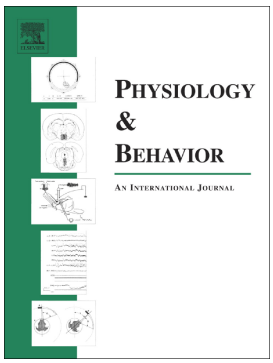

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Postischemic fish oil treatment confers task-dependent memory recovery

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Abstract

A series of our previous studies demonstrated that fish oil (FO), equivalent to 300 mg/kg docosahexahenoic acid (DHA), facilitates memory recovery after transient, global cerebral ischemia (TGCI) in the aversive radial maze (AvRM). The present study sought to address two main issues: (*i*) whether the memory-protective effect of FO that has been observed in the AvRM can be replicated in the passive avoidance test (PAT) and object location test (OLT) and (*ii*) whether FO at doses that are lower than those used previously can also prevent TGCI-induced memory loss. In Experiment 1, naive rats were trained in the PAT, subjected to TGCI (4-vessel occlusion model), and tested for retrograde memory performance 8 and 15 days after ischemia. Fish oil (300 mg/kg/day DHA) was given orally for 8 days. The first dose was delivered 4 h postischemia. In Experiment 2, the rats were subjected to TGCI, treated with the same FO regimen, and then trained and tested in the OLT. In Experiment 3, the rats were trained in the AvRM, subjected to TGCI, administered FO (100, 200, and 300 mg/kg DHA), and tested for memory performance up to 3 weeks after TGCI. At the end of the behavioral tests, the brains were examined for neurodegeneration and neuroblast proliferation. All of the behavioral tests (PAT, OLT, and AvRM) were sensitive to ischemia, but only the AvRM was able to detect the memory-protective effect of FO. Ischemia-induced neurodegeneration and neuroblast proliferation were unaffected by FO treatment. These results suggest that (*i*) the beneficial effect of FO on memory recovery after TGCI is task-dependent, (*ii*) doses of FO < 300 mg/kg DHA can protect memory function in the radial maze, and (*iii*) cognitive recovery occurs in the absence of neuronal rescue and/or hippocampal neurogenesis.

Keywords

Global cerebral ischemia, Memory deficits, Neurodegeneration, Fish oil, Memory recovery, Task-dependence

Highlights

• Global cerebral ischemia induces memory impairment in different behavioral tests.

• Fish oil task-dependently prevented memory deficits.

• The memory-protective effect of fish oil was not dose-dependent.

• The antiamnesic effect of fish oil occurred in the absence of histological neuroprotection.

• Ischemia-induced neurogenesis was unaffected by fish oil treatment.

1. Introduction

Transient, global cerebral ischemia (TGCI) is a condition that is commonly encountered in daily clinical practice, mainly because of unexpected, reversible cardiac arrest. Various other clinical conditions can also result in hypoxic/ischemic brain damage, including respiratory arrest, gas poisoning, accidental suffocation of diverse origins, perinatal asphyxia, and diagnostic and surgical procedures [1]. Depending on the duration and severity of TGCI, patients are most often left with long-lasting cognitive impairments, mainly in the domains of attention, processing speed, memory, and executive function, that render them vocationally impaired [2]. Treatments that can alleviate such cognitive deficits are urgently needed. The assessment of behavioral end-points has been recommended in the preclinical phase of neuroprotective drug development [3].

We have been investigating the effects of fish oil (FO) on the histological, biochemical, and behavioral outcomes of TGCI using the 4-vessel occlusion (VO) model. Fish oil is one of the richest natural sources of docosahexanoic acid (DHA; 22:6n-3) and eicosapentenoic acid (EPA; 20:5n-3), the two most extensively studied n-3 polyunsaturated fatty acids [4, 5]. The therapeutic potential of these fatty acids has been extended to the field of ischemic brain disease [6]. We reported that FO treatment (equivalent to 300 mg/kg DHA) that was initiated 3 days prior to ischemia prevented the consequent loss of memory (i.e., retrograde amnesia) when administered for both 28 days [7] and 10 days [8, 9] after TGCI. The antiamnesic effect of FO was also observed even when the first dose was administered at 4 h postischemia in middle-aged rats, suggesting a time window of efficacy [10]. Other data from our laboratory suggest that an antioxidant effect that occurs within the first hours of reperfusion may contribute to the memory-protective effect of FO after TGCI [9]. Interestingly, we have not observed protective effects of FO against ischemia-induced pyramidal cell death in the hippocampus or cerebral cortex, a finding that has been replicated by others [11].

Our previous studies always had two common methodological aspects. First, FO was administered at a dose equivalent to 300 mg/kg DHA which, from a clinical perspective, may be a dose that is too high. Whether a DHA dose < 300 mg/kg also effectively prevents memory loss after TGCI remains to be investigated. Second, a non-food-rewarded radial maze (i.e., the aversive radial maze [AvRM]) was used to assess remote, long-term retrograde memory. The conventional, food-rewarded, eight-arm radial maze has also been used to demonstrate the effects of DHA on TGCI-induced learning impairment [12]. The beneficial effects of FO against learning deficits after TGCI have also been evaluated in the Morris water maze [11]. These studies suggest that the radial maze and water maze are reliable behavioral paradigms to test the effects of drugs on functional recovery after ischemic brain damage. Despite the sensitivity of these behavioral models to hypoxic/ischemic brain damage [13], they are both labor-intensive and time-consuming and require several consecutive training and/or retention memory trials (RMTs). This characteristic may limit their utility for screening substances with possible neuroprotective properties. The use of different behavioral models is necessary if a battery of tests is needed to define the therapeutic efficacy of a given treatment. The beneficial effects of drugs on functional recovery after brain damage may also be task-dependent. Understanding this fact may prevent the premature invalidation of drugs that actually have neuroprotective potential.

Therefore, the present study evaluated the following: (*i*) whether the memory-protective effect of FO in the AvRM task can be replicated in the passive avoidance test (PAT) and object location test (OLT), (*ii*) whether FO that contains DHA doses < 300 mg/kg can prevent memory loss in the AvRM task after TGCI, and (*iii*) whether FO administration at different doses influences the effect of ischemia on neurodegeneration and neurogenesis.

2. Material and Methods

2.1. Subjects

Male Wistar rats (250-300 g, 3-4 months old) were used. They were maintained under controlled temperature (22°C ± 1°C) and a 12 h/12 h alternating light/dark cycle (lights on at 7:00 AM) with free access to tap water and a standard commercial chow diet (Nutrilab-CR1; Nuvital Nutrients, Curitiba, PR, Brazil). Prior to any experimental manipulations, the rats were acclimated to the laboratory vivarium for 1 week. The experimental procedures adhered to the ethical principles of the Brazilian College of Animal Experimentation (COBEA) and were approved by the local Ethics Committee on the Use of Animals (CEUA; authorization no. 157/2013).

2.2. Experimental design

Fig. 1 shows the timeline of the experiments. Experiment 1a was performed to obtain insights into the duration and consistency of passive avoidance memory in intact rats. We sought to determine the length of time we should use to test the effect of TGCI on remote, retrograde passive avoidance memory. Naive rats were trained for 3 consecutive days (day -2 to day 0) and then tested for the retention of passive avoidance memory on days 1, 7, 15, and 21 after training. Based on the results of Experiment 1a, Experiment 1b evaluated the effect of TGCI on passive avoidance memory and the effect of FO thereon 8 and 15 days after TGCI. An RMT was performed preoperatively (day -1) to confirm that the rats learned the task. Experiment 2 tested the effects of TGCI and FO in the OLT. The OLT is a one-trial spatial memory test. Naive rats were subjected to TGCI, then treated with FO or vehicle for 8 days. On day 15 postischemia they were exposed to the training condition, and again on day 16, after which the retention memory trial (RMT) was conducted. In Experiment 3, naive rats were trained for 10 days to learn the AvRM task. They were then assigned to different groups according to DHA dose. Two days later (day 0), TGCI or sham surgery was performed. In the

TGCI groups, treatment with vehicle or FO begun at 4 h after reperfusion and continued once per day during 8 days. The sham operated groups did not receive any treatment. Retention memory trials were performed on days 9, 16, and 23 postischemia. One day after the behavioral tests, the brain was removed and processed for Nissl (Experiment 1b and 2) or immunohistochemical (NeuN and doublecortin [DCX], Exp. 3) staining.

2.3. Ischemia

Transient, global cerebral ischemia was induced according the 4-vessel occlusion (4-VO) [14], with modifications. The animals were anesthetized with a halothane/oxygen mixture (Tanohalo; Cristália, Itapira, São Paulo, Brazil) that was delivered through a universal vaporizer (0.5 L/min) connected to a face mask that was affixed to the rat's nose. A longitudinal incision in the dorsal neck exposed the alar foramin at the first cervical vertebrae, through which the vertebral arteries (VA) were bilaterally electrocoagulated (unipolar current, 3-4 mA). The incision was then sutured. Another incision was made on the ventral neck to expose the common carotid arteries, which were loosely tied with silk thread. Four to 5 h later, the silk thread was carefully tightened for 15 min in conscious, spontaneously ventilating animals, thus completing the 4-VO surgical procedure. Throughout occlusion and during the first hour of reperfusion, the rats were maintained in a warming box (inner temperature, 30 ± 1°C) to avoid eventual brain hypothermia. During surgery, rectal temperature was maintained at ~37°C with a heating pad and monitored with a digital thermometer (APPA MT-520; Minipa, São Paulo, Brazil) coupled to a rectal probe (Minipa Electronics, Houston, TX, USA). Loss of the righting reflex within 2 min after carotid artery occlusion and maintenance for the entire period of occlusion were used as inclusion criteria for effective ischemia. Unresponsiveness to gentle touch, mydriasis, and tonic extension of

the paws were also monitored. Sham-operated animals underwent the same surgical procedures but without vessel occlusion.

