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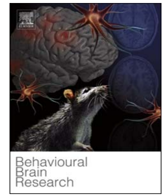
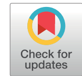


Research report

Docosahexaenoic acid (DHA) enhances the therapeutic potential of neonatal neural stem cell transplantation post—Traumatic brain injury



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ABSTRACT

Traumatic Brain Injury (TBI) is a major cause of death and disability worldwide with 1.5 million people inflicted yearly. Several neurotherapeutic interventions have been proposed including drug administration as well as cellular therapy involving neural stem cells (NSCs). Among the proposed drugs is docosahexaenoic acid (DHA), a polyunsaturated fatty acid, exhibiting neuroprotective properties. In this study, we utilized an innovative intervention of neonatal NSCs transplantation in combination with DHA injections in order to ameliorate brain damage and promote functional recovery in an experimental model of TBI. Thus, NSCs derived from the subventricular zone of neonatal pups were cultured into neurospheres and transplanted in the cortex of an experimentally controlled cortical impact mouse model of TBI. The effect of NSC transplantation was assessed alone and/or in combination with DHA administration. Motor deficits were evaluated using pole climbing and rotarod tests. Using immunohistochemistry, the effect of transplanted NSCs and DHA treatment was used to assess astrocytic (Glial fibrillary acidic protein, GFAP) and microglial (ionized calcium binding adaptor molecule-1, IBA-1) activity. In addition, we quantified neuroblasts (doublecortin; DCX) and dopaminergic neurons (tyrosine hydroxylase; TH) expression levels. Combined NSC transplantation and DHA injections significantly attenuated TBI-induced motor function deficits (pole climbing test), promoted neurogenesis, coupled with an increase in glial reactivity at the cortical site of injury. In addition, the number of tyrosine hydroxylase positive neurons was found to increase markedly in the ventral tegmental area and substantia nigra in the combination therapy group. Immunoblotting analysis indicated that DHA + NSCs treated animals showed decreased levels of 38 kDa GFAP-BDP (breakdown product) and 145 kDa αII-spectrin SBDP indicative of attenuated calpain/caspase activation. These data demonstrate that prior treatment with DHA may be a desirable strategy to improve the therapeutic efficacy of NSC transplantation in TBI.

1. Introduction

Traumatic brain injury (TBI) is considered a major health concern worldwide with an annual incidence of 150–200 cases per 100,000 individuals [1–3]. TBI represents a leading cause of disability and mortality among individuals under the age of 45 [4]. TBI is defined as the damage of the brain tissues and structures caused by mechanical forces that strike the head such as car accidents, falls, and or due to a sudden hit by a moving or stationary object [5]. TBI is characterized by both morphological and physiological changes, where it can cause

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disturbances at the level of cellular integrity and/or affect the homeostasis of the brain tissues and cell functionality; thus, causing altered cellular dysfunction [6]. Given the heterogeneous nature of brain neural structure and the complexity of brain trauma, TBI pathophysiology involves cascades of concatenated primary and secondary phases of injury. Controlled cortical impact (CCI) is among the widely used injury models of TBI [7–11] and involves long-term functional deficits of motor function as well as a decline in cognitive status [12].

Despite the wealth of information available on the molecular and cellular basis of TBI, there exists no specific effective pharmacological drug treatment for TBI [2]. Thus, effective neurotherapeutic drug intervention holds great potential to modulate TBI-induced pathological cascades in an attempt to salvage neural substrates required for neurological functional recovery and to enhance the rehabilitative capacity of surviving neural tissue [13]. To this end, several neurotherapeutic strategies-both clinical trials and experimental studies- have been proposed to ameliorate TBI outcomes, including drug administration and/or cellular interventions [2,14,15]. Among these experimental approaches, neural stem cells (NSCs) of various origins have been proposed as a next-generation neurotherapeutic targets in TBI [15–26]. These cells represent a potential therapeutic adjuvant for regenerative therapy post-TBI, due to their ability to generate mature, functional neural cells able to replace degenerated ones [24]. NSCs are mostly derived from embryonic ganglionic eminence or subventricular zone (SVZ). Transplanted cells at different locations of an injured brain show migration to the site of injury, a long-term survival post-transplantation, and differentiation into neurons capable of functionally integrating with the host tissue [27–29]. This has resulted in cognitive and functional improvement in rodents [15,30]. Recently, there has been more research devoted to increase the efficacy of NSCs, and provide a suitable microenvironment for the survival and proliferation of these transplanted cells [17,31–33].

Along the same line, Docosahexaenoic acid (DHA, 22:6n-3) is among the prevalent polyunsaturated structural lipids in the central nervous system [34]. DHA promotes brain development by promoting neuronal differentiation, neurite growth, and synapse formation [34–36]. DHA supplementation improves memory [37,38] and cognition [39,40] regulating the expression of different neurotransmitters, such as glutamate. On the other hand, it is shown that a DHA-depleted brain impedes neurite growth and synaptogenesis [41]. In addition, other studies have shown that DHA has a direct neuro-supportive effect on neural cells *in vitro* [42]. Studies investigating of DHA effect on neurogenesis using neurosphere assays have indicated that DHA promotes the maintenance of both primary neurospheres that are neurogenic NSCs and tertiary neurospheres that are gliogenic NSCs [35]. DHA treatment; however, leads to the neuronal differentiation of gliogenic NSCs but not neurogenic NSCs [43–45].

In the present study, we aimed to investigate the effect of transplanted NSCs derived from neonatal mice SVZ with or without DHA pretreatment on neural regeneration in a murine TBI model assessing neural cellular changes along with behavioral outcomes. In addition, other markers including the astrocytic (Glial fibrillary acidic protein, GFAP) and microglial (ionized calcium binding adaptor molecule-1, IBA-1) activity were assessed in the DHA/NSCs treatment to evaluate altered cellular changes. This was coupled with the quantification of neuroblasts (doublecortin; DCX) and dopaminergic neurons (tyrosine hydroxylase; TH) expression in relation to the treatment of DHA. Combined NSC transplantation and DHA injections significantly attenuated TBI-induced motor function deficits (pole climbing test), promoted neurogenesis, coupled with an increase in glial reactivity at the cortical site of injury. In addition, the number of tyrosine hydroxylase positive neurons was found to increase markedly in the ventral tegmental area and substantia nigra in the combination therapy group.

We hypothesize that DHA treatment upstream the transplantation of NSCs at the site of the injured ipsilateral cortex would be a novel strategy for NSCs survival leading to amelioration of injury severity and promoting functional recovery. Data from this work demonstrated that combined DHA injections and neonatal NSC transplantation significantly attenuated TBI-induced motor function deficits (pole climbing results) and promoted neurogenesis. These changes were accompanied by an increase in the number of dopaminergic neurons in the ventral tegmental area and the substantia nigra upon DHA-NSCs co-treatment.

2. Material and methods

2.1. Animals

The study was carried out at the Animal Care Facility of the American University of Beirut (AUB) and all animal experiments were performed in compliance with the AUB Institutional Animal Care and Use Committee (IACUC) guidelines with the reference number: (15-1-326). C57BL6 mice were obtained from Charles Rivers Laboratories and housed in a controlled environment (12 h light/dark cycles, 22 ± 2 °C). All animals were handled under pathogen-free conditions and fed chow diet *ad libitum*. A total of 31 male mice aged 7–8 weeks were subjected to TBI. One day post TBI mice were injected intraperitoneally with DMSO or DHA with a dosage of 12 mg/kg/day given on a daily basis for a period of 20 days (until sacrificed). Mice were divided into four main groups: group 1A- DMSO-injected [TBI + DMSO] (n = 12); group 1B-DHA-injected [TBI + DHA] (n = 12); group 2A-injected with DMSO and NSC transplanted [TBI + NSCs] (n = 12), group 2B-injected with DHA and NSCs [TBI + NSCs + DHA] (n = 12). Animals were randomly attributed to experimental groups in a blinded manner; in addition, the microscopic examinations were performed by two independent students in a blinded manner. Animals that showed weak health post-surgery and exhibited any disease condition were not included in the study as per the IACUC regulations. These animals were not subjected to the behavioral testing. Each group had a total number of 12 mice/group aimed at the different experiments. We had ∼3 animals eliminated from each group with approximately of 14% mortality rate. We have used 9 animals per group for each behavioral testing.

