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Repetitive and prolonged omega-3 fatty acid treatment after traumatic brain injury enhances long-term tissue restoration and cognitive recovery

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Running title: post-TBI n-3 PUFAs promote brain restoration

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ABSTRACT

Traumatic brain injury (TBI) is one of the most disabling clinical conditions that could lead to neurocognitive disorders in survivors. Our group and others previously reported that prophylactic enrichment of dietary omega-3 polyunsaturated fatty acids (n-3 PUFAs) markedly ameliorates cognitive deficits after TBI. However, it remains unclear whether a clinically relevant therapeutic regimen with n-3 PUFAs administered after TBI would still offer significant improvement of long-term cognitive recovery. In the present study, we employed the decline of spatial cognitive function as a main outcome after TBI to investigate the therapeutic efficacy of post-TBI n-3 PUFA treatment and the underlying mechanisms. Mice were subjected to sham operation or controlled cortical impact, followed by random assignment to receive the following four treatments: 1) vehicle control; 2) daily intraperitoneal injections of n-3 PUFAs for 2 weeks, beginning 2 hours after TBI; 3) fish oil dietary supplementation throughout the study, beginning 1 day after TBI; or 4) combined treatment of 2) and 3). Spatial cognitive deficits and chronic brain tissue loss, as well as endogenous brain repair processes such as neurogenesis, angiogenesis, and oligodendrogenesis, were evaluated up to 35 days after TBI. The results revealed prominent spatial cognitive deficits and massive tissue loss caused by TBI. Among all mice receiving post-TBI n-3 PUFA treatments, the combined treatment of fish oil dietary supplement and n-3 PUFA injections demonstrated reproducible beneficial effect in attenuating cognitive deficits although without reducing gross tissue loss. Mechanistically, the combined treatment promoted post-TBI restorative processes in the brain, including generation of immature neurons, microvessels, and oligodendrocytes; each of which was significantly correlated with the improved cognitive recovery. These results indicated that repetitive and prolonged n-3 PUFA

treatments after TBI are capable of enhancing brain remodeling, and could be developed as a potential therapy to treat TBI victims in clinic.

Keywords: n-3 PUFA; water maze; hippocampus; angiogenesis; oligodendrogenesis

INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of mortality and disability in children and young adults, and a major socioeconomic burden in the United States. Currently, strategies to treat severe TBI are largely limited to supportive management, *e.g.* stabilizing the function of vital organs, whereas no direct therapies are available that can effectively decelerate brain injury progression or promote brain repair[1]. Several approaches of TBI treatment, such as anti-inflammatory and anti-oxidative strategies[2], hypothermia[3] and cell-based therapies[4], demonstrate efficacy in preclinical studies; however, none of them has been successfully translated to clinical use[5,6]. TBI induces rapid and sustained pathophysiological changes in the brain, characterized by initial primary injuries resulting from mechanical forces, as well as prolonged secondary injuries through various complex cellular processes, which eventually leads to neuronal death, axonal injury, and neurological deficits[7,8]. Simultaneously, active tissue remodeling including neurogenesis[9], angiogenesis[10], and white matter repair[11], occurs in the post-injury brain, which could positively influence post-TBI functional recovery. It is imperative to develop novel TBI therapies to battle brain injury development and/or boost brain repair, thereby achieving the improvement of long-term patient outcome.

Numerous preclinical studies by our and other groups have suggested omega-3 polyunsaturated fatty acid (n-3 PUFA) as an emerging candidate for TBI therapy[12-15]. The most

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important n-3 PUFAs for human health, *i.e.* docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), must be acquired through dietary intake, with the primary sources being fish and fish oil[16,17]. n-3 PUFAs exist abundantly in the brain and play a crucial role in essential neuronal functions, such as axonal guidance, synapse and dendrite formation, neurotransmission, etc.[16,18]. Following experimental TBI, n-3 PUFAs exert potent protective effects through multifaceted actions, *e.g.* amelioration of oxidative stress[19], mitigation of endoplasmic reticulum stress[20], modulation of microglial activation[21], and improvement of white matter integrity[15]. The use of n-3 PUFAs to treat TBI has not hitherto been translated to clinic, although there are sporadic case studies using n-3 PUFAs acutely after human TBI[22,23]. A large portion of the preclinical studies on n-3 PUFAs employed a pre-TBI treatment paradigm, achieved through prophylactic dietary supplement or genetic engineering[12,15,19], which provided limited information when considering translating this treatment from bench to bedside. While some studies delivered invaluable mechanistic insights of post-TBI n-3 PUFA treatment[14,20], concerns still remain over the short delivery time window, or the lack of long-term functional evaluation. To facilitate future investigations on n-3 PUFAs toward patient use, we aim to develop a clinically feasible treatment regimen using manageable delivery routes and time window, hoping to validate the use of n-3 PUFAs post-TBI and potentially benefit the TBI victims.

In this study, we designed the post-TBI therapeutic regimens of n-3 PUFAs, in which the first intervention (daily injections) did not start until 2 h after TBI. We evaluated the efficacy of this treatment, together with fish oil dietary supplementation that started at 1 d after injury, on TBI outcome in a mouse model. Furthermore, we examined the underlying mechanisms of post-TBI n-3 PUFA treatments, with a focus on post-injury tissue remodeling processes.

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MATERIALS AND METHODS

Animals

C57BL/6J mice (male, 10-12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and used in our experiments. Mice were housed in a temperature- and humidity-controlled animal facility with a 12-h light-dark cycle. Food and water were available *ad libitum*. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Efforts were made to minimize animal suffering and the number of animals used.

Traumatic brain injury

All mice were randomly assigned into experimental groups with the use of a lottery-drawing box. TBI was induced in mice by a controlled cortical impact (CCI) as described previously[24]. Briefly, mice were anesthetized with 3% isoflurane (Butler Schein Animal Health, Dublin, OH, USA) vaporized in 67% N_2O/30% O_2 mixture until they were unresponsive to the tail pinch test. Anesthesia was maintained with 1.5% isoflurane in 67% N_2O/30% O_2 by fixing the mouse to a nose cone with consistent blowing of gas. Following a craniotomy (diameter: 3.0 cm), the CCI was performed (2.0 mm lateral to midline and 0.5 mm anterior to bregma) with a pneumatically driven CCI device (Precision Systems and Instrumentation, Fairfax, VA, USA) using a 3-mm flat-tipped impounder (velocity: 3.50 m/s; duration: 150 ms; depth: 1.5 mm). The bone flap was then replaced and sealed. Rectal temperature was maintained at 37°C ± 0.5°C during surgery and up to 30 min after TBI using a heating pad. Sham animals were subjected to all aspects of the

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surgery and handling (including the craniotomy) except for the CCI. Animals that died during or immediately after surgery (less than 5%) were excluded from the studies. All outcome measurements were carried out by investigators blinded to experimental group assignment.

Administration of n-3 PUFAs after TBI

Mice were randomly assigned to receive the following treatments of n-3 PUFAs after TBI: 1) Vehicle control. Mice were fed a regular laboratory rodent diet (Prolab Isopro RMH 3000 5P76; LabDiet, St. Louis, MO, USA), in which the n-3 PUFA content was low (0.36%), and received *i.p.* injections of 0.9% NaCl (300 µl per injection, 2 h after TBI and then daily for 14 d). 2) Injections of mixed n-3 PUFAs ("N3"). Mice were fed a regular diet, and received *i.p.* injections of EPA and DHA (7 mg EPA and 3 mg DHA per kg body weight, diluted in 300 µl 0.9% NaCl per injection, 2 h after TBI and then daily for 14 d). 3) Fish oil dietary supplementation ("FO"). Mice were fed a diet supplemented with n-3 PUFAs (DHA and EPA, triple strength n-3 fish oil, Puritan's Pride, Oakdale, NY, USA) to reach a 4% final n-3 PUFA concentration beginning 1 d after TBI and for up to 35 d, and received *i.p.* injections of 0.9% NaCl (300 µl per injection, 2 h after TBI and then daily for 14 d. 4) Combined treatment of 2) and 3) ("N3+FO"). Mice were fed with a diet supplemented with fish oil and received injections of DHA and EPA as described above. The EPA/DHA ratio (70/30%) in the injectable N3 mixture was dictated by their respective contents in the triple strength n-3 fish oil (624 mg EPA and 244 mg DHA in 1360 mg fish oil per capsule).