2.4. Fish oil preparation and treatment

The commercial formulation Omega-3 DHA 250® (Biotik do Brasil Indústria e Comércio Ltda, São Paulo, Brazil) was used. Each FO capsule (0.4 ml) contained 250 mg DHA, 50 mg EPA, and 1 mg vitamin E as an antioxidant. The total fatty acid content in each capsule was reported elsewhere [9]. The content of four capsules was diluted with 8.4 ml of extra virgin olive oil, providing a main FO solution with 100 mg/ml DHA, 20 mg/ml EPA, and 0.4 mg/ml vitamin E. From this main FO solution, 3 ml/kg was administered by gavage, which corresponded to doses of 300 mg/kg DHA, 60 mg/kg EPA, and 1.2 mg/kg vitamin E. For the other doses, to administer a constant volume (i.e., 3 ml/kg), 2 ml of the main FO solution was diluted with 1 ml of olive oil, providing doses of 200 mg/kg DHA, 40 mg/kg EPA, and 0.8 mg/kg vitamin E. Similarly, for doses of 100 mg/kg DHA, 20 mg/kg EPA, and 0.4 mg/kg vitamin E, 1 ml of the main FO solution was diluted with 2 ml of olive oil. Ischemic control rats received olive oil as the vehicle, and sham-operated rats received no treatment. Fish oil or vehicle was administered between 2:00 PM and 3:00 PM.

2.5. Behavioral analysis

2.5.1 Step-down passive avoidance test

The apparatus consisted of a Plexiglas chamber (50 cm × 26 cm × 31 cm) with an electrified grid floor (0.5-2.0 mA) and an unelectrified "safe" platform (5 cm high) that was located at one extremity of the chamber. The test procedure was performed as described elsewhere [15], with modifications. For avoidance training, the rat was placed on the unelectrified safe platform. Once it stepped down onto the electrified grid, a 5-s electric

shock (0.8 mA) was delivered to the animal's paws. If the animal remained on the grid floor after the initial electric shock, then a subsequent electric shock was delivered 30 s later, and so on for up to 2 min, until the animal jumped onto the safe platform. Using this protocol, the number of electric shocks in a training session ranged from one to three. Once the rat escaped to the safe platform and remained there for up to 5 min, then the training session ended, and the animal was considered to have learned the task. In contrast, if it stepped down onto the electrified floor before 5 min elapsed, then another electric shock was delivered. If this situation persisted for up to 2-min of intermittent shock delivery, then the training session also ended. If such poor performance persisted for two training sessions, then the animal was considered to have not learned the task and was excluded from subsequent experiments. This training protocol was performed for 3 consecutive days. The retention of passive avoidance learning was tested 1, 8, 15, and 22 days after the last training session in intact rats (Fig. 1, Experiment 1a). Based on the results, the next experiment (Fig. 1, Experiment 1b) was performed to evaluate the influence of TGCI on remote retrograde memory and the effect of FO thereon. Naive rats were trained as described in Experiment 1a, subjected to TGCI or sham surgery, treated with FO or vehicle, and then tested for memory retention on days 8 and 15 postischemia. On day 16, the brain was removed and examined for neuronal damage in the hippocampus, cerebral cortex, and amygdala (see details below).

2.5.2. Object location test

The OLT consisted of a one-trial, spatial memory test that measured the ability of rats to distinguish between one familiar spatial location and another unfamiliar spatial location [16]. Intact rats preferentially explore a familiar object that occupies a novel location more than they explore a familiar object that occupies a previous location. Here we used the procedure described elsewhere [16, 17], with modifications. The apparatus consisted of a circular arena

(83 cm diameter, 40 cm high wall) constructed of transparent polyvinylchloride, inside of which two identical objects were present. The objects (available in triplicate) consisted of glass bottles (5.5 cm maximum diameter, 15.0 cm height) filled with 200 ml of water and sand, which could not be moved by the rat. At day one (i.e., on day 15 postischemia) two identical objects were positioned equidistant (10 cm) from the open field wall. The animal was placed in the arena facing the wall and opposite to the objects, and allowed to explore them for 3 min. This process, considered as the training phase, was repeated for 5 times consecutively, with a 15-min interval between exposures [17]. On the second day of testing (i.e., on day 16 postischemia), the animal was placed in the open field for other 3 min, being the objects in the same positions they were locate in the previous day. This was considered the re-training phase (r-T). The animal was then put back in its home cage and the location of one of the objects was moved diagonally relative to the other object. Fifteen min later it returned to the open field for retention memory trail (RMT). The number of entries into the object zone and time spent exploring the two objects during the r-T and RMT phases were manually recorded. A third parameter, the discrimination index (D2), was also estimated as a measure of spatial memory, indicating whether the rat spent more time exploring the relocated object while correcting for the total exploration time in T2. D2 was calculated as the following: D2 = (exploration time in the novel location – exploration time in the familiar location) / (exploration time in the novel location + exploration time in the familiar location). Exploratory behavior was defined as when the rat directed its nose toward the object at a distance of $\leq$ 1 cm and/or touched the object with its nose. Sitting on the object was not considered exploration. To avoid the influence of olfactory cues, all of the objects and arena were thoroughly cleaned with a 70% ethanol and water solution between sessions. The object that was moved and the position to which it was moved were balanced throughout the experiment and between groups to reduce potential bias toward particular objects, sides, or

locations. One day after the OLT, the brain was removed and prepared for histological examination.

2.5.3. Aversive radial maze test

Based on the circular platform test, the AvRM works on the basis of the rat's natural behavior of avoiding open, wide, and illuminated areas and preference for darkened and enclosed shelters (i.e., the goal box). A detailed view and description of the AvRM apparatus used herein are shown elsewhere [7, 9]. Details on the original development and conceptualization of the AvRM model are reported elsewhere [18], which was later modified from an unconfined to the confined version.

Once habituated to the AvRM apparatus and testing room (4–5 min/day for 3 days), the naive rats were trained to learn the task according to the following schedule: 3 trials/session, 1 session/day, for 10 days. In each trial, a 4-min cut-off period was employed. Once the task was learned up to an asymptotic level of performance, the animals were subjected to TGCI or sham surgery and allowed to recover from surgical stress for 8 days. Retention memory trials (RMTs) were then performed 9, 16, and 23 days after TGCI to assess the ability of rats to remember the spatial location of the goal box that was learned during the training phase prior to surgery, i.e., retrograde memory. Both preoperative learning and postoperative, retrograde memory performance were measured by three parameters: (*i*) the latency to find the goal box, (*ii*) the number of reference memory errors, and (*iii*) the number of working memory errors. In each trial, a reference memory error was counted when the rat visited an arm that contained a false goal box for the first time. If the animal returned to an arm that had been previously visited during that trial, then a working memory error was counted. Accordingly, the animal exhibited reference memory by remembering the goal box location across several training days (i.e., long-term memory). In contrast, working memory performance was

evident when the rat remembered and avoided entering an arm that contained an unrewarded goal box that was previously visited within a given trial. A shorter latency and a lower number of reference/working memory errors indicated better learning/memory performance. Additional details on the procedures used in the AvRM test are described elsewhere [7-10].

2.6. Neurohistological analyses

2.6.1. Nissl staining

At the end of passive avoidance and object location tests, the animals were deeply anesthetized with 50 mg/kg sodium thiopental (Thiopentax®, Cristália, SP, Brazil), transcardially perfused with 4% paraformaldehyde in PBS 0.2 M. Their brains wee removed and postfixed in the same fixative for up to 3 days. Paraffin-embedded coronal sections (7 µm thickness, 70 µm apart) were obtained with a rotating microtome (RM2445; Leica, Goettingen, Germany), distributed into four sets of slides containing three coronal sections each, and stained with cresyl violet (Nissl). Using a 40× objective (BX41 microscope) coupled to a color, high-performance device camera (QColor3, Ontario, Canada), the number of normal-looking neurons was counted bilaterally in three coronal sections of the hippocampus, cerebral cortex, and amygdala. In the hippocampus cell count was performed manually across the pyramidal stratum of the CA1 to CA4 subfields. In the cerebral cortex, digital microscopic areas of 0.097 mm^2 were captured from the retrosplenial (RS) and parietal association (PtA) regions, under an identical microscopic light intensity. In the RS, the area of cell counting was located at the 'center' of this region. In the PtA, the cells were counted in two distinct areas that were distant from each other by the length of one microscopic field. In amygdala, viable neurons were counted in the basolateral nucleus (BLA), in a digital microscopic area of 0.097 mm^2. Neurons that showed a well-delimited, spherical form, with a

distinct nucleus and nucleolus, were counted. Neurons that appeared shrunken, with dark-stained cell bodies, sometimes with surrounding empty spaces ("ghost" cells), were considered neurons that were destined to die and therefore excluded. In each individual, the number of pyramidal cells recorded from the various measurements (2 hemispheres x 3 coronal sections) were averaged and transformed into a percentage, with the mean of the sham-operated group set to 100%. Individual normalized values were then used to represent the percentage of normal-looking neurons in each group, which values were expressed as mean ± SEM.

2.6.2. Immunohistochemistry

2.6.2.1. Tissue preparation

After behavioral testing in the AvRM task, the brains were removed as described in session 2.6.1, postfixed in the same fixative for 2 h, and then cryoprotected (30% sucrose in PBS) for at least 72 h. Frozen brains were serially sectioned on a cryostat (Criocut 1800, Reichrt-Jung, Heidelberg, Germany) into 40 µm coronal sections at coronal coordinates corresponding to 3.60 to 4.30 mm posterior to bregma [19]. The sections were collected in Eppendorf tubes containing PBS 0.1 M plus sodium azide 0.1% and stored at 4°C until immunohistochemistry reaction for adult neurons (neuronal nuclei, NeuN) and newborn neurons (doublecortin, DCX). Initially, the brain sections were successively washed in buffer A (PBS 0,1 M plus 0,15% Triton-X 100) and then incubated in citrate buffer (pH 6.0) at 50°C for 30 min for antigenic recovery. After repeated washing in buffer A, the sections were quenched in 1% H_2O_2 for 30 min and then blocked with 2% bovine serum albumin in 0.1 M PBS for 60 min at room temperature. The sections were incubated overnight with the polyclonal antibodies goat anti-DCX (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-NeuN (1:1000, Abcam, Cambridge, Massachusetts, USA) in buffer A at

room temperature. They were then incubated with the respective biotinylated secondary antibodies for 2 h and incubated in the ABC solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The peroxidase reaction was performed using 3-30-diaminobenzidine (DAB; Sigma) and 0.05% H2O2. NiCl2 was added to the DAB solution to increase the staining contrast. The sections were then mounted on gelatin-coated slides.

