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Dietary fish hydrolysate supplementation containing n-3 LC-PUFAs and peptides prevents short-term memory and stress response deficits in aged mice

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ABSTRACT

Brain aging is characterized by a decline in cognitive functions, which can lead to the development of neurodegenerative pathologies. Age-related spatial learning and memory deficits are associated with a chronic low-grade inflammation. Anxiety disorders and stress response alterations, occurring for a part of the elderly, have also been linked to an increased neuroinflammation and thus, an accelerated cognitive decline. Nutrition is an innovative strategy to prevent age-related cognitive impairments. Among the nutrients, n-3 long chain polyunsaturated fatty acids (LC-PUFAs) and low molecular weight peptides from proteins, especially those from marine resources, are good candidates for their immunomodulatory, anxiolytic and neuroprotective properties. The aim of this study is to determine the combined effect of n-3 LC-PUFAs and low molecular weight peptides on cognitive functions, and their mechanism of action. We are the first to show that a dietary supplementation with a fish hydrolysate containing n-3 LC-PUFAs and low molecular weight peptides prevented the age-related spatial short-term memory deficits and modulated navigation strategies adopted during spatial learning. In addition, the fish hydrolysate displayed anxiolytic activities with the reduction of anxiety-like behaviour in aged mice, restored the plasmatic corticosterone levels similar to adult animals following an acute stress and modulated the hypothalamic stress response. These effects on behaviour can be explained by the immunomodulatory and neuroprotective properties of the fish hydrolysate that limited microgliosis *in vivo*, decreased LPS-induced expression of pro-inflammatory cytokines and increased the expression of growth factors such as BDNF and NGF *in vitro*. Thus, n-3 LC-PUFAs and low molecular weight peptides contained in the fish hydrolysate can play an important role in the limitation of neuroinflammation and stress response alterations during aging and represent a potential strategy for the prevention of age-related cognitive decline.

1. Introduction

Brain aging is accompanied by a decline in cognitive functions which is characterized by non-pathological, but significant alterations of memory, learning abilities and spatial recognition in both humans and animals (Deary et al., 2009; Gallagher and Rapp, 1997). This cognitive decline can lead to the development of neurodegenerative diseases, such as Alzheimer's disease (AD), and can accelerate the loss of autonomy of seniors.

Age-related cognitive alterations have been associated with a chronic low-grade inflammation, in humans (Cohen et al., 1997; Dik et al., 2005; Rafnsson et al., 2007; Weaver et al., 2002) and rodents (Barrientos et al., 2009; Braida et al., 2004; Buchanan et al., 2008). Microglia, the immunocompetent cells of the brain, become sensitized to inflammation with age and highly reactive, leading to an imbalance between pro- and anti-inflammatory cytokine production (Frank et al., 2010; Perry et al., 1993; Rozovsky et al., 1998). Interestingly, pharmacological inhibition of microglial activation and cytokine production have been shown to improve memory in an AD animal model (Choi et al., 2007).

Age-related cognitive alterations are accentuated by anxiety disorders in about 3.8% of people aged 60 and over (Beaudreau and O'Hara, 2008; Gulpers et al., 2016; Kassem et al., 2017; Pietrzak et al., 2012). Indeed, older adults with elevated anxiety symptoms presented deficits in episodic memory (Bierman et al., 2005; Booth et al., 2006;

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Mantella et al., 2007). Moreover, a positive correlation has been reported between anxiety and inflammatory markers both in humans (Arranz et al., 2007; Pitsavos et al., 2006; Schneiderman et al., 2005; Vogelzangs et al., 2013) and in mice (Connor and Leonard, 1998; Fiore et al., 1998; Schrott and Crnic, 1996). Thus, targeting neuroinflammation during aging constitutes a good strategy to delay or limit the development of age-related cognitive deficits.

