Dietary supplementation with omega-3 polyunsaturated fatty acids robustly promotes neurovascular restorative dynamics and improves neurological functions after stroke

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Abstract

Stroke is a devastating neurological disease with no satisfactory therapies to preserve long-term neurological function, perhaps due to the sole emphasis on neuronal survival in most preclinical studies. Recent studies have revealed the importance of protecting multiple cell types in the injured brain, such as oligodendrocytes and components of the neurovascular unit, before long-lasting recovery of function can be achieved. For example, revascularization in the ischemic penumbra is critical to provide various neurotrophic factors that enhance the survival and activity of neurons and other progenitor cells, such as oligodendrocyte precursor cells. In the present study, we hypothesized that chronic dietary supplementation with fish oil promotes post-stroke angiogenesis, neurogenesis, and oligodendrogenesis, thereby leading to long-term functional improvements. Mice received dietary supplementation with n-3 PUFA-enriched fish oil for three months before and up to one month after stroke. As expected, dietary n-3 PUFAs significantly increased levels of n-3 PUFAs in the brain and improved long-term behavioral outcomes after

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Conflict of interest

None.

Disclosures

None.

cerebral ischemia. n-3 PUFAs also robustly improved revascularization and angiogenesis and boosted the survival of NeuN/BrdU labeled newborn neurons up to 35 days after stroke injury. Furthermore, these pro-neurogenic effects were accompanied by robust oligodendrogenesis. Thus, this is the first study to demonstrate that chronic dietary intake of n-3 PUFAs is an effective prophylactic measure to not only protect against ischemic injury for the long term but also to actively promote neurovascular restorative dynamics and brain repair.

Keywords

Docosahexaenoic acid; Eicosapentaenoic acid; Revascularization; Angiogenesis; Neurogenesis; Oligodendrogenesis; Angiopoietin 1; Oligodendrocyte precursor cell

Introduction

Ischemic stroke is the leading cause of adult disability worldwide (Demaerschalk et al., 2010). Indeed, more than 50% of its survivors suffer from long-lasting debilitation (Wiltrout et al., 2007). Tissue plasminogen activator (tPA) is the only FDA-approved therapy for stroke victims. However, clinical use of tPA is severely limited by its short temporal window of application (Zhang et al., 2011a). Finding alternative therapies that are safe to administer as long-term prophylactic measures is therefore a matter of urgency in stroke research. Although many previous studies have shown that the adult brain has the ability to try and repair itself in response to ischemic insults, there are no safe, effective therapies that boost these endogenous repair mechanisms and thereby prevent stroke-induced neurological deficits. Thus, neurorestorative therapies that promote cerebral brain repair and neurological recovery post-ischemia have gained increasing attention in recent years (Hermann and Chopp, 2012; Liu et al., 2014b; McLaughlin and Gidday, 2013).

One candidate that may possess neurorestorative properties is the administration of omega-3 polyunsaturated fatty acids (n-3 PUFAs), a major component of dietary fish oil. n-3 PUFAs are widely known for their critical role in neurodevelopment (Harris and Baack, 2015). Excessive consumption of n-6 PUFAs relative to n-3 PUFAs is thought to contribute to the higher incidence of stroke in Westernized nations (Simopoulos, 2002). In contrast, high levels of n-3 PUFAs are known to protect against ischemic brain damage in multiple animal models (Wang et al., 2014; Zhang et al., 2014; Zhang et al., 2010). However, the underlying mechanisms are not yet well-established. Multiple mechanisms have been proposed to mediate neuroprotection by n-3 PUFAs, including the activation of pro-survival signaling cascades in neurons, blunting of microglia-mediated inflammatory responses (Hu et al., 2014; Zhang et al., 2010), and suppression of oxidative stress (Begum et al., 2013; Zhang et al., 2014). The beneficial effects of n-3 PUFAs have been confirmed in *fat-1* transgenic mice expressing the *C. elegans fat-1* gene, which encodes an n-3 fatty acid desaturase that converts endogenous n-6 PUFAs into n-3 PUFAs. Our previous work has shown that overproduction of n-3 PUFAs in *fat-1* transgenic mice enhances angiogenesis, neurogenesis, and oligodendrogenesis following cerebral ischemia (Hu et al., 2013; Wang et al., 2014). However, dietary supplementation with n-3 PUFAs is more clinically relevant and whether

it can promote brain repair and long-term recovery of neurological function is not yet known.

In the present study we tested the hypothesis that dietary supplementation with n-3 PUFAs would promote revascularization, neurogenesis, and oligodendrogenesis after stroke, thereby providing long-term neurobehavioral protection. Revascularization is one of the most effective endogenous repair mechanisms in the brain after stroke injury and illustrates the importance of a patent vascular network (Badaut and Bix, 2014; Li et al., 2014).