2.2. Controlled cortical impact injury (CCI) model

An open head injury was performed to induce TBI using the electromagnetic controlled cortical impact device (Leica Impact One Angle with Leica Angle Two™ Stereotaxic Instrument, Biosystems, Buffalo Grove, IL, USA). Mice were anesthetized by intramuscular injection of Ketamine/Xylazine (90 and 10 mg/kg, respectively) and positioned in a stereotaxic frame. The target region was set parasagittally between Bregma and Lambda (somatosensory area of the parietal cortex) with standard coordinates (+1.0 mm AP, +1.5 mm ML, and −2 mm DV). After removal of the bone using a drill, unilateral mild injury of 1 mm depth was induced by activating a piston positioned vertically above the cortical tissue. For the device injury parameter, the impactor speed was adjusted to 2 m/s while the dwell time was set at 150 ms. Mice were then rapidly removed from the stereotaxic frame, sutured and kept in a holding cage until recovery from anesthesia. The tip of the impactor was 1 mm in diameter. The size of the bone flap was 1.7 mm in diameter removed using a manual trephine. TBI was performed on day 17 of cell culture in a way that the cells are ready for transplantation after 1 week of inducing TBI (Fig. 1).

2.3. Hematoxylin and eosin stain (H&E)

Fifteen weeks post-transplantation, animals were euthanized and transcardially perfused with 10 ml 0.1 M phosphate-buffered saline (PBS) followed by 20 ml 4% paraformaldehyde. Brains were removed, post-fixed in paraformaldehyde overnight at 4° C, and cut into 50 µm coronal sections on a vibratome. Tissue sections were mounted on slides

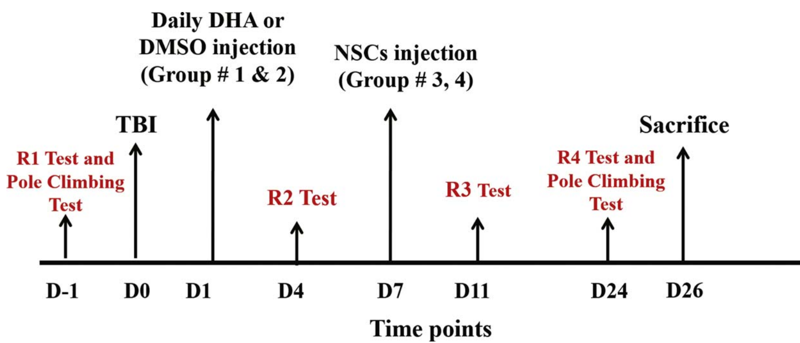

Fig. 1. A timeline representing experimental approach.
Mice were divided into four groups. Pole climbing test was performed one day before TBI and at day 24 post-TBI. Rotarod tests (R1, R2, R3 and R4) were performed at one day before TBI and at day 4 (R2), day 11 (R3) and day 24 (R4) post-TBI. The animals were sacrificed on day 26.

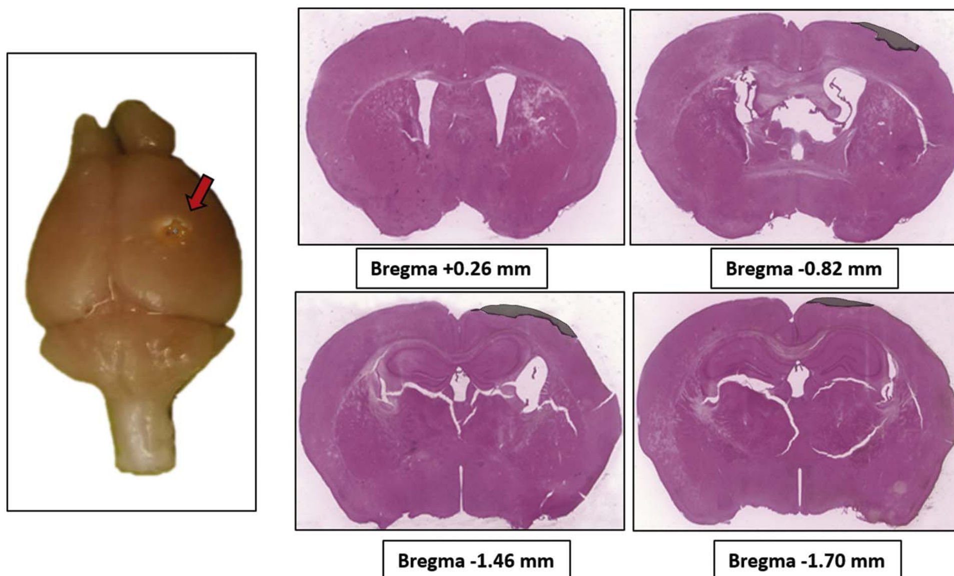

Fig. 2. Controlled cortical impact (CCI) mainly targets the somatosensory area of the parietal cortex.
Mice brain sacrificed 26 days post mild-CCI on the ipsilateral side of the parietal cortex shows the existence of a cavity in the targeted region (red arrow). Representative Hematoxylin & Eosin stained coronal brain sections are marked with their coordinates to the Bregma through the brain of an injured mouse, it shows a 1 mm maximum depth CCI injury. The black area in each section shows the contralateral hemisphere superimposed on top of the lesioned hemisphere to visualize the brain damage regions. The lesion covered the ipsilateral parietal cortex targeting mainly the somatosensory area and the secondary visual cortex V2.

and washed with distilled water for 3 min, followed by placing them in hematoxylin for 50 s to 1 min. After hematoxylin, slides were placed under running tap water and then rinsed with eosin for 50 s to 1 min. Subsequently, slides were immersed in 95% and 100% ethanol for 3 min and 5 min, respectively. Afterwards, slides were washed with xylol for 1 min. Finally, 2–3 drops of mounting solution (Aqueous Mounting Medium [sigma F4680-25ML]) were added to the slides to be visualized on the microscope (Fig. 2).

2.4. Isolation of neural stem cells (NSC)

A total of ten pups (aged 1-7 days) were used for harvesting NSCs for group 2; these are taken from C57BL6 pregnant mice. NSCs were isolated from subventricular zone (SVZ) of the lateral wall of the ventricle. This zone, on coronal brain sections, was dissected under a microscope. Tissues were collected in a tube containing DMEM/F12 medium (Gibco 31331-028) placed on ice and dissociated by adding trypsin solution (NaCl 8 g/l; KCL 0.4 g/l; glucose 1 g/l; $NaHCO_3$ 0.85 g/l, 0.5 g/l; trypsin 2 g/l) at 37° C. Deactivation of dissociation was induced by DMEM/F12, 10% Fetal Bovine Serum (FBS), and DNase (20 μg/ml). Cells were centrifuged (5 min, 1000 rpm) and maintained in a complete medium (DMEM/F12; insulin 50 mg/ml; B27; N2; glucose 16.25Mm; HEPES 1 M; Penicillin/Streptomycin). Cells were counted on hematocytometer using trypan blue then seeded in a total of 100,000 cells per T25 flask at 37° C, 5% CO_2. At this stage, primary neurospheres were obtained. EGF (20 ng/ml) and FGF (20 ng/ml) were added every 3rd, 5th, and 6th day of cell culture to maintain the cells as proliferating neurospheres that were passaged twice every 7–10 days to obtain secondary and tertiary neurospheres.

2.5. Transplantation of neural stem cells

One week post-CCI injury, all mice were anesthetized using ketamine/xylazine (90 and 10 mg/kg, respectively) and placed in a stereotaxic device. Tertiary neurospheres were co-labeled with Hoechst 33342 (10 pg/L) and cultured at 37 °C for 30 min in a humidified incubator containing 5% CO_2 prior to transplantation. Cells were dissociated into single cells and a total of 100,000 cells were stereotactically transplanted into the cortex at the injury site (coordinates: AP + 1.0 mm, ML + 1.5 mm from bregma, DV 2.5 mm from dura) via a 10 μl Hamilton syringe (Fig. 1). Cells were injected with 3 μl of complete medium using a syringe pump, which was calibrated to achieve a constant flow rate of 0.25 μl/min. Following all injections, the needle was kept in place for 5 min before being slowly withdrawn.

