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Review

Post-stroke administration of omega-3 polyunsaturated fatty acids promotes neurovascular restoration after ischemic stroke in mice: Efficacy declines with aging

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ABSTRACT

Post-stroke treatment with omega-3 polyunsaturated fatty acids (n-3 PUFAs) may be a promising therapy in young animals but this has not been tested in aged subjects, a population at most risk of ischemic stroke. Herein we examined the therapeutic efficacy of n-3 PUFAs after distal middle cerebral artery occlusion (dMCAO) in young (10–12 weeks old) and aged (18 months old) mice. Post-ischemic mice were randomly assigned to 4 groups that received: 1) regular food with low content of n-3 PUFAs, 2) intraperitoneal docosahexaenoic acid (DHA, a major component of n-3 PUFAs) injections, 3) Fish oil (FO, containing high concentration of n-3 PUFAs) dietary supplement, or 4) combined treatment with DHA and FO dietary supplement. Long-term neurorestoration induced by n-3 PUFA post-stroke administration and its underlying mechanism(s) were analyzed up to 35 days after dMCAO. Aged mice showed more severe neurological deficits than young mice after dMCAO with histological lesions extended to the striatum. Notably, post-stroke treatment with combined DHA injections and FO dietary supplementation was more effective in reducing brain injury and improving sensorimotor function in aged mice than either treatment alone, albeit to a lesser extent than in the young mice. Unlike the improvement in spatial cognitive function observed in young mice, the combined treatment regimen failed to improve cognitive function in aged mice. The reduction in stroke-induced neurological deficits with n-3 PUFA post-treatment was associated with enhanced angiogenesis, oligodendrogenesis, neuron survival and white matter restoration. Together, these results indicate that the neurological benefits of n-3 PUFA administration after stroke extend to older animals and are associated with improved neuronal survival and brain remodeling, therefore suggesting that post-stroke administration of n-3 PUFAs is a viable clinically relevant treatment option against stroke.

1. Introduction

Stroke is the 5th leading cause of death in the United States but the leading cause of serious long-term disabilities in adults (Benjamin et al., 2018). The risk for ischemic stroke and severity of stroke-related disability increase with age (Ergeletzis et al., 2002; Denti et al., 2010; Chandra et al., 2012; Suenaga et al., 2015; Yang and Paschen, 2017). Current treatments for ischemic stroke, such as intravenous thrombolysis by tissue plasminogen activator (Powers et al., 2015) and endovascular therapies (Beadell et al., 2015), show some efficacy in elderly patients; however, the benefits are far less than those observed in younger patients (Alawieh et al., 2018). Nevertheless, most interventions that have found success pre-clinically have failed to translate to viable treatment options in the clinic, partly due to the lack of preclinical testing of these therapeutic strategies in aged animals. Therefore, age as a critical biological variable needs to be considered to develop the most effective treatments for ischemic stroke.

Previous studies by us and other groups suggest that omega-3 polyunsaturated fatty acids (n-3 PUFAs) could be a potential therapy for ischemic stroke in both young and elderly patients (Eady et al., 2012; Hu et al., 2013; Eady et al., 2014; Wang et al., 2014; Zhang et al., 2015; Jiang et al., 2016; Pu et al., 2016; Cai et al., 2017). n-3 PUFAs, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), play essential roles in brain development and normal brain function through regulation of synapses, dendrite formation, and brain inflammation (Hering et al., 2003; Hasadsri et al., 2013; Igarashi et al., 2015;

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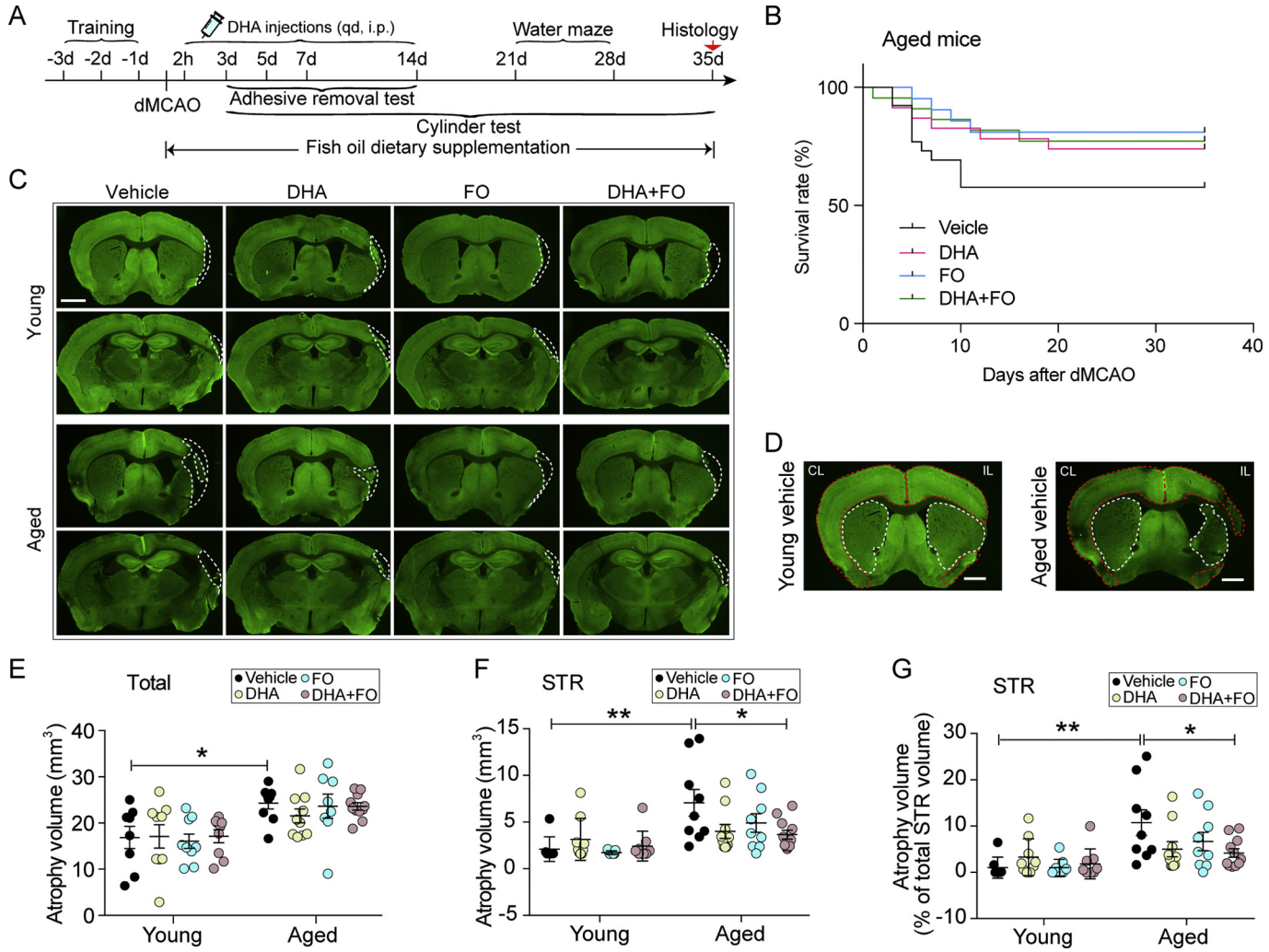


Fig. 1. Aged mice show more severe brain atrophy, especially in the striatum, than young mice 35 days after dMCAO, which can be attenuated by combined DHA and FO post-ischemic treatment. A. Illustration of experimental timeline. All mice were trained 3 days before surgery on the sensorimotor and cognitive behavioral tests. DHA was injected 2 h after dMCAO and once a day for 14 days after dMCAO. Mice were fed FO supplements every day until 35 days after dMCAO. Qd: once a day. i.p.: intraperitoneal injection. Behavioral tests were performed 3 to 35 days after dMCAO. Mice were sacrificed 35 days after dMCAO for histological examinations. B. The survival rate after dMCAO was comparable among the four groups of aged mice. C. Representative images of coronal brain sections showing MAP2 immunostaining (*green*) 35 days after dMCAO. Dashed lines show the brain atrophy. *Scale bar*: 1 mm. D. Representative images taken from (C) show brain atrophy in young and aged vehicle mice in the cortex (CTX; *red dashed line*) and striatum (STR; *white dashed line)*. IL: ipsilateral. CL: contralateral. *Scale bar*: 1 mm. E. Quantification of total brain atrophy volume in the ipsilateral hemisphere. F,G. The volume of striatal atrophy was quantified and expressed as mm^3 (F) or percentage of total striatal volume (G). n = 8–11 mice per group. *p ≤ 0.05, **p ≤ 0.01, unpaired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Thomazeau et al., 2017). In ischemia models using young mice, both pre- and post-treatment with n-3 PUFAs effectively ameliorate brain injury and promote long-term functional recovery (Hu et al., 2013; Wang et al., 2014; Zhang et al., 2015; Jiang et al., 2016; Pu et al., 2016). Furthermore, brain restoration processes such as neurogenesis, angiogenesis, and oligodendrogenesis are enhanced by n-3 PUFAs (Hu et al., 2013; Wang et al., 2014; Zhang et al., 2015). DHA content declines with aging in both human and rodent brains, which correlates with progressive deterioration of brain structure and function (Yehuda et al., 2002; McNamara et al., 2008). We previously found that long-term dietary supplementation with n-3 PUFA-enriched fish oil (FO) effectively elevated brain n-3 PUFA content in 18-month-old mice and markedly ameliorated brain infarction and neurological deficits after ischemic stroke (Cai et al., 2017). To date, whether n-3 PUFAs can improve long-term stroke outcomes in aged animals when administered post-ischemia has not been determined.

In this study, we investigated whether the functional and neuropathological protection conferred by n-3 PUFA treatment in young mice after stroke extends to aged mice. We employed three treatment regimens involving post-stroke FO dietary supplementation and/or intraperitoneal (*i.p.)* DHA injections, which demonstrated therapeutic efficacy in our previous studies performed in young adult mice (Jiang et al., 2016; Pu et al., 2016).

2. Materials and methods

2.1. Animals

Adult C57BL/6J mice (male, 10–12 weeks old; Jackson Laboratory, Bar Harbor, ME, USA) and aged C57BL/6 mice (male, 18 months old; National Institute on Aging aged mouse colony at Charles River Laboratories, Wilmington, MA, USA) were used in the present study. All animals were housed in a temperature- and humidity-controlled facility with a 12-h light/dark cycle. Food and water were available ad libitum. All experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed

in compliance with the NIH *Guide for the Care and Use of Laboratory Animals* and the *Animal Research: Reporting of In Vivo Experiments (ARRIVE)* guidelines. We made all efforts to minimize animal suffering and the number of animals used.