Revascularization not only consists of reperfusion of pre-existing vasculature in the early phase after stroke but also consists of a surge in angiogenesis through endothelial cell proliferation in late stages (Liu et al., 2014a). Post-stroke angiogenesis increases tissue perfusion with nutrients and oxygen while newly generated endothelial cells release a plethora of neurotrophic factors, thereby supporting the survival of neurons, oligodendrocytes, and their progenitor cells, and accelerating long-term recovery of neurological function (An et al., 2014; Merson and Bourne, 2014). In the past several decades, adult neurogenesis has been demonstrated in the subventricular zone (SVZ) of the lateral ventricle and the subgranular layer of the dentate gyrus. Neurogenesis can occur in response to cerebral ischemia and is characterized by the migration of SVZ-derived neural precursor cells along the blood vessels to the site of ischemic lesion (Ruan et al., 2014; Zhang et al., 2009). However, only a small proportion of these neural precursor cells survive and differentiate into mature neurons. Oligodendrocytes, the myelin forming cells of the central nervous system, are also sensitive to cerebral ischemia (Zhang et al., 2013). Stroke induces the loss of oligodendrocytes and elicits disruption of myelin, which impair axonal conductivity and exacerbate functional outcomes (Zhang et al., 2013). Oligodendrocyte precursor cells (OPCs) are known to differentiate into mature oligodendrocytes and form myelin sheaths for sprouting axons during brain repair, an important prerequisite for recovery of neurological function (Misumi et al., 2013; Zhang et al., 2011b). Hence, finding therapies that can safely boost these endogenous repair processes would greatly facilitate the clinical treatment of stroke victims. It would also benefit future stroke victims if such therapies could be administered prophylactically for the long term as a preventative measure. To accomplish this goal, n-3 PUFAs were administered through dietary intake of fish oil for three months before and up to one month after stroke injury in the present study.

Here we show that chronic dietary administration of n-3 PUFAs robustly protects the adult brain against focal cerebral ischemia up to 35 days post injury. Furthermore, n-3 PUFAs significantly enhanced post-stroke angiogenesis, neurogenesis, and oligodendrogenesis. Thus, oral administration of n-3 PUFA-enriched fish oil is a promising preventative measure with the capacity to improve natural brain repair processes and facilitate long-term recovery of neurological function.

Material and Methods

Dietary supplementation with fish oil

All experimental procedures were approved by the *Animal Care and Use Committee* at Fudan University. Four-week-old male C57BL/6J mice (Laboratory Animal LLC, Shanghai, China) were either fed a regular laboratory rodent diet with an inherently low n-3 PUFA

concentration (0.5%) or the same diet supplemented with n-3 PUFAs (docosahexaenoic and eicosapentaenoic acids, triple strength n-3 fish oil, Puritan's Pride, Oakdale, NY, USA; final n-3 PUFA concentration 4%) for 3 months before exposure to transient cerebral ischemia and up to 35 days after ischemia until sacrifice.

Transient focal cerebral ischemia model

Transient focal cerebral ischemia was induced in adult male mice (4 months old, 25–30g) by intraluminal occlusion of the left middle cerebral artery (MCA) for 60 minutes, as described previously (Wang et al., 2014). Experimental procedures were performed following Stroke Therapy Academic Industry Roundtable (STAIR) guidelines (Fisher et al., 2009). Mice were anesthetized with 3% isoflurane vaporized in 30% O₂/70%N₂ until they were unresponsive to the tail pinch test. Animals were then fitted with a nose cone blowing 1.5% isoflurane for anesthesia maintenance. A monofilament (7-0) with a silicone-coated tip was introduced into the common carotid artery, advanced to the origin of the MCA, and left in place for 60 minutes. Rectal temperature was maintained at 37±0.5°C during surgery with a temperature-controlled heating pad. Arterial blood gases were analyzed at 15 minutes after the onset of ischemia and 15 minutes after reperfusion. Regional cerebral blood flow (rCBF) was measured using laser speckle during the entire procedure. Animals that did not show an rCBF reduction of at least 75% of the baseline levels or that died after ischemia induction were excluded from further experimentation. Sham-operated mice underwent the same anesthesia and surgical procedures without MCA occlusion (MCAO).

Laser speckle imaging

Animals were anesthetized with 1.5% isoflurane. A midline incision was made on the scalp, and tissues were removed with a scalpel to expose the skull surface. All procedures were performed using sterile technique. Laser speckle images (696 × 512 pixels) were acquired at 23 fps (exposure time T = 5 ms) by the laser speckle imaging system (Dolphin BioTech Ltd. Shanghai, China) with a laser diode (780 nm; Dolphin BioTech Ltd. Shanghai, China) placed over the skull (Miao et al., 2010). Briefly, 200 consecutive frames of speckle images were recorded per trial. Image processing took place off-line with Matlab software (Mathworks Co., Ltd, USA). Raw speckle images were processed by the random process estimator (RPE) method after registration to obtain the contrast image. Changes in cerebral blood flow and the ischemic area were calculated by Matlab software.

Measurement of infarct volume

Forty-eight hours after MCAO, TTC staining was performed by a blinded investigator as we described previously (Stetler et al., 2008). Briefly, mice were anesthetized with 3% isoflurane and sacrificed. Brains were harvested and the forebrain was sliced into seven 1-mm-thick coronal sections. Sections were then stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline at pH7.4 (37±0.5°C) for 20 minutes, and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes before photography. Infarct volume was determined using the ImageJ software. To minimize the effect of edema on the measurement of infarct size, the hemispheric infarct area in each section was calculated by subtracting the area of normal, TTC-stained brain in the ipsilateral ischemic hemisphere from the

contralateral nonischemic area. Infarct volume was then calculated by summing the infarct areas over all sections and multiplied by the slice thickness.

