Neuropharmacology 108 (2016) 60–72

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Alpha-linolenic acid given as enteral or parenteral nutritional intervention against sensorimotor and cognitive deficits in a mouse model of ischemic stroke

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ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form
26 April 2016
Accepted 27 April 2016
Available online 29 April 2016

Chemical compounds:
Alpha-linolenic acid
Linolenic acid
Linolenate (PubChem CID 5280934)
Docosahexaenoic acid (PubChem CID 445580)
Eicosapentaenoic acid (PubChem CID 446284)

Keywords:
Disease modifiers
Nutraceutical
Nutrition
Polyunsaturated fatty acids
Neuroprotection
Stroke outcome and recovery

ABSTRACT

Stroke is a leading cause of disability and death worldwide. Numerous therapeutics applied acutely after stroke have failed to improve long-term clinical outcomes. An emerging direction is nutritional intervention with omega-3 polyunsaturated fatty acids acting as disease-modifying factors and targeting post-stroke disabilities. Our previous studies demonstrated that the omega-3 precursor, alpha-linolenic acid (ALA) administrated by injections or dietary supplementation reduces stroke damage by direct neuroprotection, and triggering brain artery vasodilatation and neuroplasticity. Successful translation of putative therapies will depend on demonstration of robust efficacy on common deficits resulting from stroke like loss of motor control and memory/learning. This study evaluated the value of ALA as adjunctive therapy for stroke recovery by comparing whether oral or intravenous supplementation of ALA best support recovery from ischemia. Motor and cognitive deficits were assessed using rotarod, pole and Morris water maze tests. ALA supplementation in diet was better than intravenous treatment in improving motor coordination, but this improvement was not due to a neuroprotective effect since infarct size was not reduced. Both types of ALA supplementation improved spatial learning and memory after stroke. This cognitive improvement correlated with higher survival of hippocampal neurons. These results support clinical investigation establishing therapeutic plans using ALA supplementation.

1. Introduction

Annually, 15 million people worldwide suffer a stroke. Of these, 30% die and another 30% are left permanently disabled, placing a tremendous burden on family and community. A stroke occurs on average every 40 and 90 s, in the United States and Europe, respectively (Go et al., 2014; Gustavsson et al., 2011). The estimated cost of stroke for 2010 was approximately $74 billion and €64 billion, respectively (Gustavsson et al., 2011; Lloyd-Jones et al., 2010). Apart for thrombolysis – for which inclusion criteria are fairly restrictive - clinical trials for the treatment of acute ischemic stroke have been almost all unsuccessful, leaving patients with an extremely limited repertoire of therapeutic opportunities.

Nevertheless, improvements in population health – particularly in the control of major risk factors of stroke such as hypertension, diabetes, high cholesterol levels and smoking - over the past decades have contributed to reduced stroke mortality. Unfortunately, stroke remains a substantial problem, since mortality represents, at best, a third of annual first-ever strokes. Indeed, in 2007 the

Abbreviations: ALA, alpha-linolenic acid; CA, *Cornu Ammonis*; DAB, 3,3′-diaminobenzidine; DALYs, disability-adjusted life years; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; H/I, hypoxic–ischemic; LC-omega-3, long chain omega-3; MCA, middle cerebral artery; MCAo, middle cerebral artery occlusion; MWM, Morris water-maze; NeuN, neuronal nuclei; NIH, National Institutes of Health; PBS, phosphate-buffered saline; PUFAs, polyunsaturated fatty acids; rTMS, repetitive transcranial magnetic stimulation; SNAP 25, soluble synaptosomal-associated protein of 25 kDa; TPN, total parenteral nutrition; U.S., United States.

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http://dx.doi.org/10.1016/j.neuropharm.2016.04.040

number of stroke survivors was evaluated to be 62 million, and 30–60% of these were estimated to be dependent in some aspect of daily living. These survivors have a high level of disability with more than 50% of patients being left with a residual motor or cognitive/amnesic deficit. Stroke burden was calculated to be approximately 51 million disability-adjusted life years [DALYs] (Johnston et al., 2009; Strong et al., 2007). Therefore, reducing long-term disability by improving recovery would have a substantial impact.

The major stroke-induced impairments are motor and cognitive deficits. Motor impairment is characterized by a loss or limitation of function in muscle control or a limitation in mobility (walking/gaiting) (Jorgensen et al., 1995). In 80% of patients, altered control of the face, arm, and leg of one body side are described as typical symptoms of stroke (Langhorne et al., 2009). Cognitive impairment affects multiple domains, including attention, executive function, visuo-spatial ability, memory and language. As well, focal disorders such as aphasia and neglect are common, as are more diffuse abnormalities such as reduced information processing and executive dysfunction (Cumming et al., 2013).

Confronted with a lack of treatment options, various strategies have been developed to enhance motor recovery, ranging from pharmacological agents prescribed against hypertension, depression or Alzheimer disease, to exercise training (treadmill …) and even to repetitive transcranial magnetic stimulation (rTMS) (Calautti and Baron, 2003; Cramer, 2008; Langhorne et al., 2011). Increasing physical activity and rTMS are also known to improve cognition (Cumming et al., 2013). In cognitive and memory post-stroke rehabilitation, the efficiency of task-specific training remains uncertain mainly due to a paucity of studies (Lincoln et al., 2000; Nair and Lincoln, 2007). Interestingly, most of the motor and cognitive rehabilitation approaches have been shown retrospectively to target neural plasticity and to increase levels of neurotropic factors in the brain (Di Pino et al., 2014; Johansson, 2011).

An emerging preclinical concept to support the recovery of body and brain after stroke is nutrition. Nevertheless how nutrition may affect stroke recovery has not yet been intensely investigated, which is surprising given its great influence as risk factor (Hankey, 2012). Most interventions showing that nutrition could improve the recovery of neurocognitive functions in ischemic stroke patients have been based on protein supplementation provided to counteract stroke-induced protein synthesis depression or malnutrition (Aquilani et al., 2010). Apart from supplementation with vitamins, which may reduce oxidative damage after acute ischemic stroke (B and E groups) or have statin-like effects (Vitamin D) nutritional intervention in stroke patients has been investigated to a limited extent (Pilz et al., 2011).

Severe deficiencies identified in omega-3 polyunsaturated fatty acids (PUFAs), both in the form of alpha-linolenic acid (ALA) and the long chain derivatives eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), stand out as risk factors for cardiovascular and cerebral diseases (Riediger et al., 2009; Kris-Etherton et al., 2002). A body of preclinical studies have documented omega-3 PUFAs supplementation additional benefits in the context of stroke: that as an effective and pleiotropic neuroprotective approach against ischemia (for review (Blondeau and Tauskela, 2013; Gouix et al., 2014; Nguemeni et al., 2013)). As well, fish-oil PUFAs supplemented in diet prior to ischemia was also found exerting a beneficial effect on spatial memory deficits that was not correlated with any benefit in hippocampal neurons survival suggesting that PUFAs may also promote stroke recovery (Plamondon and Roberge, 2008). During the last decade, we identified pleiotropic abilities of ALA to trigger multi-cellular and mechanistically diverse responses, resulting in neuronal protection from stroke (see: (Blondeau, 2016; Blondeau et al., 2015; Blondeau and Tauskela, 2013; Nguemeni et al., 2013)). In a mouse model of stroke, a subchronic post-treatment consisting of three sequential injections of ALA enhanced animal survival rates by three-fold, ten days following ischemia (Blondeau et al., 2009c). Several clinical studies indicated that i.v. perfusion of long chain PUFAs, delivered as parenteral supplementation with a 10% fish oil emulsion (Omegaven, Fresenius-Kabi), may reduce mortality, antibiotic use, and length of hospital stay in different diseases (Heller et al., 2002, 2006). While it seems intuitive that beneficial effects related to fish oil doses may be reliant on the pathology phenotype, it was tempting to speculate some value of omega-3 fatty acid as adjunctive therapy for stroke recovery. ALA supplementation either as an i.v. treatment or as enriched-diet also enhances brain plasticity and improves neurite growth and remodeling in hippocampal neurons (a main site for memory formation) (Blondeau et al., 2009a; Venna et al., 2009), suggesting that ALA may represent an excellent nutraceutical strategy to promote post-stroke recovery.