2.6.2.2. NeuN- and doublecortin-positive cell quantification

Sets of three random coronal sections from each animal were examined for immunoreactivity for NeuN and DCX. Using the 40× objective (optical devices described above), the number of DCX-positive cells was counted manually across the entire extent of the granular cell layer (GCL) and the subgranular zone (SGZ) of the dentate gyrus (DG) in both left and right hemispheres. The density of NeuN-positive cells was performed using integrated optical density (IOD) measurements. The regions examined were the stratum pyramidale in the CA1 and CA3 hippocampal subfields. Using a 20x objective, images were captured from the right and left CA1 and CA3 subfields and the measurements were determined in prefixed areas of 0.66 mm^2 and 0.87 mm^2, respectively. Using ImageJ software (NIH, Bethesda, MD, USA), the images were converted to 32-bit image and the background was subtracted. The threshold for positive signal was predefined, and the IOD obtained. In each rat, the number of DCX-positive neurons and the IOD values obtained from six measurements (2 hemispheres × 3 sections) were averaged and transformed into a percentage, with the mean of the sham-operated groups set to 100%. Individual normalized values were then used to represent the percentage of DCX-positive cells or IOD in each group, which values were expressed as mean ± SEM.

2.7. Statistical analysis

SAS 9.3 software was used, and the data were firstly examined for the assumptions of normality and homoscedasticity. The Linear Mixed Effects (LME) model was used for comparing memory performance longitudinally in both passive avoidance and AvRM tests. Because the data did not satisfy the assumptions of normal distribution and/or homoscedasticity, the covariance model to which the data fit better was determined using the restricted likelihood ratio test and information criteria (Akaike Information Criterion[AIC] and Bayesian Information Criterion [BIC]). Accordingly, the Toeplitz and the non-structured covariance models were used to quantify the effects of TGCI and FO on passive avoidance and AvRM memory, respectively. The F test followed by Tukey-Kramer's multiple range test was then used to quantify fixed effects (Groups and Trials) on memory performance across time, with Group as the between-subjects factor and Trial (day of testing) as the within-subjects factor [20-22]. Repeated measures one-way ANOVA with the Geisser-Greenhouse's epsilon correction factor (absence of sphericity) was used to quantify the endurancy of passive avoidance memory in intact rats. In the OLT, the number of entrance and the time spent in the objects zone were analyzed by the Generalized Linear Model (GLM) with gamma distribution. Functional spatial memory within the groups, i.e., a D2 value that differs significantly from 0, was analyzed with a two-way one-sample t-test [23]. The data of histological analysis were quantified using the one-way ANOVA or the GLM with the Poison distribution, as they fit to the assumption of homoscedasticity or not, respectively. A proportion-like t-test was used to quantify mortality data. The data are expressed as means ± SEM, and differences were considered significant at $p < 0.05$

3. Results

3.1. Animal exclusion

Of the 306 rats that were initially included in the study, 24 (7.8%) were excluded prior to any surgical procedure, since they did not learn the AvRM task (6/306 [1.9%]) or PAT (18/300 [6.0%]) during the training phase.

3.1. Mortality rate

Of the remaining 282 rats, 32 (11.4%) died during surgery, likely because of anesthesia. Of the remaining 250 rats that completed surgery, no sham-operated rats died (0%), but 57 rats (22.8%) that were subjected to TGCI died either because of rupture of the carotid artery or at some time point after completing 4-VO when the animals were already conscious, likely reflecting the fatal effect of severe TGCI ($p < 0.01$, 4-VO *vs*. sham). The mortality rate did not differ significantly between the groups that were treated with vehicle or FO ($Z = 0.0008$, $p = 0.5$).

3.2. Learning and memory performance

3.2.1. Passive avoidance

Before testing the effect of TGCI on retrograde passive avoidance memory and then the effect of FO thereon, we evaluated the consistency of retrograde memory across time in intact rats. Fig. 2 shows the endurance of passive avoidance memory that was tested after 3 days of training. On the first test day, 94.2% of the animals (16/17) exhibited a ceiling effect for latency (i.e., 300 s). This performance decreased to 70.6% (12/17) on days 7 and 15 and then to 58.8% (10/17) on day 21 of testing. The average latency did not change significantly from day 1 to day 21 of the test ($F_{2.50,\ 39.94} = 2.39$, $p > 0.05$). However, 35.3% of the rats (6/17) presented moderate to very low retention performance, the distribution of which varied over time between animals. For example, individuals *a* and *c* presented poor performance that

persisted across the entire experiment. Individuals *e*, *g*, and *h* performed well on day 7, but they presented memory loss on the subsequent testing days. In individuals *b*, *d*, and *f*, passive avoidance memory fluctuated across time. Despite of this, the data indicated that the majority of the rats remembered the task for at least 21 days after training.

Based on the results above, we choose days 7 and 15 posttraining to examine the impact of ischemia on retrograde passive avoidance memory and the effects of FO thereon (Fig. 3). We observed significant main effects of treatment ($F_{2,119}$ = 15.02, $p < 0.001$) and time ($F_{2,20}$ = 26.52, $p < 0.001$). Twenty-four hours after training and before surgery (preoperative phase), all of the rats presented the maximal step-down latency (i.e., 300 s). This performance decreased slightly but significantly 7 and 15 days after sham surgery (t_{20} = 2.46-3.14, $p <$ 0.01-0.05), thus reproducing the performance of intact rats (Fig. 2). In the postoperative phase, a significant loss of memory was observed in the vehicle-treated group both 7 and 15 days after ischemia (t_{19} = 2.44-2.55, $p < 0.05$, ischemia/vehicle *vs.* sham). This amnesic effect of ischemia was not prevented by FO, independent of whether memory was examined 7 or 15 days postischemia (t_{13} = -1.04 to -0.26, $p > 0.05$, FO *vs.* vehicle).

3.2.2. Object location test

Fig. 4 shows the effects of ischemia on object location memory, which reflects the ability of rats to discriminate changes in object location. The discrimination index (D2) was calculated based on the number of entries into and time spent in the object zone. During training (Fig. 4A, C), all of the groups similarly explored both of the objects that were in the original (old) location (χ^2 = 0.04-3.53, p = 0.06-0.84). During the RMT (Fig. 4B, D), the sham-operated group presented greater exploration of the object in the new location (χ^2 = 22.44-27.51, $p < 0.001$), indicating that they were able to remember the old position because they refocused their attention to the unfamiliar, new object location. We also expressed this

object location memory as the discrimination index D2 (Fig. 4E) compared with zero (t_{29} = 15.96, $p < 0.001$). In contrast to the sham surgery group, the ischemic vehicle-treated group explored both objects equally (χ^2 = 0.03-0.74, $p < 0.05$; Fig. 4B, D), indicating that these rats were unable to discriminate the change in object location. This was also reflected by D2, which significantly decreased compared with sham animals (χ^2 = 4.56, p = 0.033, vehicle *vs.* sham; t_{20} = 0.08, $p > 0.05$, ischemia/vehicle *vs.* zero; Fig. 4E). This memory deficit that was caused by ischemia in the OLT was not significantly alleviated by FO (χ^2 = 1.38-1.44, $p >$ 0.05, object 1 [O1]-new location *vs.* O2-old location in the ischemia/FO group, Fig. 4B, D; χ^2 = 1.78, $p > 0.05$, ischemia/FO *vs.* ischemia/vehicle; t_9 = 0.27, p > 0.05, ischemia/FO *vs.* zero, Fig. 4E).

3.2.3. Radial maze

The effect of FO on ischemia-induced memory loss in the AvRM task is shown in Fig. 5. The groups that were treated with 100, 200, and 300 mg/kg DHA are presented individually (left panels) and pooled (right panels) because they did not significantly differ from each other. Comparisons among individual groups revealed highly significant main effects of group on the latency and number of errors ($F_{4,123}$ = 6.30-13.12, $p < 0.0001$). Main effects of trial were also found ($F_{2,123}$ = 4.35-7.05, p < 0.01-0.05), indicating that retrograde memory improved across time. Notably, memory performance in the sham-operated group did not change between the pre- and postischemic phases, indicating the maintenance of retrograde memory in these animals. In contrast, the latency to find the goal box and number of reference and working memory errors significantly increased in vehicle-treated rats (t_{123} = 4.63-7.06, $p < 0.0001$, *vs.* sham), indicating that they "forgot" the task that was learned prior

to ischemia (i.e., retrograde amnesia), an effect that persisted throughout the test, despite some degree of improvement.

The memory deficit that was caused by ischemia (expressed as latency) was attenuated by all three doses of FO (t_{123} = -3.24 to -4.56, p < 0.0001-0.05, *vs.* vehicle). At DHA doses of 300 and 200 mg/kg, FO also reduced the number of working memory errors (t_{123} = -3.39 to -3.03, p < 0.01-0.05, *vs.* vehicle). The number of reference memory errors was only qualitatively mitigated by FO. The comparisons between doses revealed no dose-effect relationship, independent of the parameters that were measured (p = 0.49-0.99). The memory-protective effect of FO was more apparent when the data from the groups that were treated with different doses were pooled (latency: t_{89} = -4.53, p < 0.0001; reference memory errors: t_{91} = -2.22, p = 0.029; working memory errors: t_{91} = -2.99, p = 0.01; Fig. 5). Altogether, these data indicate that FO alleviated retrograde amnesia that was caused by TGCI, an effect that was not dose-dependent.

3.3. Neurodegeneration and neurogenesis

The effect of FO (300 mg/kg DHA) on ischemia-induced neurodegeneration (Cresyl violet [Nissl] staining) was first examined in the groups that were evaluated in the PAT (Fig. 6). The degree of neuronal death in the hippocampus, cerebral cortex, and BLA was deduced from the reduction of the number of normal-looking pyramidal neurons relative to the sham-operated group. In the ischemic vehicle-treated group, the degree of cell death ranged from severe (~80% cell loss) to no apparent lesion, indicating a highly variable pattern of neuronal death after TGCI. Compared with the sham-operated group, the ischemic vehicle-treated group presented significant neuronal death across the CA1-CA4 pyramidal stratum of the hippocampus (55.42% cell loss, χ^2_2 = 439.12, p < 0.0001), RS/PtA cortex (24.10% cell loss, χ^2_2 = 57.79, p < 0.0001), and BLA (30.50% cell loss, χ^2_2 = 97.20, p < 0.0001). Compared with vehicle, FO failed to rescue neurons in any of these structures (61.28%, 25.55%, and

33.90% cell loss, respectively; $\chi^2_1 = 0.069\text{-}4.43$, $p > 0.05$). Hstological analysis was not performed in the groups that were tested in the OLT because the treatment regimen and postischemia behavioral analysis were similar to the animals that were used in the PAT.