Assessment of neurological functions

Neurological tests were performed 1 day before and 1–21 days after ischemia. Sensorimotor deficits were assessed by the Rotarod, pole, and cylinder tests. The Rotarod test was performed as described previously (Wang et al., 2014). Briefly, mice were placed on a rotating drum accelerating from 4 to 40 rpm over 300 seconds. The latency to fall off the drum was then recorded. Preoperative training was performed for 3 days in 3 trials per day, with the last 3 trials serving as preoperative baseline. Postoperative tests were performed every two days for 14 days in 3 trials per day. The pole test was performed as described previously (Balkaya et al., 2013). Briefly, the mouse was placed face forward on the top of a vertical steel pole (height: 50 cm, diameter: 0.9 cm), which was covered with tape to create a rough surface. The latency to turn downward and the total time to reach the bottom of the cage with its front paws were recorded 4 times per day. Preoperative training was performed for 3 days in 4 trials per day. Postoperative tests were performed every other day for two weeks following surgery. The cylinder test was performed to assess asymmetries in forepaw use. The mouse was placed in a transparent cylinder (height: 15 cm, diameter: 9 cm) and videotaped for 5 minutes. Forepaw use during the first contact against the cylinder wall after rearing was then recorded. Forepaw preference was expressed as the relative proportion of right forepaw contacts, and calculated as follows: $(\text{left} - \text{right}) / (\text{left} + \text{right} + \text{both}) \times 100\%$. Neurological function tests were always performed by investigators blinded to experimental groups. Each neurobehavioral test was conducted on at least 8 animals per group.

BrdU labeling of proliferating cells

The S-phase marker 5-bromo-2-deoxyuridine (BrdU) was used to label recently proliferated cells. BrdU (50 mg/kg body weight, Sigma-Aldrich) was injected intraperitoneally daily for 3–14 days after surgery. Animals were subsequently deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in phosphate buffer. Brains were then sequentially cryoprotected in 20% and 30% sucrose, and frozen serial coronal brain sections (25 μm) were prepared on a cryostat (Leica, Bensheim, Germany). Sections were pretreated with 2N HCl for 1 hour at 37°C, followed by 0.1M boric acid (pH 8.5) for 10 minutes at room temperature. Sections were then blocked with the M.O.M kit (Vector, Burlingame, CA, USA), followed by incubation with mouse anti-BrdU antibody (1:1000, BD bioscience, San Jose, CA, USA) for 1 hour at room temperature and overnight at 4°C. After a series of washes, sections were incubated in 594 or 488-Affinipure goat anti-mouse IgGs (1:1000, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) for 1 hour at room temperature.

Vascular labeling and analysis of vascular density

Lectin was applied as described previously (Wang et al., 2014). Briefly, animals were transcardially perfused 5 minutes before euthanasia with biotin-conjugated tomato lectin (Vector labs, Burlingame, USA) at a dose of 1.25 mg/kg body weight. Coronal brain sections were prepared and incubated with FITC-streptavidin secondary antibodies to stain perfused vessels. Four sections at 0.1 mm intervals were analyzed in each brain. The

vascular surface area (mm²) and the total vascular length (mm) per volume of tissue (mm³) were calculated using ImageJ software by a blinded observer.

Lipid extraction and fatty acid analysis

Cortices were fast frozen and dried into powder in a vacuum freeze dryer until subjected to analysis. Fatty acids were extracted as described previously (Zhang et al., 2010). Fatty acid composition was determined by capillary gas chromatography using a Clarus 500 Gas Chromatograph (Perkin Elmer, Waltham, Massachusetts, USA). Tissue fatty acid methyl ester peak identification was performed by comparison to the peak retention times of a 30-component methyl ester standard (Sigma-Aldrich, St. Louis, Missouri, USA). The concentration of each fatty acid was determined by calculating peak areas. Five animals were analyzed in each group.

Immunohistochemical staining

Immunohistochemical staining was performed on 25 µm-thick free-floating sections. Briefly, coronal brain sections were blocked with 5% goat serum in phosphate-buffered saline with 0.1% Triton-X 100 for 1 hour, followed by primary antibody incubations for 1 hour at room temperature and overnight incubation at 4°C. Mature neurons were visualized using anti-NeuN (1:1000; Millipore, Billerica, MA). After a series of washes, sections were incubated for 1 hour at room temperature with goat anti rabbit secondary antibodies conjugated with DyLight 594 (1:1000, Jackson ImmunoResearch Laboratories, Inc). Sections were mounted and coverslipped with Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Immature neural progenitor cells were visualized using anti-doublecortin (DCX, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) followed by Cy3-conjugated secondary IgG antibody (1:1000; Jackson Immunoresearch Laboratories). Myelin and abnormally dephosphorylated neurofilaments were visualized with anti-myelin basic protein (MBP, 1:500; Abcam) and anti-SMI32 (1:1000; Millipore, Billerica, MA), respectively. OPCs were visualized with anti-NG2 (1:500; Millipore, Billerica, MA). Fluorescence images were captured by a blinded observer as described above.