2.2. Permanent focal cerebral ischemia model

Animals were randomly assigned to experimental groups using a lottery-drawing box. Brain cortical ischemia (referred to as "dMCAO" thereafter) was induced in the left hemisphere by permanent occlusion of the left distal middle cerebral artery (MCA) and the left common carotid artery (CCA), as previously described (Suenaga et al., 2015). Briefly, after anesthetic induction with 3% isoflurane (carrier gas: 30% O_2/70% N_2O mixture), mice were maintained under anesthesia with 1.5% isoflurane during the surgery. Rectal temperature was maintained at 37.0 °C ± 0.5 °C during surgery with a temperature-regulated heating pad. After a midline neck incision, the left CCA was exposed and occluded by suture ligation. The skin was sutured and another skin incision was made between the left eye and ear. The temporal muscle was dissected by bipolar electrocautery (Bipolar Coagulator, Codman & Shurtleff Inc., Randolph, MA, USA). After opening the burr hole and performing a craniotomy, the distal part of the MCA immediately lateral to the rhinal fissure was exposed and occluded with low-intensity bipolar electrocautery. Regional cortical cerebral blood flow (CBF) was measured in all stroke animals using laser Doppler flowmetry. Animals that did not show a regional CBF reduction to < 30% of pre-ischemia baseline levels were excluded from further experimentation. Sham-operated animals underwent the same anesthesia and surgical procedures but were not subjected to CCA ligation and MCA occlusion.

2.3. Post-stroke n-3 PUFA administration

The concentrations of DHA for injection and FO dietary supplementation were selected based on our previous studies (Jiang et al., 2016; Pu et al., 2016). Immediately after the dMCAO surgery, mice were randomly assigned to 4 groups: 1) vehicle control, 2) DHA injections, 3) FO dietary supplementation, and 4) combined treatment with DHA injections and dietary FO supplementation (Fig. 1A). Vehicle control mice were fed a regular laboratory rodent diet (Prolab Isopro RMH 3000 5P76; LabDiet, St. Louis, MO, USA), which has an inherently low n-3 PUFA concentration (0.36%) and received injections of 0.9% NaCl (300 µl per day, *i.p.* 2 h after MCAO, and then daily for 14 days). The DHA injected mice were fed a regular diet and received injections of DHA (10 mg/kg body weight, diluted with 300 µl of 0.9% NaCl, *i.p.* 2 h after MCAO, and then daily for 14 days). Mice in the FO dietary supplementation group were fed a diet supplemented with n-3 PUFAs (DHA and EPA, triple strength n-3 fish oil, Puritan's Pride, Oakdale, NY, USA; final n-3 PUFA concentration 4%) on the same day of dMCAO for 35 days post-dMCAO. The mice in the combined DHA injection and FO dietary supplementation group were fed the same n-3 PUFA-supplemented diet as group 3 and received the same DHA treatment regimen as the mice in group 2. Mice in the sham-operated groups received regular diet and injections of 300 µl of 0.9% NaCl once a day for 14 days.

2.4. Behavior tests

All sensorimotor and cognitive behavioral function tests were performed by investigators blinded to the experimental group assignments.

2.4.1. Cylinder test

The asymmetry of forelimb use was evaluated by the cylinder test before and at 3, 5, 7, 14, 21, 28, and 35 days after dMCAO as described previously (Shi et al., 2016). Briefly, the mouse was placed in a transparent cylinder (9 cm in diameter and 15 cm in height) for 10 min. A camera was fixed above the cylinder to record all the forelimb movements of the mouse. Videotapes were analyzed in slow motion, and forepaw (left/right/both) use during the first contact against the cylinder wall after rearing and during lateral exploration was recorded. Preference of the non-impaired forepaw (left) was calculated as a relative proportion of right forepaw contacts: (left − right)/(left + right + both) × 100%. Uninjured mice typically show no preference for either forepaw, whereas injured mice have increased left forepaw preference depending on the severity of the injury.

2.4.2. Adhesive removal test

Adhesive removal test was selected to examine the sensorimotor function of the mice at 3, 5, 7, and 14 days after dMCAO. As described in prior studies (Shi et al., 2017), a mouse was placed in a cage for 2 min. An adhesive tape (0.3 × 0.4 mm) was applied to the distal radial region of the right forelimb as a tactile stimulus. The time to contact and the time to remove the tape were recorded. Each animal was tested three times with a cutoff time of 2 min per trial. The data are presented as the average time to contact or remove the tape of the three trials on each testing day.

2.4.3. Morris water maze test

To examine spatial cognitive functional deficits, the Morris water maze test was applied. As previously described (Pu et al., 2016), an 11 cm square Plexiglas platform was submerged in a circular pool (109 cm diameter) and maintained 1 cm under the water surface. White, nontoxic tempera paint was added to the water to make it opaque. The water temperature was maintained at 20 ± 1 °C and the room temperature was kept at 21 ± 1 °C. This test included the acquisition phase for spatial learning and the memory test. Before dMCAO surgery or sham-operation, mice were trained for three consecutive days on the procedures in the spatial learning phase. For the acquisition or spatial learning phase, each mouse was released from one of four locations and was allotted 60 s to search for the hidden platform 22–26 days after dMCAO. At the end of each trial, the mouse was placed on the platform or allowed to remain on the platform for 20 s with prominent spatial cues displayed around the room. Data from three trials per day for 5 consecutive days were expressed as latency (in seconds) to reach the submerged platform. To evaluate the general locomotor functions of both the young and aged mice, the average swimming speed of the mice during the six testing days were recorded. Twenty-seven days after dMCAO, a single 60-s probe trial was performed for the memory test. The platform was removed and each mouse was placed in the pool once for 60 s at the same starting location as was used initially in hidden platform testing. The time spent in the goal quadrant (where the platform had been previously located) and the swim speed were recorded.

2.5. Immunohistochemistry and cell counting

All histological assessments were performed by investigators blinded to experimental group assignments. At 35 days after dMCAO, brains were harvested and coronal sections were prepared to subject to immunohistochemistry as previously described (Jiang et al., 2016). Briefly, mice were perfused with 50 ml 0.9% NaCl followed by 50 ml 4% paraformaldehyde. Brains were then removed and placed in 30% sucrose. After cryoprotection in sucrose for 2 days, the brains were sliced into 25 µm-thick coronal sections at −25 °C using a freezing microtome (model CM3050S; Leica Biosystems, Wetzlar, Germany). The sections were then subjected to immunofluorescent staining. After a series of washes, free-floating brain sections were incubated in PBST (PBS with 1% triton) for 15 min followed by blocking with 5% normal donkey serum to reduce non-specific staining. After another series of washes, brain sections were incubated with rabbit anti-NeuN (1:500; EMD Millipore, Billerica, MA, USA), rat anti-CD31 (1:200; BD Biosciences, San Jose, CA, USA), mouse anti-adenomatous polyposis coli (APC; 1:400; EMD Millipore), rat anti-myelin basic protein (MBP;

1:500; Abcam, Cambridge, MA), or mouse anti-nonphosphorylated neurofilaments (SMI-32; 1:1000; Abcam) overnight at 4 °C. After a series of washes, sections were then incubated with the appropriate donkey secondary antibodies conjugated to DyLight 488 or Cy3 (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 1 h. After washing, brain sections were than mounted and coverslipped with Fluoromount-G containing 4′,6-diamidine-2-phenylindole (DAPI; Southern Biotech, Birmingham, AL) to stain cell nuclei. Images for cell counting and the volume of immunosignal analysis were captured with an Olympus Fluoview FV1000 confocal microscope with FV10-ASW 2.0 software (Olympus America, Center Valley, PA) and analyzed by a blinded observer with ImageJ. Cell numbers were calculated from two randomly selected microscopic fields for each area in each brain.

2.6. Measurement of brain atrophy volume

Six coronal brain sections ranging from bregma 1.1 mm to −2.3 mm at 0.7-mm intervals for each mouse were chosen and immunostained with an antibody recognizing the neuron-specific marker microtubule-associated protein 2 (MAP2; 1:200; Santa Cruz Biotechnology, Dallas, TX). Images 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). Brain atrophy volume was analyzed using NIH ImageJ software. The total atrophy volume was calculated as the volume of the contralateral hemisphere minus the non-infarcted volume of the ipsilateral hemisphere. The atrophy volume in cortex (CTX) or striatum (STR) was measured as the volume of the contralateral CTX or STR minus the total volume of the remaining ipsilateral CTX or STR.

2.7. 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) as previously described (Zhang et al., 2015). Briefly, BrdU was injected (*i.p.*) twice a day at a dose of 50 mg/kg body weight 3–6 days after dMCAO. At 35 days after dMCAO, mice were sacrificed and coronal brain sections were prepared as described above. Sections were pretreated with 2 N HCl for 1 h at room temperature followed by 0.1 M boric acid (pH 8.5) for 15 min at room temperature. Sections were then blocked with M.O.M. kit (Vector, Burlingame, CA, USA) for 1 h, and incubated with purified mouse anti-BrdU antibody (1:1000; BD Biosciences) for 1 h at room temperature and then overnight at 4 °C. After a series of washes, sections were incubated with 488-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 calculated as the number of cells in the designated fields divided by the area (mm^2) of the fields. Angiogenesis was assessed by counting BrdU immunopositive cells along the microvessels in BrdU/CD31 double-stained sections. Oligodendrogenesis was assessed by counting BrdU and APC double immunopositive cells in brain sections. At least 2 microscopic fields were randomly sampled in each section.

2.8. 3D reconstruction and quantitative analyses

To quantify the expression of MBP and SMI-32 in the peri-infarct brain areas, *Z*-stack images of the CTX, STR and external capsule (EC) were taken with an Olympus Fluoview FV1000 confocal microscope. A total of 12 consecutive images with the interspace of 2 μm and total volume of 9.68×10^5 μm^3 were taken for each microscopic filed. The imaging processing software Imaris (Bitplane; Belfast, United Kingdom) was used to reconstruct three-dimensional images of MBP and SMI-32 immunofluorescence to calculate their volumes. Briefly, images were imported into Imaris, and the surface of MBP and SMI-32 immunosignals were remodeled to 3D images. The surface remodeling was processed with the four default steps in Imaris, including algorithm, selecting source channel, adjusting threshold and classifying surfaces. All the images were processed with the same adjusting parameters. The volumes of MBP and SMI-32 immunofluorescence were then calculated by Imaris based on the reconstructed 3D images. Similar to the MBP and SMI-32 reconstruction, newly generated microvessels were reconstructed with images that were double stained for BrdU and CD31.

2.9. Data analysis

All data are presented as mean ± SEM. Individual data points are presented where applicable. The statistical differences among means of multiple groups within an age cohort were assessed by one- or two-way ANOVA followed by the Tukey *post hoc* test. An unpaired *t*-test was used when comparing two groups. Pearson product linear regression analysis was used to correlate the multiple histological parameters and sensorimotor behaviors. A p value of < 0.05 was deemed statistically significant.

3. Results

Forty-two young and 106 aged mice were used in the present study. For young mice, nine mice were assigned to each stroke group and six mice to the sham group. Two mice died during surgery. The remaining 40 mice from all four groups survived to the end of the experimental protocol (i.e. 35 days after dMCAO or sham-operation), resulting the mortality rate of 0% in all groups. For aged mice, 20–25 mice were assigned to each stroke group, whereas the sham group consisted of eight mice. Among the 98 mice that received dMCAO, 5 mice died during surgery, 27 mice died within 35 days after the surgery. The survival rates of each group are shown in Fig. 1B. Data from mice that did not survive to the end of the experimental protocol were excluded from subsequent data analyses.