2.6. Behavioral tests

2.6.1. Rotarod performance test

The rotarod performance test was used to assess balance, grip strength and motor coordination of the mouse [5,46], especially post-TBI utilizing an automated rotarod instrument (UgoBasile, 47750 – Rat Rota-Rod NG, Comerio, Italy). The mouse was placed on a rotating rod under continuous acceleration and the time it took the mouse to fall was recorded. Rotarod test was performed four times for each group: 4 days before TBI (R0), 3 days after TBI (R1), 3 days after stem cell injection (R2), and 10 days after stem cell injection (R3) (Fig. 1). Each test was performed for 4 consecutive days, starting with 3 trials of 5 min each with an acceleration speed of 4–40 rpm in the first three days with an inter-trial time of 15 min. The fourth day (challenging

session) consisted of one trial of 8 min with an acceleration speed of 4–60 rpm.

2.6.2. Pole climbing test

Fourteen days after NSC injection, pole climbing test was performed to study motor coordination of the mice (Fig. 1) assessing motor performance and coordination [47,48]. Each mouse was trained for three consecutive trials followed by 5 consecutive experimental trials. The mouse was placed on the tip of a vertical rod (height = 60 cm, diameter = 1 cm) and the time at which the mouse reached the bottom (t-total) was recorded; in addition the t-turn (time at which the mouse turned on the rod to head down), the t-half (the time at which the mouse reached half of the rod) and the t-stop (the time at which the mouse stopped on the rod), were also recorded.

2.7. Immunofluorescence

Brain floating sections (50 µm thick) were washed with PBS and PBST (0.1% Triton in PBS), then incubated for 2 h in a solution of 10% heat-inactivated fetal bovine serum (FBS) in PBST. Thereafter, tissues were incubated with primary antibodies diluted in PBST-1% FBS overnight at 4° C. The following antibodies were used: GFAP (1/1000; Abcam 7260) for astrocytes; DCX (1/500; Santa Cruz sc-8066) for neuroblasts; IBA1 (1/1000; Wako 091-19741) for microglia and macrophages. Tyrosine hydroxylase (TH) (mouse monoclonal antibody, 1/1000; Millipore MAB318) was used to assess the number of dopaminergic neurons at SN and VTA. Then sections were rinsed in PBST, and incubated with the appropriate fluorochrome-conjugated secondary Ab for 1 h at room temperature, followed by three washes of 20 min each in PBST. All samples were counterstained in 1 µg/ml Hoechst (Sigma) in PBS, and mounted in Fluoromount (F4680-25ML).

2.8. SDS-polyacrylamide gel electrophoresis and immunoblotting technique

Immunoblotting technique was performed according to standard procedures using ECL detection as performed in our laboratory. In summary, Tissue samples (20 µg) samples were run on SDS–PAGE (4–20% acrylamide) with a Tris–glycine running buffer system and transferred onto a PVDF membrane using a wet transfer unit (Bio-Rad) at 30 V overnight. The blots were probed with the primary antibodies used including monoclonal mouse anti αII-spectrin (BML-FG6090-0500, ENZO, 1/4000), rabbit monoclonal anti GFAP (Abcam #7260, 1/10000). Uneven loading of samples onto different lanes might occur despite careful protein concentration determination and careful sample handling and gel loading (20 µg per land). B3-tubulin (monoclonal #sc-58888; Santa Cruz Biotechnology, 1/2000) was used as protein loading evenness control. The blots are washed with TBST and exposed to HRP coupled secondary antibodies then results revealed using the Chemidoc machine (Bio-Rad, Ca). The molecular weights of intact proteins and their potential BDPs were assessed by running alongside all blue molecular markers (#161-0373/BIO-RAD).

2.9. Quantification and imaging

All the quantification was done using the NIH-Image J program. DCX-positive area was quantified along the lateral wall of the SVZ with respect to the total length of the SVZ. IBA-1 and GFAP quantifications were performed by determining the total area covered by these markers at the TBI site with respect to the area of the injury. Quantification of dopaminergic neurons at the SN and VTA was determined by counting individual TH-positive cells. At least three sections per mouse were used for quantification. Microscopic imaging was done using Zeiss LSM 710 confocal microscope. Images were acquired as tile scans with 40 X-oil objectives and analyzed using the Zeiss ZEN 2009 image-analysis software. Images for the different experimental interventions were acquired under the same laser and microscopic parameters for the purpose of consistency.

2.10. Statistical analysis

Statistical analysis was conducted using Statistical Package of Social Science SPSS 22.0 software (SPSS Inc., Chicago, IL, USA), and GraphPad Prism 6. The measurement data are expressed as mean ± SEM and were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple group comparisons. When two groups were compared independent Student's *t*-test was applied. Pole climbing and rotarod data were analyzed using two-way repeated measures ANOVA (RM ANOVA) followed by post hoc contrasts or pairwise comparisons (with Bonferroni adjustment for multiple comparisons).

Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Establishing the CCI model of mild injury targeting the somatosensory area of the parietal cortex

All mice in the 4 groups sustained the same injury with the same coordinates (Fig. 2). Hematoxylin and eosin (H&E) staining of coronal brain sections from CCI animals revealed a cortical cavity spanning from the lateral ventricles to the beginning of the hippocampus. The impact produced a 1 mm depth tissue deformation, which is considered a mild TBI of the first layers of the parietotemporal cortex. The lesion occurred mainly in the primary somatosensory cortex (S1) and the second visual cortex (V2) and was associated with cortical swelling and herniation as documented in previous studies [49].

3.2. Effects of NSC transplantation and DHA daily injection on motor coordination

To monitor the effect of DHA and NSC treatments on motor impairment in injured mice, pole climbing and accelerating rotarod tests were performed. Pole-climbing test consisted of placing mice at the top of a vertical rod, head upwards, where time-to-turn and time-to-descend the rod are recorded (Fig. 3). Prior to TBI, all the mice in the 4 different groups exhibited similar behavioral results, with the total time needed to cross the rod (T-total) of ~18 s. Three weeks post injury (TBI), a significant difference in the performance was detected in T-total among the 4 tested groups. The TBI group exhibited a motor

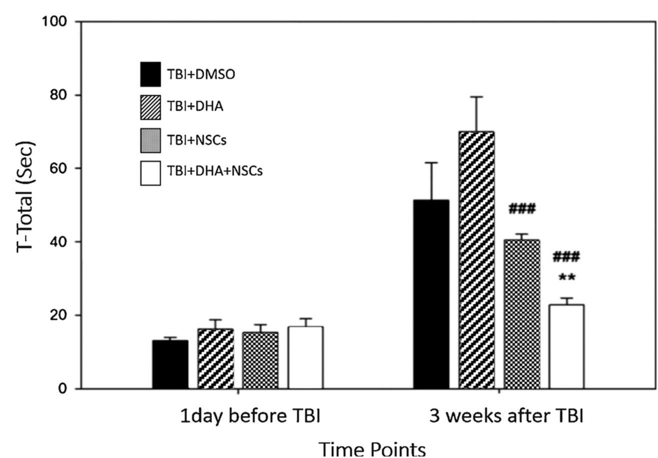

Fig. 3. Significant improvement in motor coordination was observed between groups tested on pole climbing. Motor balance assessment using pole climbing test was measured 1 day before TBI and 3 weeks after TBI. Data are presented as means ± S.E.M. Significant differences between the groups were evaluated using two-way ANOVA. Symbols indicate the statistical difference between NSCs + DHA group and the DMSO group, (**) $p < 0.01$.