To provide insight into the potential of ALA on post-stroke rehabilitation, we explored whether ALA supplementation delivered by i.v. subchronic treatment or modification of the daily diet could facilitate recovery of motor and cognitive functions after stroke in the 30 min MCAo model (which has been described as the gold standard for characterization of long-term function in mice (Balkaya et al., 2013)). We first evaluated the effect of the ALA supplementation on the ischemic brain lesion and long-term mortality rate. We further determined motor deficits by the rotarod and pole test and cognitive deficits using the Morris Water-Maze test. Finally, we evaluated the effect of ALA supplementation on selective neuronal lesions that are known to important for stroke rehabilitation.

2. Material and methods

2.1. Animals, diets and treatments

Male 4-week old C57BL/6 J mice (Janvier France Breeding) were housed under standardized conditions and received care according to the policies of European Community Directive 86/609/EEC. The Institutional Animal Care and Use Committees (CIEPAL) approved the study (French Authorization N°06-118). Mice were fed a regular rodent diet (SAFE, France) with an ALA concentration of 0.25% (0.25 g in 100 g of regular diet, a proportion matching the "murine" recommended intake (Bourre et al., 1993; Pudelkewicz et al., 1968)) or an experimental diet enriched in ALA by a factor of three compared to regular chows, for 6 weeks. The content of proteins, minerals, micronutrients, vitamins and the metabolizable energy density provided by the different formulations are as previously described (Nguemeni et al., 2010). Briefly, in the ALA enriched diet (ALA-diet) lipids from rapeseed oil accounts for 10% and ALA for 0.75% by weight. The ALA-diet did not contain any EPA or DHA, in contrast to the regular chow. Body weight, and water and food consumption were monitored through the entire experiments (Nguemeni et al., 2010). With respect to parenteral supplementation (ALA, Enzo Life Sciences), animals were injected in the penil vein 2 h, 3 d, 7 d, 10 d, 14 d, 17 d and 21 d after the occlusion of the middle cerebral artery (MCAo). The timing and dose (500 nmol/kg as a bolus of 50 μl) selected for sequential injections of ALA were based on our previous studies in the mouse model of focal ischemia induced by a longer duration of MCAo (Blondeau et al., 2009c; Heurteaux et al., 2006).

2.2. Middle cerebral artery occlusion

Focal ischemia was induced by a 30 min of transitory MCAo

using an intraluminal filament technique (Blondeau et al., 2009b; Nguemeni et al., 2010). Cerebral blood flow measurements by laser Doppler flowmetry (Perimed, Craponne, France) confirmed an ischemic occlusion (reduced by 85% of baseline) during MCAo and restoration of blood flow during reperfusion, conditions required for mice being included in the study. Physiological parameters, including weight and survival rate were assessed (Nguemeni et al., 2010).

2.3. Evaluation of brain ischemic lesions

The infarct volume was determined 24 h after MCAo on coronal frozen brain sections stained using a solution of 1% cresyl violet in 0.25% acetic acid. The striatal and cortical areas of infarction were measured on each section using a computer image analysis system and corrected for brain edema according (Golanov and Reis, 1995). Infarct volume (in mm^3) was calculated by a linear integration of the corrected lesion areas (Blondeau et al., 2009b; Nguemeni et al., 2010). Late infarct consolidation was assessed 21 days post-stroke by NeuN (neuronal nuclei) immunohistochemistry. Briefly, brain sections were incubated overnight with anti-NeuN antibodies (mouse monoclonal, 1:200; Chemicon) and a 3-stage avidin-biotin method with corresponding biotinylated secondary antibody. NeuN expression was visualized with 3,3′-diaminobenzidine (DAB as chromogene). NeuN-positive cells were counted in the cortex and striatum, including the caudate-putamen area at the level of the lesion (bregma level 0.3 mm; magnification × 40) (Blondeau et al., 2009b; Heurteaux et al., 2006; Nguemeni et al., 2010). Data were expressed as number of NeuN-positive cells per mm^2.

In a separate group of mice, we blindly assessed cellular status at 3 weeks post-MCAo. Tissue characteristics were quantified by measuring ipsilateral and contralateral hemispheric volumes and calculating tissue loss and atrophy using coronal cryosections stained with cresyl violet (Lee et al., 2014). Calculations of neuronal density of hippocampal CA1, CA2 and CA3 (*Cornu Ammonis*) subfields were evaluated on coronal sections of the dorsal hippocampus stained with cresyl violet, corresponding to brain sections located between 1.46 and 2.18 mm posterior to bregma. The number of living neurons in the stratum pyramidal within the CA1, CA2 and CA3 area (μm^2 measured with a digitizer) was counted using a Leica DMD108 microscope.

2.4. Behavioral tests

2.4.1. Rotarod

The rotarod test has been reported to be effective for evaluating motor coordination and balance alterations after MCAo in rodent (Rogers et al., 1997). The rotarod apparatus (Ugo Basile, France) consists of a striated rod (diameter 3 cm) subdivided into 5 areas of 5 cm width by disks 25 cm in diameter. The days preceding the day of surgery, mice were trained on the rotarod for 5 min at a constant speed (4 rpm) that was followed by a single trial on the rotarod accelerating from 4 to 40 rpm over a period of 10 min (maximal scores at 10 min) after 30 min rest. The latency of mice to fall from the rotarod was recorded and this score was considered as the baseline (pre-operative score). Mice were tested during the first week post-stroke at Day 2, 3, 4, 7, to detect the effect of ALA supplementation on motor deficits (Fig. 2). Rotarod test was also carried out at Day 10, 14 and 21 post-MCAo to confirm the occurrence of spontaneous recovery of motor impairments (Freret et al., 2011), when the effect of ALA supplementation on spatial memory using the Morris water-maze task was studied (data not shown).

2.4.2. Pole test

The vertical pole test is a motor task reported to be effective for evaluating motor coordination, forelimb strength, and ability to grasp and balance in MCAo mice (Ji et al., 2009; Matsuura et al., 1997). A vertical wood pole of 50 cm was covered with tape to create a rough surface. The animal was placed with the head facing upward near the top of the pole. The time taken to make a complete U-turn downward (time to turn) and the total time to touch the ground with all four paws were recorded. If unable to turn completely, the time to reach the ground was also included in the time to turn. Each animal was tested over 5 trials and the average score was recorded as the final pole test score.

2.4.3. Morris Water-Maze (MWM) test

The water-maze test is a spatial memory task reported to be effective for evaluating learning and memory deficits after MCAo. Experiments were closely adapted from previously published protocols with minor modifications (Quintard et al., 2011; Winter et al., 2004). A 120-cm-diameter, 60-cm-high circular swimming pool was filled to a depth of 32 cm with 21 ± 2 °C opaque water. Visible cues were placed on the walls of the pool remained in fixed position throughout experiments. A clear plexiglas platform with a diameter of 11 cm was submerged 1 cm below the water level. Swimming performance (eg, path, speed, latency, distance) was tracked with a computer-based system (ANYmaze, Smart, Bioseb, France). A full experiment had two phases: a learning period (place task) for 4 consecutive days and a probe trial on day 5, two and three weeks after ischemia. Phase 1: for the learning period, each mouse was subjected to four trials a day in a pool divided into four equal hypothetical quadrants. The position of platform was constant throughout the training session, while the starting position on each of the four training trials day was changed (Rehni and Singh, 2012). If a mouse did not find the platform within 60 s, it was guided to the platform. After reaching the platform, mice were allowed to stay there for 30 s. Phase 2: for the probe trial, mice were allowed to swim freely for 60 s in the absence of the platform. The time spent in each quadrant and crosses through the location of the former platform was measured. Absence of visual or motor impairment was controlled without specific visual cues placed around the outside of the tank but a visible platform (identified by an object on the platform). This test was performed the day after the probe trial.