3.1. Aged mice show more severe brain atrophy than young mice after dMCAO, which could be attenuated by combined DHA and FO treatment

To examine whether post-stroke administration of n-3 PUFAs confers prolonged neuroprotection in aged mice, we first examined brain atrophy in coronal brain sections immunostained with MAP2 35 days after dMCAO (Fig. 1C,D). dMACO induced significantly larger volumes of total brain atrophy (Fig. 1E) and cortical atrophy (Fig. S1A) in aged mice than young mice. After expressing the cortical atrophy volume as a percentage of the total cortical volume to allow for differences in total brain volume between young and aged mice, we found a trend towards aged mice having a larger cortical atrophy volume (Fig. S1B). Remarkably, we observed significant striatal atrophy in aged mice but not in young mice 35 days after dMCAO (Fig. 1F, G). Even though the dMCAO model is supposed to restrict brain damage to the CTX by blocking blood flow to the distal cerebral branch of MCA, striatal injury in aged mice may result from increased vulnerability of aged white matter to ischemic injury (Rosenzweig and Carmichael, 2013) and poorer collateral blood flow in the aged brain after ischemia (Arsava et al., 2014).

Post-stroke treatment with DHA injections alone, or FO supplementation alone did not rescue total brain, cortical or striatal atrophy in either young or aged mice (Fig. 1E–G and Fig. S1). However, combined DHA and FO treatment significantly alleviated the atrophy volume in the STR of aged mice (Fig. 1F,G). These data show for the first time that the dMCAO model induces striatal atrophy in addition to cortical atrophy in aged mice and that post-stroke combined treatment with DHA and FO is able to attenuate this injury.

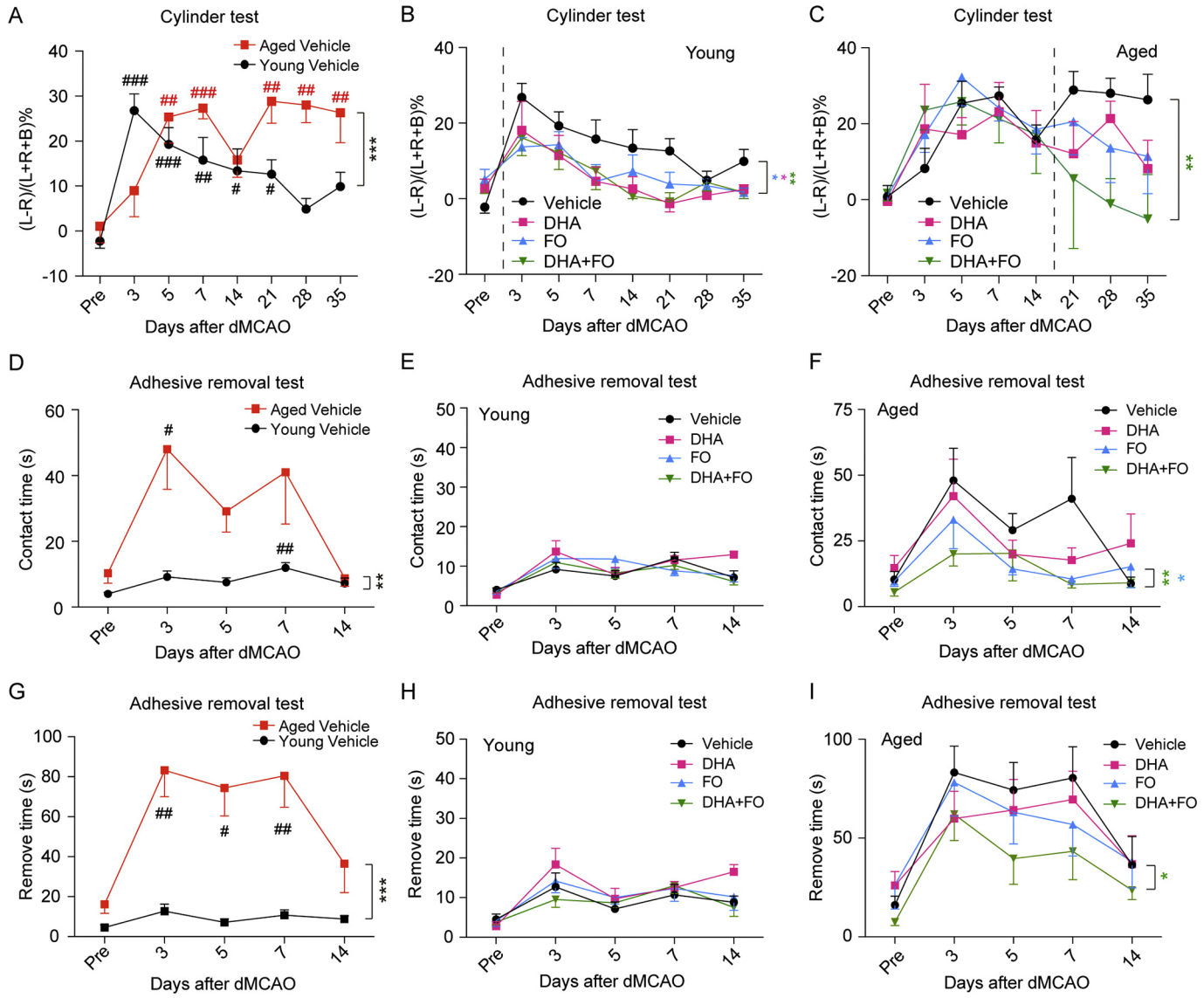


Fig. 2. Aged mice develop more severe sensorimotor deficits after dMCAO, and n-3 PUFA-induced protection is limited with aging. Mice were subjected to dMCAO and received different n-3 PUFA treatments. Sensorimotor functions were assessed before (Pre) and up to 35 days after dMCAO by the cylinder test (A–C) and adhesive removal test (D–I). Dashes lines indicate the portion of data for statistical analyses (3–35 days in B and 21–35 days in C). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 by two-way ANOVA. #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001 vs. pre-dMCAO levels by one-way ANOVA. n = 8–9 (cylinder test) and n = 6 (adhesive removal test) in young dMCAO groups. n = 8–12 (cylinder test) and n = 9–11 (adhesive removal test) in aged dMCAO groups.

3.2. Aged mice show more severe sensorimotor deficits than young mice after dMCAO, and n-3 PUFA-afforded protection declines with aging

We further examined the ability of n-3 PUFA post-stroke administration to attenuate long-term neurological deficits after dMCAO as a function of age. The cylinder test detected more severe and persistent asymmetric forelimb use in aged mice than young mice after dMCAO (Fig. 2A). Treatment with DHA only, FO only and DHA + FO all significantly alleviated forelimb asymmetry in young mice (Fig. 2B). However, only DHA + FO treatment reduced forelimb asymmetry in aged mice, and this reduction did not manifest until 21 days and onwards after dMCAO (Fig. 2C).

In the adhesive removal test, aged mice exhibited readily detectable deficits in both contact time and removal time at 3–7 days after dMCAO; both parameters showed nearly complete recovery at 14 days after dMCAO (Fig. 2D,G). Young mice only showed deficits at day 7 in contact time in the adhesive removal test after dMCAO (Fig. 2D,G), and none of the three n-3 PUFA treatments further altered the contact time and removal time (Fig. 2E,H). In aged mice, FO alone or DHA + FO treatment significantly mitigated the deficits in contact time (Fig. 2F), whereas only combined DHA + FO treatment alleviated deficits in the removal time (Fig. 2I).

These findings demonstrated more severe long-term sensorimotor deficits in aged mice than young mice after dMCAO. Administration of n-3 PUFAs post-stroke improved sensorimotor function in the aged mice, though to a lesser extent than in the young mice.

3.3. n-3 PUFA post-stroke treatment enhances long-term spatial cognitive function in young mice but not in aged mice

Improvement in cognitive functions after stroke as the result of therapeutic treatment would greatly improve the quality of life of stroke patients (Tatemichi et al., 1994). Therefore, we performed the Morris water maze test to assess hippocampus-dependent and –independent (Liu and Bilkey, 1998; Miyoshi et al., 2012) spatial cognitive deficits and age-related efficacy of n-3 PUFA post-stroke treatments