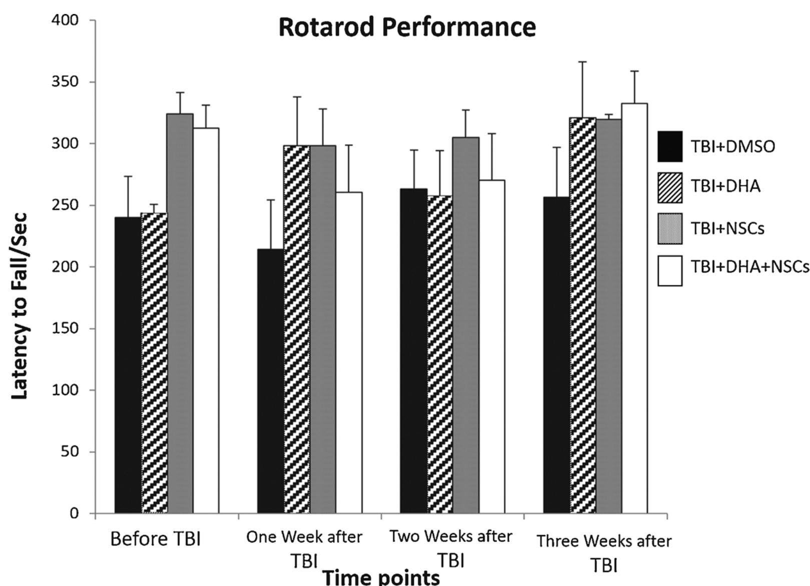

Fig. 4. Rotarod Data on Motor Coordination among the Different Groups. Rotarod performance was measured using the accelerating rotarod test (4–60 rpm/min) in the 4 groups of mice. The latency to fall is indicated. Mice were tested once a week before TBI and for 3 weeks following TBI. No significant improvement in motor coordination was observed among the groups tested on rotarod. Data represent means ± S.E.M. of each group. Statistical analysis was performed using independent student *t*-test.

deficit with an increased T-total (51 s ± 10). Similarly, both the (TBI + DHA) and the (TBI + NSCs) group showed a similar deficit (70 s ± 9; 40 s ± 2, respectively). On the other hand, the (TBI + DHA + NSCs) group showed a significant decrease in the T-total (23 s) compared to DMSO group ($p < 0.001$), indicative of improved motor coordination. Lastly, utilizing an accelerating rotarod test, no significant statistical difference in motor coordination was detected among all groups as shown in Fig. 4.

3.3. Increased neurogenesis in the lateral SVZ after NSC transplantation coupled with DHA injections

It has been shown that, after injury, neuroblasts migrate from the subventricular zone (SVZ) to the lesion site. The level of neurogenesis in the SVZ was assessed by quantifying DCX positive cells, a specific marker of neuroblasts (Fig. 5A). Quantification of DCX positive cells in the lateral SVZ (Fig. 5B) showed no significant increase in neurogenesis in TBI + DHA (12.74 ± 1) compared to DMSO injected group (11.78 ± 4). While a significant increase of DCX positive cells was observed in the TBI + NSCs group (18.73 ± 0.6) compared to DMSO injected and this increase was higher in the TBI + DHA + NSCs group (26.06 ± 2.32; $p < 0.05$).

3.4. Increased glial reactivity at the cortical site of injury after NSC transplantation coupled with DHA injections

Our results have shown that there is an increased inflammatory response apparent by an elevation in the number of glial cells, including astrocytes and microglia. Astroglial reactivity was assessed by glial fibrillary acidic protein (GFAP) immunolabeling, an astrocytic marker known to be upregulated in reactive astrocytes (Fig. 6A). GFAP, on the other hand, increased in the TBI + DHA + NSCs group. This observation was reflected by the quantification of GFAP positive cells in the ipsilateral cortex over the intact cortical area compared to the control group (Fig. 6B). GFAP quantification showed similar levels of astrogliosis in both TBI + DHA (0.06 ± 0.003) and TBI + NSCs (0.06 ± 0.009) groups compared to the DMSO group (0.084 ± 0.016). While in the TBI + DHA + NSCs group, astrogliosis increased significantly (0.10 ± 0.013; $p < 0.05$) compared to the control group (0.084 ± 0.016). Similarly, reactive astrogliosis due to brain damage is associated with microglial activation. We examined the expression of IBA1, a microglial marker at the cortical site of injury (Fig. 7A). Quantification of the IBA1 positive cells in the injured cortical area (Fig. 7B) showed a similar level of microgliosis in TBI + DHA group (0.081 ± 0.019) and TBI + NSCs group (0.098 ± 0.014) compared to the DMSO group (0.069 ± 0.014). On the other hand, a significant expansion in microglial cells was observed in the TBI + DHA + NSCs group (0.13 ± 0.008; $p < 0.05$).

3.5. Quantification of GFAP and αII-spectrin breakdown products (BDPs)

In addition, we assessed for both GFAP fragmentation and αII-spectrin proteolysis as they have been considered biomarkers of TBI as discussed later. Both GFAP and αII-spectrin proteins have been shown to be vulnerable to calpain-mediated truncation resulting in molecular signature breakdown products (BDPs) of 38 kDa (GFAP–38 kDa); this 38 kDa GFAP breakdown product has been recently validated as a potential biomarker of (necrotic) calpain-mediated neural injury and which has been assessed in both clinical and experimental brain injury studies [50–59]. Similarly, this 38 kDa (GFAP–38 kDa) has been compared to the αII-spectrin BDPs of 120, 145 and 150 kDa (αII-spectrin SBDPs) [56,59–64]. Analysis of these BDPs, the 38 kDa GFAP BDP as well as the SBDP-150 levels were significantly higher in TBI + DMSO and TBI + DHA compared to the TBI + NCS + DMSO and TBI + NCS + DHA (Fig. 8A&B). The 145 and 120 kDa SBDPs showed no significant difference among the groups (Fig. 8A&B). Similarly, the 38 kDa calpain associated GFAP-BDP was detected in the TBI groups with the lowest level detection in the TBI + NCS + DHA (Fig. 8A&B). It is well established that these specific markers (BDPs) are useful biomarkers indicative of the level and mechanism of TBI (50–54, 60–64). The presence of GFAP–38 kDa and 150 kDa SBDP evident in the TBI groups (indicative of calpain activation) conversely was minimally detected in the TBI + NCS + DHA group. These findings demonstrate that calpain activation was ameliorated in the combined DHA + NSCs treatment.

3.6. Increased TH positive neurons in the midbrain after NSC transplantation coupled with DHA injections

It is well-established that, in addition to the initial cortical damage, TBI induces a series of secondary insults, including a rapid loss of the dopaminergic neurons of the ventral tegmental area (VTA) and substantia nigra (SN). For this reason, we examined the effect of NSC transplantation and DHA injection on TH-positive (TH +) neurons in the SN and VTA (Fig. 9A). TBI + DHA group and TBI + NSCs group showed comparable numbers of TH+ neurons (447 ± 40 and 482 ± 58, respectively) compared to DMSO group (363 ± 80)