Nutrition as a potential innovative way to prevent or delay age-related cognitive decline, is of growing interest (Allès et al., 2016). Different kinds of nutrients have been shown to have beneficial effects on age-related cognitive decline. Indeed, the consumption of n-3 long chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA) has been associated with a decrease in cognitive deficits or linked to better cognitive abilities in the elderly (Devore et al., 2009; González et al., 2010; Titova et al., 2013; Whalley et al., 2004; Yurko-Mauro et al., 2010). Similarly, in aged animals, dietary supplementation with n-3 LC-PUFAs improved spatial learning and memory performances (Cutuli et al., 2014; Hashimoto et al., 2015; Kelly et al., 2011; Labrousse et al., 2012). This beneficial effect may be linked to the n-3 LC-PUFAs immunomodulatory properties. Indeed, several studies have reported that older individuals with higher blood levels of n-3 LC-PUFAs presented a lower cytokine production (Farzaneh-Far et al., 2009; Ferrucci et al., 2006; Tsitouras et al., 2008). Similar results have been shown in aged rodents, with decreased plasma and brain expression levels of pro-inflammatory cytokines and microglial activation markers following supplementation with n-3 PUFA precursor or n-3 LC-PUFAs (Hashimoto et al., 2015; Labrousse et al., 2012; Lynch et al., 2007; Maher et al., 2004; Moranis et al., 2012). DHA exerts anti-inflammatory effects *via* several pathways including *via* the inhibition of the transcription factor nuclear factor Kappa-B (NFκB), which is involved in pro-inflammatory cytokine production (Ajmone-Cat et al., 2012; Chang et al., 2015; De Smedt-Peyrusse et al., 2008; Fourrier et al., 2017; Pettit et al., 2013). Low molecular weight peptides (< 1000 Da) included in protein hydrolysates are also of particular interest since they can present anti-inflammatory and anxiolytic properties. Indeed, orally-administered milk peptides suppressed the expression of inflammatory factors in the hippocampus of AD mice (Min et al., 2017). Moreover, at the periphery, peptides from soy, milk, salmon and lupine decreased inflammation in mouse intestine (Kovacs-Nolan et al., 2012) and abdominal aorta (Nakamura et al., 2013). In addition, at the cellular level, they exerted anti-inflammatory effects on murine macrophages (Ahn et al., 2015), and on human primary monocytes (la Paz et al., 2019). Low molecular weight peptides also display anxiolytic properties. Notably, small peptides from milk (Lactium®), cod, mackerel (Gabolysat® PC60) and from salmon hydrolysate have anxiolytic effects in humans (Lanoir et al., 2002) and in animals (Belhaj et al., 2013; Bernet et al., 2000). Hence, the combination of both peptides and DHA contained in fish hydrolysate could have beneficial effects on age-related inflammation and on anxiety related to aging. To our knowledge, only Le Poncin-Séac'h and Le Poncin-Lafitte (2010) have shown that the consumption of fish hydrolysate containing both low molecular weight peptides and DHA, prevented from memory complaint and led to better memorization performances in healthy individuals from 45 to 65 years old. However, the mechanisms of action of a fish hydrolysate containing low molecular weight peptides and n-3 LC-PUFAs have never been studied.

Thus, we investigated the effects of a marine by-products-derived hydrolysate supplementation, mainly containing low molecular weight peptides and n-3 LC-PUFAs, on the prevention of age-related cognitive decline in mice. We examined the effects of fish hydrolysate on spatial learning and memory tasks using respectively the Y-maze and the Morris water maze with the analysis of navigation patterns, known to be modified with aging (Bensalem et al, 2016). We also evaluated stress reactivity response and the biological processes associated with aging, neuroinflammation and neuroprotection.

2. Methods

2.1. Animals

7-week old and 12-month old male C57Bl/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) were raised under normal 12 h-12 h light/dark cycle on cellulose litter in controlled environment (21–23 °C, 40% of humidity), with *ad libitum* access to food and water. Animal husbandry and experimental procedures were done in accordance with the EU Directive 2010/63/EU for animal experiments and were approved by the national ethical committee for the care and use of animals (approval ID A16320).