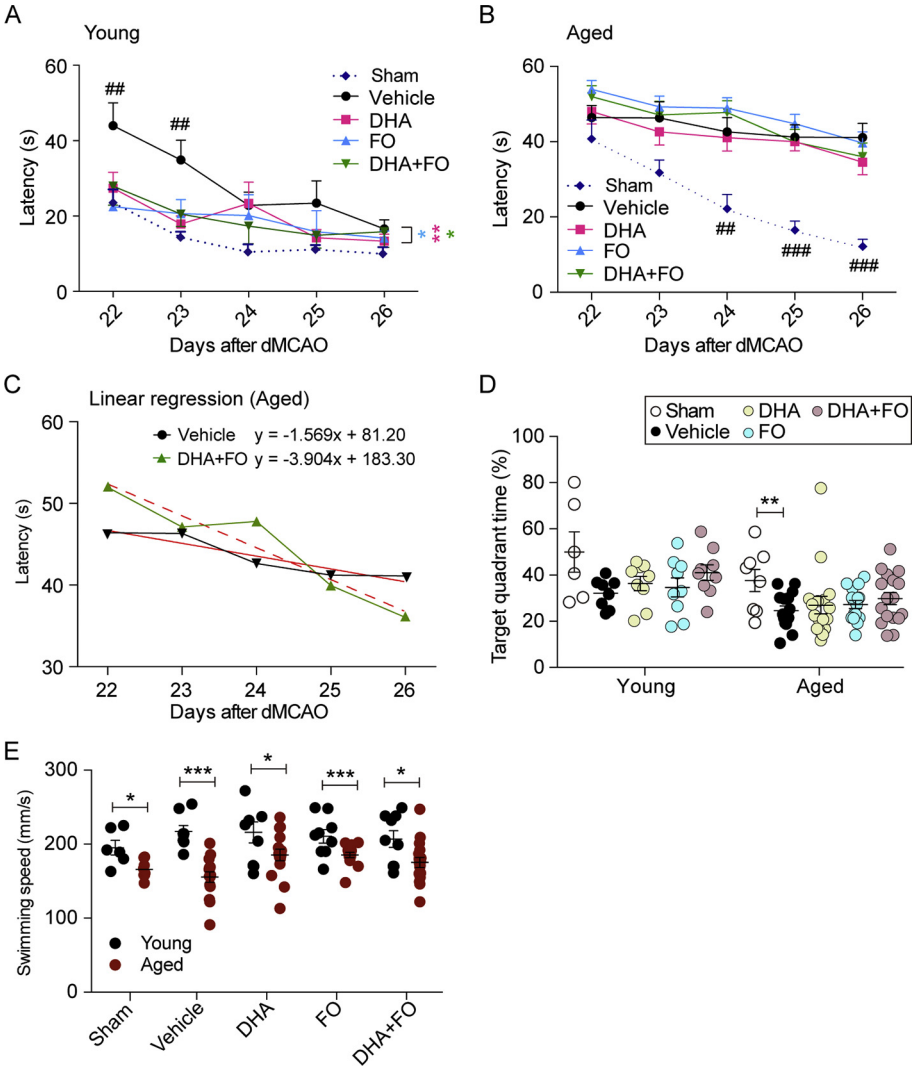


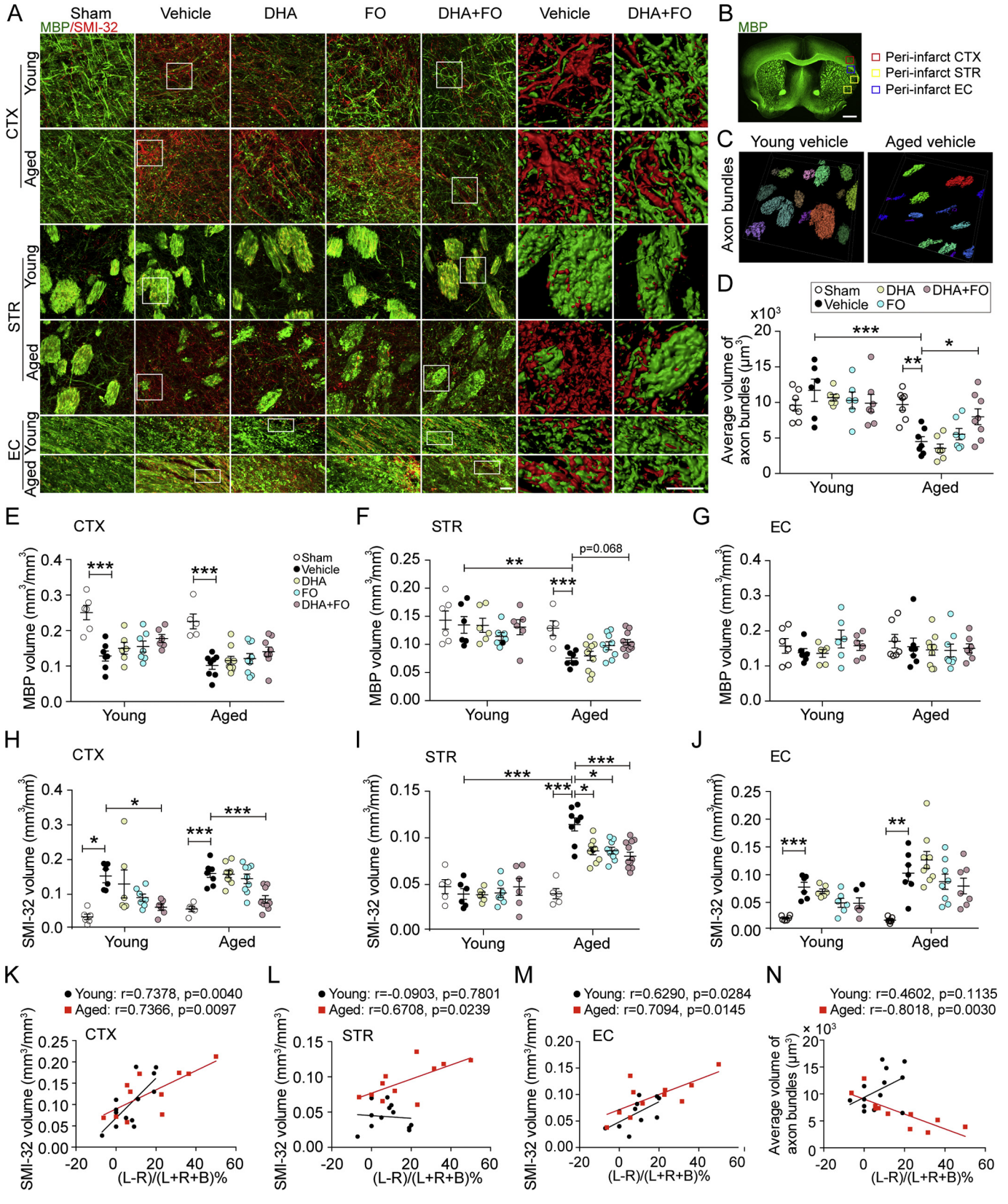
Fig. 3. Combined DHA and FO treatment promotes cognitive functions in young mice but not in aged mice after dMCAO. Morris water maze test was performed at 22 to 27 days after dMCAO. A,B. Spatial learning was assessed by the latency to locate the submerged platform in seconds (s). *p ≤ 0.05, **p ≤ 0.01 vs. vehicle by two-way ANOVA. ##p ≤ 0.01, ###p ≤ 0.001 vs. sham by two-way ANOVA. C. Linear regression of the latency to locate the submerged platform in aged mice in vehicle (*solid read line*) and DHA + FO (*dashed red line*) groups. D. Spatial memory was assessed 27 days after dMCAO by the time the mouse spent in the target quadrant. **p ≤ 0.01 by one-way ANOVA. E. Average swimming speed 22–27 days after dMCAO. *p ≤ 0.05, ***p ≤ 0.001, unpaired t-test. n = 6 in young sham group, n = 8–9 in young dMCAO groups; n = 8 in aged sham group, n = 15–17 in aged dMCAO groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from day 22 to day 27 after dMCAO. As sensorimotor function after stroke gradually recovers over several weeks, we chose this time frame to reduce the likelihood that deficits in swimming ability derived from acute effects of stroke on sensorimotor function would be a confounding factor in this test. In young mice, all three n-3 PUFA treatment groups showed decreased escape latency to find the submerged platform during the 5 days of training, indicating substantially improved spatial learning ability (Fig. 3A). In aged mice, none of the n-3 PUFA post-stroke treatments significantly mitigated learning deficits (Fig. 3B). However, combined DHA + FO treatment showed a trend towards improved escape latency compared to vehicle treatment during the 5 days of training, as reflected by a larger absolute value of slope in the linear regression analysis (Fig. 3C). Ischemia induced deficits in spatial memory in aged mice but not in young mice, which could not be alleviated by n-3 PUFA treatments (Fig. 3D). Swimming speed was not altered by dMCAO or by either n-3 PUFA treatment regimen in young or aged mice (Fig. 3E), thus excluding the possibility that the differences observed in young and aged mice in the learning and memory tasks resulted from variations in gross motor functions. Although all five groups of aged mice showed comparable swimming speeds, they swam significantly slower than the young mice (Fig. 3E). In summary, these data indicate that post-stroke administration of n-3 PUFAs improves spatial learning function in young mice but not in aged mice.

3.4. Aged mice exhibit more extensive white matter injury, especially in the STR, than young mice after dMCAO, which can be ameliorated by n-3 PUFA treatment

We examined the mechanism(s) underlying the histological and neurological improvements induced by post-stroke n-3 PUFA administration. Preservation of both white matter and grey matter integrity is essential to maintaining sensorimotor and cognitive functions and highly correlates with stroke outcomes (Pu et al., 2013; Chen et al., 2017; Rost et al., 2018). We initially examined the microstructural changes in white matter components in the peri-infarct CTX, STR and EC 35 days after dMCAO by double-label MBP (marker for myelin) and SMI-32 (marker for demyelinated axons) immunostaining (Fig. 4A,B). In sham-operated mice, MBP was abundantly expressed in CTX, STR and EC, while SMI-32 was barely observed in STR and EC, and only slightly expressed in CTX in both young and aged mice (Fig. 4A). Interestingly, in accordance with the brain atrophy observed in the STR of vehicle treated aged mice, dMCAO induced gross impairments of myelinated axon bundles (with MBP wrapped) in the peri-infarct STR as shown by the decrease in the size of the axon bundles (Fig. 4 C-D, Movie S1). Combined DHA and FO treatment significantly increased the size of the axon bundles in aged animals (Fig. 4D). This phenomenon was not observed in young mice (Fig. 4C-D, Movie S2).

In the post-ischemic ipsilateral hemispheres, young mice did not show myelin sheath injury in STR (Fig. 4F, I) but showed injury in CTX and EC, which was reflected by the reduced volume of MBP



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immunosignals in CTX and increased volume of SMI-32 immunosignals in both CTX and EC (Fig. 4E, H, J). As expected, aged mice showed more extensive myelin sheath injury as visualized by decreased expression of MBP in both the peri-infarct CTX and STR (Fig. 4E, F), and increased SMI-32 immunosignals in all three regions (Fig. 4H-J). Combined DHA + FO significantly decreased SMI-32 expression in the peri-infarct CTX both in young and aged mice (Fig. 4H). In the STR, DHA + FO significantly attenuated SMI-32 expression in aged mice

Fig. 4. White matter injury deteriorates in aged mice, especially in the striatum after dMCAO, and n-3 PUFAs attenuate myelin injury in both young and aged mice. A. Representative images of MBP and SMI-32 immunofluorescence in the peri-infarct cortex (CTX), striatum (STR), and external capsule (EC). Boxes illustrate areas that were enlarged and 3D remodeled in the 6th and 7th columns. *Scale bar*: 20 μm. B. A representative image of MBP immunofluorescence in a coronal brain section at 35 days after dMCAO. *Boxes* illustrate the peri-infarct CTX, STR and EC where images in A were taken. *Scale bar*: 1 mm C. 3D rendering of myelinated axon bundles from the peri-infarct STR 35 days after dMCAO. Axon bundles were reconstructed and isolated from 3D surface rendering based on MBP immunofluorescence by Imaris. Axon bundles within different range of volumes were labeled with different colors. D. Quantification of the average volumes of axon bundles in the peri-infarct STR. E–J. The volume of MBP (E–G) and SMI-32 (H–J) immunofluorescence in the peri-infarct CTX, STR and EC. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 by one-way ANOVA when comparing across groups of the same age or unpaired t-test was used when comparing two groups between young and aged mice. n = 5–7 in sham groups, n = 6–11 per group in dMCAO groups. K—N. Pearson product linear regression analysis was performed to correlate the forelimb asymmetric rate 35 days after dMCAO with the average volume of axon bundles (K), and with the volume of SMI-32 fluorescence in CTX, STR and EC (L-N).

(Fig. 4I), yet only showed a trend towards enhancing MBP expression (Fig. 4F).

To investigate whether the improved white matter integrity contributes to post-stroke sensorimotor recovery, Pearson product linear regression analysis was performed to assess their correlation. The asymmetric rate in the cylinder test at 35 days after dMCAO were significantly and negatively correlated with the average volume of axon bundles in aged mice but not in young mice (Fig. 4K). In addition, the asymmetric rate was positively correlate with the volume of SMI-32 immunosignals in all three regions in aged mice, and in both CTX and EC in young mice (Fig. 4L-N). As young mice did not exhibit STR injury, we did not observe correlation between behavior outcomes and white matter pathological changes in STR (Fig. 4K, M). These results suggest that restoration of white matter integrity may be an important factor of n-3 PUFA-induced neurological protection against dMCAO in both young and aged mice.