Neurohistological analysis was also performed in a subset of animals that were tested in the AvRM task and treated with vehicle or FO at doses of 100, 200, and 300 mg/kg DHA. Neurodegeneration was deduced from the reduction of the integrated optical density (IOD) that was generated by NeuN-positive neurons in the CA1 and CA3 subfields of the hippocampus. Fig. 7 (upper left panel) shows that the IOD in ischemic vehicle-treated rats significantly decreased in the CA1 subfield (64.64% average cell loss; $\chi^2 = 8.70$, $p < 0.01$, *vs.* sham), an effect that was not prevented by FO ($p > 0.05$), although some individuals presented no lesions. Ischemia caused much less neuronal death in the CA3 subfield (18.31% cell loss), although it was still statistically significant ($F_{4,25} = 3.47$, $p < 0.05$, *vs.* sham). This effect was also not prevented by FO ($p > 0.05$), independent of the dose used, thus replicating the lack of neuronal rescue by FO that is shown in Fig. 6 (Nissl staining). In the same animals, the impact of ischemia on the proliferation of newborn cells in the dentate gyrus (DG) and the effect of FO thereon were estimated by counting the number of DCX-immunoreactive neurons (Fig. **7**, bottom left). The number of DCX-positive neurons increased in the ischemic vehicle-treated group ($F_{4,25} = 6.33$, $p < 0.01$, *vs.* sham). This stimulating effect of ischemia on newborn cell proliferation was not significantly changed by any dose of FO ($p > 0.05$, *vs.* vehicle).

4. Discussion

The present study extends our previous work that evaluated FO-mediated memory protection in the AvRM test after TGCI [7-9, 20]. In the present study, we sought to replicate our findings in the PAT and OLT. We evaluated whether FO that contains DHA doses < 300

mg/kg prevents ischemia-induced memory deficits in the AvRM test. Histologically, the effect of FO on ischemia-induced cell death was evaluated by both Nissl staining and NeuN immunohistochemistry. Doublecortin immunohistochemistry was used to evaluate the neurogenic response to ischemia and the effect of FO thereon. The behavioral analyses showed that all the three tests (AvRM, PAT, and OLT) were sensitive to TGCI, but only the AvRM task was able to detect the effect of FO on reducing memory deficits that were caused by TGCI, suggesting that this beneficial effect of FO is task-dependent. Fish oil-mediated memory protection in the AvRM was more consistently observed after 300 mg/kg DHA treatment, although the DHA doses of 200 and 100 mg/kg also alleviated memory deficits, reflected by latencies and working memory errors. However, a dose-effect relationship was not evident. Histologically, FO failed to reduce neuronal death, independent of the dose and staining method used. Finally, TGCI increased the number of DCX-immunoreactive neurons in the hippocampal DG, an effect that was not influenced by FO.

4.1 TGCI impairs memory in the AvRM, PAT, and OLT

The sensitivity of the AvRM test to TGCI (Fig. 5) replicated our earlier findings [7-10, 20]. The AvRM is also sensitive to chronic cerebral hypoperfusion (CCH) in middle-aged rats [10, 21, 22] but not young rats [10]. Moreover, the AvRM was able to distinguish the effects of different FO treatment regimens after TGCI [8, 9] and the lack of memory recovery by FO after CCH [10]. The AvRM also distinguished between the effects of sildenafil (phosphodiesterase-5 inhibitor) and cilostazol (phosphodiesterase-3 inhibitor) after CCH [21] and detected the long-lasting memory-protective effect of atorvastatin after CCH in middle-aged rats [22]. These studies indicate that the AvRM is a reliable paradigm for investigating the effects of drugs on learning and memory after hypoxic/ischemic insult. With regard to the effect of TGCI in the PAT, our present results agree with previous findings in gerbils [24,

25], rats [23, 26, 27], and mice [28]. In these previous studies, the impact of TGCI on passive avoidance behavior was investigated using both acquisition (learning) and retention (memory) tests after ischemia, in which retention was assessed 24 h after training (i.e., recent, anterograde memory). To test whether the memory-protective effect of FO in the AvRM [7-10, 20] could be replicated in the PAT, we used an experimental timeline that was similar to the one that was used for the AvRM to assess remote, retrograde memory as the functional end-point (see Fig. 1, Experiments 1 and 3). This protocol has also been successfully used to demonstrate the deleterious effects of focal brain ischemia (or stroke) on retrograde memory performance in mice [15]. Retrograde amnesia implies that the memory trace that was formed and consolidated during preoperative training was lost after TGCI. This protocol has the advantage of identifying and excluding individuals that are unable to learn the task during the training phase, thus eliminating the influence that such individuals would impose on retention data (e.g., overestimating the effect of either ischemia or sham surgery). For example, individuals *a*, *b*, *c*, *d*, and *f* that are represented in Fig. 2 are examples of such animals, either because they did not learn (*a*) or retain (*c*) the task or because their step-down responses fluctuated dramatically over time (*b*, *d*, *f*). Despite the presence of such animals, however, the majority of the rats were able to consistently retain the passive avoidance memory for at least 21 days posttraining. Therefore, we consider that the PAT protocol that was used herein reliably evaluated the impact of TGCI on passive avoidance memory and the effect of FO thereon and allowed comparisons with the data from the AvRM (discussed below).

The memory-disruptive effect of TGCI that was observed in the OLT (Fig. 4) is also consistent with other studies that used rats [29], gerbils [30], and mice [23]. Deficits in the memory of object locations were also observed in mice after focal cerebral ischemia (stroke) [31]. Furthermore, the OLT has been successfully used to diagnose stroke-induced memory impairment in humans [32]. Differently from the AvRM and PAT, in which remote

retrograde memory was assessed, recent memory was measured in the OLT, since memory retention was assessed 15 min after the re-training session (see, Fig. 1). Consistent with these studies, the present results indicate that all three tests that were used herein were sensitive to TGCI. However, differential task sensitivity was observed with regard to the effect of FO on ischemia-induced memory deficits.

4.2. Fish oil prevented ischemia-induced memory loss in the AvRM but not in the PAT or OLT

We investigated the effects of FO on ischemia-induced memory loss in the PAT and OLT. The treatment regimen relative to TGCI onset and the behavioral analyses were similar to those that were used in the AvRM experiments, in which ischemia-induced retrograde amnesia has consistently been shown to be prevented by FO (see references above). In the present study, FO prevented retrograde amnesia in the AvRM, which was expected, but not in the PAT or OLT. However, other studies that evaluated the effects of cerebral ischemia on performance in the PAT reported the memory-protective effects of such drugs as the monoamine oxidase-B inhibitor L-deprenyl [26], phosphodiesterase-4 inhibitor rolipram [25 27], and escin (a natural mixture of triterpenoids) [28]. Similar findings have also been reported in the OLT, which was able to detect the memory-protective effects of the *Ginkgo biloba* extract EGb761 [30] and rolipram [23] after cerebral ischemia. These studies suggest that both the PAT and OLT are sensitive to the effects of drugs on memory recovery after TGCI.

In the present study, however, both the PAT and OLT failed to detect the memory-protective effect of FO after TGCI, in contrast to the AvRM, indicating that this effect of FO is task-dependent. The task-dependent effects of drugs on learning and memory have often been reported in intact animals [33-36]. However, no other study of which we are aware has

evaluated similar effects under conditions of cerebral ischemia; thus, we are unable to make comparisons with our present findings. The reasons why FO prevented memory loss after TGCI in a task-dependent manner are unknown, and we can only offer speculations. One possible reasons may be related to the period of treatment relative to the behavioral tests. In the present PAT experiment, FO administration commenced 2 days after training. This is different from the studies cited above that reported the memory-protective effects of L-deprenyl [26], rolipram [27], and escin [28] in the PAT after TGCI. In those studies, the drugs were administered prior to training, including the day of training [27]. Moreover, the retention test occurred 24 h after training (i.e., not long after the end of drug administration). Sarter et al. [38] reviewed several studies that used the PAT as a model to screen drugs with memory-enhancing properties. These authors stated, "drug-induced prolongation of the response latencies has been shown to depend on the length of the interval between training and treatment," and "post-training drug administration is believed to have clear advantages over pre-training drug administration as it precludes the non-cognitive components of a drug's effects which can interact with the aversive, stressful, or anxiogenic components of a passive avoidance learning trial." Therefore, in the studies that tested L-deprenyl, rolipram, and escin, pretraining drug administration may have interacted with the non-cognitive components of passive avoidance behavior, thus contributing to the longer latency in the retention test. If so, then such an influence did not occur in the present study, in which FO was administered 2 days after training, and the retention trial was performed 8 and 15 days later.

The same interpretation may also apply to the failure of FO in preventing memory deficits in the OLT. Although FO was administered pretraining, the training session occurred 1 week after the end of drug treatment (Fig. 1, Experiment 2). This experimental protocol is different from the one that was used in other studies that reported the memory-protective effects of

EGb761 [30] and rolipram [23] after ischemia, in which the drug treatments covered the entire period of training, and the retention test was performed as soon as 1 min [30] or 4 h [23] after training. Under such conditions, some effects of the drugs on acquisition may have contributed to the increase in the discrimination index in the retention test. If so, then this might not have occurred in the present study because a relatively long time (1 week) elapsed between the end of FO treatment and training in the OLT. Future studies should determine whether pre- *vs.* posttraining FO administration or whether the length of time between the end of treatment and the retention test actually influences the effect of FO in the PAT and OLT after TGCI.