2.2. Diet

Mice were fed a control diet (INRAE Jouy-en-Josas, France) for 12 weeks and were then ascribed to different groups: one group of adult mice (n = 12) and one of aged mice (n = 11) received the control diet, whereas the two other groups of adult mice (n = 12) and aged mice (n = 13) received the hydrolysate-enriched diet (INRAE Jouy-en-Josas, France) containing 0.19% of the fish hydrolysate (Table 1). Diets started at the age of 5 months for adult mice and 15 months for aged mice and continued throughout the entire experiment (11 weeks) (Fig. 1). Thereby, at the end of the experiment, adult and aged mice were approximately 8 and 18 months old respectively. The fish hydrolysate was provided by the Brain Booster Consortium. It was obtained from marine by-products and contained mostly low molecular weight peptides (< 1000 Da) and n-3 LC-PUFAs such as DHA and eicosapentaenoic acid (EPA). The specific composition of the fish hydrolysate is detailed in patent number B251427FR. The low molecular weight peptides dose (5.55 mg of low molecular weight peptides/mouse/day) was determined by a literature review (Belhaj et al., 2013; Le Poncin-Séac'h and Le Poncin-Lafitte, 2010). The dose of n-3 LC-PUFAs was 280 µg/mouse/day (of which 143 µg/mouse/day of DHA and 70 µg/mouse/day of EPA).

2.3. Behavioural tests

2.3.1. Spatial recognition short-term memory in the Y-Maze

Six weeks after the beginning of the supplementation, spatial recognition memory was evaluated using the Y-maze test as described by Dellu et al. (1992) and reported in Dinel et al. (2016) (Fig. 1). The apparatus consisted in a Y-shaped maze with 3 arms (35 cm long and 10 cm deep), illuminated at 15 lx. Extra-maze visual cues are placed on the walls, allowing mice to locate themselves in space. In the first trial, one arm of the Y-maze was closed, and mice were allowed to visit the two other arms for 5 min. After a 1 h inter-trial interval (ITI), mice were placed again in the start arm for the second trial and allowed to explore all three arms for 5 min. Start and closed arms were randomly assigned for each mouse. The animals were video-tracked (SMART system; Bioseb, Vitrolles, France) to analyze the time spent in the different

Table 1
Composition of the control and hydrolysate-enriched diets.

Components	Percent (%)	
	Control diet	Hydrolysate-enriched diet
Hydrochloric casein	18	18
Corn starch	45,9	45,71
Sucrose	24	24
Cellulose	2	2
Peanut oil	5	5
Mineral Mix	4	4
Vitamin Mix	1	1
+ DL methionine	0.1	0.1
+ Vitamin A 5 UI/g	5 UI/g	5 UI/g
Hydrolysate	0	0,19

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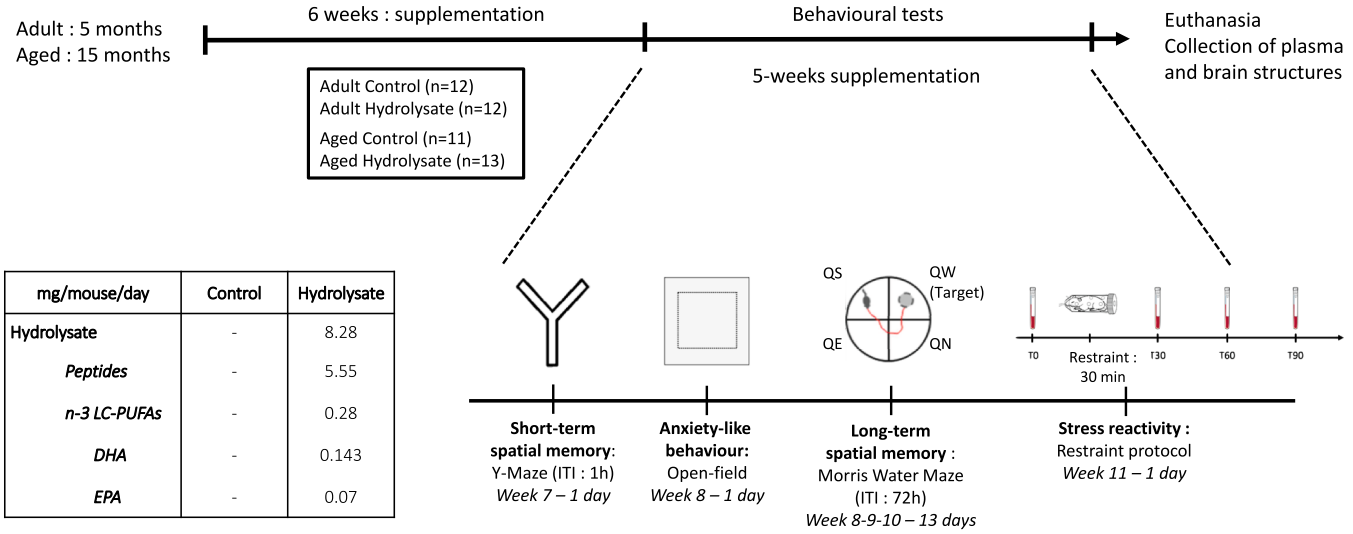