3.5. Post-stroke n-3 PUFA treatments promote neuron survival but not neurogenesis at 35 days after dMCAO

Next, we examined whether neurological improvement afforded by post-stroke administration of n-3 PUFAs was associated with preservation of grey matter integrity. Thus, we assessed the total number of viable neurons ($NeuN^+$ cells) and neurogenesis ($BrdU^+/NeuN^+$ cells) in the peri-infarct CTX and STR 35 days after dMCAO (Fig. 5A,B). dMCAO induced significant neuronal loss in the peri-infarct CTX in both young and aged mice (Fig. 5C). However, significant striatal neuronal loss was only observed in aged mice (Fig. 5C). FO dietary supplementation alone or combined with DHA injections significantly increased the number of viable neurons in the CTX of young mice (Fig. 5C). In aged mice, only DHA + FO treatment increased the number of viable neurons in both the CTX and STR (Fig. 5C,D). The increased number of viable neurons elicited by n-3 PUFA treatments may account for the improved neurological functions. In CTX, the number of viable neurons showed significant negative correlation with the asymmetric rate in the cylinder test 35 days after dMCAO in both young and aged mice (Fig. 5D). Young mice did not show neuron loss in STR, and only aged mice exhibited a trend ($p = 0.0504$) towards a high of correlation between asymmetric rates and viable neuron numbers (Fig. 5E). Thus, functional protection induced by n-3 PUFA post-stroke administration may partially derive from an increase in viable neurons in both young and aged mice.

To further access whether attenuation of neuronal loss resulted from treatment-induced increases in neuronal survival or neurogenesis, we assessed neurogenesis by double labeling cells with BrdU (marker for recently proliferated cells) and NeuN (marker for mature neurons) in the peri-infarct CTX and STR 35 days after dMCAO. Despite the easily visible $BrdU^+$-labeled cells in the peri-infarct CTX or STR in mice after dMCAO (Fig. 5A), there was barely any detectable $BrdU^+/NeuN^+$ cells. Moreover, many of the $BrdU^+$ cells were also co-labeled with DCX, a maker for neural precursor cells (data not shown). These results suggest that dMCAO-induced neurogenesis in peri-infarct CTX or STR did not lead to formation of mature neurons within the 35 days experimental time frame. Therefore, n-3 PUFA administration may attenuate neuronal loss by promoting neuronal survival.

3.6. Post-stroke n-3 PUFA treatment promotes angiogenesis in the peri-infarct CTX and STR after dMCAO, and the efficacy declines with aging

The formation of new blood vessels through angiogenesis is linked to improved histological and neurological function after stroke (Liu et al., 2007; Yu et al., 2007; Zhang and Chopp, 2009). Therefore, to assess whether angiogenesis plays a role in n-3 PUFA-induced neuroprotection, we examined double-label immunostaining of BrdU and CD31 (marker for endothelial cells of microvessels) 35 days after dMCAO (Fig. 6A,B). In the peri-infarct CTX, combined treatment with DHA + FO increased the number of double-labeled BrdU/CD31 cells in both young and aged mice (Fig. 6C) compared to the vehicle control group. Whereas FO alone and FO combined with DHA increased the number of BrdU/CD31 double-labeled cells in the peri-infarct STR of young mice, only DHA + FO increased the number of BrdU/CD31 double-labeled cells in the peri-infarct STR in aged mice (Fig. 6D). The efficacy of combined DHA and FO treatments on angiogenesis in the peri-infarct STR was significantly greater in young than in aged mice (Fig. 6E). The number of $BrdU^+/CD31^+$ cells in both CTX and STR were negatively correlated with the asymmetric rate in cylinder test 35 days after dMCAO in mice of both ages (Fig. 6F,G). These findings suggest that the decreased protective effect observed in the aged mice given n-3 PUFAs may be related to limited induction of angiogenesis by n-3 PUFAs in the brains of aged mice.

3.7. Post-stoke n-3 PUFA treatment enhances oligodendrogenesis and promotes the survival of oligodendrocytes in both young and aged mice at 35 days after dMCAO

Oligodendrogenesis is an important repair mechanism for white matter restoration and correlates with improved neurological function recovery after stroke (Zhang et al., 2011; Itoh et al., 2015; Zhang et al., 2015). The improvement in white matter integrity with n-3 PUFA administration in both young and aged mice suggests the potential involvement of oligodendrogenesis. Therefore, we examined the impact of n-3 PUFAs on oligodendrogenesis by double labeling cells with BrdU and APC (marker for mature oligodendrocytes). Immunostaining for double-labeled BrdU/APC was barely observed in the non-injured contralateral hemisphere in both young and aged mice (Fig. S2). In the injured hemisphere, dMCAO increased the number of double-labeled BrdU/APC cells in both young and aged mice (Fig. 7B–D). In the peri-infarct CTX and EC, treatment with DHA + FO augmented the number of $BrdU^+/APC^+$ cells in both young and aged mice (Fig. 7B,D). As dMCAO did not cause striatal infarction in young mice, $BrdU^+/APC^+$ cells were barely observed in the STR in young mice of all treatment groups. In contrast, in aged mice DHA + FO combined treatment significantly enhanced the number of $BrdU^+/APC^+$ cells in the peri-infarct STR (Fig. 7C),

There were no treatment-related effects on the number of APC^+ cells in the contralateral non-injured hemisphere in young or aged mice (Fig. S2). However, the total number of APC^+ cells was significantly higher in the peri-infarct CTX after DHA + FO treatment in both young and aged mice when compared to vehicle treated mice (Fig. 7E). Age-related differences in the number of APC^+ cells in the STR after dMCAO were observed, such that the number of APC^+ cells was significantly

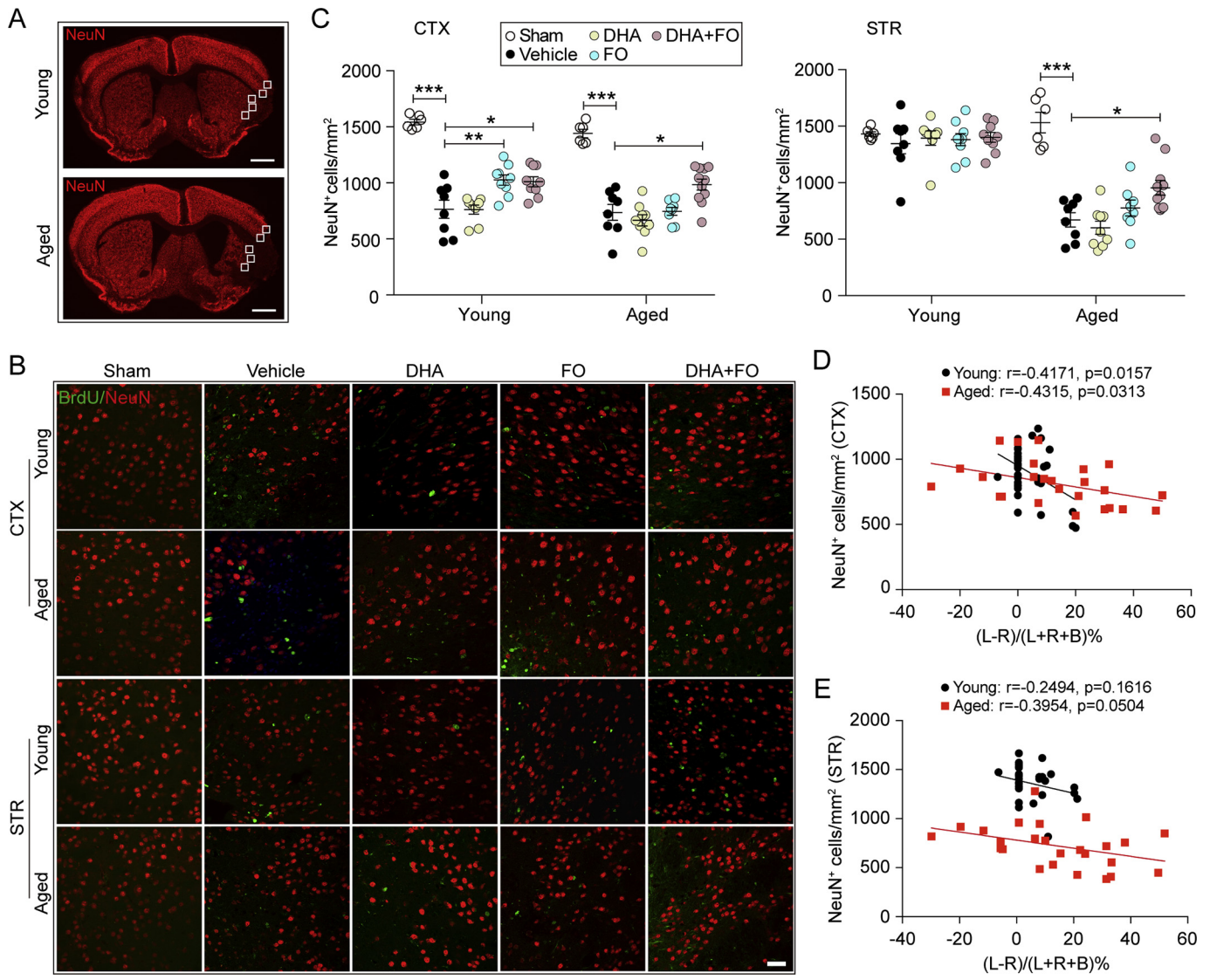

Fig. 5. Post stroke n-3 PUFA treatment reduces neuron loss but does not promote the generation of mature neurons in young and aged mice after dMCAO. A. Representative images of NeuN immunofluorescence in coronal brain sections 35 days after dMCAO. Boxes illustrate the peri-infarct CTX and STR where images in B were taken. *Scale bar*: 1 mm. B. Double-label immunostaining of BrdU and NeuN in the peri-infarct CTX and STR 35 days after dMCAO. *Scale bar*: 50 μm. C. Quantification of NeuN-positive cells in the peri-infarct CTX and STR. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; one-way ANOVA. n = 6 in sham groups, n = 8–11 in dMCAO groups. D-E. Pearson coefficient correlating the asymmetric rate in the cylinder test 35 days after dMCAO with the number of viable neurons in CTX and STR.

higher in young compared to aged mice (Fig. 7F). This discrepancy in the number of striatal APC$^+$ cells between the young and old animals may be caused by the infarct-related loss of APC$^+$ cells in the STR of aged mice in the dMCAO model, which did not occur in the young mice. Only combined treatment with DHA and FO enhanced the number of striatal APC$^+$ cells in aged mice. There was no further increase in the number of APC$^+$ cells in the STR with any treatment in young mice (Fig. 7F). In contrast, only the young mice displayed an increase in the number of APC$^+$ cells in the EC with DHA + FO treatment (Fig. 7G). Importantly, the number of APC$^+$ cells in CTX displayed a strong correlation with sensorimotor function in the cylinder test 35 days after dMCAO in aged mice (Fig. 7H). The number of APC$^+$ cells in STR in exhibited a moderate but still statistically significant correction with sensorimotor function in aged mice (Fig. 7I).

These data suggest that the post-stroke enhancement of oligodendrocytes survival, as well as enhanced oligodendrogenesis, with n-3 PUFA administrations may also play a role in its long-term histological and neurological protection. However, given the modest effect that n-3 PUFAs had on generating new oligodendrocytes compared with the total number of oligodendrocytes present, n-3 PUFAs are more likely to provide neuroprotection by promoting the survival of oligodendrocytes in both young and aged mice, rather than through oligodendrogenesis.