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Morris Water Maze

The Morris water maze test was performed at 29-34 d after TBI to evaluate long-term spatial cognitive deficits as previously described[25]. Briefly, a pool (diameter: 109 cm) with opaque water was divided into four quadrants by phantom lines. A square platform (11 cm × 11 cm) was submerged under the water for 1 cm in one of the four quadrants. Three trials were performed on each testing day. For each trial, mouse was placed into the pool from each of the other three quadrants without the platform, and allowed 60 s to swim and locate the hidden platform (learning phase of the test). The time at which the mouse found the platform (escape latency) was recorded for each trial. At the end of each trial, the mouse was placed on the platform or allowed to stay on the platform for 10 s with prominent spatial cues displayed around to memorize the position of the platform. Mice were pre-trained for 3 consecutive days before TBI (3 trials on each day; Fig. 1A). After TBI, three trials were performed daily for 5 consecutive days from day 29 to 33, and the average latency to escape was calculated. At day 34 after TBI, a single, 60-s probe trial was performed in which the platform was removed. The time the mouse spent in the target quadrant where the platform was previously located was recorded (memory phase of the test). Swim speed was recorded on each testing day from day 29 to 34 after TBI as a parameter to assess the gross locomotor function of the mouse.

Immunohistochemistry and image analysis

At day 35 after TBI, mice were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were collected and cryoprotected in 30% sucrose in PBS. Frozen serial coronal brain sections (25 μm thick) were cut by a cryostat (Microm HM450, Thermo scientific, Florence, KY, USA).

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Immunohistochemistry was performed on free-floating sections. Briefly, sections were blocked with 5% donkey serum in PBS for 1 h, followed by overnight incubation (4°C) with the following primary antibodies: rabbit anti-NeuN (1:500; EMD Millipore, Billerica, MA, USA), goat anti-doublecortin (DCX; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat anti-CD31 (1:200; BD Biosciences, San Jose, CA, USA), mouse anti-adenomatous polyposis coli (APC; 1:400; EMD Millipore). After a series of washing, sections were incubated for 1 h at 37ºC with the appropriate donkey secondary antibodies conjugated with Alexa Fluor 488 or Cy3 (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Alternate sections from each experimental condition were incubated in all solutions except the primary antibodies to assess nonspecific staining. Sections were then mounted and coverslipped with Fluoromount-G containing 4', 6-diamidino-2-phenylindole (DAPI; Thermo Scientific, Pittsburgh, PA, USA). Fluorescence images were captured with an Olympus Fluoview FV1000 confocal microscope using FV10-ASW 2.0 software (Olympus America, Center Valley, PA, USA). Alternatively, whole-brain images of NeuN fluorescence were acquired with an inverted Nikon Diaphot-300 fluorescence microscope equipped with a SPOT RT slider camera and Meta Series Software 5.0 (Molecular Devices, Sunnyvale, CA, USA).

Brain tissue loss was analyzed with ImageJ as described previously[15]. Briefly, six equally-spaced NeuN-stained coronal brain sections encompassing the CCI territory (from bregma 1.10 mm to bregma -1.34 mm) were selected. The tissue loss in each section was calculated by subtracting the NeuN immunosignal positive area in the contralateral hemisphere from that in the ipsilateral hemisphere. The volume of tissue loss was calculated by multiplying the mean area of tissue loss in each section by the thickness of the tissue evaluated.

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Examination of recently proliferated cells

Recently proliferated cells were labeled with the S-phase marker 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, Missouri, USA) as previously described[26]. Briefly, BrdU was injected *i.p.* twice a day at a dose of 50 mg/kg body weight at 3-6 d after TBI. At 35 d after TBI, mice were sacrificed and coronal brain sections were prepared as described above. Sections were treated with 2N HCl for 1 h at 37ºC followed by 0.1 M boric acid (pH 8.5) for 10 min at room temperature. Sections were then blocked with M.O.M. kit (Vector, Burlingame, CA, USA) for 1 h, followed by incubation with purified mouse anti-BrdU antibody (1:1000; BD Biosciences) for 1 h at room temperature and then overnight at 4ºC. After a serial of washing, sections were incubated with the Alexa Fluor 488-conjugated AffiniPure donkey anti-mouse IgG (1:1000; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Fluorescence images were captured as described above. BrdU immunopositive cells were counted using ImageJ and expressed as the number of cells *per* mm^2 or *per* microscopic field (0.16 mm^2 at ×40 magnification). Post-TBI neurogenesis, angiogenesis, and oligodendrogenesis in the peri-lesion areas (within 300 μm to the lesion edge) were evaluated on BrdU/NeuN, BrdU/DCX, BrdU/CD31 and BrdU/APC double-stained sections. Two microscopic fields from the cerebral cortex and striatum, and one microscopic field in each of the subventricular zone (SVZ), hippocampal CA1, CA3 and dentate gyrus (DG) areas were randomly sampled in every section.

Statistical analyses

All data are presented as mean ± SEM. The differences between means of multiple groups were assessed by one- or two-way ANOVA followed by the Bonferroni *post hoc* test. The Pearson

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product linear regression analysis was used to correlate the mouse's spatial learning performance (at 33 d after TBI) and memory performance in the Morris water maze with various histological parameters. $p < 0.05$ was considered to be statistically significant.

RESULTS

Post-TBI treatment with n-3 PUFAs was effective in improving long-term cognitive recovery

Cognitive impairments are common complications caused by TBI, and are closely related to daily functioning of the TBI victims[7,27]. We previously reported that n-3 PUFA-enriched diets can substantially elevate brain n-3 PUFA content when applied to mice on a long-term basis[26]. This prophylactic treatment before the onset of TBI, in combination with the continuous n-3 PUFA supplement after injury, was able to ameliorate long-term spatial learning and memory deficits in a mouse CCI model[15]. To test whether n-3 PUFAs hold potential to be used on TBI victims, we designed an n-3 PUFA interventional regimen starting 2 h after TBI via *i.p.* injections of EPA and DHA mixture (Fig. 1A). The EPA/DHA ratio (7:3) of the injection mixture was dictated by their respective compositions in the triple strength n-3 fish oil. This acute treatment, together with long-term FO dietary supplement that started 1 d after TBI, features a potentially manageable therapeutic window and delivery route in clinical settings. To determine the efficacy of n-3 PUFA treatment against TBI-induced cognitive decline, we assessed the long-term spatial learning and memory performance by mice in the Morris water maze at 29-34 d after TBI (Fig. 1B). Spatial learning was observed in all groups during the 5-day testing period, as demonstrated by the gradually decreased latency for the mice to find the submerged platform at 29-33 d after TBI (Fig. 1C). Compared to sham-operated mice, TBI

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induced prominent spatial learning deficits in the vehicle group (Fig. 1C; $p \leq 0.001$ vehicle vs. sham by two-way ANOVA). Post-TBI N3 injections did not reduce the escape latency compared to vehicle group. In contrast, FO dietary supplementation alone or combined with N3 injections significantly facilitated the recovery of spatial learning, compared to either vehicle- or N3-injected group (Fig. 1C). Consistently, N3+FO-treated mice, but not either treatment alone, also improved the spatial memory 34 d after TBI compared to vehicle- or N3-injected group (Fig. 1D). All five groups showed comparable swimming speed during the 6-day testing (Fig. 1E). Therefore, the observed difference in the mice's learning and memory performance was not a consequence of varied gross locomotor function.