2.5. Lipid extraction and fatty acid analysis

Fatty acids were extracted from cortex samples and analyzed as described previously (Awada et al., 2013). Tissue fatty acid methyl ester peak identification was performed by comparison with peak retention times of a 30-component methyl ester standard (Sigma). The concentration of each fatty acid was determined by calculating the areas of peaks.

2.6. Statistical analysis

Statistics were carried out in GraphPad Prism. Data were expressed as mean ± SEM. Survival curves were compared by log-rank test. Statistical analysis of differences between groups was performed by using unpaired *t*-test or ANOVA. Where F ratios were significant, statistical analyses were extended and post-hoc comparisons were made by using Tukey's test for multiple comparison tests. Pearson correlation analysis was used to study the correlation between the time in target zone and the hippocampal neuronal density. The level of significance was set at $p < 0.05$.

3. Results

3.1. ALA dietary supplementation is superior to i.v. treatment in improving motor coordination, but does not correlate with neuronal protection 3 days post-MCAo

During the 3 weeks following a 30 min MCAo, the mortality rate was not influenced by ALA supplementation achieved through diet modification or i.v. subchronic treatment (Fig. 1A and B). Mortality evaluated 7 days post-MCAo was 25% and 16% for mice fed the regular diet and the ALA-diet, respectively, which was not significantly different from the 16% mortality observed when animals were i.v. treated with ALA. Quantitative assessment of infarct volumes 24 h reperfusion after 30 min MCAo revealed that mice fed regular or ALA-diet displayed similar cerebral infarction volumes (29.2 ± 6.4 mm^3 versus 28.8 ± 5.5 mm^3, Fig. 1C). ALA treatment by i.v. subchronic treatment did not influence cerebral infarction induced by 30 min MCAo. The infarct volumes were of 26.7 ± 2.0 mm^3 and 25.5 ± 2.2 mm^3, respectively in the vehicle and ALA treated-group (Fig. 1D).

The percentage change in body weights post-MCAo compared with values recorded immediately prior to undergoing MCAo were used as primary indicators of general well-being. Mice were weighed daily for 7 days post-surgery for the short-term study and

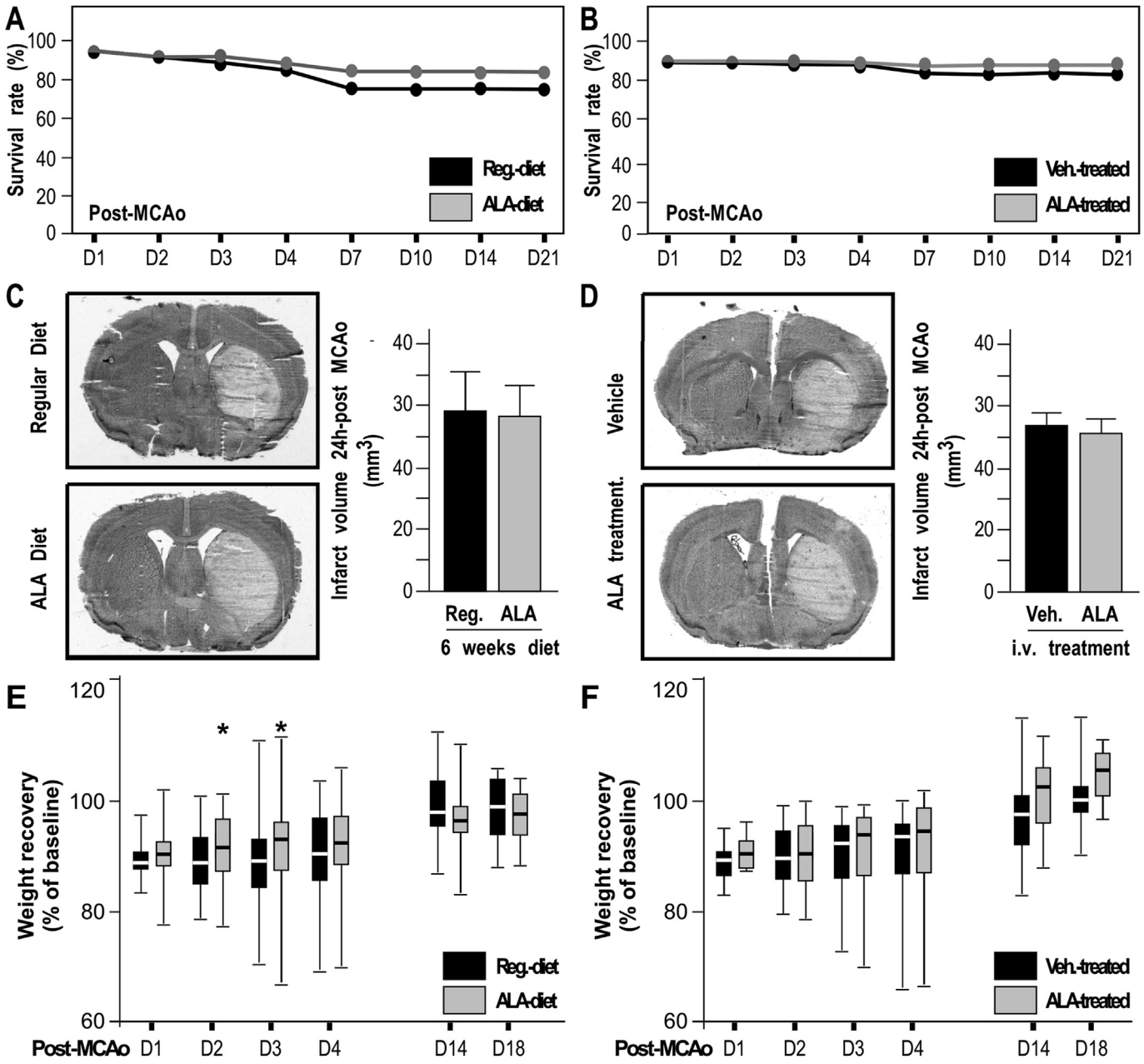

Fig. 1. Effects of α-linolenic acid supplementation on long-term survival, infarct size and body weight after 30 min MCAo. Long-term survival rate after 30 min of MCAo was not affected by ALA supplementation, by modification of the daily diet (**A**) or by i.v. subchronic treatment (**B**), (n = 35, n = 35, n = 30 and n = 28 in the regular diet, ALA-diet, vehicle-treated and ALA-treated groups, respectively). Twenty-four hours after 30 min MCAo, the infarct volume was similar between all groups, showing no effect of the ALA supplementation (**C** and **D**). Data are represented by mean ± SEM (n = 11 per group). (**E** and **F**) During the first week post-MCAo corresponding to the period of motor deficit evaluation, weight loss was lower in animal feds the ALA-diet following 30 min of MACo. During the cognitive deficit evaluation, animals did not differ in weights between each group. Data are represented by boxes, median, and interquartile range; whiskers, min–max; +, mean ($^*p < 0.05$; $^{**}p < 0.01$; n > 30 per group; unpaired *t*-test).

at D7, D14 and D18 in the longer-term study. All animals lost weight over the first 2 days post-MCAo (Fig. 1E and F) after which they began to regain weight until reaching their baseline after a week. Interestingly, mice fed the ALA-diet gained weight at a significantly faster rate than mice fed the regular-diet (Fig. 1E), while there was no differences in weight gain between groups treated with subchronic treatment (Fig. 1F). In the longer-term study, weight did not significantly differ between experimental groups 21 days post-MCAo.