mg/mouse/day	Control	Hydrolysate
Hydrolysate	-	8.28
Peptides	-	5.55
n-3 LC-PUFAs	-	0.28
DHA	-	0.143
EPA	-	0.07

Fig. 1. Experimental design. Adult (5 months) and aged (15 months) mice were fed the control diet or the hydrolysate-enriched diet for 6 weeks. Behavioural tests were performed during the next 5 weeks. Total supplementation duration was 11 weeks. DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ITI: inter-trial interval; LC-PUFAs: long chain polyunsaturated fatty acids; QE: quadrant east, QN: quadrant north; QS: quadrant south; QW: quadrant west.

arms. In addition, a recognition index was calculated to compare the animals' performance against chance (33%): time spent in the new arm/(time spent in the new arm + time spent in the familiar arm + time spent in the starting arm).

2.3.2. Spatial learning and reference memory in the Morris water maze

Spatial learning and memory were assessed in the Morris water maze as previously detailed in Bensalem et al. (2016) and Morris (1984). Briefly, two familiarization days occurred. Mice had to find a visible platform in a small pool (60 cm diameter) surrounded by white curtains to familiarize with water and swimming (3 consecutive trials a day; 60 s cut-off). Then, visuomotor deficits were evaluated during a day of cued learning in the Morris water maze where mice had to find a visible platform pointed out with a cue (6 trials a day; 90 s cut-off). During spatial learning, mice were trained during four consecutive days to learn the location of the submerged platform by using distal extra-maze cues (6 trials a day; 90 s cut-off). For each trial, the latency and distance travelled to reach the platform as well as the swim path were recorded by Imetronic videotracking system (Pessac, France).

72 h after the last training session, spatial memory was evaluated during the probe test for 60 s in the maze without platform. The distance travelled in the four quadrants was recorded using the SMART system (Bioseb, Vitrolles, France). The quadrant where the platform was located during spatial learning is referred to as target quadrant.

2.3.3. Analysis of navigation strategies in the Morris water maze

The navigation path was analyzed for each trial of the spatial learning test. The categorization scheme was adapted as described by Bensalem et al. (2016) (Fig. 2). Navigation strategies were divided into two main categories: non-spatial vs. spatial strategies. Non-spatial strategies included first "global search" strategies: "peripheral looping" (persistent swimming around the outer 15 cm of the pool), "random" (searching the entire tank, > 75% surface coverage), "circling" (swimming in tight circles, possibly with some net directional movement), and then "local search" strategies: "scanning" (searching restricted to a limited, often central, portion of the tank, > 15% and < 75% of surface coverage), "chaining" (circular swimming at an approximately fixed distance greater than 15 cm from the wall), "repeated incorrect" (swimming in a precise direction that does not contain the platform and repeating the same trajectory several times), and "focal incorrect" (searching intently a small portion of the tank that does not contain the platform). Spatial strategies included "repeated correct" (swimming in direction of the platform and repeating the same trajectory several times), "focal correct" (swimming and searching intently in the zone containing the platform), "spatial indirect" (swimming indirectly to the platform with eventually 1–2 loops) and "spatial direct" (swimming directly to the platform).