We next examined whether the improved cognitive function elicited by n-3 PUFA treatment was associated with less neuronal tissue loss after TBI, as determined on NeuN-immunostained coronal sections (Supplementary Fig. 1A, Supplementary material available at, https://pitt.app.box.com/v/PuCellTransplantSuppleFigs). However, the volumes of the tissue loss in all four groups of TBI mice were comparable (Supplementary Fig. 1B). In summary, these data indicate that long-term FO dietary supplement after TBI, especially when combined with acute N3 injections, significantly improves cognitive recovery. Mechanisms other than the reduction of gross neuronal tissue lost might underlie this improved cognitive function.

Post-TBI n-3 PUFA treatment does not alter histological hippocampal integrity

We further investigated the underlying mechanisms accounting for the improved cognitive functions observed in mice receiving post-TBI n-3 PUFA treatments. Since spatial learning and memory are believed to largely rely on the histological and functional integrity of the hippocampus[25,28], we measured the number of viable neurons ($NeuN^+$ cells) in the ipsilesional

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hippocampal CA1, CA3 and DG at 35 d after TBI (Fig. 2A). The results revealed that TBI caused significant neuronal loss in the ipsilateral CA1 and CA3, but not in the DG (Fig. 2B). Post-TBI N3 injections, FO supplements, or combined N3 and FO treatment did not elicit significant protection on hippocampal integrity in CA1 or CA3 when examined at 35 d after TBI (Fig. 2B). Furthermore, neurogenesis was barely observed in the entire hippocampus, shown by very few $BrdU^+$ cells or $BrdU^+/NeuN^+$ cells in CA1, CA3 and DG (Fig. 2A, C). Pearson product linear regression analysis demonstrated moderate but yet statistically significant correlations between the numbers of CA3 viable neurons, but not CA1 viable neurons, and the mice's performance in the water maze learning and memory test (Fig. 2D, E). In summary, these data suggest that the post-TBI n-3 PUFA treatment did not significantly preserve hippocampal neuronal integrity, although the CA3 viable neurons are partially linked to improved spatial cognitive functions. Other mechanisms thus must contribute to the cognitive improvement in mice receiving N3+FO treatment.

Delayed n-3 PUFA treatment enhances the survival of cortical and striatal neurons

Despite the absence of direct protection on hippocampal neurons, post-TBI n-3 PUFA treatment elicited dramatic improvement of cognitive recovery, suggesting the participation of non-hippocampal mechanisms. It is a generally acceptable concept that a functionally integrated neural network is required to properly navigate the Morris water maze, which is dependent not only on the hippocampus but also other brain regions such as the cerebral cortex and striatum[29-31]. Since the present CCI model produced substantial lesion in both the cortex and striatum, we examined the neuronal survival in these regions at 35 d after TBI (Fig. 3A, B). Post-TBI FO treatment alone resulted in significant increase of viable neurons in the peri-lesion (within 300

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μm from the injury border) striatal areas compared to mice receiving vehicle treatment (Fig. 3C, D). Combined N3+FO treatment further enhanced the protection and significantly increased viable neurons in both the peri-lesion cortex and striatum (Fig. 3C, D). In comparison, N3 injections alone did not produce a significant effect on neuronal loss (Fig. 3D; N3 943.60 ± 48.38 cells/mm^2 vs. vehicle 938.72 ± 45.79 cells/mm^2 in the cortex and N3 1185.30 ± 47.02 cells/mm^2 vs. vehicle 1087.65 ± 82.65 cells/mm^2 in the striatum, $p>0.05$). Some BrdU$^+$ cells were observed in the peri-lesion cortex and striatum, but only a very small portion expressed the mature neuronal marker NeuN (Fig. 3C,E). Furthermore, n-3 PUFA treatment did not alter the number of BrdU$^+$/NeuN$^+$ cells in either the cortex or striatum (Fig. 3E). These data suggest that increased viable neurons in FO or N3+FO-treated mice may be a result of protection against cell death rather than increased neurogenesis. The number of viable neurons in both the cortex and striatum showed moderate but statistically significant negative correlation with the escape latency in the water maze learning task (Fig. 3F), and positive correlation with the spatial memory (Fig. 3G). Together, these results suggest that the combined FO dietary supplementation and N3 injections may improve post-TBI cognitive functions partially through promotion of neuronal cell survival in the cortex and striatum.

Combined N3 and FO treatment after TBI stimulates the generation of immature neurons

TBI has been shown to accelerate neurogenesis in the hippocampus and SVZ, which may contribute to the functional recovery of the brain[9,32,33]. In the present study, very few BrdU$^+$/NeuN$^+$ cells were detected throughout the brain after TBI (Fig. 2, 3). To determine whether this was due to a deficiency of neural stem/progenitor cell (NSC/NPC) generation *per se*, we examined the expression of DCX, a marker for neuronal precursor cells and immature

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neurons, at 35 d after TBI (Fig. 4A). TBI stimulated endogenous neurogenesis, shown by increased $BrdU^+/DCX^+$ cells in the peri-lesion cortex (Fig. 4B; vehicle 1.94±0.20 cells/field vs. sham 0 cells/field, p=0.009). Among all four treatments, FO dietary supplementation alone or combined with N3 injections increased the $BrdU^+/DCX^+$ newly generated immature neurons in both the peri-lesion cortex and striatum (Fig. 4B). Some $DCX^+/BrdU^-$ cells were also present, indicating that the antibody used exhibited some non-specificity. Nevertheless, there was no difference in $BrdU^+/DCX^+$ cell numbers among all groups in the SVZ at 35 d after TBI (Supplementary Fig. 2), indicating that the newborn immature neurons in the SVZ largely migrated to the cortex and striatum at earlier stages after TBI. Importantly, the increase of newborn immature neurons in the striatum significantly correlated with the mice's spatial learning and memory performance in the Morris water maze (Fig. 4C, D). These results suggest that post-TBI n-3 PUFA supplementation was capable of stimulating the generation of immature neurons and their migration towards the peri-lesion brain regions. Although not fully differentiated into mature neurons, these immature cells might play a role in the improvement of post-TBI cognitive recovery.

Delayed n-3 PUFA treatments promote angiogenesis after TBI

Angiogenesis is an essential restorative mechanism in the brain after TBI, and supplies a "vascular niche" that is necessary for the survival of surrounding cells[34,35]. We examined the impact of delayed n-3 PUFA treatment on angiogenesis in the peri-lesion regions in the cortex and striatum 35 d after TBI by double-label immunostaining of BrdU and the endothelial marker CD31 (Fig. 5A). TBI stimulated endogenous angiogenesis in the peri-lesion cortex (Fig. 5B; vehicle 56.01±7.92 cells/mm^2 vs. sham 13.97±2.59 cells/mm^2, p=0.011). FO dietary

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supplementation alone or combined with N3 injections significantly increased the numbers of newly generated microvessels in both the cortex and striatum (Fig. 5B), whereas N3 injections alone did not alter angiogenesis compared to vehicle treatment (Fig. 5B; N3 53.59 ± 4.35 cells/mm^2 vs. vehicle 56.01 ± 7.92 cells/mm^2 in the cortex; N3 59.63 ± 4.39 cells/mm^2 vs. vehicle 47.55 ± 6.27 cells/mm^2 in the striatum; $p>0.05$). Meanwhile, the spatial learning and memory ability in the water maze showed a significant correlation with angiogenesis (Fig. 5C, D). Together, these results further demonstrated that the beneficial effect of n-3 PUFA treatments on post-TBI cognitive function was partially achieved through promoting angiogenesis in the surviving brain tissues.