Another possibility is that the mechanisms by which FO prevents ischemia-induced memory loss in the AvRM are not present in the PAT or OLT. This speculation is based on the observation that a given drug can differentially affect various components of learning and memory (i.e., acquisition, consolidation, and retrieval), and different molecular mechanisms are time-dependently involved in each of these components in different brain structures (e.g., hippocampus *vs.* amygdala) [39]. For example, the efficacy of propranolol in impairing memory consolidation and/or reconsolidation clearly depends on the task that is used. Effects on memory consolidation are more evident in non-aversive tests, whereas effects on reconsolidation (which is important in cases of posttraumatic stress) are more evident in moderately to highly aversive tasks. This suggest that such differential task sensitivity to propranolol may be related to the emotional valence of the memory that is evaluated and the state of noradrenergic neurotransmission in each task [40]. In another study, the nonselective monoamine oxidase inhibitor phenelzine improved the retention of passive avoidance memory but impaired memory retention in a water maze task. These effects were associated with changes in the activity/availability of biogenic amines and the γ-aminobutyric acid neurotransmitter, respectively [41]. Pretraining inactivation of the hippocampus by lidocaine

impaired the acquisition of place learning but facilitated the acquisition of response learning in the same behavioral model, indicating that the same brain structure can interfere with learning in either a negative or positive way, depending on the specific brain structure (e.g., hippocampus or striatum) that governs specific learning processes [35]. In a model of aged-related memory decline, the phosphodiesterase-5 inhibitors sildenafil and vardenafil improved memory retention but not acquisition, even 1 week after drug removal, suggesting that the drugs interfered with processes associated with long-term memory consolidation but not acquisition [42]. Furthermore, estrogens are known to either improve or impair cognition according to the behavioral task and neural system that is engaged. This dissociation may be related to the specific or preferential activation of estrogen α or β receptors that are differentially localized in various brain structures [43].

Altogether, these studies suggest that (*i*) there may be differential sensitivity to a given drug when two or more behavioral models of learning and memory are used or when two components of the same cognitive function are assessed in the same behavioral task and (*ii*) task-dependent effects of drugs may be related to selective interactions with specific neurobiological mechanisms that underlie each particular function within the domain of cognition. The sensitivity of the AvRM but not PAT or OLT in detecting the memory-protective effect of FO after TGCI may depend on the mechanisms by which FO exerts its effects on memory in these behavioral tasks. Notably, DHA is likely the most active constituent of FO, and it has been reported to exert more than 40 distinct actions at the molecular, cellular, and integrative levels. Each of these levels play specific roles in the formation and maintenance of learning and memory [4]. Therefore, the lack of a memory-protective effect of FO in the PAT and OLT does not necessarily invalidate the results from the AvRM task. Instead, the present data suggest that the beneficial effect of FO on memory recovery (or preservation) after TGCI may be restricted to certain types of memory.

4.3. Lack of dose-effect relationship

The antiamnesic effect of FO in the AvRM test was not dose-dependent (Fig. 5), which is consistent with previous studies. DHA treatment at doses of 10, 100, and 200 mg/kg for 21 days prior to TGCI facilitated learning performance in the conventional (appetitive) radial maze, with no dose-effect relationship [12]. Our data may be at least partially explained by the pattern of intestinal absorption of FO in rats. In an *in vitro* study, intestinal segments were immersed in FO concentrations that ranged from 1.25 to 80 mg/10 ml of Krebs-Ringer medium. Under these conditions, the rate of FO absorption progressively increased when intestinal segments were incubated in FO concentrations that ranged from 1.25 to 5 mg. At higher concentrations (10-80 mg), the rate of absorption reached a steady state around 8.62 mg. When the intestinal segments were incubated with increasing concentrations of FO, the amount of EPA+DHA that was absorbed also reached a maximum of 1.02 mg when 10 mg FO was provided in the incubation medium. This value did not increase further when the FO concentration in the medium was > 10 mg [44]. These results indicate that the intestinal absorption of FO or its main constituents follows zero-order kinetics (i.e., it is saturable). Consistent with these data, the blood concentrations of EPA and DHA increased but did not differ significantly between rats that received 30 or 300 mg/kg FO. Moreover, the inflammatory response that was induced by carragenin was equally reduced by all doses of FO studied (18.75-300 m/kg), thus indicating no dose-effect relationship (Ciomar Bersani Amado, personal communication). Therefore, it is possible that the brain concentration of DHA was similar in the groups that received FO at doses that were equivalent to 100, 200, and 300 mg/kg DHA. Considering the broad spectrum of action of DHA [4, 5] and assuming that DHA is mainly responsible for the memory-protective effect of FO, the pharmacokinetic

findings mentioned above could explain the lack of a dose-effect relationship for the antiamnesic effect of FO after TGCI.

4.4. Fish oil-mediated memory protection occurs in the absence of neuronal rescue after TGCI

Fish oil did not prevent ischemic neuronal death in the hippocampus, cerebral cortex, or amygdala in either the Nissl staining (Fig. 6) or NeuN-immunoreactivity (Fig. 7) assay. These results replicate our previous findings in the hippocampus and cerebral cortex that were stained with Nissl [7-10, 20]. Previous studies reported that FO [45] and DHA [12] conferred histological neuroprotection after TGCI, but high interindividual variability is seen in the magnitude of neuronal death after TGCI. Such variability was observed herein (Fig. 6 and 7) and in previous studies from our laboratory. This did not occur in the sham-operated groups, thus discarding possible methodological flaws. Indeed, large inter-animal variance in rats that were subjected to TGCI was also reported after careful, unbiased stereological analysis [46]. This pattern of neurodegeneration may reflect an inherent characteristic of each individual or group (e.g., the size and competence of collateral circulation), and it may lead to possible misinterpretations of the data when the putative neuroprotective effect of a drug is examined. Notably, neuronal death does not necessarily occur in all animals that are subjected to TGCI, despite an appropriate level of vessel occlusion (e.g., 4-VO model). This implies that the presence of minor lesions or even the absence of lesions in a few animals that receive a given drug may not reflect drug-induced neuroprotection. Instead, neuronal death did not occur in those individuals. This interpretation is supported by the present results that are shown in Fig. 7. In fact, nearly 80% cell loss occurred in the CA1 subfield in all of the animals that received FO at a dose of 300 mg/kg DHA, whereas five animals that were treated with FO at doses of 100 and 200 mg/kg DHA presented no lesions compared with sham animals. In these cases,

our interpretation is that instead of FO-mediated neuronal rescue, these animals had no lesions after TGCI, which is further supported by our findings in the CA3 (Fig. 7) and CA1-C4 as a whole (Fig. 6). Similar variability in the degree of hippocampal lesion after TGCI and DHA treatment has also been reported by others and interpreted as DHA-mediated neuroprotection [12, 45]. The problem of assessing histological neuroprotection after ischemic brain damage has been critically reviewed, and our present and previous findings indicate that caution needs to be taken when basing the results on the number of "viable" neurons or the volume of infarct as the main endpoint to infer the neuroprotective effects of drugs [46, 47]. We contend that the most important end-point for assessing the neuroprotective potential of drugs should be functional, despite the obvious importance of preserving as many neurons as possible after ischemic insult [47].

4.5. Fish oil did not alter ischemia-induced hippocampal neurogenesis

Cerebral ischemia is reported to stimulate neurogenesis in the hippocampal DG as a compensatory, adaptive response to brain injury [48]. During the course of neurogenesis, DCX immunoreactivity identifies a microtubule-associated protein that is transiently expressed in newborn neurons [49]. In the present study, we observed an increase in the number of DCX-immunoreactive cells in the hippocampus 24 days after the ischemic insult, which agrees with other studies in mice [23], gerbils [50], and rats [51]. This reactive neurogenesis in response to ischemia was unaffected by FO treatment. Although the numerical density of DCX-expressing neurons has been used to indicate changes in the population of neuroblasts, unclear is whether DCX-labeled neurons represent neurons that are generated in the last few days before or many days after TGCI. The visualization of new neurons with DCX in sections that are thicker than those that were used herein may help identify the maturation stage of neuronal differentiation of DCX-immunoreactive neurons,

including the phase of dendritic branching [52] and consequently successful neuronal integration and functionality [53]. Mice that were fed a mixture of n-3 polyunsaturated fatty acids, especially EPA, DHA, and docosapentenoic acid, for 8 weeks presented an increase in the dendritic arborization of newborn neurons and improvements in memory performance [54]. Fish oil also restored dendritic density in the hippocampus in rats [20] and mice [55] that were subjected to global and focal ischemia, respectively. Therefore, FO may facilitate memory recovery after TGCI by stimulating dendritic neuroplasticity, mainly in the CA3 subfield of the hippocampus [20]. Future studies are needed to elucidate the effects of FO on all phases of neurogenesis after TGCI.

Possible limitations of the present study should be pointed out. Firstly, one might question that the influence of gavage-induced stress was not controlled, since a vehicle-treated, sham-operated group was lacking. We have reported, however, that sham operated rats treated by gavage with vehicle or atorvastatin for up to 43 days, including the days of behavioral testing, did not differ from each other in the AvRM task; also their memory performance did not differ between the post- and pre-treatment phases [22]. This finding was reproduced in other study using chronic roflumilast administration by gavage after chronic cerebral hipoperfusion in aged rats (unpublished). Protective effects of eicosapentaenoic acid [56] or omega-3 lipid emulsion [57] on memory impairments induced by cerebral ischemia were observed and interpreted in the absence of a vehicle-treated sham group. Furthermore, inclusion of a control group was not considered necessary for evaluating the effects of drugs on learning and memory performance on passive avoidance [28], even after a long lasting treatment that coincided with the testing day [27]. These data suggest that the absence of a vehicle-treated, sham operated group does not exclude a reliable interpretation of the present results. Also, the lack of a vitamin E-treated group could be questioned, since it would control for the effects of vitamin E as an antioxidant. However, a dose much greater than those that were present in FO

used here (0.4 to 1.2 mg/kg) was required to change the outcomes of brain ischemia. In a rat model of stroke, vitamin E was effective to prevent both oxidative stress and neurological deficits when given at a dose of 250 mg/kg, but not 150 mg/kg [59]. Similarly, vitamin E that was given at a dose of 100 mg/kg, for five days consecutive, failed to prevent learning deficit caused by chronic cerebral hypoperfusion in rats [60]. Therefore, the amount of vitamin E present in FO should not be sufficient to influence the effect of FO on memory after TGCI. Finally, a question may also arise regarding the possible influence of cerebral ischemia on tactile and/or nociceptive sensibility of rats in response to electric shocks during the passive avoidance test. Indeed, ischemia-induced analgesia has been observed in rats that were evaluated in the hot plate test [61]. Unknown is whether similar effect occurred under the present experimental conditions. Moreover, mice that were subjected to focal cerebral ischemia (MCAO model) were as able as their sham-operated counterparts to learn the passive avoidance task [15]. This indicates that the deficit of memory retention that was observed after MCAO [15] was not disturbed by other complicated factors outside of the effect of ischemic brain damage on memory. It may be, therefore, that the memory deficit of passive avoidance observed here after TGCI, and the lack of FO effect thereon, were not influenced by an eventual disturbance in tactile and/or nociceptive sensibility.