Western blot

Protein isolation from brain tissues was performed as described previously (Stetler et al., 2008). Western blot was performed using the standard SDS-PAGE method. PVDF membranes were blocked by 5% non-fat dried milk in TBS-T (10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween) at room temperature for 1 hour. Then membranes were incubated with rabbit polyclonal antibodies directed against angiopoietin 1 (1:500, Abcam), angiopoietin 2 (1:500, Abcam), meteorin (1:500, R&D) and mouse monoclonal anti-β-actin antibody (1:1500, Sigma-Aldrich) overnight at 4°C. Chemiluminescence was detected with luminescence detection system (Pierce, Rockford, USA) and semi-quantitatively analyzed by Quantity One 4.5.2 software (Bio-Rad, Hercules, USA). Data were quantified from 4 animals per group.

Statistical analysis

All data are reported as the mean $\pm$ SEM. Significant differences between means were assessed by ANOVA and *post hoc* Scheffe tests for multiple comparisons, unless otherwise indicated.

Results

Chronic administration of fish oil increases n-3 PUFAs in the brain

Fatty acids were analyzed in the forebrains of n-3 PUFA-supplemented (N3 high or N3H) and control diet-fed (N3 low or N3L) mice using gas chromatography 3 months after the onset of the change in diet. As shown in Fig S1A, no significant difference was detected between N3H and N3L mice in the proportions of total saturated fatty acids and mono-unsaturated fatty acids. In contrast, the n-3 PUFA fraction from N3H mice was significantly elevated relative to N3L mice, whereas the n-6 PUFA fraction was profoundly decreased, leading to an overall increase in the n-3/n-6 ratio (Fig S1B). Three major n-3 fatty acids, including EPA (eicosapentaenoic acid, C20:5), DPA (docosapentaenoic acid, C22:5) and DHA (docosahexaenoic acid, C22:6) were all significantly increased in N3H mice compared to N3L mice (Fig S1C). Two major n-6 fatty acids, LA (linoleic acid, C18:3) and AA (arachidonic acid, C20:4) were significantly decreased in N3H mice compared to N3L mice, with a concomitant increase in DPA (docosapentaenoic acid, C22:5, n-6) (Fig S1D). These results demonstrate that dietary intake of n-3 PUFAs is an effective means of increasing the n-3/n-6 ratio in the brain.

n-3 PUFAs reduce infarct volumes without affecting regional cerebral blood flow

To determine the neuroprotective effect of n-3 PUFAs, adult male mice from both groups were subjected to MCAO and sacrificed 48 hours later. As shown in Fig 1, N3H mice developed a significantly smaller infarct volume than N3L mice (Fig 1B–D). Furthermore, the 7 day survival rate of N3H mice was approximately 13% higher than N3L mice (Fig 1A). To rule out the possibility that protection was associated with differences in regional cerebral blood flow (rCBF), we monitored rCBF using laser speckle imaging before and after the onset of MCAO as well as 10 minutes after reperfusion. As shown in Fig S2, no significant difference in rCBF was detected in the ipsilateral hemisphere between N3H and N3L mice. The area of the ischemic core (rCBF reduction $> 70\%$ of baseline values) and ischemic penumbra ($30\% < \text{rCBF} < 50\%$ of baseline values) was measured in the ipsilateral hemisphere and found to be equivalent in N3H and N3L mice (Fig S2C, $p=0.08$ for core and $p=0.46$ for penumbra by two-way ANOVA, $n=9$ per group). Furthermore, no significant alterations in arterial blood pressures or blood gases were found during MCAO (data not shown). These findings demonstrate that n-3 PUFAs protect against cerebral ischemia entirely independent of changes in rCBF during ischemia or other physiological variables that are known to affect stroke outcome.

n-3 PUFAs improve neurological function and confer long-term neuroprotection against cerebral ischemia

To investigate the impact of n-3 PUFAs on neurobehavioral outcomes after stroke, three independent measures of neurological function - the cylinder, Rotarod and pole tests - were performed up to 21 days following the ischemic insult. Animals with sham operations displayed similar neurobehavioral performance regardless of group (Fig 2). After ischemia, N3L and N3H mice both showed spontaneous recovery in sensorimotor function during the 2–21 days of reperfusion. However, neurological outcomes were significantly better in N3H mice compared to control mice, according to all three behavioral tests. Thus, n-3 PUFAs robustly improve long-term sensorimotor function after cerebral ischemia.

n-3 PUFAs enhance post-ischemia revascularization in the ischemic penumbra

Previous studies suggest that long-term neuroprotective effects of n-3 PUFAs are associated with angiogenesis and neovascularization (Wang et al., 2014). To determine whether n-3 PUFAs enhance angiogenesis following ischemia, we examined revascularization in the ischemic penumbra (Fig 3A), a region with active vascular remodeling during the post-ischemia recovery stage. The number of functional vessels was decreased 1 d after MCAO, as shown by reductions in fluorescent lectin signal (Fig 3B–C). Revascularization developed gradually during the 35 days following transient ischemia in both groups. However, vascular length and surface area were both significantly increased by n-3 PUFAs (Fig 3D–E). There was no difference in vasculature in sham-injured N3H and N3L animals (Fig 3C–E). To further determine the effect of n-3 PUFAs on angiogenesis, we calculated the number of BrdU⁺ cells in functional vessels 7 to 35 days after stroke (Fig 4A). Spontaneous recovery of vasculature was significantly enhanced by n-3 PUFAs (Fig 4A–B). Only a few BrdU⁺ cells were present in vessels of non-injured sham animals from both N3H and N3L groups.