Post-TBI n-3 PUFA treatments boost oligodendrogenesis

TBI-induced demyelination and axonal injury are important contributors to cognitive decline[7,11]. Oligodendrogenesis, including the proliferation of oligodendrocyte progenitor cells (OPCs) and their differentiation into myelinating oligodendrocytes, facilitates remyelination, white matter repair, and neurological recovery after TBI[11]. We therefore examined post-TBI oligodendrogenesis in the peri-lesion cortex and striatum 35 d after TBI by double-label immunostaining of BrdU and APC, a marker for mature oligodendrocytes[36]. In vehicle-treated mice, few BrdU$^+$/APC$^+$ cells were observed in the peri-lesion cortex or striatum (Fig. 6A, B; $p>0.05$ vehicle vs. sham), indicating negligible post-TBI oligodendrogenesis. While N3 injections did not cause an increase in BrdU$^+$/APC$^+$ cell numbers compared to vehicle group, long-term FO dietary supplement led to a significant increase of the newly-generated mature oligodendrocytes in both cortex and striatum (Fig. 6A, B; FO 82.20 ± 10.10 cells/mm^2 vs.

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vehicle 28.20 ± 4.38 cells/mm^2 in the cortex; FO 74.94 ± 8.95 cells/mm^2 vs. vehicle 29.01 ± 4.87 cells/mm^2 in the striatum; $p \leq 0.001$ and $p \leq 0.05$, respectively). Notably, when N3 injections were combined with FO supplementation, oligodendrogenesis was further enhanced (151.04 ± 18.33 cells/mm^2 in the cortex and 144.59 ± 18.33 cells/mm^2 in the striatum; $p \leq 0.001$ vs. vehicle and N3). It is likely that the enhanced oligodendrogenesis associated with N3+FO treatment contributed to the improved spatial cognitive function, as the mice's performance in the learning tests of the Morris water maze showed moderate but statistically significant correlations, whereas the memory ability exhibited strong correlations with the extent of oligodendrogenesis in both cortex and striatum (Fig. 6C, D). These results suggest that improved oligodendrogenesis might be another underlying mechanism in improving cognitive function by n-3 PUFA treatments.

DISCUSSION

The present study characterized the therapeutic efficacy of n-3 PUFAs against TBI-induced cognitive deficits, using an administration paradigm that combines repeated post-TBI DHA/EPA injections with long-term FO dietary supplementation. The results demonstrated that the combined N3 and FO treatment significantly improved spatial cognitive recovery even without affecting the tissue contusion after TBI. Further mechanistic explorations revealed that combined N3 and FO treatment not only protected neurons against TBI-induced cell death, but also promoted multiple endogenous restorative mechanisms, such as generation of immature neurons, microvessels, and oligodendrocytes, all of which could contribute to the improved cognition in TBI mice.

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The extent of TBI-induced neuronal death strongly correlates with the development of cognitive deficits[37,38]. TBI leads to rapid neuronal cell death resulting from direct contusion, followed by progressive secondary cell death in surrounding tissues[8]. n-3 PUFAs have been shown to exert potent protection against oxidative stress, glutamate-induced excitotoxicity, apoptotic cell death, and inflammation[16,39,40]. In mice receiving 2 months of prophylactic n-3 PUFA dietary supplementation, TBI caused less amount of loss of CA3 neurons compared to mice on a regular diet[15]. In the present study, we confirmed that delayed n-3 PUFA treatment after TBI also provided significant protection against neuronal loss in the cerebral cortex and striatum. Interestingly, long-term dietary supplement of FO (for up to 35 d after TBI) effectively reduced neuronal loss (Fig. 3). It is worth noting that although N3 injections alone were not sufficient to be neuroprotective in the long run, they appeared to boost the beneficial effect of FO supplement (Fig. 3). Future research is needed to further determine how the delayed n-3 PUFA treatments alter the spatial and temporal profile of neuronal cell death after TBI and the underlying signaling mechanisms.

Importantly, the number of viable neurons in the CA3, cortex and striatum were in significant correlation with post-TBI cognitive functions. We speculate that the increased viable neurons might largely result from neuroprotection, because seldom any $BrdU^+/NeuN^+$ cell was present in TBI brains with or without FO treatment. TBI was shown to stimulate neurogenesis in the granule cell layer of the DG, and the new cells were thought to be able to mature into neurons and contribute to the restoration of hippocampal functions[32,41]. The absence of robust neurogenesis in DG after TBI in the present study may be partially explained by the differences in injury sites of the CCI model between our study and the previous studies by others. Compared to the model used in the literature[32,42], our CCI model produces a lesion at more anterior coronal

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levels, which may trigger less amount of neurorestorative responses in the DG (Fig. 2C). Nevertheless, the lack of $BrdU^+/NeuN^+$ cells throughout various brain regions in our study suggest two possibilities, *i.e.* these was a failure of NSC/NPC generation *per se*, or the NSCs/NPCs did not fully differentiate into mature neurons.

We therefore examined the $BrdU^+/DCX^+$ cells and found that n-3 PUFA treatments did promote the proliferation of NSCs in post-TBI cortex and striatum. However, these immature neurons apparently failed to differentiate into mature neurons during the 35 d post-TBI period. Interestingly, the number of $BrdU^+/DCX^+$ immature neurons was nevertheless significantly correlated with post-TBI cognitive recovery, suggesting that the induced neurogenesis could contribute to functional recovery through mechanisms independent of neuronal replacement. For instance, immature neurons generated via neurogenesis can produce and release a plethora of trophic factors[43], thereby supporting the survival of surrounding neurons and nourishing a favorable microenvironment toward tissue repair.

The brain has some plasticity for spontaneous functional recovery after injury, through multiple endogenous restorative processes such as angiogenesis, axonal sprouting, and white matter repair[44-47]. In addition to the increase of immature neurons discussed above, we observed enhanced angiogenesis and oligodendrogenesis in the peri-lesion brain tissue upon post-TBI n-3 PUFA treatment (Fig. 5, 6). The generation of new microvessels helps to form the neurovascular niches, facilitates tissue remodeling such as neurogenesis and synaptogenesis, and supports the survival and migration of NSCs/NPCs, all of which may lead to functional recovery[44,48]. Another important restorative mechanism is oligodendrogenesis, which is essential for remyelination and white matter repair after TBI[49]. Following ischemic brain injury, delayed n-3 PUFA administration is capable of promoting angiogenesis and oligodendrogenesis, which are

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associated with improved functional recovery[50,51]. In the present study, we noticed an enhancement of post-TBI angiogenesis and oligodendrogenesis upon n-3 PUFA treatment that lasted up to 35 d after injury. Interestingly, our analysis shows that although all these restorative responses, *e.g.* generation of immature neurons, vessels, and oligodendrocytes, correlated with cognitive recovery, the correlations were all just moderate. Such observation may imply that any single component of post-TBI restoration may be necessary, but not sufficient enough to lead to an overall functional improvement. To this extent, n-3 PUFAs target multiple aspects of tissue remodeling simultaneously and for an extended period of time after injury, which could suggest the therapeutic potentials in clinical use. We found that for multiple histological parameters, including neuroprotection, angiogenesis, and oligodendrogenesis, FO supplements demonstrated more significant effects than N3 injections alone, and combined N3 and FO treatment have the most consistent beneficial effect among all treatment paradigms. Together, these results suggest that combined acute injections of n-3 PUFAs, together with chronic supplementation, may be a promising strategy to treat TBI victims.

