sham operated group. 1 week after hemisection, there was a loss of serotonin but by 3 weeks levels had returned to values similar to sham operated animals. $N = 5$ or 6 per group. #$p < 0.05$, 1 week hemisection versus sham group. *$p < 0.05$, 3 week hemisection versus sham group. (***C***, ***D***) The images were captured 1750 μm caudal to the lesion site. Double labelling shows serotonin fibres (green) in contact with ChAT immunoreactive motor neurons (red) in the ventral horn of vehicle- and DHA-treated rats. Insets, dashed boxes at higher magnification. (***E***) Quantitative analysis reveals that there is a significant increase in the density of serotonin fibres contacting motor neurons in the DHA-treated group compared with the control group. N = 5 or 6 per group. *$p < 0.05$, DHA versus vehicle group. †$p < 0.05$, sham versus vehicle group. #$p < 0.05$, DHA versus sham group. Scale bar, 100 μm. Reproduced from [42] with permission.

2.3. Pyramidotomy

The CST axons in rodents run in the medullary pyramids, before decussating and descending at the base of the dorsal columns. Unilateral pyramidotomy transects these CST axons and

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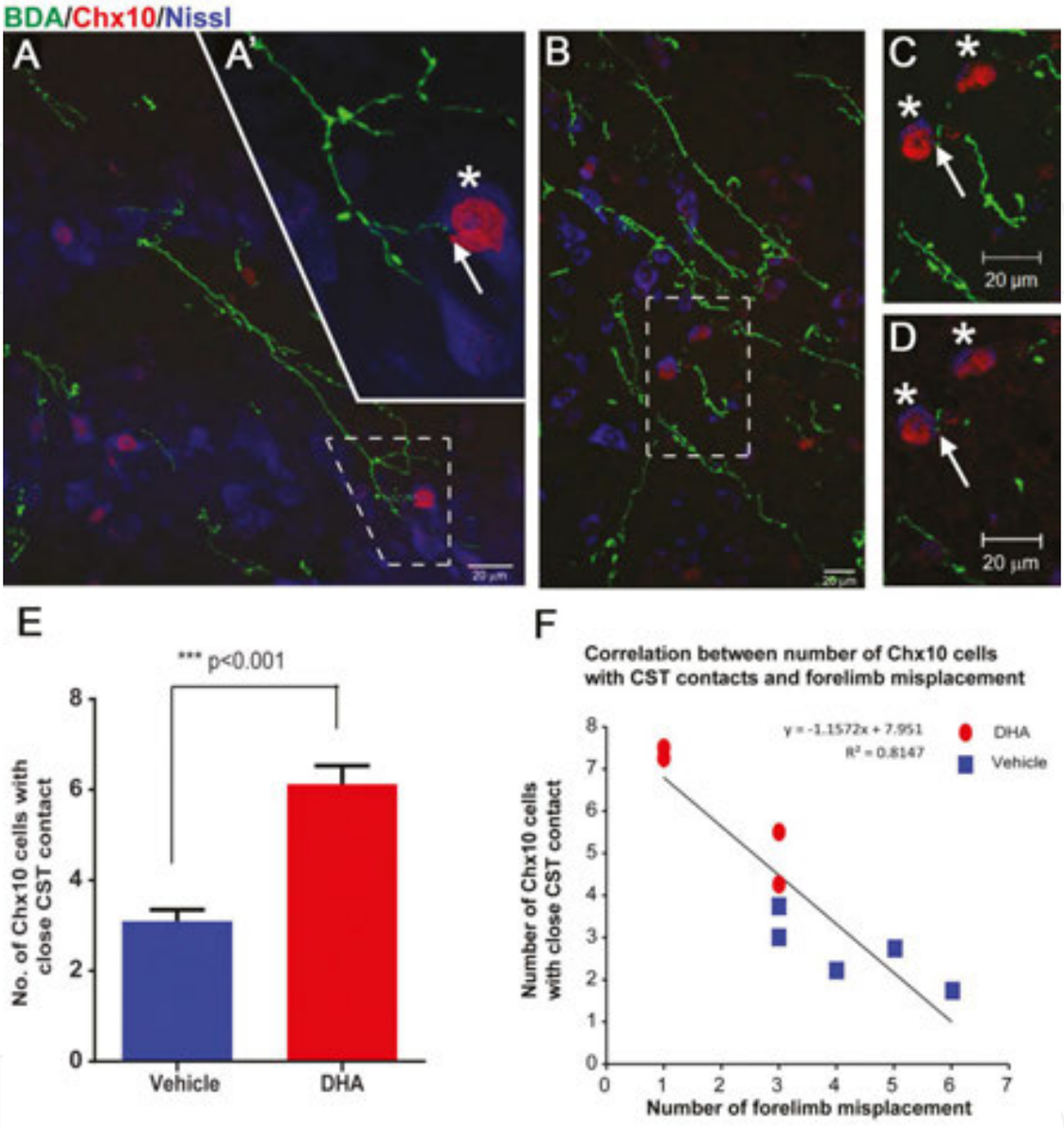