4. Discussion

The present study is the first to investigate the therapeutic efficacy of post-stroke n-3 PUFA treatment against long-term neurological deficits in aged mice (18 months old) and to compare that efficacy to that observed in young adult mice (10–12 weeks old). Our results demonstrate that aged mice exhibit more severe neurological deficits and pathological changes, especially in the STR, than young mice after dMCAO. FO supplementation combined with DHA injections after stroke attenuated brain damage and improved sensorimotor function in both ages; however, aged mice benefitted less from this treatment regimen than young mice. Neurological improvement conferred by n-3 PUFA correlated with increased neuronal survival in the peri-infarct regions and enhanced white matter restoration and brain repair processes, including angiogenesis and oligodendrogenesis. Notably, the

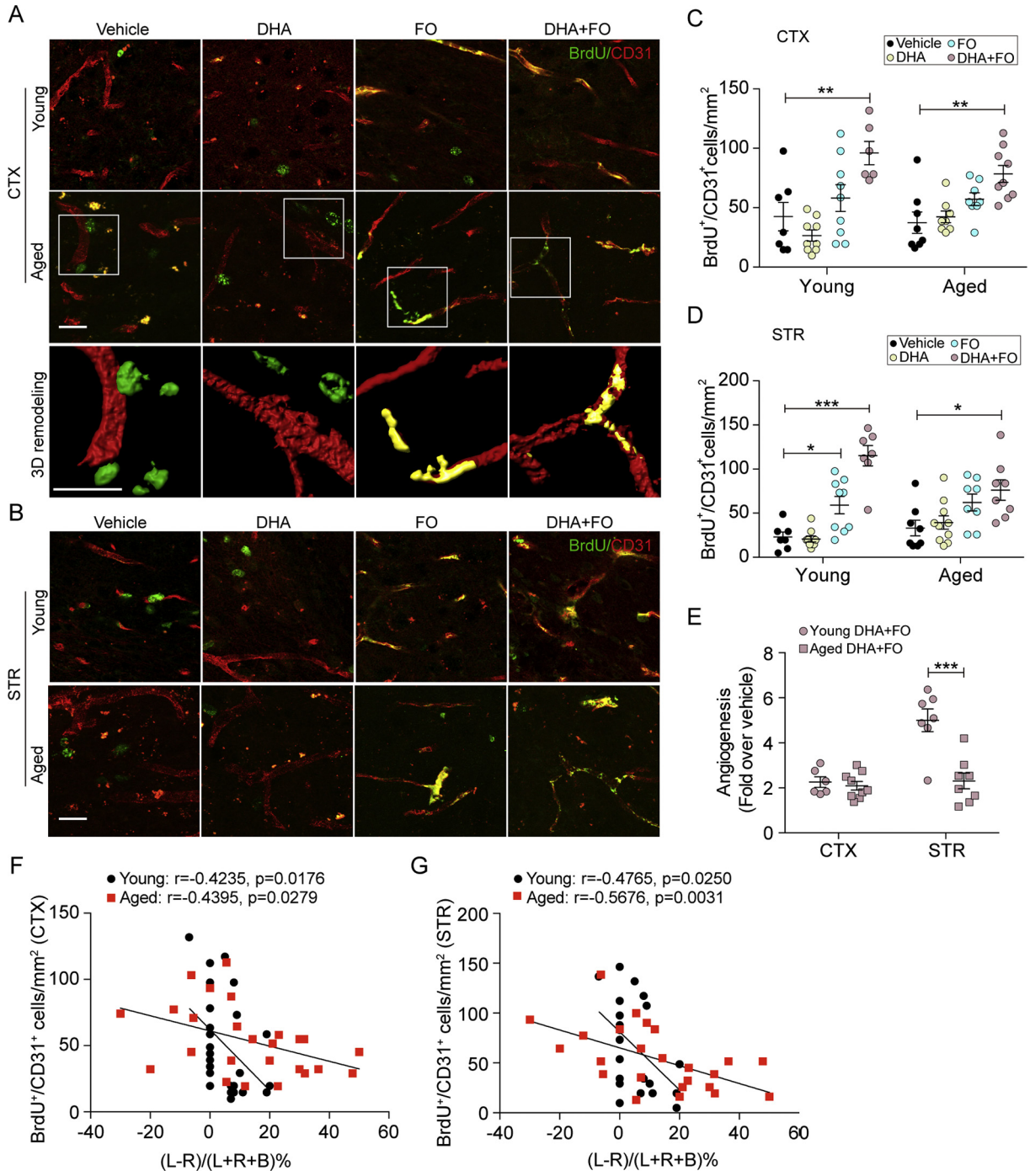

Fig. 6. Post stroke DHA and FO combined treatment boosts angiogenesis in both young and aged mice after dMCAO. A-B. Representative images of double-label immunostaining of BrdU and CD31 in the peri-infarct CTX (A) and STR (B) 35 days after dMCAO. *Scale bar*: 20 μm. *Boxes* indicate areas enlarged and 3D remodeled in the 3rd row in A. C–D. Quantification of BrdU and CD31 double-positive cells (*yellow*). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 by one-way ANOVA. E. Angiogenesis induced by DHA and FO combined treatment was greater in the STR of young compared to aged mice. Data are expressed as the density of $BrdU^+/CD31^+$ cells in DHA + FO group vs. that in vehicle group. ***p ≤ 0.001, unpaired *t*-test. n = 6–10 per group. F-G. Pearson correlation between asymmetric rate in cylinder test 35 days after dMCAO and $BrdU^+/CD31^+$ cells in CTX and STR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reduced or lack of protection in sensorimotor and cognitive function, respectively, in the aged mice was associated with more limited effects on white matter restoration and brain repair when compared with young mice.

Prophylactic treatment with n-3 PUFAs is known to protect against ischemic stroke and models of other neurological disorders (Pu et al., 2013; Zhang et al., 2014; Zhang et al., 2015; Zugno et al., 2015). Furthermore, n-3 PUFAs effectively improve long-term neurological functions in young adult animals when administered after stroke onset (Jiang et al., 2016; Pu et al., 2016). However, stroke is more prevalent in the elderly (Favate and Younger, 2016). Further, brain damage and neurological deficits incurred from stroke are more severe in the elderly, and their functional recovery from stroke is more protracted (Ergeletzis et al., 2002; Denti et al., 2010; Suenaga et al., 2015). So far, only a small fraction of preclinical neuroprotection studies (< 0.01%) have been done in aged animals, and this fact may be a contributing

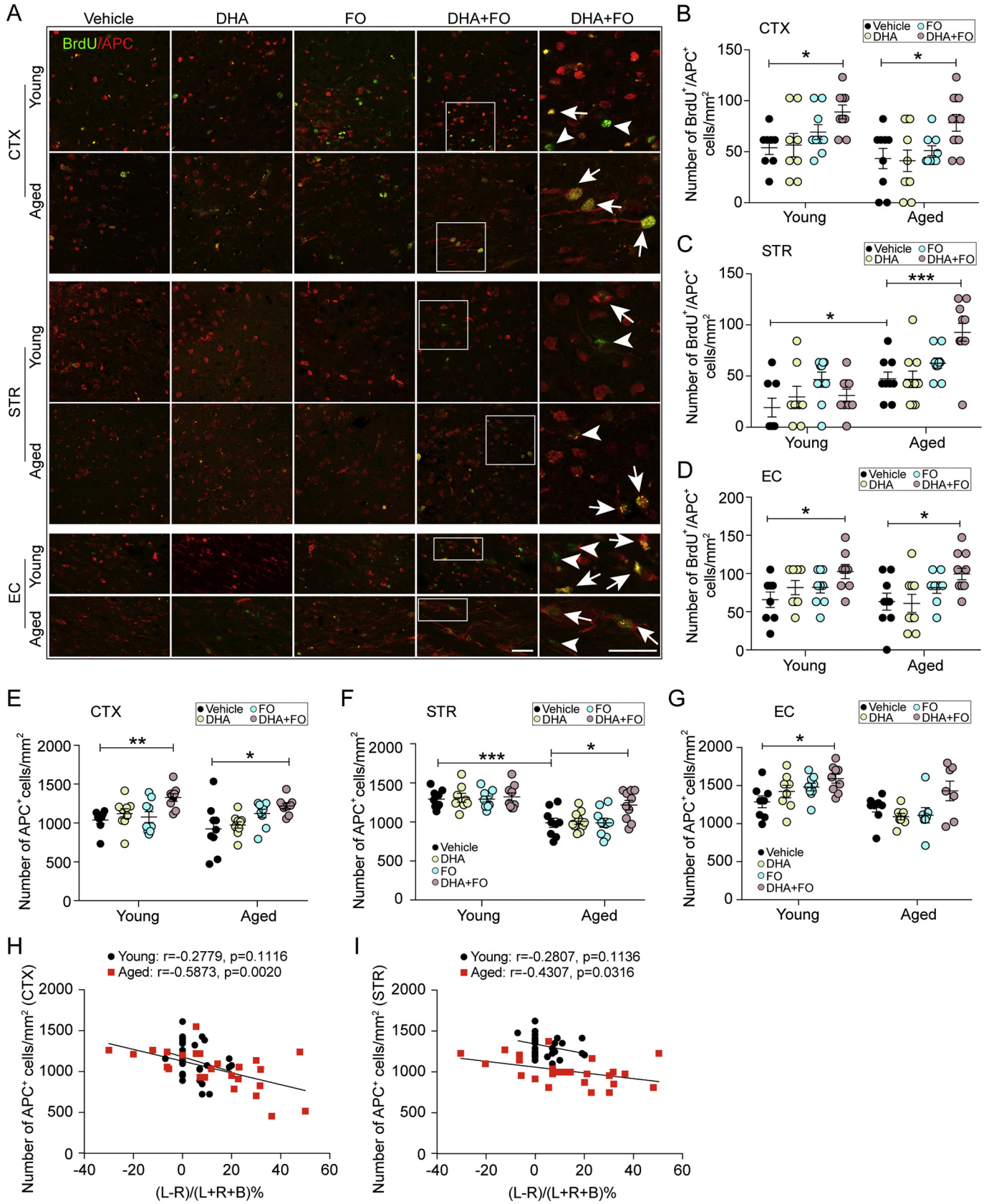

Fig. 7. Post-stroke n-3 PUFA treatment promotes the survival of oligodendrocytes and oligodendrogenesis in both young and aged mice after dMCAO. A. Representative images of BrdU and APC immunofluorescence in the peri-infarct CTX, STR and EC. *Boxes* illustrate areas that were enlarged in the 5th column. *Arrowheads*: BrdU$^+$ cells, *arrows*: BrdU$^+$/APC$^+$ cells. *Scale bar*: 40 μm. B–D. Quantification of newly generated mature oligodendrocytes (BrdU$^+$/APC$^+$ cells) in the CTX, STR and EC. E–G. Quantification of total mature oligodendrocytes (APC$^+$ cells) in the peri-infarct CTX, STR and EC. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. One-way ANOVA was applied among groups of the same age. Unpaired t-test was used when comparing two groups between young and aged mice. n = 6–10 per group. H—I. Pearson correlation between asymmetric rate in cylinder test 35 days after dMCAO and APC$^+$ cells in CTX and STR.

factor to the lack of success of preclinically validated neuroprotectants in clinical trials of stroke (Shi et al., 2017). In this study, 18-month-old mice were tested, which is approximately equivalent to 65 years old man based on the presence of senescent changes in biomarkers (Dutta and Sengupta, 2016). We used the dMCAO stroke model, which induces highly reproducible sensory, motor and cognitive dysfunction and ischemic infarct in both young and aged mice (Kuraoka et al., 2009; Suenaga et al., 2015). In addition, the dMCAO model with no acute reperfusion is clinically relevant, as the majority of stroke patients do not receive reperfusion therapy. Brain infarct and atrophy was significantly larger in both CTX and dorsal-lateral STR in aged mice, in remarkable contrast to young mice where brain atrophy was limited to the CTX, providing further evidence that aging increases the brain's vulnerability to ischemic damage. Total brain atrophy in young and aged mice was not altered by n-3 PUFAs post-stroke treatment, but combined DHA and FO treatment significantly reduced striatal atrophy in aged mice 35 days after dMCAO. Thus, rapid and persistent enhancement of n-3 PUFAs in the brain after stroke is capable of prolonging tissue protection in aged mice.