5. Conclusion

All three behavioral tests that were used in the present study (AvRM, PAT, and OLT) were sensitive to TGCI, but only the AvRM was able to detect the antiamnesic effect of FO, indicating that FO-mediated memory recovery (or its preservation) after TGCI is task-dependent. Despite the lack of a dose-effect relationship, the memory-protective effect of FO in the AvRM was seen at DHA doses < 300 mg/kg, which may have clinical relevance. Ischemia-induced neuronal death in the hippocampus, cerebral cortex, and amygdala was not

prevented by FO, independent of the dose and staining method used, indicating that FO-mediated memory protection after TGCI is dissociable from histological neuroprotection. Fish oil also did not influence the effect of ischemia on the proliferation of newborn cells, but the possible effect of FO on subsequent phases of neurogenesis (i.e., maturation, migration, and integration) cannot be discarded. Supporting previous studies, the present results suggest that FO may have therapeutic utility in the field of cerebral ischemia.

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Contributors

Humberto Milani and Rúbia M.W. de Oliveira designed the study and wrote the manuscript. Janaína N. de Oliveira performed the surgery, fish oil treatment, and behavioral analysis with the radial maze and passive avoidance tests. Luane de Oliveira Reis was responsible for the object location test. Jacqueline Godinho, Cristiano C. Bacarin, and Emilene D.F. Ferreira

performed the neurohistological and neurogenesis analysis. Ligia M. Soares assisted with the statistic analysis and drafting of the figures.

Conflict of interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. Schematic representation of each experimental protocol (Experiments 1a, 1b, 2, and 3), outlining the phases of training, FO administration, retention memory trials (RMTs), and histological (Nissl) and immunohistochemical (NeuN, DCX) staining relative to the onset of TGCI.

Figure 2. Stability of memory retention in intact rats in the PAT. The animals were trained for 3 consecutive days and then tested for the retention of passive avoidance memory 1, 7, 15, and 21 days later. Individuals *a-f* presented either consistent of fluctuating step-down latencies across days of testing.

Figure 3. TGCI-induced memory impairment in the PAT and the effect of FO thereon. Twenty-four hours after training and before surgery (preoperative phase), all rats exhibited the maximal latency (i.e., 300 s). They were then subjected to TGCI and treated with vehicle or FO (300 mg/kg DHA) for 8 days. Retrograde memory performance was assessed on days 8 and 15 postischemia (postoperative phase). The data are expressed as mean ± SEM. The sample sizes are shown in parentheses. $*p < 0.05$, $*p < 0.05$, $***p < 0.01$, within-group comparisons between the pre- and postoperative phases; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, between-group comparisons with sham in the postoperative phase.

Figure 4. TGCI-induced memory impairment in the OLT and the effect of FO thereon. (Top) Schematic representation of the identical objects (O1 and O2) in the open field during re-training (r-T) and the retention memory trial (RMT), with a 15-min intrval between r-T and RMT. During r-T, both O1 and O2 were located in the old position. During the RMT, O1 was moved diagonally. (A, C) Number of entries into and time spent in the object zone, respectively, during T. (B, D) Same measures as in the RMT. (E) Discrimination index (D2). The data are expressed as mean ± SEM. The sample sizes are shown in parentheses (panel B). $***p < 0.001$, within-group comparison of new location *vs.* old location; $^{\#\#\#}p < 0.001$, within-group comparison *vs.* zero; $^{\$\$\$}p < 0.001$, between-group comparison of ischemia/vehicle *vs.* sham.

Figure 5. TGCI-induced memory impairment in the AvRM task and the effect of FO thereon. Naive rats learned the AvRM task over 10 days of training (T) and then were subjected to sham surgery or TGCI (day 0). Fish oil or vehicle treatment began 4 h postischemia and continued daily for 8 days. The retention memory trials (RMT) began the day after the end of treatment and were performed once per week for 3 weeks (days 9, 16, and 23). The data show the temporal distribution of memory

performance, expressed as latency, the number of reference memory errors, and the number of working memory errors for individual groups (left panels) or after pooling the groups that received different doses of FO (right panels). Preischemia training performance (T) is expressed as the mean of the last 3 days of training (days -3 to -1). ***$p < 0.001$, **$p < 0.01$, *vs*. sham; $^{###}p < 0.001$, $^{##}p < 0.01$, *vs*. vehicle. The data are expressed as mean ± SEM. Sham, $n = 11$; vehicle, $n = 11$; 300 mg/kg FO, $n = 11$; 200 mg/kg FO, $n = 11$; 100 mg/kg FO, $n = 10$.

Figure 6. (Top) Number (%) of normal-looking neurons counted across the CA1-CA4 subfields of the hippocampus, retrosplenial/parietal association cortex (RS/PtA), and basolateral nucleus of the amygdala (BLA) in rats that were subjected to ischemia, FO/vehicle treatment, and the passive avoidance test (Experiment 2b, Fig. 1). The data are expressed as mean ± SEM. ***$p < 0.001$, *vs*. sham. Sham, $n = 21$; ischemia/vehicle, $n = 20$; ischemia/FO, $n = 7$. (Bottom) Representative photomicrographs (400× magnification) of normal-looking neurons in the CA1 subfield, cerebral cortex, and BLA. The arrow and arrowhead indicate normal-looking and degenerating neurons, respectively.

Figure 7. Effects of FO (100, 200, and 300 mg/kg DHA) on neurodegeneration and newborn cell proliferation assessed by NeuN and DCX immunoreactivity, respectively. Rats were treated with FO for 8 days after TGCI and tested for retrograde memory performance in the AvRM task (see Fig. 1). (Upper left) Integrated optical density of NeuN-positive neurons in the CA1 and CA3 subfields of the hippocampus. (Bottom left) Number of DCX-positive newborn neurons in the dentate gyrus. The data are expressed as mean ± SEM. ***$p <$ 0.0001, *vs*. sham; $^{##}p < 0.01$, $^{###}p < 0.0001$, *vs*. vehicle. (Right) Representative photomicrographs of coronal brain sections assayed for NeuN immunoreactivity in the CA1 subfield or DCX immunoreactivity in the DG.

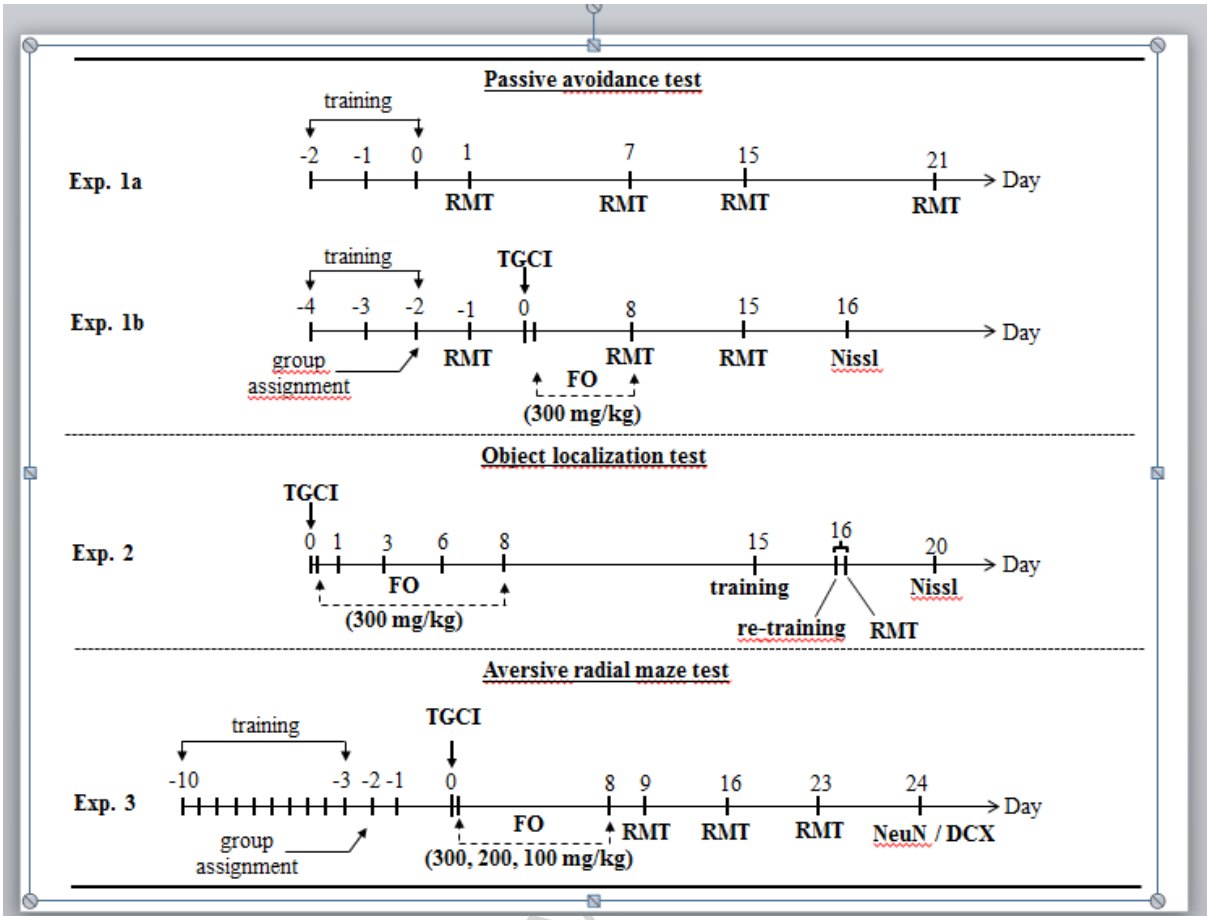
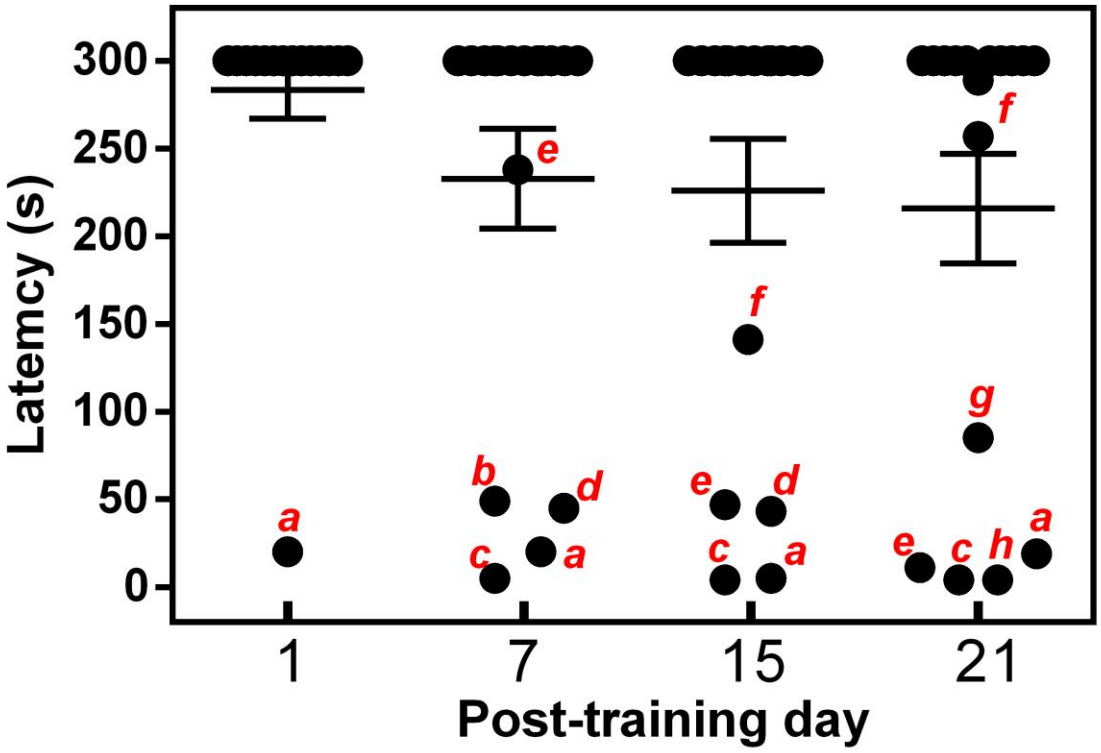