Figure 6. DHA enhances functional recovery via connection with Chx10 interneurons in pyramidotomy mice. (***A***–***D***), Confocal images of mouse cervical spinal cord transverse sections at the intermediate laminae of the CST-denervated side, showing examples of BDA-labelled CST collaterals (green) in the vicinity of blue fluorescent Nissl-stained cells (Neurotrace 435/455), some of which are V2a interneurons identified by immunostaining for Chx10 (red). (***A***, ***B***) Dashed boxes represent high magnification in ***A′***, and in ***C***, ***D***, which reveal contacts (arrows) between BDA-labelled collaterals and Chx10 interneurons (asterisks). (***A′***) *A Z*-stack comprising 10 × 0.66 μm optical images. (***C***), A stack comprising 25 × 0.72 μm optical images. (***D***, A) Single 0.72 μm optical image. Examination of the single optical image confirms that the BDA-labelled CST fibre contacts (arrow) one of the Chx10 interneurons. (***E***) Quantitative analysis revealed a significant increase in the number of Chx10 interneurons contacted by BDA-labelled CST collaterals following DHA treatment (red bar) compared with vehicle treatment (blue bar). (***F***) A strong negative correlation was observed between the numbers of Chx10 interneurons with BDA-labelled CST contacts and the numbers of forelimb misplacements. Data were taken from DHA-treated (red circle) and vehicle-treated (blue square) animals. ***$p<0.001$, DHA versus vehicle group. Scale bar, 20 μm. Reproduced from [42] with permission.

results in the loss of CST axons in the spinal cord on the contralateral side. Using BDA to label the CST axons on the non-lesioned side, we observed that a small number of CST axons respond to the pyramidotomy by sprouting across the midline to innervate the lesioned side (**Figure 6A**). The number of sprouting CST axons is doubled following DHA treatment [42]. Furthermore, we have shown that the sprouting axons contact a particular class of interneuron, namely the V2a propriospinal neurons described above. V2a neurons were identified by their expression of the Chx10 transcription factor (**Figure 6A–D**). V2a interneurons have been shown to play an important role in skilled reaching [22]. Following DHA treatment and pyramidotomy, an increased number of V2a interneurons receive contacts from CST axons (**Figure 6E**), and the recovery of forelimb movement shows a tight correlation with the number of V2a neurons that receive contacts (**Figure 6F**). It therefore appears that DHA treatment after pyramidotomy results in the formation of a novel circuit (CST to contralateral V2a interneurons) that promotes recovery of skilled reaching. This is in addition to the effects of DHA on sprouting 5-HT axons, and neuroprotective effects on neuronal cell bodies and axons.