To determine whether daily increased intake of ALA improved functional recovery after stroke, mice were preventively fed an ALA-diet for 6 weeks before MCAo. Pre-operative performance in the rotarod test did not differ significantly between animals fed the regular or ALA-diet. As expected, postoperative performance of MCAo animals was significantly impaired (Fig. 2A). Spontaneous recovery from the motor coordination impairment was faster in the group fed the ALA-diet compared to the animals fed the regular diet (Fig. 2A). The post-MCAo performance of animals fed the ALA-diet was significantly improved by an average of 30% during the first week of recovery (2 W ANOVA: effect of group: $F = 21.53$, $\$\$\$p = 0.00001$). Single time-point analysis showed significant improvement in the performances on the rotarod in MCAo-mice fed the ALA-diet as compared to animals fed the regular diet ($^{*}p < 0.05$, Fig. 2A). Compared to their pre-injury baseline on post-MCAo day 2, 3, and 4, mice fed the ALA-diet were able to perform at 69%, 74%, and 100% ($\#\#p < 0.01$, $\#p < 0.05$) while mice fed the regular diet only performed at 50%, 58%, and 76% ($\#\#\#p < 0.001$, $\#\#p < 0.01$ and $\#p < 0.05$), respectively. Surprisingly, the performance of vehicle-treated animals and ALA-treated animals were similar for the rotarod (Fig. 2B). Since the two sequential injections of ALA were insufficient in improving motor recovery, we then explored whether the improvement of motor coordination caused by the ALA-diet would be confirmed in the pole test. Again, when tested before MCAo, no difference was observed in this effect between the different diets. Between day 3–4 post-MCAo, the average performance for the "time to turn" task of animals fed the ALA-diet was significantly improved as compared to animal fed the regular diet (Fig. 2C, RM ANOVA: effect of group: $F = 4.35$, $\$\$p = 0.0397$). Single time-point analysis revealed that mice fed the ALA-diet took less time to finish a complete turn only on day 3 post-MCAo. The time to turn the head downwards was 2.9 ± 0.7 s for the ALA-diet group compared to 6.6 ± 1.8 s for the regular diet group (Fig. 2C, $^{*}p < 0.05$). In the "latency to descend" task, MCAo animals showed significant impairment for 4 days (up to 14 days – not shown) as compared to their pre-operative performance (Fig. 2D), except at day 3 post-stroke for the mice fed the ALA-diet, which is probably correlated with the marked reduction of their time to turn. Overall, the ALA-diet did not have any significant quantitative effects on their rapidity to reach the ground, while the quality of the descent was clearly visually abnormal (falling or slipping down backward) in the group fed the regular diet (data not shown). This predicted that analysis of the mice motion in fine behavioral assessment protocols to discern improvement in the range of motion, grasping and supination from compensation by postural adjustments may reveal improved recovery of the group fed the ALA-diet (Murphy and Corbett, 2009). A detailed analysis of the neural loss confirmed that the improved motor recovery did not correlate with better neuronal survival. Three days post-MCAo, brains of animal fed the ALA and regular diet exhibited a similar subcortical lesion as well as of the caudate putamen of the ipsilateral hemisphere, characterized microscopically by the loss of 26% of the NeuN-positive cells reflecting a marked neuronal destruction (Fig. 2E and F). We further explored whether the disparity in efficiency between dietary supplementation and subchronic treatment extended to cognitive recovery post-stroke.

3.2. ALA supplementation improves spatial learning and memory after MCAo

We first evaluated the effects of an ALA-diet on functional recovery within 3 weeks after ischemic brain injury in a MWM paradigm. The acquisition procedure did not reveal any significant difference between groups in swimming speed (Fig. 3A) or any significant reduction in the distance to find the hidden platform (Fig. 3B). Nonetheless, mice fed the ALA-diet had significantly improved latency to the hidden platform compared with mice fed the regular diet (Fig. 3C), illustrating that the ALA-diet improved learning performance of MCAo mice (2 W ANOVA: effect of group: $F = 10.78$, $\$\$p = 0.0014$). Results of probe trial testing showed that MCAo mice fed the ALA-diet performed significantly better compared with MCAo mice fed the regular diet. During the probe trial, time in the target zone compared to the other three quadrants was highest for mice fed the ALA-diet compared to the regular diet (Fig. 3D, $p < 0.05$). During the 60 s probe trial, the number of platform crossings was significantly different between mice fed the ALA- and regular diet (9.6 ± 2.0 and 3.1 ± 1.4, respectively, $p < 0.05$, Fig. 3E). The ALA-diet group performance was significantly better during both periods (0–30 s and 30–60 s, $p < 0.05$, Fig. 3E). A representative swim pattern of the probe test for each group clearly demonstrated the difference in the search process 18 days post-MCAo (Fig. 3F), whereas both groups exhibited similar performance in visible platform tests (Fig. 3G).

Using the same MWM paradigm to investigate the effect of ALA-treatment on cognitive function, we observed similar levels of improvement using the repeated ALA injections. No difference between the ALA and vehicle-treated group was observed in swimming speeds and distance to find the hidden platform (not shown), but the ALA-treatment displayed improved learning performance (2 W ANOVA: effect of group: $F = 4.96$, $\$p = 0.04$, Fig. 4A). ALA-treated mice spend 25% more time in the target zone (Fig. 4B, $p < 0.05$) compared to the vehicle-treated mice. Again, ALA supplementation positively impacted the search process 18 days post-MCAo (Fig. 4C), while ALA- and vehicle treated groups displayed similar performance in visible platform tests (data not shown).

3.3. The ALA-induced cognitive improvement is supported by an increased survival of neuronal cell of the hippocampus

To determine whether ALA improves cognition, we investigated particular aspects of the brain lesion linked to spatial memory deficit 3 weeks post-MCAo.

At late time points, tissue loss and atrophy are accurate estimations of the maturation of the ischemic lesion. The profound (12%) tissue loss that was observed in the ipsilateral hemisphere of the animals fed the regular diet was not detected in the ipsilateral hemisphere of the animals fed the ALA-diet ($\#, p < 0.05$) (Fig. 5A). A profound (12%) tissue loss was also observed in the ipsilateral hemisphere of the vehicle-injected animal. The atrophy was reduced to 9% in the ALA-treated mice, but did not reach statistical significance (Fig. 5B). As a more straightforward endpoint, we assessed neuronal density of the ipsilateral and contralateral hippocampi. Thirty minutes MCAo induced significant neuronal damage in the hippocampal sector. Compared with their respective contralateral sides, 45% and 30% of hippocampal neurons of animals fed the regular diet and vehicle-treated groups, respectively, were destroyed (not shown). An ALA-diet significantly reduced neuronal loss by 42% in the ipsilateral hippocampus compared with regular diet when measured 3 weeks after ischemia (Fig. 5C). Similarly, the ALA subchronic treatment also reduced neuronal loss throughout the different hippocampal layers compared with the vehicle treatment (Fig. 5C). The reduced number of necrotic cells observed