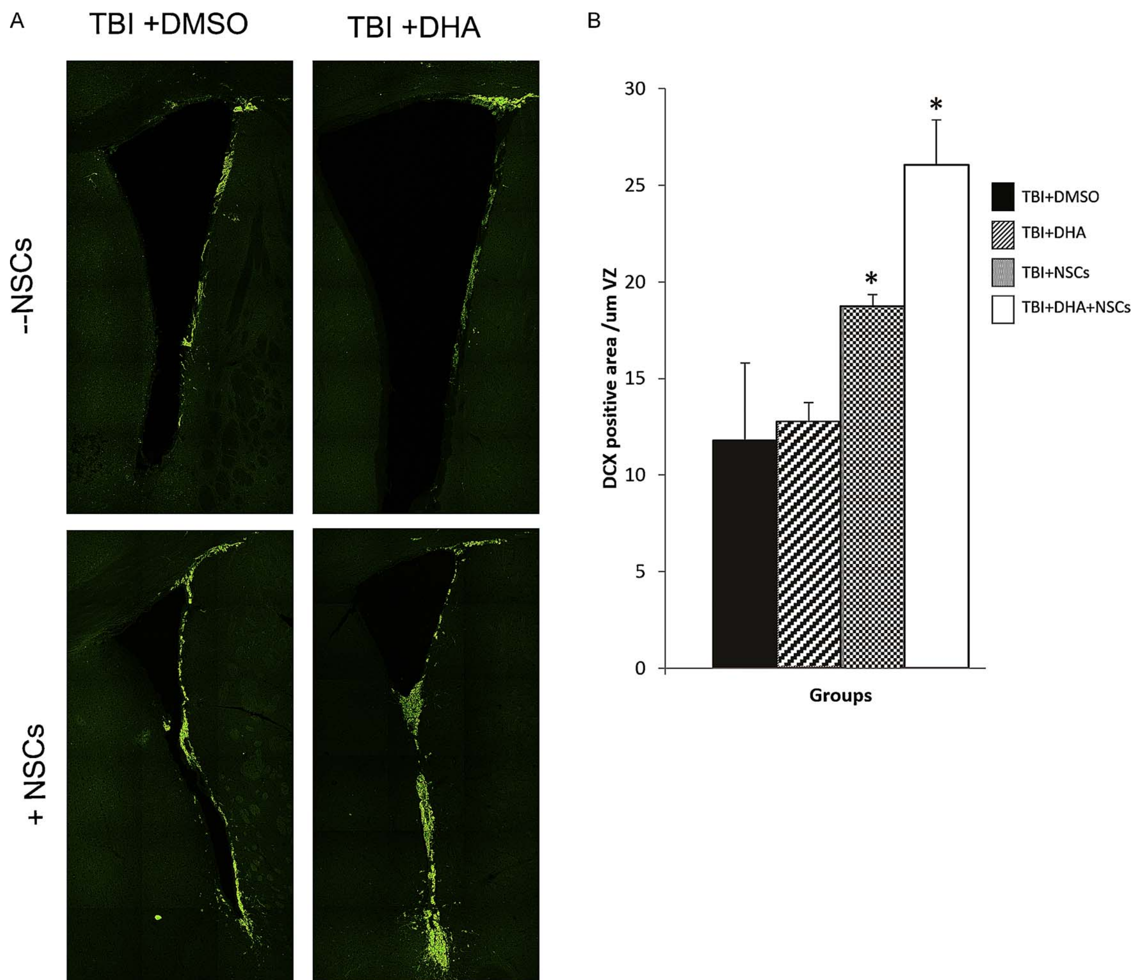

Fig. 5. Increased DCX-positive neuroblasts at the lateral SVZ in mice given a dual neurotherapeutic treatment of NSCs and DHA. (A) In the lateral SVZ, DCX labeling reflects neuroblast production. Representative confocal tile scan images (40 X-oil objective) of 40 μm mice brain coronal sections immunostained with anti-DCX antibody (green) and counterstained with Hoechst (blue) show the area of DCX positive cells. Scale bars 100 μm. (B) Quantification of DCX-positive area is shown in the histogram. No difference in DCX expression is observed between DMSO and DHA groups. NSC transplantation induces expansion of DCX expression in the SVZ (NSC + DMSO group). This expansion is increased when NSC transplantation is combined with daily DHA injection (NSCs + DHA group). Each bar represents the area of DCX-positive cells per VZ length (mean ± S.E.M., $n = 3$ in each cohort). Symbols indicate statistical differences between groups vs. the DMSO group (*) $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 9B). on the other hand, TBI + DHA + NSCs group showed a synergistic effect on the number of nigrostriatal dopaminergic neurons, leading to a significant increase of TH+ neurons (905 ± 67), compared to the other groups.

4. Discussion

In this work, we hypothesized that the co-treatment of transplanted NSCs derived from the neonatal mice SVZ with DHA pretreatment would carry neurotherapeutic potential in an experimental model of chronic mild TBI. For this purpose, NSCs were transplanted in the injured cortical region 1 week post injury based on the best available literature evidence demonstrating that the 7-day time frame would be optimal for blood vessel vascularization along with enhanced survival and proliferation of grafted cells [65,66]. Although mild CCI was induced, in some animals a modest herniation was observed further damaging the surrounding brain tissue. This particular time point of 7 days would provide a better environmental “niche” compared to early time points (1 day, 2 days and 5 days) where primary and secondary brain injury phases are still active [67]. These unfavorable survival conditions would not be ideal for NSC survival and axonal sprouting due to the lack of significant blood supply coupled with an active pro-cell death machinery. Data from this work demonstrated that combined DHA injections and neonatal NSCs transplantation significantly attenuated TBI-induced motor function deficits (pole climbing results) and promoted neurogenesis which was accompanied by an increase in the number of dopaminergic neurons as discussed below.

4.1. Behavioral recovery

Rotarod and pole climbing tests were used to study the effect of stem cells transplantation and DHA injection on motor recovery. Rotarod test is widely used to assess motor and balance coordination in studies including various TBI mouse models and treatments [68–70]. Climbing tests are used to monitor sensorimotor impairments associated with hypothalamic and nigrostriatal motivational systems in rats and mice [71]. In our study, rotarod test did not show any significant changes in the motor behavior pre- and post-TBI induction with or without DHA treatment (Fig. 4). However, pole climbing reflected high sensitivity enabling us to detect motor deficits and motor recovery post-treatment. This discrepancy in results could be related to the nature of the CCI injury level where mild cortical injury was performed (a mild injury of 1 mm depth). The use of pole climbing test may represent an ideal marker to detect behavioral deficit recovery in assessing mild severity of TBI used in our study. In addition, it is possible that the mild injury may have not led to sufficient neuronal loss that could generate

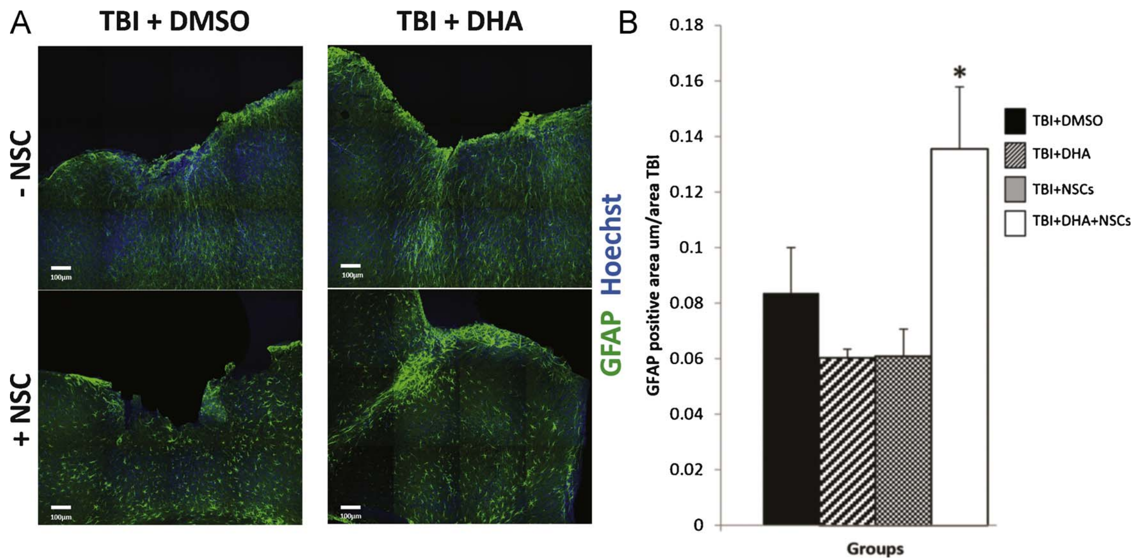

Fig. 6. Increased glial reactivity at the TBI region in mice given a dual neurotherapeutic treatment of NSC and DHA.
(A) Representative confocal tile scan images (40 X-oil objective) of 40 μm mice brain coronal sections immunostained with anti-GFAP antibody (green) and counterstained with Hoechst (blue) show an increased number of GFAP+ cells in the NSCs + DHA group compared to the other 3 groups. Scale bars 100 μm. (B) Quantification of GFAP-positive area is shown in the histogram. No difference in GFAP expression is observed among the DMSO, DHA and NSC + DMSO groups. NSC transplantation coupled with daily DHA injection (NSCs + DHA group) increases the GFAP positive cell area in the TBI region. Each bar represents the area of GFAP-positive cells per area of TBI (mean ± S.E.M., $n = 3$ in each cohort). Symbols indicate statistical differences between the NSCs + DHA group and the NSC + DMSO group (*) $p < 0.05$.

motor deficits that are detectable with the Rotarod testing.