Figure 1



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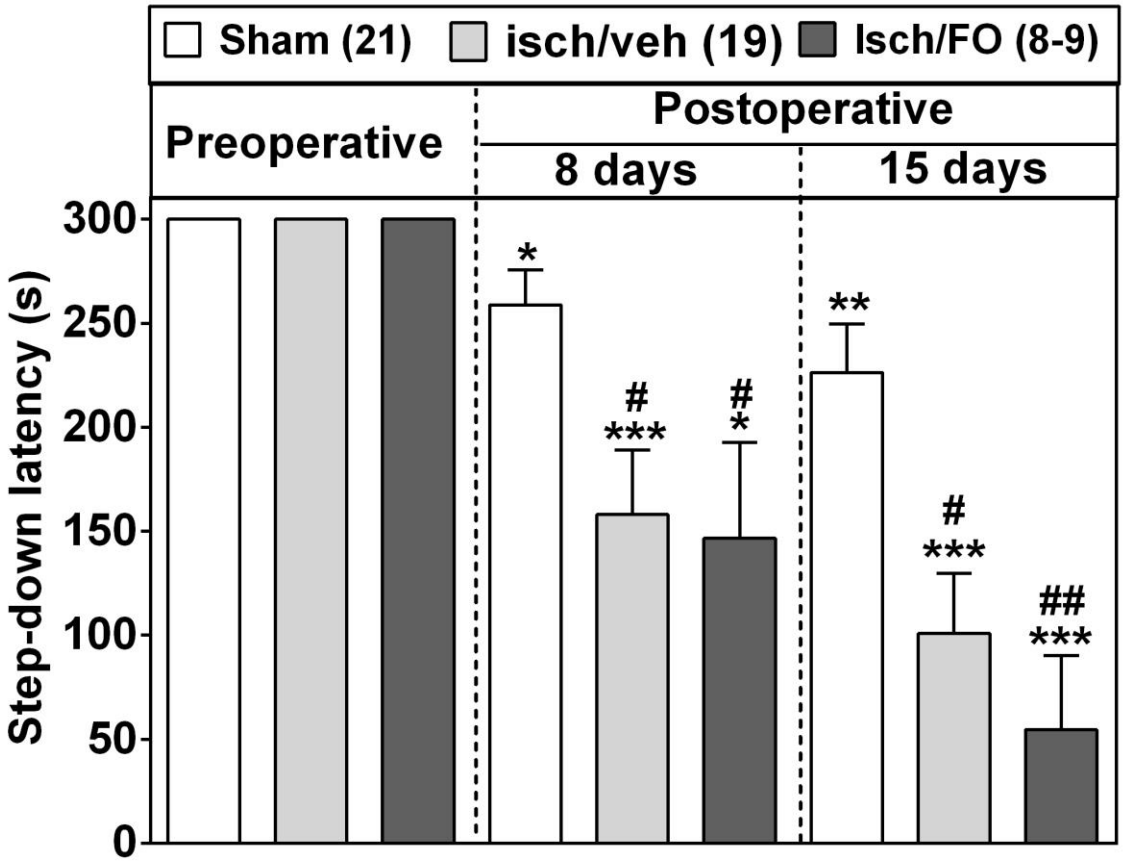

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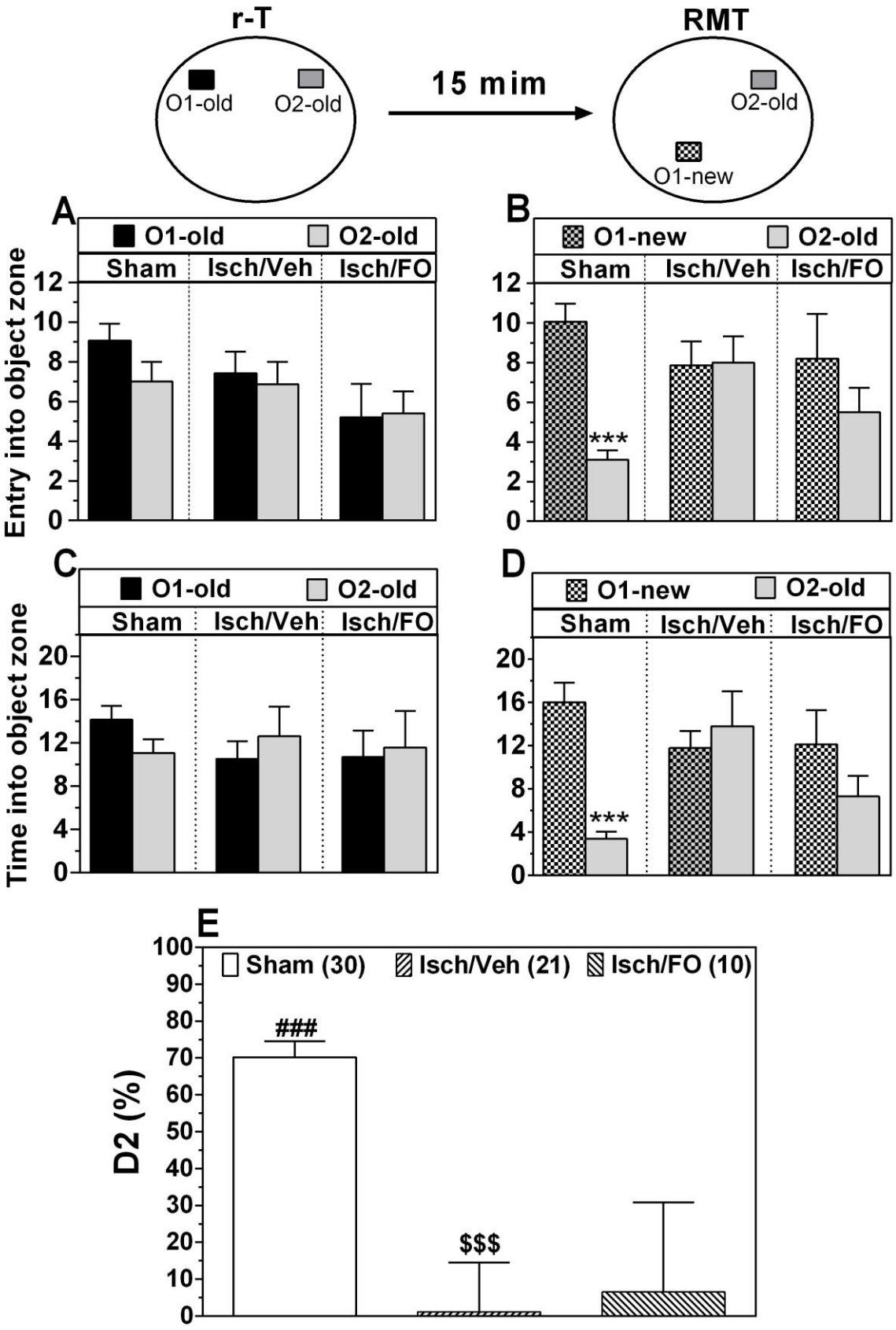