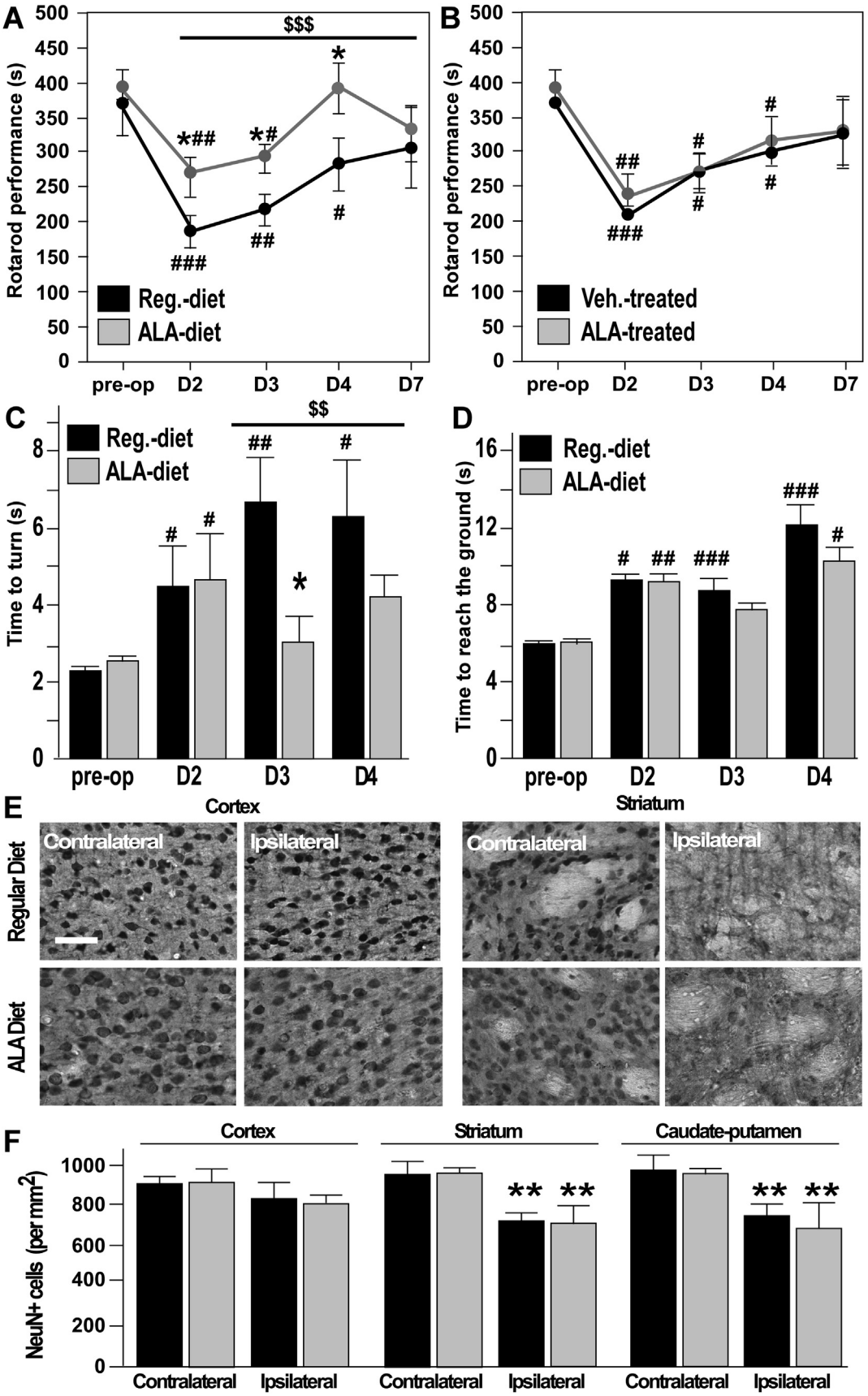

Fig. 2. Effects of α-linolenic acid supplementation on motor deficits and neuronal loss induced by 30 min MCAo. (**A**) Mice fed the ALA-diet displayed better rotarod performance within the first week post-MCAo as compared to mice fed the regular diet. (**B**) The post-MCAo recovery of motor coordination was similar for the mice injected with ALA subchronic treatment or vehicle. Data are represented by mean ± SEM (n = 15 per group at each time point). $^{*}p < 0.05$ versus respective control; $\#p < 0.05$; $\#\#p < 0.01$; $\#\#\#p < 0.001$ versus

in tissue samples obtained from animals treated with the ALA-diet and ALA subchronic treatment is highlighted on photomicrographs shown in Fig. 5D. A correlation between hippocampal neuron loss and time spent in the target zone was observed in the control groups (regular diet and vehicle treated animals), which might explain why ALA-supplemented animals presenting with less hippocampal damage exhibited better spatial memory recovery (Fig. 5E).

In addition, the analysis of cortical content of omega-3 PUFAs indicated that the ALA-diet and a subchronic treatment of 3 sequential ALA injections did not change the content of the major omega-3 fatty acids of the brain, DHA, compared to their respective control (Fig. 5F). No difference was detected between the groups in the overall n-6/n-3 PUFA ratio (Fig. 5G) and the omega-3 PUFA fraction as well as in the proportions of saturated fatty acids, monounsaturated fatty acids, and n-6 polyunsaturated fatty acids (Fig. 5H). Altogether, these data suggest the existence of a protective effect of ALA on the different hippocampal layers, which cannot be attributed to a potential bioconversion of ALA to DHA. This sustained neuroprotection into late recovery stage may account for the superior performance in the MWM cognitive test.

4. Discussion

Malnutrition is often observed in stroke patients because of increased metabolic demands due in part to stroke complications (Dennis et al., 2005). Consequently, on this basis alone, a nutritional support should probably lead to improved clinical outcomes. Unfortunately, the effect of such nutritional intervention is still unclear for stroke patients and understudied in preclinical research. The risk for severe deficiency in omega-3 PUFAs, known as risk factors for stroke in the general population, often occurs during post-stroke rehabilitation, while a body of preclinical evidence documents the benefits of ALA supplementation either as an i.v. treatment or as enriched-diet in animal models of stroke (Blondeau, 2016). Therefore we believe that an emerging direction in nutritional intervention post-stroke stands in omega-3 ALA supplementation. Since successful translation of putative therapies will depend on demonstration of robust efficacy on common deficits resulting from stroke like loss of motor control and memory/learning, the central question of this study was to evaluate the influence of nutritional support in omega-3 ALA in a murine model of ischemic stroke. The 30 min MCAo produces a consistent selective lesion (mainly striatal) with a low mortality in mice (Balkaya et al., 2013), thereby allowing characterization of long-term functional outcome. The infarct size and the related striatal damage observed at 2 and 3 days post-MCAo were similar to those described for this short-duration of ischemia (Katchanov et al., 2001; Kilic et al.). In a clinical setting, the choice of nutritional support lies between oral diet supplementation, enteral feeding (for patients who suffer for dysphagia or other feeding difficulties, supplementation can be achieved *via* a nasogastric or percutaneous endoscopic gastrostomy tubing) or intravenous feeding (supplementation is delivered *via* central venous line or by a less invasive option known as peripheral total parenteral nutrition -TPN- *via* peripheral venous line). Interestingly, a neuroprotective effect of ALA has been observed when given by intravenous injection or after a 6-week feeding of an ALA enriched diet in the acute phase of ischemia in several animal models of brain ischemia (Blondeau et al., 2001, 2009c, 2002b; Heurteaux et al., 2006; Lauritzen et al., 2000; Nguemeni et al., 2010). In addition, LC-omega-3 DHA also displayed neuroprotective effects on acute and long-term ischemic brain damage (Belayev et al., 2011), improving functional outcomes up to 5 weeks after neonatal hypoxia/ischemia (Zhang et al., 2010). However the effect of ALA on long-term brain damage and neurological functional recovery was unknown.

In the 30 min MCAo model, where the infarct is mainly striatal, no striking protective effect in reducing the lesion volume and the mortality was detected. This result contrasts with our previous observations in the more stringent model of 60 min MCAo, where robust protection of the cortex was observed (Nguemeni et al., 2010). Indeed, the evolution of loss of striatal neurons was observed in all animal groups, being similar three days post-MCAo between mice feed the ALA-enriched and regular diet. Similarly, the neuroprotective effects of ALA observed with the single or subchronic i.v. treatment in different models of brain ischemia (Blondeau et al., 2002b, 2009c, 2003; Heurteaux et al., 2006; Lauritzen et al., 2000) were not observed with the milder ischemia of 30 min. It is known that the caudate-putamen, which is more damaged by a mild stroke, has inherited motor and cognitive functions (Katchanov et al., 2003), probably due to the interconnections of this structure with several brain regions.

This study highlights the importance of diet supplementation in the loss of body weight after stroke and its link with the recovery of motor functions. While the exact etiology of weight loss after ischemia is not completely understood, cerebral infarction itself clearly represents one major cause. Interestingly, this work shows that the extent of weight loss and the lesion volumes were similar in all groups the first post-operative day. Nevertheless, the time to regain baseline levels (which usually depend on the duration and severity of ischemia) was improved in the animals fed the ALA-diet within the first week post-stroke, which coincides with the spontaneous recovery of motor function. This information is of importance by providing an indication on how animals may better recover from ischemia and even reflect a better prognostic for functional outcome. The weight recovery after MCAo is a good and independent indicator of the animal welfare. Indirectly, it shows the general physiological condition of ischemic mice with an extremely good correlation with clinical observations.