Our previous study on *fat-1* mice suggested that n-3 PUFAs promote brain repair by stimulating endogenous cerebral angiogenesis after ischemia (Wang et al., 2014). To identify the molecular mechanism underlying the angiogenic effect of n-3 PUFAs, we compared the expression of three proteins essential for angiogenesis, angiopoietin 1 (Ang 1), Ang 2, and meteorin, at 7 and 14 days after ischemia. Ang 1 is known to recruit and sustain periendothelial support cells. Ang 2, a natural Ang 1 antagonist, induces the destabilization required for additional vessel sprouting after ischemia (Qin et al., 2013). Cooperation between VEGF and Ang 2 is also known to enhance vessel maturation and vascular remodeling (Brudno et al., 2013). As expected, we observed pronounced elevations in Ang 1 in the ipsilateral hemisphere after transient cerebral ischemia (Fig 4C–D). Although Ang 1 may play an essential role in angiogenesis after ischemia, it is not likely to be sufficient to support extensive angiogenesis under normal conditions. Furthermore, decreases in Ang 2 after MCAO in control mice may actively inhibit angiogenesis (Fig 4D). However, n-3 PUFAs robustly stimulated the expression of both Ang 1 and Ang 2, which may help accelerate angiogenesis following stroke. Although n-3 PUFAs also appeared to increase meteorin 7 days after ischemia, no significant differences were detected between N3H and N3L groups.

n-3 PUFAs stimulate neurogenesis following cerebral ischemia

Neurogenesis induced by ischemic injury involves the proliferation of neural stem cells, migration of neuroblasts toward the ischemic area, and differentiation of neural progenitor cells. Sufficient blood flow is critical for supporting the migration of neuroblasts to the ischemic penumbra and for their subsequent maturation (Iwai et al., 2002). To determine the role of revascularization on neuroblast migration, we stained for DCX, a marker of immature neural progenitor cells, in lectin-perfused brains. The number of migrating neuroblasts was significantly higher in N3H mice, and these neuroblasts were found within migratory paths along lectin-stained blood vessels approaching the ischemic area (Fig 5A–B). These results indicate that n-3 PUFAs improve the migration and survival of neuroblasts.

The SVZ is a critical source of neurogenesis in the adult brain. However, no significant differences between N3H and N3L mice were detected in SVZ DCX⁺ cells (data not shown). Neuroblasts are known to mature and integrate into parenchymal tissue after migration is complete (Chu et al., 2012). Therefore, we performed BrdU and NeuN double staining to determine the effect of n-3 PUFAs on the maturation of newly generated neural precursor cells (NPCs) after ischemia. In the ischemic cortex and striatum, SVZ-derived mature neurons were detected in N3H mice, whereas very few neuroblasts and mature neurons survived in N3L brains up to 35 days after ischemic injury (Fig 5C–E).

n-3 PUFAs enhances oligodendrogenesis and white matter recovery

White matter injury is an important component of brain damage after stroke and detrimental to the normal electrophysiological communication between brain regions (Mifsud et al., 2014). OPCs are present in the adult brain, and provide the opportunity for oligodendrocyte replenishment after injury. To characterize the effect of n-3 PUFAs on oligodendrogenesis after cerebral ischemia, we investigated the proliferation of OPCs in the striatum and corpus callosum (Fig 6A) by double labeling for BrdU and NG2. n-3 PUFAs exerted no significant impact on the survival and proliferation of NG2 cells under sham physiological conditions (Fig 6 B–C). However, cerebral ischemia activated NG2 cells, so that they acquired an amoeboid shape and developed stout processes. Furthermore, there was a dramatic decrease in NG2 cell numbers in the penumbra at 35 days after ischemia. The proliferation of BrdU⁺/NG2⁺ OPCs was not able to fully compensate for the loss of OPCs in post-stroke N3L mice. However, n-3 PUFAs significantly enhanced the proliferation of NG2 cells and helped preserve the number of OPCs at 35 days after stroke injury (Fig 6B–C).