Despite the lack of effect of n-3 PUFAs on atrophy volume, all n-3 PUFA treatment regimens improved sensorimotor function and learning ability after dMCAO in young mice. n-3 PUFA post-treatment was more effective against the dMCAO, as oppose to the MCAO model (Jiang et al., 2016; Pu et al., 2016). This may possibly be due to the more limited damaged induced by dMCAO (Fluri et al., 2015) and/or the smaller time interval between stroke induction and therapeutic intervention. As expected, sensorimotor deficits in the aged mice were greater and more protracted than the young mice, and the therapeutic efficacy, which was typically only observed with DHA + FO, declined and took longer to manifest. Although we did not see obvious hippocampal damage in either young or aged mice after dMCAO, prominent cognitive deficits were observed in both young and aged mice and were more severe in the aged mice, and these deficits were not attenuated by any n-3 PUFAs treatment regimen in aged mice. In addition to the major influence from the histological and functional integrity of the hippocampus, cognitive function is a complex process that is also affected by neuronal connection and projection in multiple brain regions (Okada et al., 1995). The more severe cognitive functions in aged mice may come from the greater histological damage and the worse restorations in the brain. Further studies are warranted to examine the subtle changes of the hippocampus at both histological and functional levels.

The effects of n-3 PUFAs prior to induction of stroke involve neuronal and non-neuronal mechanisms, including enhanced neurogenesis, angiogenesis, oligodendrogenesis and white matter integrity in young and old mice (Cai et al., 2017; Hu et al., 2013; Zhang et al., 2015). These repair processes were also augmented in young mice when n-3 PUFAs were administered after stroke, and correlated with reduced functional deficits (Pu et al., 2016; Jiang et al., 2016). Cortical neuronal survival increased with FO and FO + DHA treatment in young mice, but only combined treatment increased cortical and striatal neuronal survival in aged mice, further indicating that a rapid increase in n-3 PUFAs after stroke is required for protection. Neurogenesis as reflected by newly-generated mature neurons was not observed in young or aged animals, suggesting that protection against cell death, not the formation of mature neurons, contributed to the ameliorating effects of n-3 PUFAs in the current study. However, previous studies suggest that n-3 PUFA promotes the generation of neuronal progenitor cells (Hu et al., 2013). Thus, further studies of extending the study time frame or examining the newly generated unmatured neurons are warranted.

Angiogenesis was induced in the CTX and STR in both age groups after dMCAO with DHA + FO treatment. Angiogenesis was greater in the STR of young mice, where no cell death occurred, compared to aged mice that did experience neuronal death in the STR. Thus, increased nutrient support provided by angiogenesis-related tissue perfusion, may have played a role in greater neuronal survival with DHA + FO treatment (Zhang and Chopp, 2009; Tang et al., 2014). In addition to its effects on neuronal survival, angiogenesis facilitates brain recovery after ischemia by promoting neurovascular remodeling (Arai et al., 2009), remyelination (Yuen et al., 2014; Cai et al., 2017), neurite outgrowth (Lin et al., 2013) and white matter recovery (Yuen et al., 2014; Cai et al., 2017). The mechanism by which n-3 PUFA increase angiogenesis may involve upregulation and extracellular release of angiopoietin 2 in astrocytes, which further facilitates endothelial proliferation and barrier formation by enhancing the vascular endothelial growth factor, the phospholipase Cγ1, and the Src signaling pathways (Wang et al., 2014; Zhang et al., 2015).

Aged mice are more sensitive than young mice to ischemia-induced white matter injury, (Rosenzweig and Carmichael, 2013; Munoz Maniega et al., 2017). Consistent with these studies, we found that aged mice exhibited more severe myelin sheath injury, more severe impairment of striatal MBP^+ axon bundles and decreased survival of mature oligodendrocytes in STR after dMCAO. Decreased ischemia-induced demyelination and increased axon bundle size in the striatum after n-3 PUFA treatment was associated with improved survival of APC^+ cells and oligodendrogenesis. Interestingly, although oligodendrogenesis was enhanced by n-3 PUFAs post-stroke treatment, the number of newly regenerated oligodendrocytes was only a small part of the total number of the increased oligodendrocytes in both young and aged mice. These results suggested that promotion of oligodendrocytic cell survival rather than oligodendrogenesis might be the primary mechanism underlying the improved white matter integrity and the enhanced neurological functions. Possible mechanism(s) underlying increased oligodendrocytic cell survival include activation of Akt and cyclic AMP responsive element binding protein (CREB) pro-survival/anti-apoptosis pathways, as observed with DHA induced improvement of axonal preservation and survival of oligodendrocytes after spinal cord injury (Figueroa et al., 2012), and reduction of ischemia-induced excitotoxicity (Keleshian et al., 2014).

One limitation of this study is that CBF was not measured in deep brain structures after dMCAO. We have measured cortical CBF by Doppler cytometry and laser speckle contract imaging in our current and previous studies, confirming that the dMCAO model is highly reproducible (Suenaga et al., 2015). However, both Doppler cytometry and laser speckle contrast imaging can only measure blood flow of the overlying CTX (Shi et al., 2016; Cai et al., 2017), whereas the biggest difference in ischemia-induced brain damage between the young and aged mice in the current study was in the deeply embedded STR. This limitation warrants further examination of CBF in deep brain structures by exploring other methods of measurement, such as MRI.

In summary, our study demonstrates that administration of n-3 PUFAs after ischemia promotes histological and neurological function in both young and aged mice, especially when DHA and FO dietary supplementation are combined. Histological and functional neuroprotection induced by n-3 PUFAs is present, albeit declined in the aged mice, and includes significant attenuation of white matter injury and neuron loss associated with enhanced angiogenesis and oligodendrogenesis. Interestingly, as with functional protection, enhancement of brain repair mechanisms in the aged mice was more restricted. Therefore, the results of this study provide evidence that n-3 PUFA post-stroke treatment is a promising therapy with high translational value against stroke in the most vulnerable aged population.

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Declarations of interest

None.

References

Alawieh, A., Chatterjee, A., Feng, W., Porto, G., Vargas, J., Kellogg, R., Turk, A.S., Turner, R.D., Imran Chaudry, M., Spiotta, A.M., 2018. Thrombectomy for acute ischemic stroke in the elderly: a 'real world' experience. J. Neurointerv. Surg. https://doi.org/10.1136/neurintsurg-2018-013787.

Arai, K., Jin, G., Navaratna, D., Lo, E.H., 2009. Brain angiogenesis in developmental and pathological processes: neurovascular injury and angiogenic recovery after stroke. FEBS J. 276, 4644–4652.

Arsava, E.M., Vural, A., Akpinar, E., Gocmen, R., Akcalar, S., Oguz, K.K., Topcuoglu, M.A., 2014. The detrimental effect of aging on leptomeningeal collaterals in ischemic stroke. J. Stroke Cerebrovasc. Dis. 23, 421–426.

Beadell, N.C., Bazan, T., Lutsep, H., 2015. The year embolectomy won: a review of five trials assessing the efficacy of mechanical intervention in acute stroke. Curr Cardiol Rep 17, 102.

Benjamin, E.J., et al., 2018. Heart disease and stroke statistics-2018 update: a report from the American Heart Association. Circulation 137, e67-e492.

Cai, M., Zhang, W., Weng, Z., Stetler, R.A., Jiang, X., Shi, Y., Gao, Y., Chen, J., 2017. Promoting neurovascular recovery in aged mice after ischemic stroke - prophylactic effect of omega-3 polyunsaturated fatty acids. Aging Dis 8, 531–545.

Chandra, R.V., Leslie-Mazwi, T.M., Oh, D.C., Chaudhry, Z.A., Mehta, B.P., Rost, N.S., Rabinov, J.D., Hirsch, J.A., Gonzalez, R.G., Schwamm, L.H., Yoo, A.J., 2012. Elderly patients are at higher risk for poor outcomes after intra-arterial therapy. Stroke 43, 2356–2361.

Chen, C., Bivard, A., Lin, L., Levi, C.R., Spratt, N.J., Parsons, M.W., 2017. Thresholds for infarction vary between gray matter and white matter in acute ischemic stroke: A CT perfusion study. J. Cereb. Blood Flow Metab., 271678X17744453. https://doi.org/10.1177/0271678X17744453.

Denti, L., Scoditti, U., Tonelli, C., Saccavini, M., Caminiti, C., Valcavi, R., Benatti, M., Ceda, G.P., 2010. The poor outcome of ischemic stroke in very old people: a cohort study of its determinants. J. Am. Geriatr. Soc. 58, 12–17.

Dutta, S., Sengupta, P., 2016. Men and mice: relating their ages. Life Sci. 152, 244–248.

Eady, T.N., Belayev, L., Khoutorova, L., Atkins, K.D., Zhang, C., Bazan, N.G., 2012. Docosahexaenoic acid signaling modulates cell survival in experimental ischemic stroke penumbra and initiates long-term repair in young and aged rats. PLoS One 7, e46151.

Eady, T.N., Khoutorova, L., Obenaus, A., Mohd-Yusof, A., Bazan, N.G., Belayev, L., 2014. Docosahexaenoic acid complexed to albumin provides neuroprotection after experimental stroke in aged rats. Neurobiol. Dis. 62, 1–7.

Ergeletzis, D., Kevorkian, C.G., Rintala, D., 2002. Rehabilitation of the older stroke patient: functional outcome and comparison with younger patients. Am J Phys Med Rehabil 81, 881–889.

Favate, A.S., Younger, D.S., 2016. Epidemiology of ischemic stroke. Neurol. Clin. 34, 967–980.

Figueroa, J.D., Cordero, K., Baldeosingh, K., Torrado, A.I., Walker, R.L., Miranda, J.D., Leon, M.D., 2012. Docosahexaenoic acid pretreatment confers protection and functional improvements after acute spinal cord injury in adult rats. J. Neurotrauma 29, 551–566.

Fluri, F., Schuhmann, M.K., Kleinschnitz, C., 2015. Animal models of ischemic stroke and their application in clinical research. Drug Des. Devel. Ther. 9, 3445–3454.