Weight loss after stroke is common even in patients in good physical condition before stroke onset, for whom no less than a quarter also undergo weight loss in the short and long term (Jonsson et al., 2008). It is also associated with poor nutritional status that is considered as a marker of poor outcome after stroke (Collaboration, 2003). Consistent with clinical observations, MCAo-induced weight loss has been associated with poorer recovery of motor function (Dittmar et al., 2003). This could explain why we found that, by comparing the two ALA supplementations, only oral supplementation (achieved through the modification of the daily diet) improved motor recovery post-MCAo, while no dissimilarities were found in infarct size and caudate-putamen lesion. Several studies on basal ganglia dysfunction have implicated the putamen in the control of motor coordination: these include motor learning, motor performance and tasks, motor preparation, specifying

pre-MCAo performance and ($) indicates a group effect ($$$$p < 0.001$). (**C** and **D**) Similar results were obtained in the pole test that only showed an improvement of balance and forelimb strength post-MCAo in mice fed the ALA-diet. Data are represented by mean ± SEM (n = 15 per group at each time point). $^{*}p < 0.05$ versus respective control, $\#p < 0.05$; $\#\#p < 0.01$; $\#\#\#p < 0.001$ versus pre-MCAo performance and ($) indicates a group effect ($$$p < 0.001$). (**E**) Representative photographs of NeuN immunohistochemistry performed 3 days after 30 min MCAo illustrate that neuronal sparing was confined to striatum. (**F**) Quantification of NeuN + cells confirmed that the neuronal loss confined to striatum and caudate-putamen was not averted by consumption of the ALA-diet. This suggests that the improvement in motor function by the ALA-diet is not correlated to any preservation of brain region engaged in motor function. Data are represented by mean ± SEM (n = 6 per group). $^{**}p < 0.001$ versus respective contralateral hemisphere. Scale bar is 50 μm, applicable to all sections.

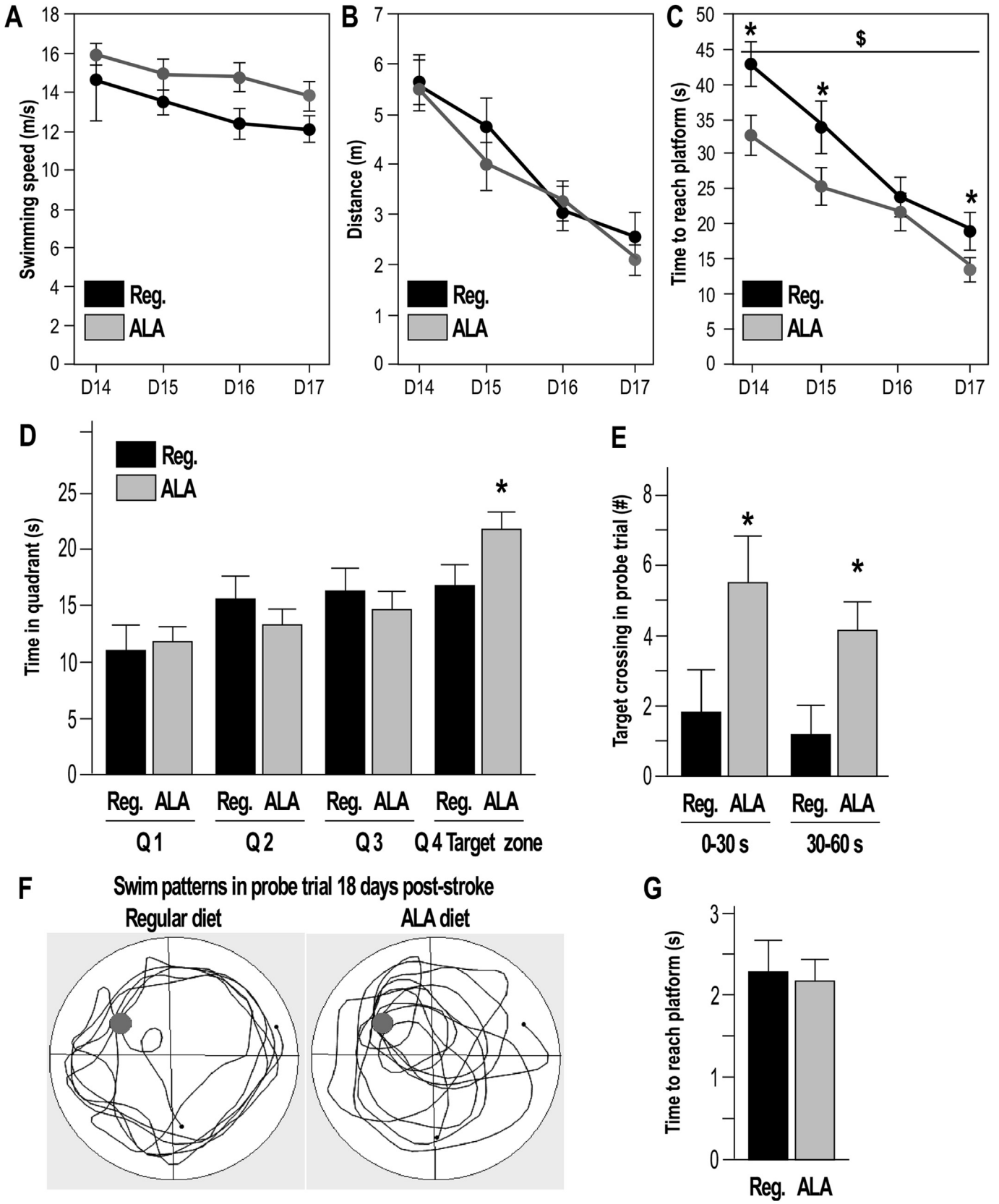

Fig. 3. ALA-Diet improves post-MACo cognitive recovery evaluated by the Morris water-maze test. Between the 14 and 17 days after stroke, while no significant difference were found in the swimming speed (**A**) and the distance traveled (**B**), the time to reach the hidden platform (**C**) of the mice fed the ALA-diet was improved as compared to those fed the regular diet. In addition to displaying a better learning, the mice fed the ALA-diet also showed a better recovery of spatial memory 18 days post-MCAo, as indicated by an increased time spend in the target quadrant (**D**) and number of target crossings than the regular-diet group (**E**). (**F**) Representative swim patterns 18 days post-stroke illustrate the better probe strategy of the mice fed the ALA-diet. (**G**). In the cue test performed the following day after for controlling the absence of visio-motor deficits, the time to reach the visible platform was similar between the mice fed the ALA-and regular diet. Data are represented by mean ± SEM (n = 15 per group). $^{*}p < 0.05$ versus regular diet and ($) indicates a group effect ($\$p < 0.05$) during the learning period.