4.2. Neurogenesis mechanism

Recently, a number of studies have utilized different modes of neural stem cell therapy as a treatment of TBI [15,27,29,31,32,72]. These studies have mainly used stem cells derived from embryonic tissue or fetal stem cell lines [15,72–77]. Cells were engrafted at different locations in the brain, in the ventricles or at the cortical site of injury. Varying degrees of cell survival after transplantation into the injured brain have been reported and results indicate that transplanted cells were able to differentiate into neurons, astrocytes and oligodendrocytes precursors. Sun et al., had successfully used adult-derived NSCs that were transplanted into experimental injured rat brain [78]. Many cells migrated out of the injection site into surrounding areas expressing markers for mature astrocytes or oligodendrocytes. Electrophysiological studies have shown that the transplanted cells possessed typical mature glial cell properties demonstrating that the adult-derived NSCs became region-specific functional cells [78]. In our work, we utilized neonatal NSCs, which have advantages over embryonic stem cells given their “limited clinical translatability” due to ethical and technical obstacles. Injected NSCs with DHA treatment showed a marked neurogenesis both in the SVZ region as being assessed by DCX immunostaining (Figs. 5 A and 4 B). The exact fate of transplanted NSCs remains unknown in our study. While some of these cells may have died, our data suggest that NSCs are still alive and may have

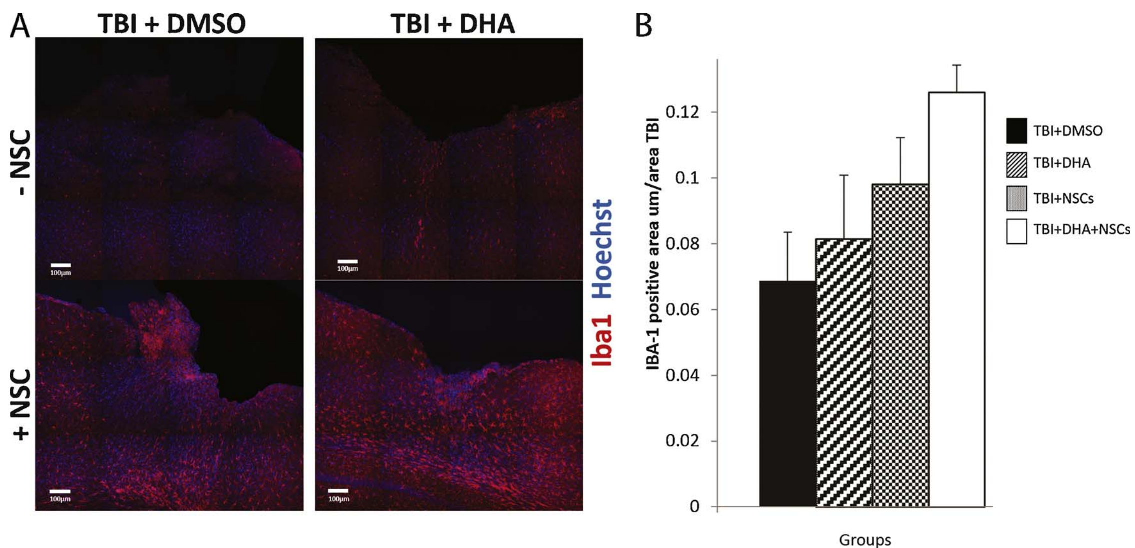

Fig. 7. Increased microglial reactivity at the TBI region in mice given dual neurotherapeutic treatment of NSC and DHA.
(A) Representative confocal tile scan images (40 X-oil objective) of 40 μm mice brain coronal sections immunostained with anti-IBA1 antibody (red) and counterstained with Hoechst (blue) show an increased number of IBA+ cells in the NSC s + DHA group compared to the other 3 groups. Scale bars 100 μm. (B) Quantification of the IBA1-positive area is shown in the histogram. No difference in IBA1 expression is observed between DMSO, DHA and NSC + DMSO groups. NSC transplantation coupled with DHA daily injection (NSC + DHA group) increases the IBA1 positive cell area in the TBI region. Each bar represents the area of IBA1-positive cells per area of TBI (mean ± S.E.M., n = 3 in each cohort). Symbols indicate statistical differences between NSCs + DHA group vs. DMSO group (*) $P < 0.05$.