Promoting long-term neurological recovery has been a major focus in rehabilitation for TBI victims. Cognitive deficits are core characteristics as a result of TBI and can persist for a long period of time. To date, the development of effective neurotherapeutic interventions against TBI has been largely fruitless, in spite of the growing public awareness and research endeavors. Preclinical studies should take into consideration using clinically manageable methods to eventually translate the therapy to TBI clinics. Fish oil is a safe and economical prophylactic supplement that has been shown to protect against brain disorders in many animal models. In our experiments, we began repetitive EPA/DHA injections at 2 h after TBI, however, EPA/DHA injections alone did not improve cognitive functions. Compared to previous studies by other

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groups showing neuroprotection of DHA when delivered 5 min after TBI[20], we might have missed the therapeutic time window for n-3 PUFAs to exert acute protection. Nevertheless, when FO dietary supplementation was given to mice even as late as 1 d after TBI, most outcome parameters examined (*e.g.* spatial learning and memory, neuronal viability in the cortex and striatum, regeneration of immature neurons, angiogenesis and oligodendrogenesis) were improved. When FO dietary supplementation combined with acute N3 injections, substantial therapeutic effects were achieved. These data suggest the importance of long-term supply of n-3 PUFAs to promote tissue repair and functional recovery, even the treatment starts as late as 1 d after TBI. With long-term brain tissue restoration as the objective, the n-3 PUFA treatment regimen described here has a clinically relevant time window of efficacy, making it a legitimate candidate for testing in TBI clinical trials. However, some caveats exist in our study. For example, some of the neurovascular restorative processes that could be affected by n-3 PUFAs were not examined in the present study, *e.g.* the effect of n-3 PUFAs on microglia activation, inflammatory responses, and white matter integrity[52-55]. Another limitation is the use of the CCI model. Despite being one of the most widely used TBI model, the CCI model replicates some, but not all, aspects of human TBI pathology. The diffusive effects of CCI are not as obvious as in several other models (*e.g.* closed head injury, blast, fluid percussion injury)[56]. Future preclinical research thus is warranted to test the therapeutic efficacy of post-TBI n-3 PUFA delivery using other neurotrauma models, in order to facilitate the clinical translation of this promising therapeutic intervention for TBI.

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Author contributions

J.C., L.C. and H.P. designed the research. H.P., X.J., Z.W., S.H. and W.Z. performed the research. X.J. and Y.S. analyzed the data. H.P., X.J., Y.S., D.H. and J.C. wrote the manuscript. All authors reviewed and approved the manuscript.

Disclosure

The authors declare no competing financial interests.

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Figure legends

Figure 1. Combined treatment of n-3 PUFAs after TBI elicits long-term improvement of spatial cognitive function.

(A) Experimental timelines. Mice were pre-trained for the Morris water maze test for 3 d before TBI. Mixed DHA and EPA (N3) were injected *i.p.* 2 h after TBI and then daily for 14 d. Fish oil (FO) dietary supplements started 1 d after TBI. qd: once a day. bid: twice a day. Water maze learning test were performed at day 29 to 33 and memory test at day 34 after TBI. Mice were sacrificed at 35 d after TBI for histological examinations. (B) Representative images showing mice swim paths from each group at the learning or memory phase of the Morris water maze. (C) The latency for mice to escape to the submerged platform in the learning test at 29-33 d after TBI. (D) Spatial memory was measured at 34 d after TBI by the time spent in the target quadrant where the platform was previously located. (E) Average swimming speed at 29-34 d after TBI indicates no significant difference of gross motor function among all groups. n=6 mice for sham group, n=8 mice for vehicle, N3 and FO groups, n=7 mice for N3+FO group. *$p \leq 0.05$, ***$p \leq 0.001$ FO or N3+FO vs. vehicle, $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ FO or N3+FO vs. N3 by two-way ANOVA (C) or one-way ANOVA (D).

Figure 2. Post-TBI n-3 PUFA treatment does not improve hippocampal integrity.

(A) Representative images showing double-label immunofluorescence of BrdU and NeuN in the CA1, CA3 and DG of the ipsilesional hippocampus at 35 d after TBI. *Scale bar*: 50 µm. (B) Quantification of total surviving neurons ($NeuN^+$ cells) in the ipsilesional CA1, CA3 and DG. (C) Quantification of newly-generated mature neurons ($BrdU^+/NeuN^+$ cells) in the ipsilesional CA1, CA3 and DG. Barely any newly-generated mature neurons were present in all regions of the

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hippocampus in all groups. $^{\#}p \leq 0.05$ N3+FO vs. N3 by one-way ANOVA. (D,E) Pearson correlation between the mice's spatial learning (D) and memory (E) performance in the Morris water maze and the numbers of total viable neurons in the ipsilesional CA1 and CA3. n=6 mice for sham group, n=8 mice for vehicle, N3 and FO groups, n=7 mice for N3+FO group.

Figure 3. Post-TBI n-3 PUFA treatment attenuates cortical and striatal neuronal death.

(A) A representative image of one coronal brain section showing NeuN immunofluorescence at 35 d after TBI. *Boxes* demonstrate the ipsilateral peri-lesion areas in the cortex (CTX) and striatum (STR) where images in (C) were taken. *Scale bar*: 1 mm. (B) A representative image from the peri-lesion cortex showing typical double-label immunofluorescence of BrdU and NeuN. *Arrow*: $BrdU^+/NeuN^+$ cell (*yellow*). *Arrowheads*: $BrdU^+/NeuN^-$ cells (*green*). *Scare bar*: 50 μm. (C) Representative images of BrdU (*green*) and NeuN (*red*) immunofluorescence in the peri-lesion cortex and striatum at 35 d after TBI. *Scale bar*: 50 μm. (D,E) Quantification of total viable neurons (D) and newly-generated mature neurons (E) in the peri-lesion cortex and striatum. $^*p \leq 0.05$, $^{***}p \leq 0.001$ vs. vehicle, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ vs. N3 by one-way ANOVA. (F,G) Pearson correlation between the mice's spatial learning (F) and memory (G) performance in the Morris water maze and the numbers of total viable neurons in the cortex and striatum. n=6 mice for sham group, n=8 mice for vehicle, N3 and FO groups, n=7 mice for N3+FO group.

Figure 4. Post-TBI n-3 PUFA treatment stimulates the generation of neural progenitor cells.

(A) Representative images of BrdU and DCX immunofluorescence in the ipsilateral peri-lesion cortex and striatum at 35 d after TBI. *Boxes* illustrated the regions enlarged in high-power images (the 6th column). *Arrows*: $BrdU^+/DCX^+$ cells (*yellow*). *Arrowheads*: $BrdU^+/DCX^-$ cells

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(*green*). *Scale bar*: 50 μm. (B) Quantification of $BrdU^+/DCX^+$ cells in the peri-lesion cortex and striatum. $^{\$\$}p\leq0.01$ vs. sham. $^*p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$ vs. vehicle, $^{\#}p\leq0.05$, $^{\#\#}p\leq0.01$, $^{\#\#\#}p\leq0.001$ vs. N3 by one-way ANOVA. (C,D) Pearson correlation between the mice's spatial learning (C) and memory (D) performance in the Morris water maze and the numbers of $BrdU^+/DCX^+$ cells in the cortex and striatum. n=6 mice for sham group, n=8 mice for vehicle, N3 and FO groups, n=7 mice for N3+FO group.

Figure 5. Delayed n-3 PUFA treatment promotes post-TBI angiogenesis.

(A) Representative images of BrdU and CD31 immunofluorescence in the ipsilateral peri-lesion cortex and striatum at 35 d after TBI. *Boxes* indicate areas that were enlarged in the high-power images (the 6th column). *Arrows*: $BrdU^+$ cells on vessels (*yellow*) *Arrowheads*: $BrdU^+/CD31^-$ cells (*green*). *Scale bar*: 50 μm. (B) Quantification of newly generated endothelial cells ($BrdU^+/CD31^+$ cells) in the peri-lesion cortex and striatum. $^{\$}p\leq0.05$ vs. sham. $^*p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$ vs. vehicle, $^{\#}p\leq0.05$, $^{\#\#}p\leq0.01$ vs. N3 by one-way ANOVA. (C,D) Pearson correlation between the mice's spatial learning (C) and memory (D) performance in the Morris water maze and the numbers of $BrdU^+/CD31^+$ cells in cortex and striatum. n=6 mice for sham group, n=8 mice for vehicle, N3 and FO groups, n=7 mice for N3+FO group.

Figure 6. Post-TBI n-3 PUFA treatment enhances oligodendrogenesis.