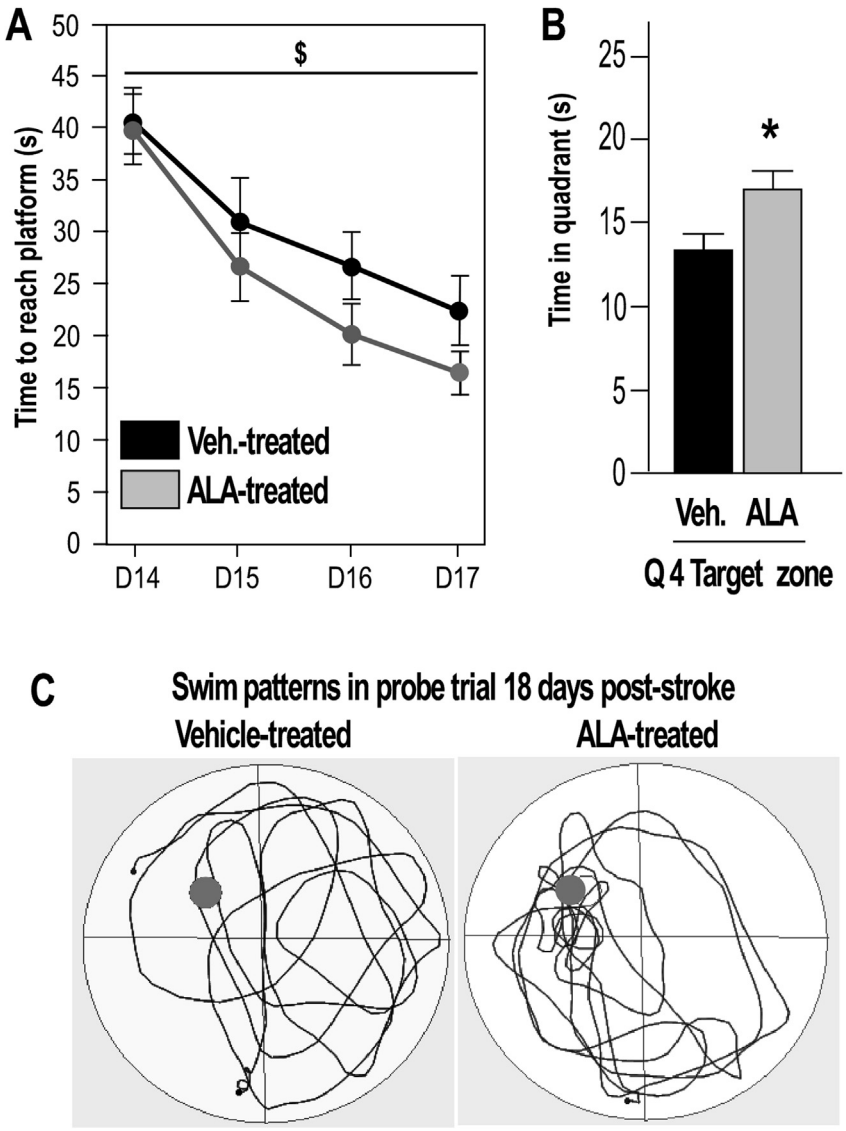

Fig. 4. ALA subchronic i.v. treatment also improves post-MACo cognitive recovery. (**A**) Between the 14 and 17 days after stroke, ALA-subchronic treatment post-MCAo improves the time to reach the hidden platform as compared to vehicle. (**B**) The mice injected with the subchronic treatment also displayed a better recovery of spatial memory 18 days post-MCAo, as indicated by an increased time spent in the target quadrant. (**C**) Representative swim patterns 18 days post-stroke illustrate the better probe strategy of the mice fed the ALA-diet. Data are represented by mean ± SEM (n = 15 per group). $^{*}p < 0.05$ versus vehicle and ($) indicates a group effect ($\$p < 0.05$) during the learning period.

amplitudes of movement, and movement sequences (Evarts and Wise, 1984; Ward et al.). Nevertheless, in the present study the ischemic damage does not seem to be involved in the difference of motor recovery observed. Although we did not investigate whether post-stroke metabolism was improved by the ALA-diet, it is known that stroke alters a number of metabolic factors and pathways due to a global catabolic/anabolic imbalance. Indeed, the increased catabolic and failing anabolic activities induce the depletion of energy stores leading to muscle functional decline and fat wasting, both of which contribute to the clinical manifestation of weight loss (Gariballa et al., 1998). Indeed, lessons from “the obesity paradox” suggesting that patients who are mildly obese may actually have a better stroke outcome underline the requirement for monitoring changes in body weight and composition after stroke. Treating the catabolic/anabolic imbalance after stroke could significantly improve long-term outcome (Scherbakov et al., 2011). During short periods of insufficient energy supply, the human body will burn primarily free fatty acids from body fat stores, and therefore will use ALA as an energy source. Alpha-linolenic acid is integrally involved in the metabolism and could interact directly with nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR) (Forman et al., 1997), liver X receptor and hepatocyte nuclear factor-4 and therefore be implicated in glucose and lipid homeostasis and for metabolic adaptation (Contreras et al., 2013). Although further studies are required, it is tempting to conclude that post-stroke nutritional support in ALA may be an important and required source of energy and metabolic support that could improve the short-term post-stroke body weight and motor recovery.

Since 30 min MCAo in adult mice not only causes short-term motor deficits but also long-term impairment of the spatial learning and memory, we evaluated the effect of ALA supplementation on spatial memory using the Morris water-maze task at 3 weeks after surgery, a time when MCAo animals display spontaneous recovery of body weight and motor impairment (Freret et al., 2011). Indeed, eventual secondary motor impairments and body

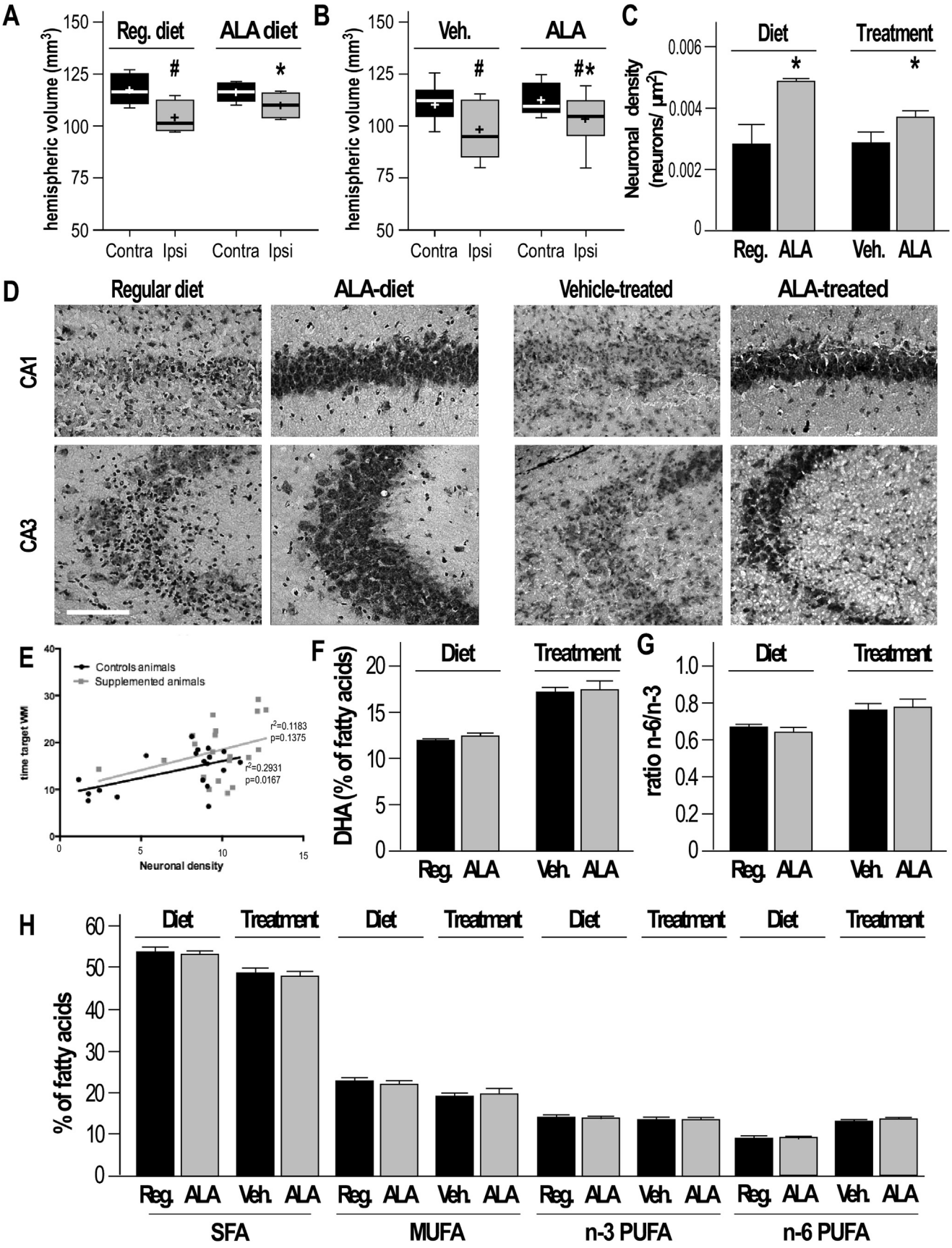

Fig. 5. ALA supplementation reduces parenchymal atrophy and loss of hippocampal neurons. (**A** and **B**) Twenty-one days post-MCAo, analysis of hemispheric volume identified a substantial atrophy of the ipsilateral hemisphere compared to its contralateral in the control groups (e. g. regular diet fed and vehicle treated animals) that was circumvented by ALA supplementation by consumption of ALA-diet or subchronic treatment. Data are represented by Boxes, median, and interquartile range; whiskers, min–max; +, mean (n = 6 per

weight did not influence performance in the water-maze task, as swimming speed did not differ between the different groups before or after MCAo. Nevertheless, ALA-diet fed mice and ALA-injected mice took significantly less time and exhibited optimized searching behaviors to reach the platform compared with the mice fed the regular diet or treated with the vehicle, respectively. These findings demonstrate that nutritional support in omega-3 ALA achieved by oral or intravenous supplementation improves the spontaneous recovery of spatial learning and memory in a mouse model of ischemic stroke.