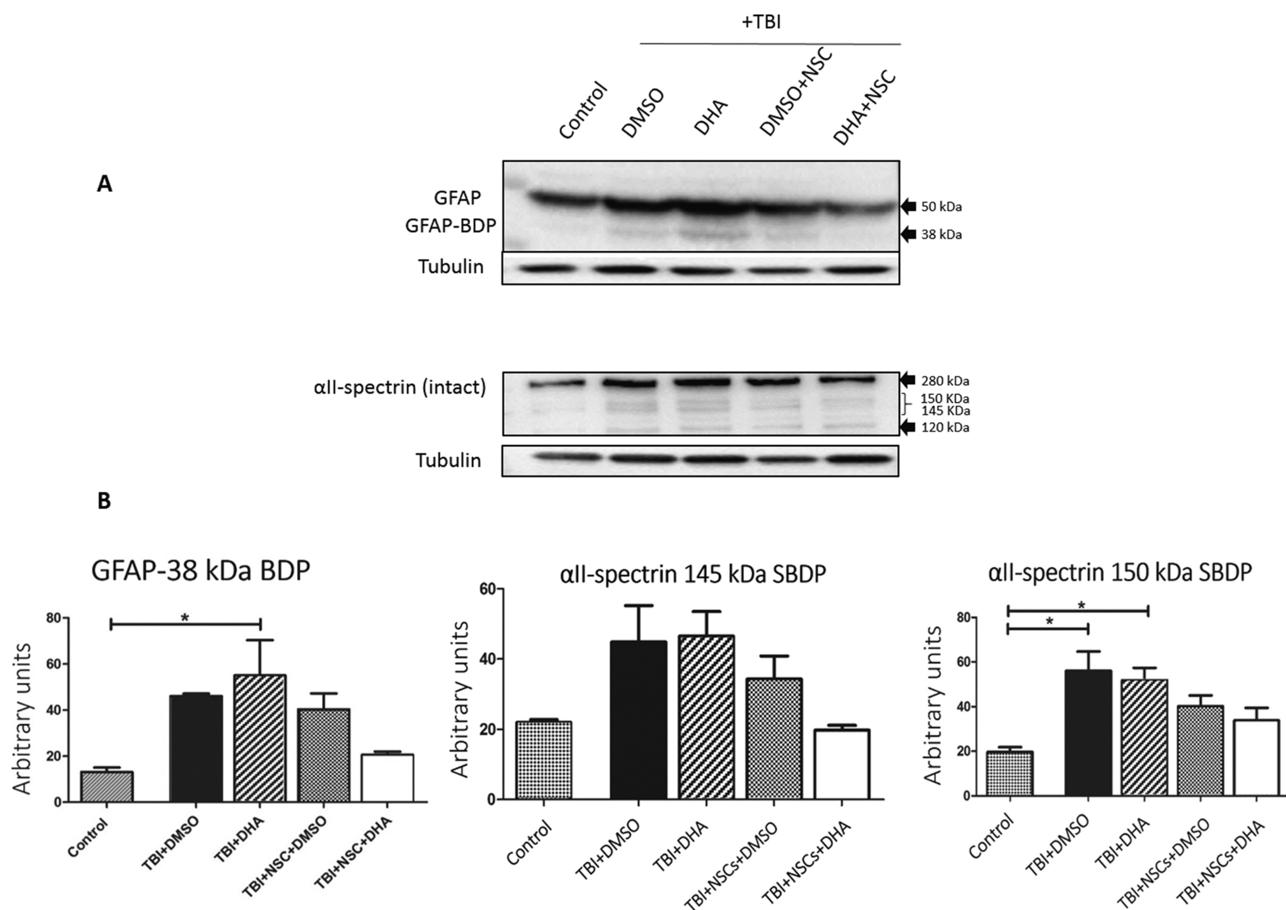

Fig. 8. Evaluation of TBI Biomarkers: GFAP 38-BDP, SBDP145 and SBDP150.
A: Assessment of Western blot analysis of the GFAP 38-BDP, SBDP145 and SBDP150.
Shown is the Western blot analysis of intact GFAP 38-BDP (1), SBDP145 (2) and SBDP150 (3). These blots show altered protein abundance among the different treatment of TBI treated with DHA/NSCs. Analysis of these BDPs, SBDP-150 levels were significantly higher in the TBI + DMSO and TBI + DHA compared to the TBI + NCS + DMSO and TBI+ NCS + DHA. The 145 and 120 kDa SBDPs showed no significant difference among the groups. Similarly, the 38 kDa calpain associated GFAP-BDP was detected in the TBI groups with the lowest level detection in the TBI+ NCS + DHA.
B: Bar graph of mean ± SEM of the GFAP 38-BDP (1), SBDP145 (2) and SBDP150 (3) as a function of injury condition and treatment. Shown are the Bar graph of mean ± SEM of the different BDPs associated with GFAP and αII-spectrin proteins. (1) The 38 kDa GFAP-BDP was higher in TBI + DHA groups compared to control group (0 < 0.05), while the levels were not significantly elevated in TBI + DMO, TBI + NCS + DMO and TBI + NCS + DHA animals (n = 3). (2) Levels of SBDP145 were not significantly different across treatments and compared to controls, (3) while SBDP150 levels were significantly elevated in TBI + DMSO and TBI + DHA animals compared to controls but TBI + NCS + DMSO and TBI+ NCS + DHA animals did not exhibit significant differences (n = 3). Values represent arbitrary densitometry units means. *: $p < 0.05$.

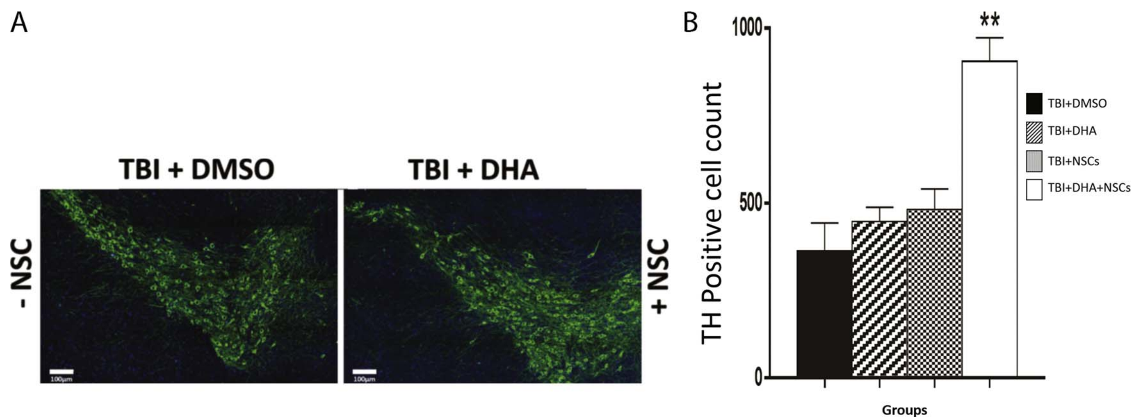

Fig. 9. Increased Tyrosine hydroxylase positive neurons in the midbrain in mice given a dual neurotherapeutic treatment of NSCs and DHA. (A) Representative confocal tile scan images (40 X-oil objective) of 40 µm mice brain coronal sections immunostained with anti-TH antibody (green) and counterstained with Hoechst (blue) show an increased number of TH+ cells in the NSCs + DHA group compared to the other 3 groups (shown is a representative image of TBI + DMSO vs. the NSCs + DHA group). Scale bars 100 µm. (B) Quantification of TH-positive cells is shown in the histogram. No difference in TH+ numbers is observed among DMSO, DHA and NSC + DMSO groups. NSC transplantation coupled with daily DHA injection (NSCs + DHA group) increase the TH- positive cell number in the SN and VTA. Each bar represents the mean of TH-positive cell number in 2–9 sections per mouse brain (mean ± S.E.M., n = 3 for DMSO group NSC + DHA group, n = 3 for DHA group and NSC + DMSO group). Symbols indicate statistical differences between the NSC + DHA group and the DMSO group (**) $P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differentiated into mature cells. However, it is unknown whether these cells have functionally integrated into the neural circuit and how DHA may have contributed to the therapeutic outcome warrant further investigation.

Based on previous studies, Katakura et al. reported that DHA-induced neuronal differentiation by decreasing Hes1 expression and increasing p27 expression, which caused cell cycle arrest in NSCs [45]. In addition, Sakayori et al. demonstrated that DHA increased the neuronal differentiation of gliogenic tertiary NSCs *in vitro* [43]. DHA has been shown to maintain NSCs during embryogenesis. It is also essential for maintenance and proliferation of NSCs in adult rat hippocampi and fat-1 transgenic mice. Furthermore, DHA depletion in the brain by chronic omega-3 fatty acid deficiency exacerbates injury levels and impedes the functional recovery. DHA has been shown to promote neurite outgrowth, synaptogenesis and glutamatergic neurotransmission by increasing the glutamate receptor subunit.

In their study, Kawakita et al., explored whether DHA affects NSCs' potential to differentiate into neurons. Rat neurospheres were cultured under differential conditions with or without DHA. DHA promoted differentiation into neural stem cells and increased newborn neurons modulating the hippocampal function through enhancement of neurogenesis [35]. Several lines of evidence have indicated that DHA modulates the expression of the brain plasticity-related protein BDNF (Brain-derived neurotrophic factor) and have associated CREB (cAMP response element binding protein)- (a transcription factor involved in learning and memory)-and synapsin 1 with reduced oxidative damage and increased neural plasticity [79,80]. Could DHA facilitate the survival, growth, and differentiation into neurons of these transplanted NSCs in vivo? In our study, we hypothesize that these neuroprotective data and rehabilitative effects observed post stem cell transplantation/ DHA co-treatment arise from multiple events as discussed later.

In addition, several studies have been able to confirm that brain injury by itself is capable of inducing endogenous neurogenesis in different brain regions, including SVZ, dentate gyrus and hippocampus [81–83]. This injury-induced neurogenesis contributes considerably to post-injury recovery [84,85]. Neuroblasts can migrate from the SVZ to the injured striatum and cortex in an attempt to enhance the outcomes of injury recovery and to replace damaged neurons [86]. Similar results to ours have been reported in other studies showing that stem cell transplantation can affect endogenous stem/precursors neurogenesis [87,88], increase endogenous neurogenesis in the hippocampus [89,90] and provide a neuroprotective environment for cell population at the site of transplantation [91,92]. In our study, this observed increase in endogenous neurogenesis is enhanced for the DHA-treated NSCs group, which is translated in enhancing motor recovery post-TBI. These results complement different studies that show that either DHA or NSCs would induce neural repair and behavioral recovery [16,22,33,35,37,41,44,45,79,93–97]. To the best of knowledge, this is the first study to utilize such dual treatment of DHA and NSCs for neural repair and functional outcomes. In our work, the only neurotherapeutic outcome was observed when NSCs and DHA were simultaneously rather than separately applied as a treatment.

We postulate that the higher number of DCX positive cells in the group receiving the dual therapy compared to those with DHA or stem cells transplantation could be attributed to the ability of DHA to influence positively the survival and proliferation of the exogenous transplanted stem cells and promote their survival by modulating the microenvironment via neurotrophic factor secretion.

Studies have documented that endogenous stem cells interact with the transplanted NSCs and modulate the microenvironment of the injured brain by secreting different chemokines, and growth factors [98]. These, in turn, may enable the transplanted stem cells to survive and migrate to the damaged brain part where they settle near blood vessels and interact with GFAP and IBA-1 positive cells [99]. Alternatively, neurogenesis and behavioral recovery have been attributed to neurotrophic factors triggering endogenous repair on the premise that the percentage of stem cells capable to differentiate into neurons is believed to be negligible [100,101].