(A) Representative images of BrdU and APC immunofluorescence in the ipsilateral peri-lesion cortex and striatum at 35 d after TBI. *Boxes* indicate areas that were enlarged in the high-power images (the 6th column). *Arrows*: $BrdU^+/APC^+$ cells (*yellow*). *Arrowheads*: $BrdU^+/APC^-$ cells (*green*). *Scale bar*: 50 μm. (B) Quantification of newly generated mature oligodendrocytes

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(BrdU$^+$/APC$^+$ cells) in the peri-lesion cortex and striatum. *p≤0.01, ***p≤0.001 vs. vehicle, $^{\#\#}$p≤0.01, $^{\#\#\#}$p≤0.001 vs. N3 by one-way ANOVA. (C,D) Pearson correlation between the mice's spatial learning C) and memory (D) performance in the Morris water maze and the total number of BrdU$^+$/APC$^+$ cells in the cortex and striatum. n=6 mice for sham group, n=8 mice for vehicle, N3 and FO groups, n=7 mice for N3+FO group.

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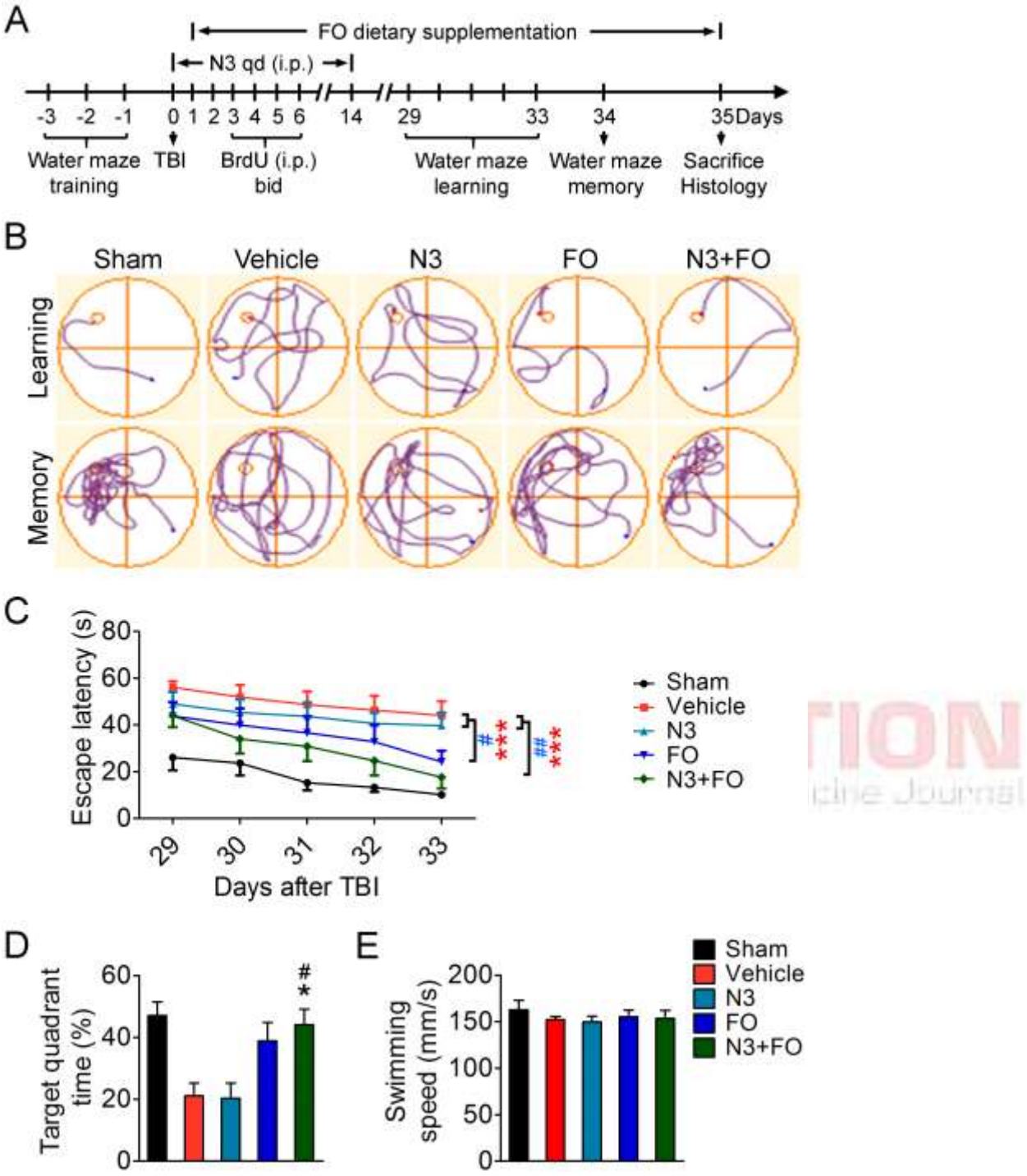