2.3.4. Anxiety-like behaviour in the open-field test

Anxiety-like behaviour was evaluated using the open-field test. Mice were exposed to an unfamiliar squared (40 × 40 cm) open-field surrounded by walls (16 cm high) illuminated at 30 lx. The apparatus was virtually divided into two parts: the central area and the peripheral area. A decreased time spent in the centre was considered as an increased anxiety-like behaviour. Each mouse was placed in a corner and allowed to explore the open-field for 10 min. Time spent in the central area, locomotor activity, centre crossings and thigmotaxis were recorded to evaluate anxiety-like behaviour (SMART software, Bioseb, Vitrolles, France).

2.3.5. Stress reactivity in the restraint test

To assess stress reactivity, mice were subjected to a restraint test during 30 min. Blood was drawn from the mandibular vein before the beginning of the restraint protocol (T0), at 30 min (T30), 60 min (T60) and finally by a transcardiac puncture at 90 min (T90).

2.4. Tissue preparation

Ninety minutes after the beginning of the restraint test, mice were euthanized by isoflurane inhalation. After transcardiac perfusion with phosphate buffered saline (PBS), brains structures of interest and plasma were isolated and frozen at −80 °C until analysis. Concerning the preparation of brains for immunohistochemistry, transcardiac perfusion with PBS was followed by a 4% paraformaldehyde (PFA) perfusion. Then, brains were removed, post-fixed in PFA overnight at 4 °C, cryoprotected in 30% sucrose during 48 h at 4 °C, rapidly frozen with isopentane and stored at −80 °C. For the corticosterone measurements, plasma was isolated from the blood by centrifugation at 3000*g* for 20 min.

2.5. In vitro measurements

In order to study interactions between cells and to have a better understanding of signaling pathways involved, a so-called "sandwich" co-culture was performed between microglial-like cells (BV2) and neural cells (HT22). However, the 2 cell types were cultured separately on the first place.