3. Discussion

Motor pathways are so complex that it is difficult to establish a direct causal relationship between the effects of a therapeutic agent on spinal circuitry and an improvement in a particular motor task. However, the data reviewed above indicates that there is a strong correlation between the neuroprotective and neuroplasticity effects of DHA and the recovery of motor function. This dual effect of DHA, together with its established safety profile and the recent demonstration of the efficacy in SCI of a multinutrient combination containing DHA and EPA [44], makes DHA a particularly promising candidate for development as a therapeutic agent in SCI. There is a substantial literature on the neuroprotective effects of DHA (reviewed above) but the effects on neuroplasticity are more novel and less studied. We have shown that DHA treatment following SCI upregulates the microRNA miR-21 and suppresses phosphatase and tensin homolog (PTEN), a central negative regulator of the phosphatidylinositol 3-kinase (PI3K) signalling pathway [42]. However, these effects are quite widespread and it is not known how they result in beneficial axon sprouting. Bareyre and colleagues [45] have shown that new connections form after SCI, but inappropriate connections are lost because they are not used. A similar mechanism may mould the sprouting and connections promoted by DHA, in which case, there may be great benefit in combining DHA treatment with rehabilitation and task specific training.

3.1. Therapeutic implications

The magnitude of the effects induced by the acute administration of DHA and reported in various models of SCI is comparable with that described with various other therapeutic approaches which are already being explored in clinical trials in neurotrauma, such as progesterone, erythropoietin, riluzole and minocycline [46]. DHA has clear potential for

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clinical translation in SCI; therefore, it is appropriate to consider the issues which remain to be clarified in order to improve the chances of translational success.

It is important to note that in the studies published so far with DHA and reporting beneficial effects of this fatty acid in a variety of conditions, there are two types of administration: DHA administered using a chronic oral supplementation route and DHA administered as an acute bolus, using an injectable route. Are the cellular targets and mechanisms activated by DHA the same in the two types of treatment? When DHA is administered chronically, one of the consequences of this regime is the structural enrichment in DHA in membranes, and because DHA has unique molecular structural characteristics, this influences the dynamics of membrane components, and may change the activity of ion channels and G-protein-coupled receptors (GPCR). DHA incorporation in membranes changes the properties of specialized domains such as the lipid rafts and caveolae, and this can modify signalling [47]. When DHA is administered acutely as a bolus, its half-life in plasma is very short (approximately 2 min), as shown in PET imaging studies carried out in healthy volunteers [48]; therefore, efficacy may be due to the activation of different mechanisms. Long-chain omega-3 PUFA such as DHA and EPA have various specific molecular targets, including ion channels, GPCR and nuclear receptors [17, 47]. They include voltage-sensitive Na^+ and Ca^{2+} channels and two-pore domain background K^+ channels, such as TREK-1 (which is also a target for riluzole), the GPCRs GPR40 and GPR120, and transcription factors such as the retinoid X receptors (RXR) and peroxisome proliferator-activated receptors (PPAR). RXR can heterodimerize with PPAR or with retinoic acid receptors (RAR), and changes in the expression of these receptors have been reported after SCI [49]. Several studies indicate that the activation of retinoid signalling supports axonal regeneration [50].

The spontaneous partial recovery of function which occurs after SCI may be due to neuroplasticity, compensation and repair [51]. Rehabilitation through training is at present the most successful treatment for patients with SCI, to enhance the recovery of some neurological function. Rehabilitation enhances the spontaneous plasticity changes that occur after injury and enhances the activity of sensorimotor pathways, as documented in experimental SCI [52]. Exercise leads to a reconfiguration of cortical representation maps, it modifies the biophysical properties of motoneuron membranes, changes the activity of spinal inhibitory circuits and also increases the levels of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) [53]. Liu and collaborators [54] have shown that exercise increases the expression of miR21 and decreases PTEN mRNA levels, a profile of effects similar to our observations with DHA. Considering this similarity of action, a goal of future studies should be the exploration of a combination of DHA and exercise, to establish whether what could be achieved is true synergism or only additivity. The concept of combining treatment with exercise is supported by examples such as the successful combination of exercise with strategies such as the reduction in the glial scar components using chondroitinase ABC [55]. However, not all combinations achieve an optimum effect, as shown by the negative results obtained by combining exercise with antibodies against the myelin-derived inhibitor Nogo A [56]. Therefore, it will be essential to continue to characterize the targets and mode of action of DHA, so that it becomes clear what mechanisms could be harnessed and amplified by

combining DHA (acute administration and chronic exposure) and rehabilitation training, to optimize outcome in SCI patients.

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