Figure 1

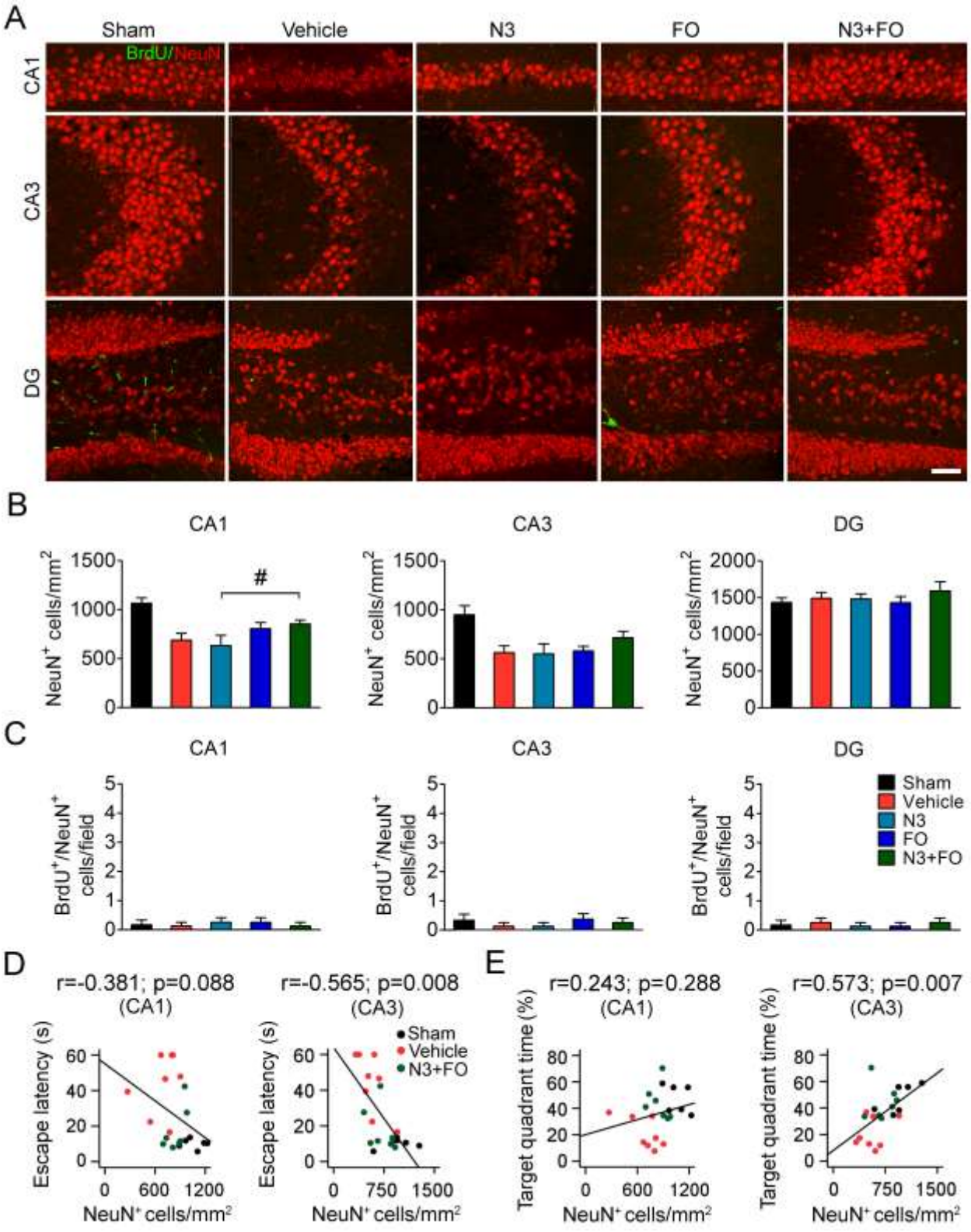

Figure 2

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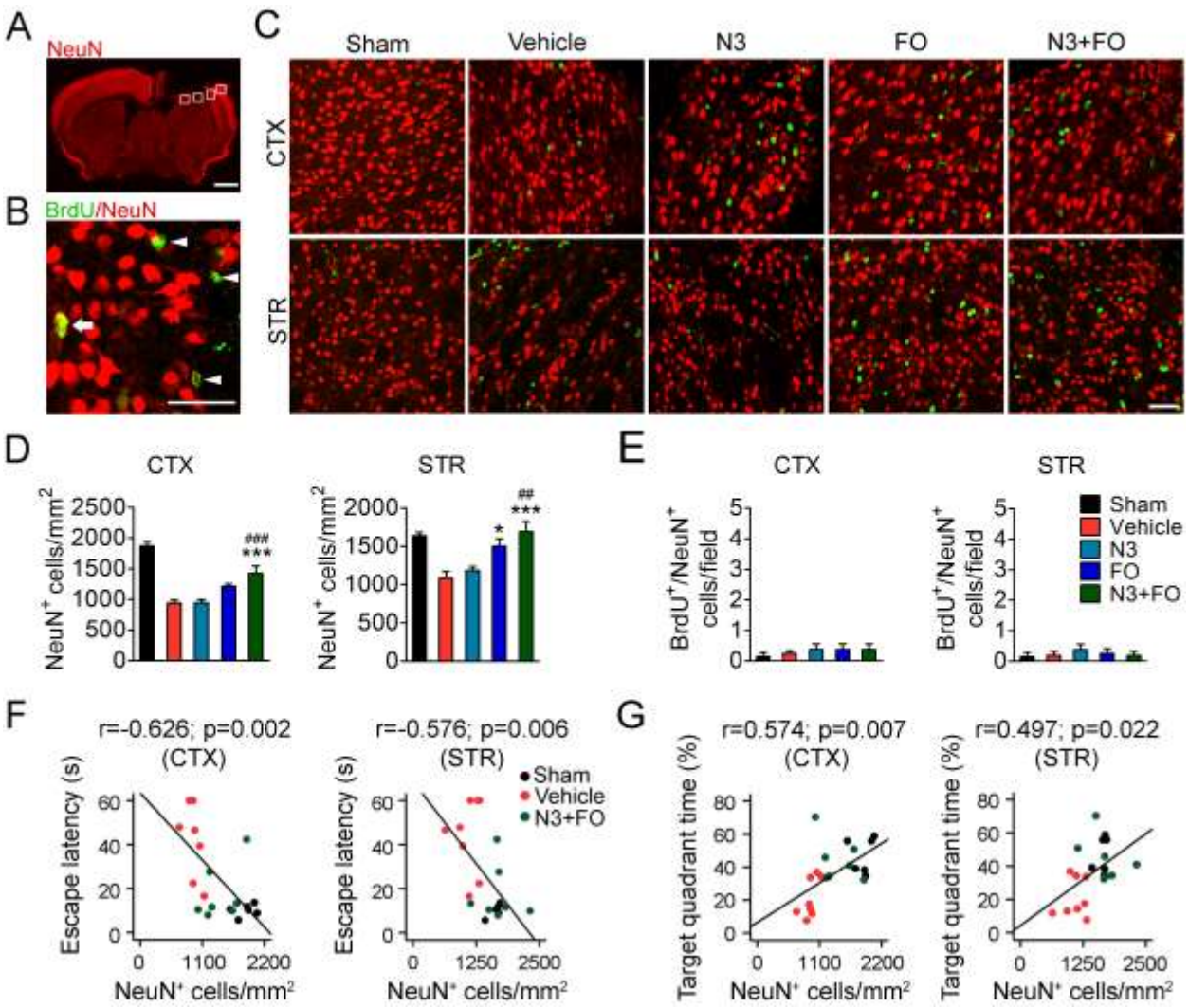

Figure 3

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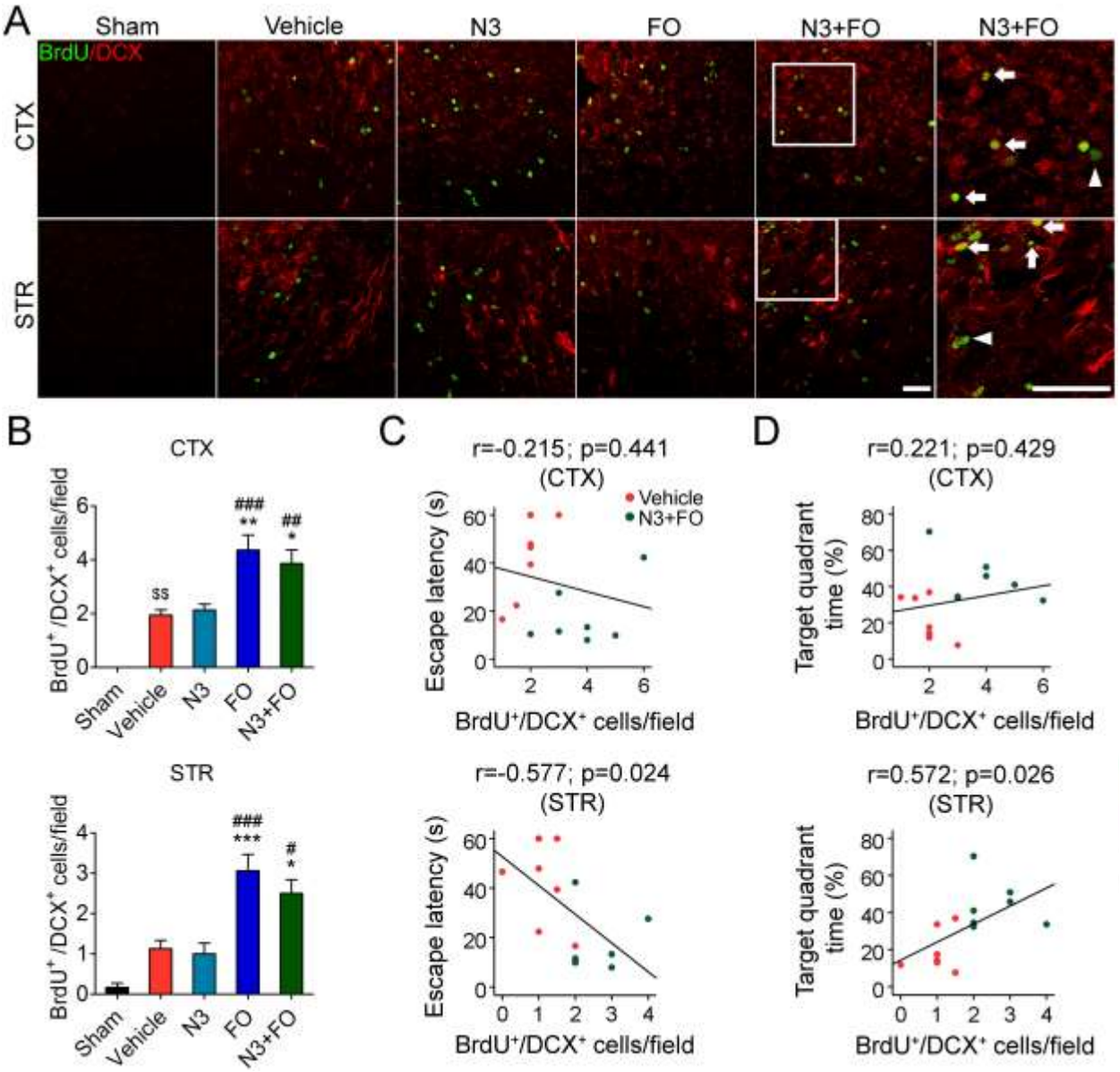