Because the MWM is known to detect impairment in special memory function associated with hippocampal damage (Morris et al., 1982), we investigated the long-term damage specific to this region. In the MCAo model used in this study, the cerebral cortex was mostly spared and the striatum was infarcted. Here we observed a correlation between hippocampal neurons loss and the time spent in the target zone in the control groups (regular diet and vehicle treated animals) suggesting that spatial cognitive impairment in the MWM task could be correlated with the degree of damage of the hippocampus. How MCAo induces brain lesions in the hippocampus (this work) or thalamus and hypothalamus (Hata et al., 1998), areas that are not directly supplied by the MCA, remains unclear. Recently, Castel's group proposed that damage in these deep regions might be due to the alteration of tissue perfusion by deep and small cerebral arteries arising directly from the internal carotid artery, at the proximity of the origin of MCA during the intraluminal filament insertion (El Amki et al., 2015). Therefore, vasoactive properties of ALA (Blondeau et al., 2007) could support the anastomotic network, which supplies a part of the hippocampus and the thalamus, and may participate in preservation of such areas known to be crucial to spatial learning and memory. Both types of ALA supplementation drastically reduce ischemic hemisphere atrophy and ALA induces direct *in vitro* and *in vivo* neuroprotective effects (Blondeau et al., 2001, 2002a; Heurteaux et al., 2006; Lang-Lazdunski et al., 2003; Lauritzen et al., 2000; Nguemeni et al., 2010), likely accounting for reduced damaged projections from infarcted cortex, thalamic atrophy, or denervated basal nucleus cholinergic fibers to the cortex (Fujie et al., 1990; Kataoka et al., 1991). Similarly, ALA protection from secondary damage induced in neocortical sites within the MCA territory known to also disturb spatial learning and memory independently from hippocampal lesion (Frankland and Bontempi, 2005) could account for the improvement of spatial learning and memory. However the robust hippocampal protective effect of both types of ALA supplementation against MCAo-induced hippocampal lesion observed 3 weeks after the ischemic insult is undoubtedly a determining factor in improving the recovery of MCAo-induced deficits identified in the MWM. The growing understanding of the mechanisms that underlie recovery of the damage brain involves neural plasticity (Kleim and Jones, 2008; Murphy and Corbett, 2009). Therefore functional outcome may be improved by restorative therapies through stimulation of synaptogenesis in the ischemic brain (for review see (Chopp et al., 2009; Murphy and Corbett, 2009)). Modification of the omega-3 content of dietary regimens change the genes brain expression of genes controlling synaptic plasticity, cytoskeleton and membrane association, signal transduction, ion channel formation (Kitajka et al., 2002). We and others have respectively shown that ALA injections and ALA-diet triggered neurogenesis and synaptogenesis in the adult brain (Blondeau et al., 2009a; Venna et al., 2009). Thus a casual relationship between ALA-induced synaptogenesis and cognitive recovery may be surmised. While the precise cellular and molecular targets of ALA on stroke recovery will require further investigations before being sorted out, an evident but under-studied link between ALA–induced protection and stroke recovery, that is cerebral inflammation should be considered. Clinical and experimental evidence have implicated cerebral inflammation in several aspects of stroke: neuroinflammation may not only lead to irreversible neuronal alterations, but probably contribute to worsen outcome and recovery in stroke (Allan et al., 2005; Drake et al., 2011). In contrast, several anti-inflammatory actions of omega-3 PUFAs have been proposed, including in neonatal Hypoxic–Ischemic (H/I) brain injury (Zhang et al., 2010). Indeed, Chen's laboratory has recently shown that LC-omega-3 supplementation through an EPA/DHA-enriched diet improves long-term neurological outcomes after neonatal H/I injury. This prolonged protection was attributed to an anti-inflammatory effect on microglia leading to a robust suppression of proinflammatory mediator release (Zhang et al., 2010). Our ongoing studies will determine whether suppression of neuroinflammation and release of proinflammatory cytokines (IL1-β, IL-6, TNF-a) and chemokines (MCP-1, MIP-1-alpha, IL-8) (Conductier et al., 2010; Le Thuc et al., 2015) play a critical role in mediating the neuroprotective effect of ALA supplementation after ischemic stroke.

With respect to efficiency of the oral and intravenous approach, the overall benefits of the subchronic treatment we used were less impressive than those observed with the ALA-diet. Originally, this subchronic treatment was only designed for promoting a better survival against 60 min MCAo (Blondeau et al., 2009c; Heurteaux et al., 2006). Therefore, such beneficial effects on long-term hippocampal and cognitive deficits were unanticipated and since we can not exclude that an optimization of subchronic treatment with a daily delivery may offer results of similar amplitudes as the diet enriched in ALA, it reinforces the idea that studies testing different protocols and approaches are still needed in the field.

5. Conclusions

In summary, in one of a minor number of studies concerning nutritional intervention in the critically post-stroke recovery period, we propose that nutritional intervention with ALA would help counteract stroke-induced deficits including impairments of cognition that occur in patients (Stephens et al., 2004) This case for nutritional support in ALA is also reinforced by the demonstration of an association between hippocampus and memory in humans (Burgess et al., 2002) and the correlation observed by MRI between cognitive impairments to hippocampal alteration (Lin et al., 2014) While insights into the precise mechanisms requires further studies, we identify that hippocampal preservation has an intrinsic effect of ALA treatment leading to reduced cognitive deficits, which is not due to bioconversion to DHA. Therefore, such work holds promise as an instigator of further randomized, controlled studies

group). #$p < 0.05$ versus respective contralateral hemisphere, and *$p < 0.05$ versus respective control. (**C**) Neuronal loss observed in the hippocampus 21 days post-MCAo in the regular-diet and vehicle treated groups was significantly reduced in the animal supplemented in ALA by modification of the diet or subchronic treatment. Data are represented by mean ± SEM (n = 6 per group). *$p < 0.05$. (**D**) The better preservation of the hippocampal neurons is illustrated by representative cresyl violet staining of the CA1 and CA3 neuronal layers. Scale bar is 100 μm, applicable to all sections. (**E**) A correlation that was analyzed by Pearson's analysis between hippocampal neurons loss and the time spent in the target zone was found in the control groups (regular diet and vehicle treated animals), confirming why the ALA-supplemented animal presenting less hippocampal damage had a better spatial memory recovery. The consumption of ALA-diet and the subchronic treatment did not modify in the cortex, the concentration of DHA, the major omega-3 PUFA of the brain (**F**), the pre-MCAo omega-6/omega-3 ratio (**G**), and of saturated fatty acids (SFAs), mono unsaturated fatty acids (MUFAs) and PUFAs (**H**). Data are represented by mean ± SEM (n = 5 per group).

that will be required to confirm the value of ALA in adjunctive therapy for one of medicine's most urgent priorities.

Funding sources

This work was supported by the CNRS, the Fondation pour la Recherche Médicale DRM20101220421, ONIDOL (n°889P00-01) and St Hubert grants (n°087696) and the SFNEP Bernard Beaufrère Prize.

Disclosures

None.

Acknowledgments

We thank Dr C. Rovere, O. Le Thuc, C. Widmann, N. Simon-Rousseau, C. Simonneau, A. Renault for many helpful discussions and Dr J. Tauskela for his critical reading of the manuscript. We are very grateful to Pr M. Guichardant and P. Daira (lipidomics IbiSA platform of the LISA Carnot institute), and Drs N. Combe, L. Fonseca, B. Buaud and C. Vaysse (Iterg, Equipe Nutrition, Métabolisme et Santé) for Fatty Acid Analysis.

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