Figure 4

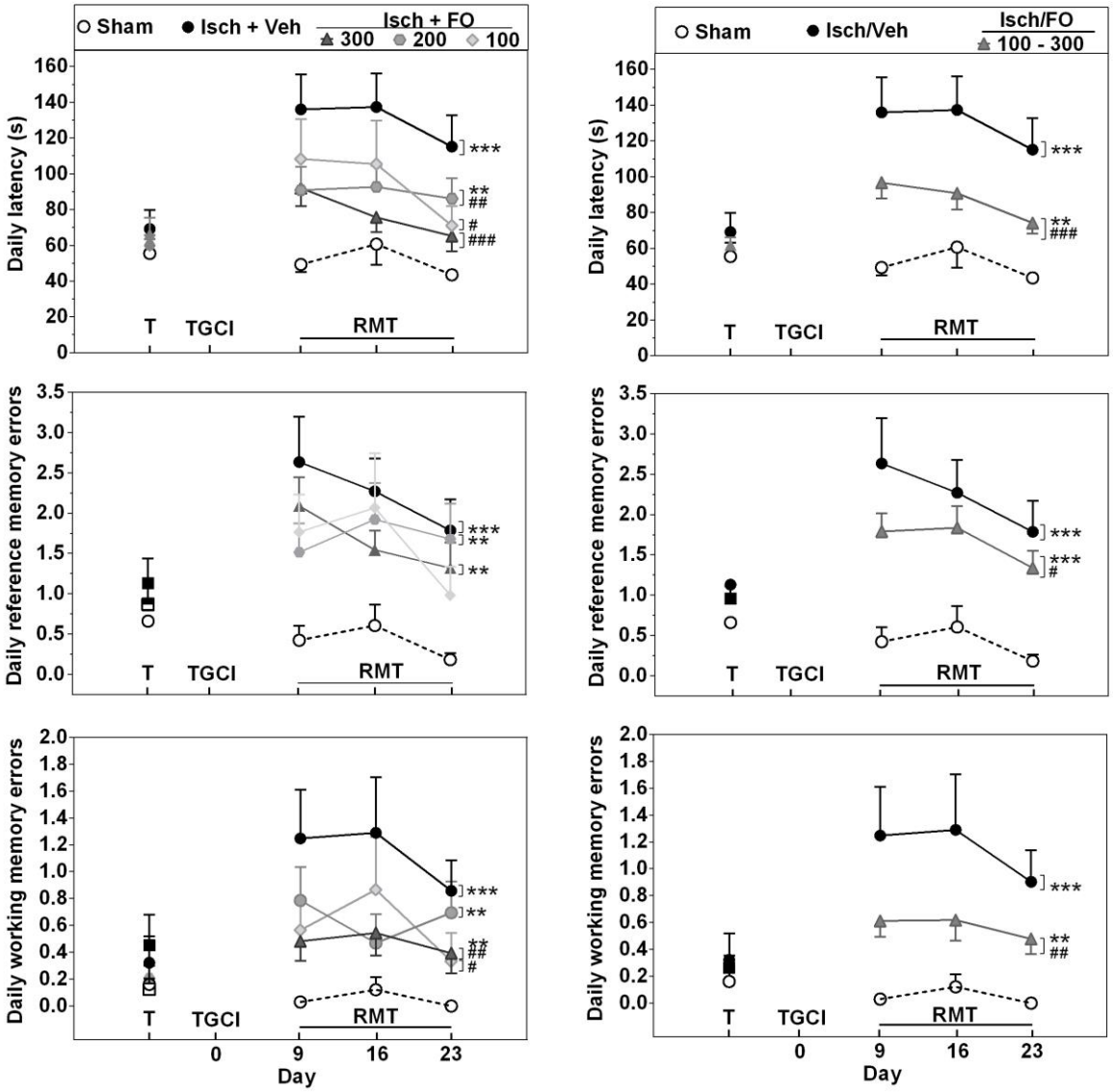

Figure 5

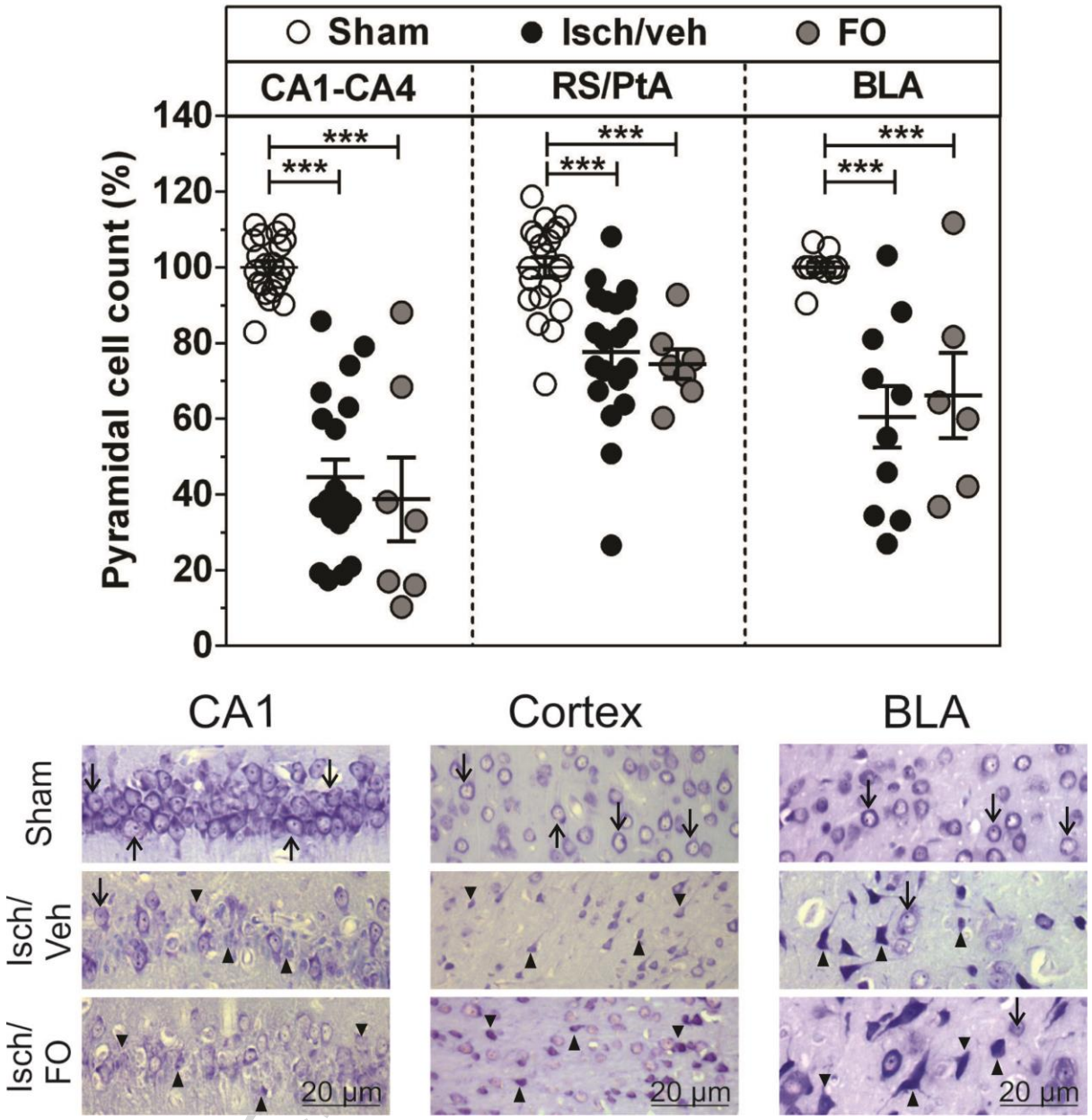

Figure 6

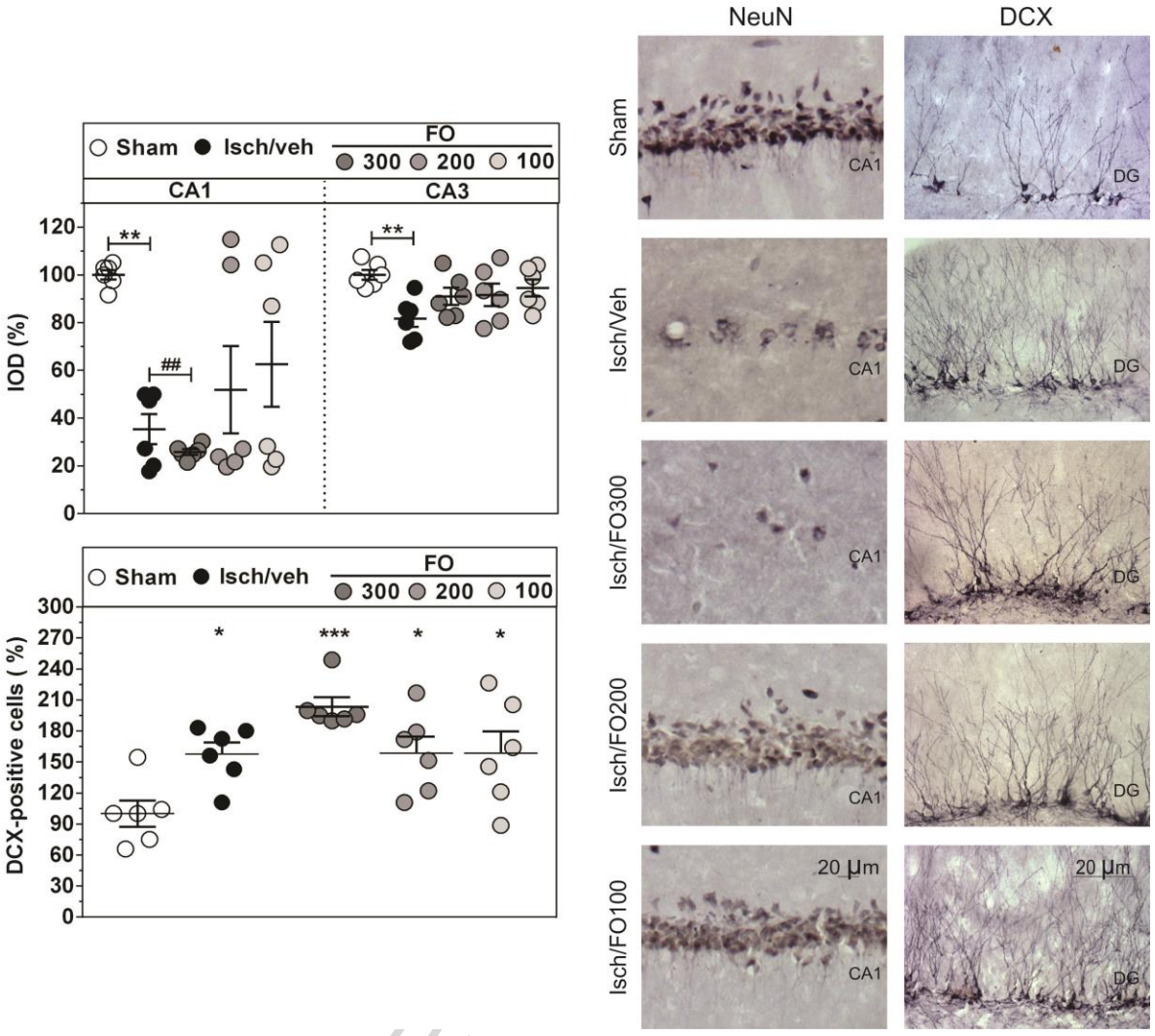

Figure 7

Highlights

- Global cerebral ischemia induces memory impairment in different behavioral tests.
- Fish oil task-dependently prevented memory deficits.
- The memory-protective effect of fish oil was not dose-dependent.
- The antiamnesic effect of fish oil occurred in the absence of histological neuroprotection.
- Ischemia-induced neurogenesis was unaffected by fish oil treatment.