Figure 4

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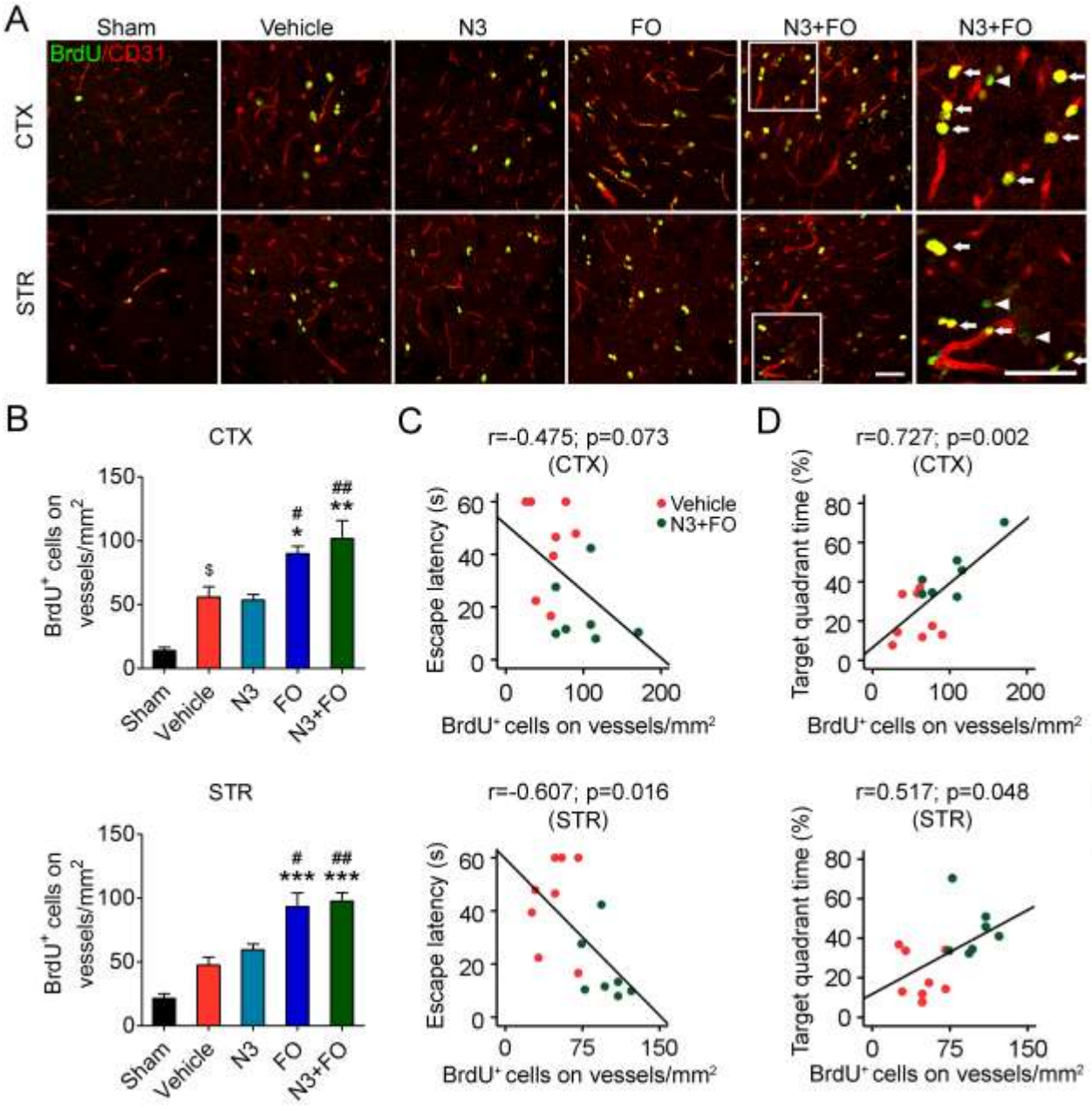

Figure 5

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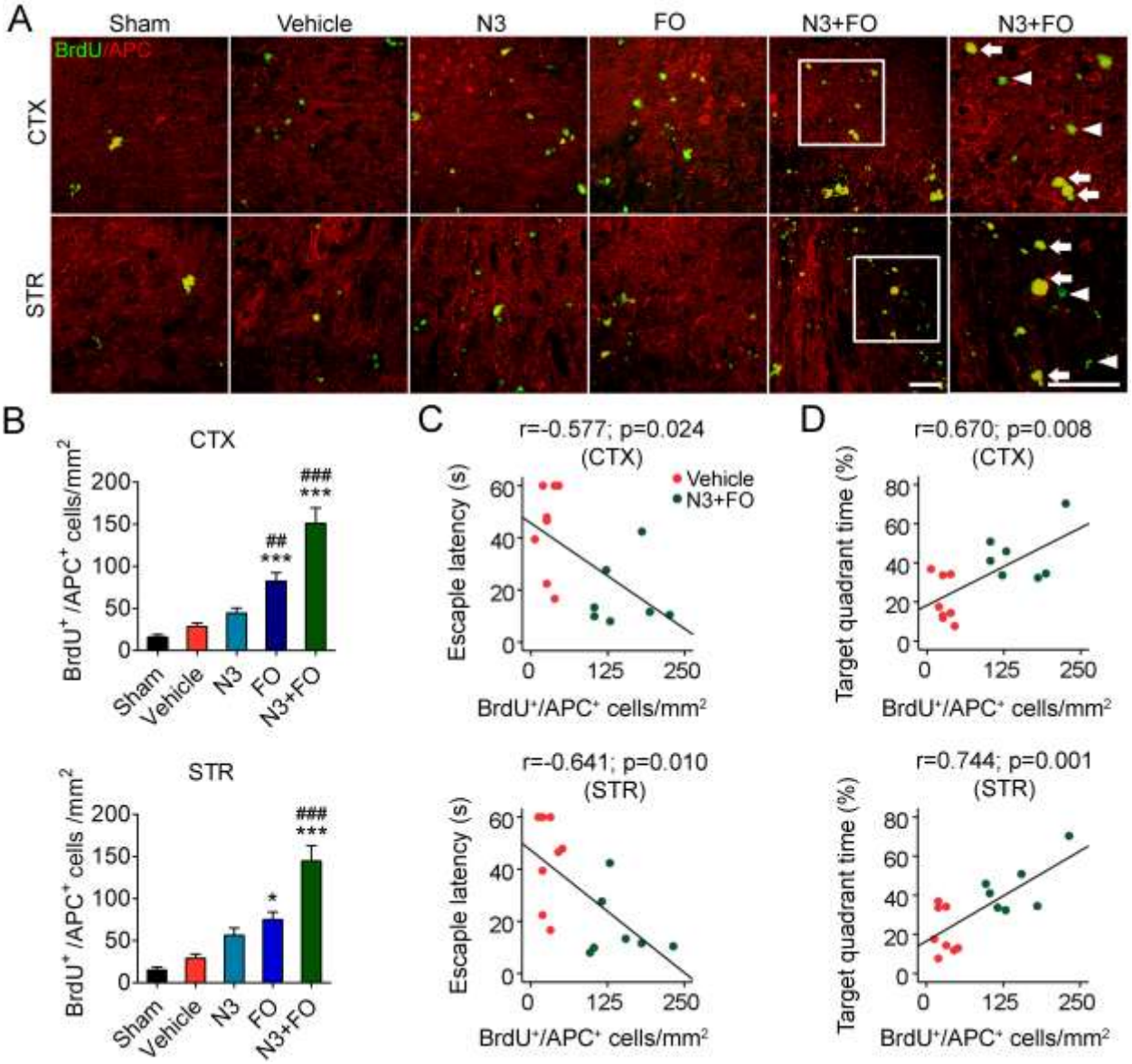

Figure 6

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