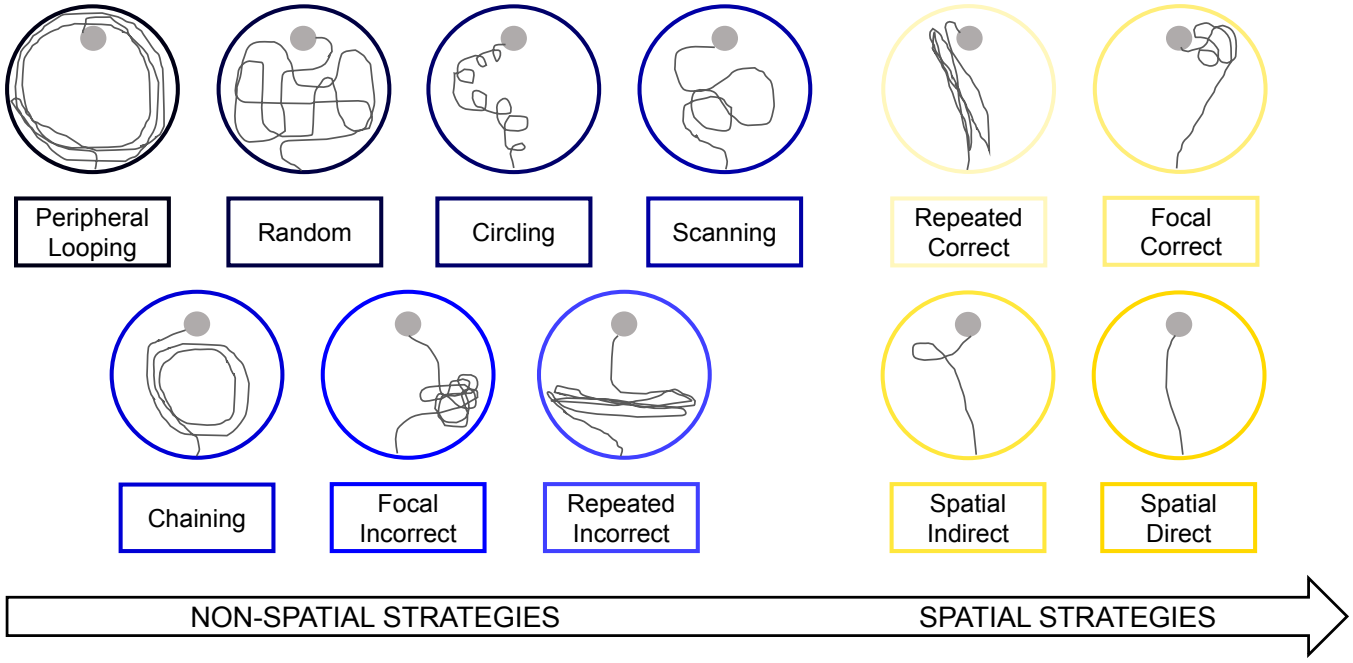

Fig. 2. Categorization scheme of navigation paths. Representative navigation path patterns of "non-spatial" (in blue) and "spatial" (in yellow) strategies used to locate the platform. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BV2 cells (a murine microglial cells provided by Dr. Watterson (North Western University, USA)) were grown in RPMI Glutamax (Invitrogen, Life Technologies, Saint Aubin, France) supplemented with 10% inactivated fetal calf serum and 1% of a penicillin (100 U/mL)-streptomycin mixture (100 µg/mL; Sigma-Aldrich, Lyon, France) in 5% CO_2 wet atmosphere at 37 °C as described by De Smedt-Peyrusse et al (2008). BV2 were seeded at 2 $\times 10^5$ cells/well and cultured in RPMI complete medium in 6 well-plates.

HT22 cells (a hippocampal mouse cell line, provided by Dr. Maronde (Lippstadt, Germany)) were grown in DMEM (Invitrogen, Life Technologies, Saint Aubin, France) with 10% inactivated fetal calf serum and 1% of a mixture of penicillin (100 U/mL)-streptomycin (100 µg/mL; Sigma-Aldrich, Lyon, France) in a 5% CO_2 wet atmosphere at 37 °C. HT22 were seeded at 1x10^5 cells/lamellae and are cultured in DMEM complete medium on lamellae.

After 24 h of culture, the HT22 cells were put in contact with the microglial mat to treat both cell lines for 16 h with the hydrolysate (16 µM of DHA, 10 µM of EPA and 0.27 mg/mL of peptides). According to Debbabi et al (2017) and preliminary work in the lab, the concentration of DHA has been validated to induce anti-inflammatory effect. The cells were then treated during 6 h with 1 µg/mL lipopolysaccharide (LPS; Sigma-Aldrich, Lyon, France) to induce inflammation and collected in TRIzol (n = 5; Invitrogen, Life Technologies, Saint Aubin, France).

2.6. Biochemical measurements

2.6.1. Measurement of corticosterone

Corticosterone was measured in plasma before and 30, 60 and 90 min after initiation of the stress protocol using the ELISA DetectX® corticosterone immunoassay kit (Euromedex, Strasbourg, France). The corticosterone concentration (ng/ml) of each sample was calculated according to the standard range provided by the supplier by spectrophotometry (Victor3V, PerkinElmer, France).