Remyelination of axons is essential for the recovery of neurological function after ischemia. To characterize the effect of n-3 PUFAs on white matter recovery, two markers were used: MBP (a marker of mature oligodendrocytes) and SMI32 (a marker of nonphosphorylated neurofilaments) (Thangavel et al., 2009). The SMI32/MBP ratio in the ipsilateral hemisphere was then compared with that of the contralateral hemisphere. Almost all axons in the striatum and corpus callosum are myelinated under normal conditions and little SMI32 signal was present in sham-operated animals (Fig 7A). Stroke led to oligodendrocyte injury and progressive demyelination of axons, as shown by increased SMI32 staining and decreased MBP staining in the striatum and corpus callosum (Fig 7A). As a result, the SMI32/MBP ratio dramatically increased in N3L mice starting at 3 days after transient

ischemia, and continued up to 35 days post-ischemia (Fig 7B). n-3 PUFAs significantly preserved myelin in the striatum and corpus callosum of N3H mice at 3–35 days after ischemia, as reflected by more MBP and reduced SMI32 staining compared to N3L mice. The SMI32/MBP ratio was reduced 3–35 days after ischemia by n-3 PUFAs (Fig 7B). Unlike the striatum and corpus callosum, high SMI32 staining (shown as red signal) in the cortex of sham animals indicates that the about one half of axons in this region are unmyelinated under normal conditions (Schirmer et al., 2011). Transient ischemia reduced both MBP and SMI32 staining in the cortex, indicating the post-ischemic loss of both axons and myelin in control N3L mice (Fig 7). In N3H mice, both cortical axons and myelin were preserved by n-3 PUFA treatment (Fig 7A and C). These data suggest that endogenous proliferation of OPCs after stroke is not sufficient to repair severe white matter damage in control N3L mice. However, n-3 PUFAs significantly preserved myelin integrity and promoted oligodendrogenesis, thereby facilitating the recovery of white matter after ischemia.

Discussion

The present study is the first report to provide direct evidence that dietary supplementation with n-3 PUFAs promotes long-term neurological recovery up to 21 days after ischemia. n-3 PUFAs also robustly promote post-stroke revascularization and boost endogenous angiogenesis, neurogenesis, and oligodendrogenesis. Thus, prophylactic supplementation with n-3 PUFAs is a highly promising neuroprotective strategy for ischemic stroke.

The brain is enriched in long chain polyunsaturated fatty acids, especially DHA, relative to many other tissues (Lauritzen et al., 2001). Due to deficiencies in Δ -12 and Δ -15 desaturases, mammalian organisms depend predominantly on dietary sources of polyunsaturated fatty acids. Epidemiological observations have suggested that lower n-3 PUFA intake increases the risk of many neurological disorders, including neurodegenerative diseases and ischemic stroke (He et al., 2002; Mohajeri et al., 2015; Mozaffarian et al., 2005). Excessive consumption of n-6 PUFAs contribute to an elevation in the n-6/n-3 ratio, leading to poor neural development and lack of functional recovery after damage (King et al., 2006). In the present study, dietary supplementation with n-3 enriched fish oil increased DHA levels in the brains of adult mice, whereas the major n-6 PUFAs, linoleic acid and arachidonic acid, were greatly decreased, leading to a net elevation in the n-3/n-6 ratio. Our results therefore demonstrate that dietary supplementation with n-3 PUFAs can indeed impact cerebral fatty acid content in adulthood. Consistent with previous research on *fat-1* transgenic mice, dietary supplementation with n-3 PUFAs not only protected against cerebral ischemia in the acute phase (within 48 hours) after reperfusion but also in the delayed phase (up to 35 days), as manifested by reductions in infarct volume and improvements in neurological functions. Thus, the present study strongly supports the hypothesis that prophylactic supplementation with n-3 PUFAs is an effective strategy to enhance functional recovery and promote long-lasting rehabilitation following stroke.

n-3 PUFAs possess a unique ability to modulate capillary integrity and neovascularization in the retina (SanGiovanni and Chew, 2005). In the present study, n-3 PUFAs exerted no significant effect on cortical blood flow during ischemia and after reperfusion. Nevertheless,

the density of lectin perfused cerebral microvessels was significantly higher in the N3H group than the N3L group within 24 hours after ischemia. Post-stroke vascular remodeling consists of revascularization of pre-existing vasculature at early phase, and neovascularization through angiogenesis at chronic phase (Liu et al., 2014a). Our data revealed that n-3 PUFA supplementation improved post-stroke revascularization, starting as early as 1 day after MCAO when much less amounts of functional (lectin perfused) microvessels could be detected in the N3L groups (Fig. 3). This rapid revascularization in n-3 PUFA-treated brain was likely due to reopening of pre-existing microvasculature rather than increased generation of new vessels. n-3 PUFAs preserved the integrity of blood vessels, possibly supporting the survival of endothelial cells during ischemia and reperfusion. Although our studies are consistent with many reports of the stimulatory effects of n-3 PUFAs on angiogenesis, n-3 PUFAs are nevertheless able to suppress tumor vasculature formation through multiple mechanisms, including a decrease in VEGF (Matesanz et al., 2010), PDGF (Basak et al., 2013), and MMPs (Tsuzuki et al., 2007). These results suggest that the impact of n-3 PUFAs on microvasculature may be disease- and context-dependent. In the context of cardiovascular disease, DHA and EPA may reduce the production of VEGF to suppress endothelial wound repair (Zhuang et al., 2013). However, in *fat-1* mice, we recently demonstrated that n-3 PUFAs induce the production of Ang 2 in astrocytes to stimulate endothelial cell proliferation after stroke (Wang et al., 2014).