Hasadsri, L., Wang, B.H., Lee, J.V., Erdman, J.W., Llano, D.A., Barbey, A.K., Wszalek, T., Sharrock, M.F., Wang, H.J., 2013. Omega-3 fatty acids as a putative treatment for traumatic brain injury. J. Neurotrauma 30, 897–906.

Hering, H., Lin, C.C., Sheng, M., 2003. Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. J. Neurosci. 23, 3262–3271.

Hu, X., Zhang, F., Leak, R.K., Zhang, W., Iwai, M., Stetler, R.A., Dai, Y., Zhao, A., Gao, Y., Chen, J., 2013. Transgenic overproduction of omega-3 polyunsaturated fatty acids provides neuroprotection and enhances endogenous neurogenesis after stroke. Curr. Mol. Med. 13, 1465–1473.

Igarashi, M., Santos, R.A., Cohen-Cory, S., 2015. Impact of maternal n-3 polyunsaturated fatty acid deficiency on dendritic arbor morphology and connectivity of developing Xenopus laevis central neurons in vivo. J. Neurosci. 35, 6079–6092.

Itoh, K., Maki, T., Lok, J., Arai, K., 2015. Mechanisms of cell-cell interaction in oligodendrogenesis and remyelination after stroke. Brain Res. 1623, 135–149.

Jiang, X., Pu, H., Hu, X., Wei, Z., Hong, D., Zhang, W., Gao, Y., Chen, J., Shi, Y., 2016. A post-stroke therapeutic regimen with omega-3 polyunsaturated fatty acids that promotes white matter integrity and beneficial microglial responses after cerebral ischemia. Transl. Stroke Res. 7, 548–561.

Keleshian, V.L., Kellom, M., Kim, H.W., Taha, A.Y., Cheon, Y., Igarashi, M., Rapoport, S.I., Rao, J.S., 2014. Neuropathological responses to chronic NMDA in rats are worsened by dietary n-3 PUFA deprivation but are not ameliorated by fish oil supplementation. PLoS One 9, e95318.

Kuraoka, M., Furuta, T., Matsuwaki, T., Omatsu, T., Ishii, Y., Kyuwa, S., Yoshikawa, Y., 2009. Direct experimental occlusion of the distal middle cerebral artery induces high reproducibility of brain ischemia in mice. Exp. Anim. 58, 19–29.

Lin, C.H., Lee, H.T., Lee, S.D., Lee, W., Cho, C.W., Lin, S.Z., Wang, H.J., Okano, H., Su, C.Y., Yu, Y.L., Hsu, C.Y., Shyu, W.C., 2013. Role of HIF-1alpha-activated Epac1 on HSC-mediated neuroplasticity in stroke model. Neurobiol. Dis. 58, 76–91.

Liu, P., Bilkey, D.K., 1998. Perirhinal cortex contributions to performance in the Morris water maze. Behav. Neurosci. 112, 304–315.

Liu, X.S., Zhang, Z.G., Zhang, R.L., Gregg, S., Morris, D.C., Wang, Y., Chopp, M., 2007. Stroke induces gene profile changes associated with neurogenesis and angiogenesis in adult subventricular zone progenitor cells. J. Cereb. Blood Flow Metab. 27, 564–574.

McNamara, R.K., Liu, Y., Jandacek, R., Rider, T., Tso, P., 2008. The aging human orbitofrontal cortex: decreasing polyunsaturated fatty acid composition and associated increases in lipogenic gene expression and stearoyl-CoA desaturase activity. Prostaglandins Leukot. Essent. Fat. Acids 78, 293–304.

Miyoshi, E., Wietzikoski, E.C., Bortolanza, M., Boschen, S.L., Canteras, N.S., Izquierdo, I., Da Cunha, C., 2012. Both the dorsal hippocampus and the dorsolateral striatum are needed for rat navigation in the Morris water maze. Behav. Brain Res. 226, 171–178.

Munoz Maniega, S., Chappell, F.M., Valdes Hernandez, M.C., Armitage, P.A., Makin, S.D., Heye, A.K., Thrippleton, M.J., Sakka, E., Shuler, K., Dennis, M.S., Wardlaw, J.M., 2017. Integrity of normal-appearing white matter: Influence of age, visible lesion burden and hypertension in patients with small-vessel disease. J. Cereb. Blood Flow Metab. 37, 644–656.

Okada, M., Nakanishi, H., Tamura, A., Urae, A., Mine, K., Yamamoto, K., Fujiwara, M., 1995. Long-term spatial cognitive impairment after middle cerebral artery occlusion in rats: no involvement of the hippocampus. J. Cereb. Blood Flow Metab. 15, 1012–1021.

Powers, W.J., Derdeyn, C.P., Biller, J., Coffey, C.S., Hoh, B.L., Jauch, E.C., Johnston, K.C., Johnston, S.C., Khalessi, A.A., Kidwell, C.S., Meschia, J.F., Ovbiagele, B., Yavagal, D.R., American Heart Association Stroke C, 2015. 2015 American Heart Association/American Stroke Association Focused Update of the 2013 guidelines for the early management of patients with acute ischemic stroke regarding endovascular treatment: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke 46, 3020–3035.

Pu, H., Guo, Y., Zhang, W., Huang, L., Wang, G., Liou, A.K., Zhang, J., Zhang, P., Leak, R.K., Wang, Y., Chen, J., Gao, Y., 2013. Omega-3 polyunsaturated fatty acid supplementation improves neurologic recovery and attenuates white matter injury after experimental traumatic brain injury. J. Cereb. Blood Flow Metab. 33, 1474–1484.

Pu, H., Jiang, X., Hu, X., Xia, J., Hong, D., Zhang, W., Gao, Y., Chen, J., Shi, Y., 2016. Delayed docosahexaenoic acid treatment combined with dietary supplementation of omega-3 fatty acids promotes long-term neurovascular restoration after ischemic stroke. Transl Stroke Res 7, 521–534.

Rosenzweig, S., Carmichael, S.T., 2013. Age-dependent exacerbation of white matter stroke outcomes: a role for oxidative damage and inflammatory mediators. Stroke 44, 2579–2586.

Rost, N.S., Cougo, P., Lorenzano, S., Li, H., Cloonan, L., Bouts, M.J., Lauer, A., Etherton, M.R., Karadeli, H.H., Musolino, P.L., Copen, W.A., Arai, K., Lo, E.H., Feske, S.K., Furie, K.L., Wu, O., 2018. Diffuse microvascular dysfunction and loss of white matter integrity predict poor outcomes in patients with acute ischemic stroke. J. Cereb. Blood Flow Metab. 38, 75–86.

Shi, Y., Zhang, L., Pu, H., Mao, L., Hu, X., Jiang, X., Xu, N., Stetler, R.A., Zhang, F., Liu, X., Leak, R.K., Keep, R.F., Ji, X., Chen, J., 2016. Rapid endothelial cytoskeletal reorganization enables early blood-brain barrier disruption and long-term ischaemic reperfusion brain injury. Nat. Commun. 7, 10523.

Shi, L., Rocah, M., Leak, R.K., Zhao, J., Bhatia, T.N., Mu, H., Wei, Z., Yu, F., Weiner, S.L., Ma, F., Jovin, T.G., Chen, J., 2018. A new era for stroke therapy: integrating neurovascular protection with optimal reperfusion. J. Cereb. Blood Flow Metab. https://doi.org/10.1177/0271678x18798162.

Suenaga, J., Hu, X., Pu, H., Shi, Y., Hassan, S.H., Xu, M., Leak, R.K., Stetler, R.A., Gao, Y., Chen, J., 2015. White matter injury and microglia/macrophage polarization are strongly linked with age-related long-term deficits in neurological function after stroke. Exp. Neurol. 272, 109–119.

Tang, Y., Wang, J., Lin, X., Wang, L., Shao, B., Jin, K., Wang, Y., Yang, G.Y., 2014. Neural stem cell protects aged rat brain from ischemia-reperfusion injury through neurogenesis and angiogenesis. J. Cereb. Blood Flow Metab. 34, 1138–1147.

Tatemichi, T.K., Desmond, D.W., Stern, Y., Paik, M., Sano, M., Bagiella, E., 1994. Cognitive impairment after stroke: frequency, patterns, and relationship to functional abilities. J. Neurol. Neurosurg. Psychiatry 57, 202–207.

Thomazeau, A., Bosch-Bouju, C., Manzoni, O., Laye, S., 2017. Nutritional n-3 PUFA deficiency abolishes endocannabinoid gating of hippocampal long-term potentiation. Cereb. Cortex 27, 2571–2579.

Wang, J., Shi, Y., Zhang, L., Zhang, F., Hu, X., Zhang, W., Leak, R.K., Gao, Y., Chen, L., Chen, J., 2014. Omega-3 polyunsaturated fatty acids enhance cerebral angiogenesis and provide long-term protection after stroke. Neurobiol. Dis. 68, 91–103.

Yang, W., Paschen, W., 2017. Is age a key factor contributing to the disparity between success of neuroprotective strategies in young animals and limited success in elderly stroke patients? Focus on protein homeostasis. J. Cereb. Blood Flow Metab. 37, 3318–3324.

Yehuda, S., Rabinovitz, S., Carasso, R.L., Mostofsky, D.I., 2002. The role of polyunsaturated fatty acids in restoring the aging neuronal membrane. Neurobiol. Aging 23, 843–853.

Yu, S.W., Friedman, B., Cheng, Q., Lyden, P.D., 2007. Stroke-evoked angiogenesis results in a transient population of microvessels. J. Cereb. Blood Flow Metab. 27, 755–763.

Yuen, T.J., Silbereis, J.C., Griveau, A., Chang, S.M., Daneman, R., Fancy, S.P.J., Zahed, H., Maltepe, E., Rowitch, D.H., 2014. Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. Cell 158, 383–396.

Zhang, Z.G., Chopp, M., 2009. Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. Lancet Neurol. 8, 491–500.

Zhang, R.L., Chopp, M., Roberts, C., Jia, L., Wei, M., Lu, M., Wang, X., Pourabdollah, S., Zhang, Z.G., 2011. Ascl1 lineage cells contribute to ischemia-induced neurogenesis and oligodendrogenesis. J. Cereb. Blood Flow Metab. 31, 614–625.

Zhang, M., Wang, S., Mao, L., Leak, R.K., Shi, Y., Zhang, W., Hu, X., Sun, B., Cao, G., Gao, Y., Xu, Y., Chen, J., Zhang, F., 2014. Omega-3 fatty acids protect the brain against ischemic injury by activating Nrf2 and upregulating heme oxygenase 1. J. Neurosci.

34, 1903–1915.
Zhang, W., Wang, H., Zhang, H., Leak, R.K., Shi, Y., Hu, X., Gao, Y., Chen, J., 2015. Dietary supplementation with omega-3 polyunsaturated fatty acids robustly promotes neurovascular restorative dynamics and improves neurological functions after stroke. Exp. Neurol. 272, 170–180.
Zugno, A.I., Canever, L., Mastella, G., Heylmann, A.S., Oliveira, M.B., Steckert, A.V., Castro, A.A., Dal Pizzol, F., Quevedo, J., Gama, C.S., 2015. Effects of omega-3 supplementation on interleukin and neurotrophin levels in an animal model of schizophrenia. An. Acad. Bras. Cienc. 87, 1475–1486.