4.3. Microglial and astroglial activation

TBI induces reactive gliosis characterized by hypertrophic and proliferating astrocytes, proliferating microglia and NG2-positive oligodendrocytes, which eventually form a bordering glial scar around the damaged area [102]. This general reactive gliosis can exert protective functions essential for neuronal survival during the acute stress-handling phase of neurotrauma [103]. However, these benefits seem to be counterbalanced by restricted regenerative potential at a later stage [104,105]. This neuroprotective or neurotoxic ability depends on specific molecular stimuli, resulting in containment or aggravation of disease progression [106]. Along the same line, the exact role of microglia has been under investigation due to its diverse roles. Although microglial cells were considered to impede injury-induced neurogenesis [107], recent studies have shown that microglia can enhance neurogenesis upon activation by IL-4 and IFN-gamma [108]; while under chronic activation, microglia was shown to adopt an anti-inflammatory response [109]. In an elegant study by Deierborg et al., the role of microglia in the proliferation of neural stem/progenitor cells (NSPCs) harvested from the SVZ region 1–2 weeks was investigated following excitotoxic striatal model of brain injury [20]. It was found that there was a direct relation between microglia from the injured brain and the increased proliferation of NSPCs along with an increased ability of NSPCs to differentiate into neurons and oligodendrocytes rather than astrocytes. This has been attributed to secreted microglial trophic factors [20]. In addition, microglial-induced NSPCs neurogenesis was observed *in vivo* as well, which highlights the importance of microglial temporal activation on the dual role that microglia perform (Fig. 7). Similarly, we have also observed an increased astrocytosis in the injured cortical vicinity in the DHA-NSCs treated groups. This upregulation in GFAP positive cells has been suggested to induce blastogenesis giving rise to NSCs that can differentiate into neurons and glia (Fig. 6). [110–112]. This is of high significance to us that −along with the elevated DCX positivity- astrogliosis was also detected. Our study doesn't answer whether these astrocytes may have undergone blastogenesis differentiating into functional neuronal cells. Further investigation is needed to assess the exact role exerted by the GFAP positive reactivity.

In addition, it was shown that transplanted NSCs in an injured brain can interact with microglia and reactive astrocytes secreting neuroprotective substances to rescue host neurons [91,92], and can also increase endogenous neurogenesis. In one study, Harvey et al. assessed DHA for inflammatory indices in a rat CCI model. DHA was administered intraperitoneally post-TBI for 3–21 days [93]. It was shown that DHA ameliorated neuronal endoplasmic reticulum stress and interfered with the microglial conversion from the TBI-observed activated amoeboid shape to the surveillance state expressing the anti-inflammatory marker CD206 [93]. In our study, neither DHA nor transplanted NSCs administered separately resulted in an increase in reactive gliosis at the site of injury. We hypothesize that the increased gliosis observed upon NSC transplantation coupled with DHA treatment, result in a crosstalk between glial cells and NSCs which may be mediated via secreted factors (cytokines and/or trophic factors) [98]. Thus, neurogenesis, along with the observed behavioral recovery, may highlight a neuroprotective role of microglial activation which may be mediated by DHA supportive environment. Definitely, further studies are needed to assess the exact function(s) of such an activation may have.

4.4. Tyrosine hydroxylase cell enhancement & neural injury markers

In addition to the above events, we have observed that the dual treatment has increased the nigrostriatal TH-positive neuronal cells in the ventral tegmental area (VTA) and substantia nigra 3 weeks post

-treatment compared to either treatment (Fig. 9A and B). It has been previously shown that there is a marked decrease in the dopaminergic neuron survival in rats raised on n-3 PUFA-deficient diet which has been attributed to a decrease in dopaminergic cell number, as well as function [113]. Loss of dopaminergic innervating fibers from the ventral tegmental area and substantia nigra alter the synaptic structure and dendritic complexity within the striatum and frontal cortex [114,115]. Similarly, other reports have indicated that NSCs are potentially capable of differentiating into dopaminergic neurons [116,117]. In one study by Chang et al., the effect of DHA treatment on Induced pluripotent stem (iPS) cells indicated that DHA treatment facilitated iPS differentiation into TH positive neurons both in vitro and in vivo. This was coupled with an enhanced functional recovery along with an increase in dopamine release in case of Parkinson's disease [94]. This TH conversion co-occurred with upregulation in the expression of neuroprotective pro-survival genes, including Bcl-2, Bcl-xl, BDNF and glial cell-derived neurotrophic factor [94]. We hypothesize that the combined effect of DHA and NSC transplantation has contributed to the elevated numbers of TH-positive neurons of the VTA and the SN (Fig. 9).

Along the same line, we have quantified TBI-associated biomarkers that are indicative of calpain/caspase activation assessed via GFAP and αII-spectrin proteolysis resulting in defined BDPs. It is well established that these specific BDPs are useful biomarkers indicative of the injury level and mechanism of TBI (50–54, 60–64). In this work, the presence of the GFAP–38 kDa and the 150 kDa SBDP in the TBI groups (indicative of calpain activation) was minimally detected in the TBI+ NCS + DHA group (Fig. 8). These results that indicate that calpain activation was ameliorated in the combined DHA + NSCs treatment resulting in a decreased neural injury. Thus, this combination of DHA would represent a desirable strategy to improve the therapeutic efficacy of NSC transplantation post-TBI.

4.5. Limitation and future directions

This work builds on previous and current recent studies from our and other labs that have assessed the capabilities of neural stem cells in ameliorating neural injury post-TBI [118,119]. Implanted NSCs would modulate existing pathological conditions replacing injured neurons via proliferation and/or inducing trophic effects. To the best of the knowledge, this is the first study to assess the role of combined treatment of DHA and NSCs injection in an experimental model of TBI We planned our study based on the complementary neurotherapeutic roles and potential incremental effect of DHA and NSCs on promoting cellular repair and behavioral recovery. Data from this work demonstrated that NPCs-DHA treatment would promote cellular neurogenesis and facilitate functional recovery with no overt behavioral or morphological abnormalities. However, one should be cautious in interpreting the data as they carry a number of limitations that future work needs to address.

First, this research utilized mild TBI model of CCI (1 mm) which does not cause pronounced cellular injury and behavioral/cognitive deficits as compared to moderate or severe experimental brain injury models. This model carries a number of inherent limitations where a greater part of the injury will be due to the herniation that naturally occurs; thus, imposing variation and inconsistency in the injury level. The exact effect of NSCs implementation with DHA would be better assessed in much severe CCI model rather than the mild TBI which may be too mild to fully assess our hypothesis explaining the unaltered rotarod behavioral testing among the groups. Second, this work would have benefitted from the use of GFP-transgenic mice where implemented NSCs are traced depicting better assessment for proliferation and neurogenesis as well as comparing the M1 M2 microglial population to precisely determine their neuroprotective or detrimental roles. Third, our work did not include a sham surgery or naive surgery cohorts with the treatments alone as they may offer better analysis on the obtained data. The sham surgery itself with DHA would assess the role of DHA and/or NSCs treatment on naïve animals; this needs to be addressed in future work as it will highlight on the cellular and molecular mechanisms of DHA/NSCs treatment. Finally, a major concern is raised concerning the time frame of NSC transplantation; the literature is rich in different studies and clinical trials with conflicting optimal time point for NSCs implementation; each with its own rationale which needs to be addressed in future work. Our work has relied on previous work showing that NSC-transplanted at 7 days would result in enhanced cellular response and improved cognitive outcomes and would be clinically more relevant. Several researchers would debate that this time point has resulted in the observed gliosis/astrogliosis data which warrants further optimization. In conclusion, our humble work demonstrated encouraging results for NSCs complemented with DHA treatment in terms of behavioral and molecular endpoints. Future work is required to answer further gaps raised in this work.

Competing financial interests statement

The authors declare no financial or any other conflicts of interest pertaining to this work.

Author contribution

WAA, JS and FK conceived the study design, obtained funding for the study, and revised and edited the manuscript. JS, HG & NR harvested and assessed the neural stem cells used for the experiments. JS, HG, NR, & SM, conducted the experimental design of the TBI. WAK, JS, FK, KZ, FS and HD wrote the manuscript. FK, JS, SM and WAA assisted in the study design. FK, RMB, JS and WAA helped analyze the output data. SS, NR, HG performed the behavioral testing. All authors assisted in the final assessment of data and reviewed the manuscript. All authors have read and approved the final manuscript.

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