Angiopoietins are a family of growth factors that regulate the formation and function of the vascular system (Augustin et al., 2009). Ang 1 facilitates angiogenesis by acting on the endothelial cell-specific tyrosine kinase receptor Tie2 (Zacharek et al., 2007). n-3 PUFAs robustly increased the expression of Ang 1 in the present study, which is likely to have stimulated post-stroke angiogenesis. Ang 1 can also work directly on neurons to support neurite outgrowth and synaptogenesis (Chen et al., 2009; Kosacka et al., 2006; Zhang et al., 2010), suggesting that n-3 PUFAs may boost neurogenesis through Ang 1.

Ang 2 was initially described as an endogenous antagonist of Ang 1 at the Tie2 receptor, which disrupts blood vessel formation in Ang 2-overexpressing embryos (Qin et al., 2013). Ang 2 treatment alone does not increase microvessel density *in vivo*, but it is essential for vessel destabilization during post-ischemic angiogenesis and sensitizes endothelial cells to VEGF-mediated angiogenesis (Zhu et al., 2005). We discovered that ischemia-induced downregulation of Ang 2 was reversed by n-3 PUFAs. This effect may work in concert with VEGF to stimulate endothelial proliferation (Wang et al., 2014).

Meteorin is a newly identified neurotrophic factor involved in cerebral angiogenesis and developmental maturation (Park et al., 2008). Although n-3 PUFAs did not significantly increase meteorin levels 7 days after ischemia, we cannot rule out its role in the recovery process. An increase in meteorin is known to occur in neural progenitor cells during the early phases of stroke, and may promote neurogenesis in the SVZ and neuroblast migration towards the ischemic striatum (Wang et al., 2012).

Stroke induces the proliferation and migration of NSC/NPCs in the SVZ. Revascularization is critical for the migration of immature neuroblasts and important for adequate blood flow and nutrition (Ohab et al., 2006; Thored et al., 2007). However, most of the newly generated

neurons are known to die shortly after stroke. Indeed, less than 20% of new neurons in the ischemic striatum survive longer than 2 weeks and only a very small proportion eventually replace the lost mature neurons (Ohab et al., 2006). n-3 PUFAs not only increased the survival of immature neurons (DCX⁺ cells) along the perfused vessel walls but also promoted their maturation in cortical parenchyma up to 35 days after stroke injury. These findings support the hypothesis that neurogenesis is enhanced after stroke injury by dietary fish oil supplementation. One limitation of the current study for using BrdU to label newly proliferated cells is that BrdU may cause random mutations to cells and label dying cells as well (Duque and Rakic, 2011). Although this effect of BrdU labeling has not been reported in rodents so far, it should be taken into consideration in our data interpretation.

Regenerated axons must be adequately myelinated for full restoration of neurological function after ischemic stroke (Franklin and Kotter, 2008). However, oligodendrocytes are exquisitely sensitive to ischemia and new OPCs therefore have to proliferate to repair the ischemic brain. Consistent with our previous findings in *fat-1* mice, we discovered that n-3 PUFAs may improve the proliferation of OPCs after ischemic stroke, thereby facilitating axonal repair. In concert with the improvements in angiogenesis and neurogenesis, the rescue of OPCs by n-3 PUFAs is expected to improve long-term neurological performance after cerebral ischemia.

In conclusion, our data demonstrate that chronic administration of fish oil elevates cerebral n-3 PUFA content and confers long-lasting neurological and histological protection against ischemic brain damage. One of the major novel findings of the present study is that dietary supplementation with fish oil not only reduces ischemic brain injury but also actively promotes brain repair. n-3 PUFAs may achieve this goal by promoting the revascularization of the brain after stroke and boosting neurogenesis and oligodendrogenesis. The advantage of dietary supplementation with nontoxic n-3 PUFAs over riskier treatments such as tPA is evident. Thus, considered together with a vast body of literature on fish oil, our studies place n-3 PUFAs in the unique position of being a natural, safe treatment that can be readily administered as a prophylaxis for long periods. Further studies on the mechanisms underlying n-PUFA-induced neurogenesis, oligodendrogenesis, and revascularization are highly warranted. Studies to investigate whether late administration of n-3 PUFAs after the onset of stroke injury is also therapeutic are also currently underway in our laboratory. Altogether, these studies may reveal fish oil to be a versatile treatment for two separate clinical populations: individuals at risk for stroke and individuals with a recent history of stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	arachidonic acid
ALA	α -linolenic acid
Ang	angiopoietin
BrdU	5-bromo-2-deoxyuridine
DAPI	4,6-diamidino-2-phenylindole
DCX	doublecortin
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DTA	docosatetraenoic acid
EPA	eicosapentaenoic acid
LA	linoleic acid
MBP	myelin basic protein
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MMP	matrix metalloproteinase
NPC	neural precursor cell
OPC	oligodendrocyte precursor cell
PDGF	platelet-derived growth factor
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene fluoride
rCBF	regional cerebral blood flow
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SVZ	subventricular zone
tMCAO	transient middle cerebral artery occlusion
VEGF	vascular endothelial growth factor

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Highlights

- n-3 PUFAs improve long-term neurobehavioral outcomes after stroke
- n-3 PUFAs stimulate post-stroke revascularization and angiogenesis
- n-3 PUFAs upregulate angiopoietin 1 and angiopoietin 2
- n-3 PUFAs promote the survival of newborn neurons after stroke
- n-3 PUFAs enhance post-stroke oligodendrogenesis and axonal remyelination

Figure 1. n-3 PUFAs protect against transient focal cerebral ischemia

(A) Seven-day survival rates of N3L and N3H groups after 60-min MCAO. (B) Infarct volume calculated on TTC-stained coronal brain sections in N3L and N3H mice at 48 hours after MCAO. n-3 PUFAs almost completely abolished the infarct. (C) Infarct area in coronal sections collected every 1 mm through the MCA territory at 48 hours after MCAO. Data are mean $\pm$ SEM. n=7 per group. *p 0.05, **p 0.01 vs. N3L. (D) Representative TTC-stained sections showing smaller infarct size in N3H mice than N3L mice at 48 hours after MCAO.

Figure 2. n-3 PUFAs improve long-term neurological functions

(A) Cylinder test at 3–21 days after MCAO or sham operation, n=8 per group. (B) Rotarod test before and 3–14 days after sham operation or MCAO. n=8 per group. (C) Pole test before and 3–14 days after sham operation or MCAO. The latency to turn on the pole and latency to descend the pole were recorded. n=10 per group. Shown are mean $\pm$ SEM.

*p 0.05, **p 0.01 vs. N3L sham, # p 0.05, p 0.01 vs. N3H MCAO, ^p 0.05, ^^p 0.01 vs. N3H sham.

Figure 4. n-3 PUFAs upregulate the expression of angiopoietin 1 and stimulate angiogenesis
 (A) Representative images of BrdU immunofluorescent signal (red), double labeled with lectin (green) in the ischemic penumbra of N3L and N3H brains 7–35 days after MCAO or sham operation. Scale bar = 25 μ m. (B) Quantification of BrdU cells along vessels in the cortex (CTX) and striatum (STR) at 7–35 days after MCAO. Data are presented as mean $\pm$ SEM, n=4 per group at each time point. *p 0.05, **p 0.01 vs. N3L at the same time point. (C) Representative Western blots showing levels of angiopoietin 1 (Ang 1), angiopoietin 2 (Ang 2), and meteorin in the ipsilateral hemispheres of N3L and N3H mice after 7 and 14 days of reperfusion or after sham surgery. β -actin was used as an internal loading control. (D) Protein expression of Ang 1, Ang 2, and meteorin was quantified and expressed relative to N3L sham mice. Data are presented as mean $\pm$ SEM, n=4 per group at each time point. *p 0.05, **p 0.01 vs. N3L sham, #p 0.05 vs. N3L MCAO at the same time point.

Figure 5. n-3 PUFAs enhance post-stroke neurogenesis

(A) Representative images of doublecortin (DCX) cells (red) along functional vessels (green) in the striatum after MCAO. Scale bar = 25 μ m. (B) Quantification of DCX cells at 35 days post ischemia. Data are presented as mean $\pm$ SEM, n=4 per group, **p 0.01 vs. N3L. (C) Representative images of BrdU/NeuN double labeling in the cortex (CTX) and striatum (STR) at 35 days after MCAO. Arrows: NeuN /BrdU cells. Scale bar = 25 μ m. (D) 3D confocal scan of the area in the inset in panel C. Scale bar = 100 μ m. (E) Quantification of NeuN/BrdU⁺ cells in CTX and STR. Data are presented as mean $\pm$ SEM, n=4 per group, * p 0.05, ** p 0.01 vs. N3L MCAO.

Figure 6. n-3 PUFAs enhance oligodendrogenesis after cerebral ischemia

(A) Immunostaining for NG2 (green) and BrdU (red) in the ipsilateral striatum (STR) and corpus callosum (CC) of N3L and N3H mice at 35 days after MCAO or sham operation. Arrows: NG2 /BrdU cells. Nucleus are labeled with DAPI (blue). Scale bar = 25 μ m. (B) Quantification of NG2⁺ and NG2 /BrdU cells, expressed as the number of positive cells per mm² in the striatal peri-infarct region. (C) Quantification of NG2 and NG2 /BrdU cells in the peri-infarct region of the corpus callosum. Data are mean $\pm$ SEM, n=4 per group; *p < 0.05 vs. N3L MCAO, #p < 0.05 vs. sham.

Figure 7. n-3 PUFAs decrease demyelination after MCAO

(A) Representative images of SMI32 (red) and MBP (green) double staining in N3L and N3H mice 3–35 days after MCAO or sham operation. Nuclei are labeled with DAPI (blue). Arrows: demyelinated axons. Scale bar = 25 μ m. (B) Quantification of SMI32/MBP ratio in the striatum and corpus callosum. (C) Quantification of SMI32/MBP in the cortex. Data are presented as mean $\pm$ SEM, n=5 per group, *p 0.05, **p 0.01, ***p 0.001 vs. N3L sham. ##p 0.01, ###p 0.001 vs. N3L tMCAO. ^^p 0.01 vs. N3H sham.