2.6.2. Quantitative real-time PCR

The expression of the different genes of interest was evaluated by real-time quantitative PCR as described by Rey et al. (2016). These analyses were performed on BV2 and HT22 cells, and on hippocampus and hypothalamus. Briefly, total RNAs were extracted from cells, hippocampus and hypothalamus using TRIzol (Invitrogen, Life Technologies, Saint Aubin, France). The purity and quantity of RNA for each sample were measured by spectrophotometry (Nanodrop, Life technologies, Saint Aubin, France). One or two micrograms of RNA were reverse transcribed into complementary DNA (cDNA) using Superscript III (Invitrogen, Life Technologies, Saint Aubin, France). TaqMan® specific primers were used to amplify genes of interest as previously described (Madore et al., 2013; Mingam et al., 2008; Rey et al., 2016). We focused on IL-6 (Mm00446190_m1), IL1β (Mm00434228_m1), TNF-α (Mm00443258_m1), BDNF (Mm04230607_s1) and NGF (Mm00443039_m1) for co-culture; IL-6, IL-1β, TNF-α, CD11b (Mm00434455_m1) for hippocampus; β-Actin (Mm02619580_g1) as housekeeping gene. Specific primers for the hypothalamic stress responsive genes CrhR1, CRHBP, HSD11β1, GR, MR and CRH were designed using PrimerBlast (NCBI, Bethesda, MD) and purchased from Eurogentec (Eurogentec, Seraing, Belgium). Specificity of the primers was confirmed by nucleotide BLAST (NCBI, Bethesda, MD). Primer sequences are provided in Table 2. Fluorescence was determined on an ABI PRISM 7500-sequence detection system (Applied Biosystems, Villebon sur Yvette, France). Data were analyzed using the comparative threshold cycle (Ct) method and results were expressed as relative fold change (Madore et al., 2013; Mingam et al., 2008; Rey et al., 2016) to control target mRNA expression.

2.6.3. Immunohistochemistry

Free-floating coronal sections of 40 µM through the hippocampus were collected on a cryostat (Leica Biosystems, Nanterre, France). After being incubated for 30 min with H_2O_2 0.3% and washed in PBS 1X, sections were blocked in a buffer containing 3% bovine serum albumin (BSA), 0.3% Triton in PBS 1X for 45 min at room temperature (RT). Sections were then immunolabelled with a rabbit polyclonal antibody against Iba1 (1:1000; Wako #019-19741, Plaisir, France) in blocking buffer over night at 4 °C and incubated with biotinylated goat anti-rabbit secondary antibody (1:2000; Vector, Les Ulis, France) for 2 h at RT. All sections were processed in parallel. Secondary antibody was revealed using the biotin-streptavidin-immunoperoxidase technique (1:1000; ABC Biovalley, Nanterre, France) for 1 h at RT. Staining was visualized using 3,3′diaminobenzidine (DAB) labelling. The number of Iba1-positive cells in the hippocampus was estimated using the optical fractionator method, with systematic random sampling (Mercator software, La Rochelle, France), and counted by an experimenter blinded

Table 2
Primer sequences for stress responsive genes.

Gene (Full name)	Primer	Sequence
CrhR1 (Corticotropin releasing hormone receptor 1)	Forward	5′-GGAGCATCCGGTGCCTG-3′
	Reverse	5′-AAAGCCGAGATGAGGTTCCA-3′
CRHBP (Corticotropin releasing hormone binding protein)	Forward	5′-TGGTTCCATACCAGCACCAAAAC-3′
	Reverse	5′-AGCTCCACAAAGTCACCAGTCC-3′
HSD11β1 (11β-Hydroxysteroid dehydrogenase type 1)	Forward	5′-GGAAGGTCTCCAGAAGGTAGTGTC-3′
	Reverse	5′-GAGGCTGCTCCGAGTTCAAG-3′
GR (also called Nr3c1; Nuclear receptor subfamily 3, group C, member 1)	Forward	5′-GTGGAAGGACAGCACAATTACCT-3′
	Reverse	5′-GCGGCATGCTGGACAGTT-3′
MR (also called Nr3c2; Nuclear receptor subfamily 3, group C, member 2)	Forward	5′-GCCGTGGAAGGACAACACA-3′
	Reverse	5′-CCTAAGTTCATGCCGGCTTG-3′
CRH (Corticotropin releasing hormone)	Forward	5′-CAGCCCTTGAATTTCTTGCA-3′
	Reverse	5′-TCACCCATGCGGATCAGA-3′
β-actin (Housekeeping gene)	Forward	5′-TACAATGAGCTGCGTGTGGC-3′
	Reverse	5′-ACATGGCTGGGGTGTTGAAG-3′

to the group assignments with a 40× microscope objective (Nikon Corporation, Champigny-sur-Marne, France).

2.7. Statistical analysis

Statistical analyses were conducted with GraphPad Prism 7 (GraphPadSotfware, San Diego, USA), except for repeated measure analysis we used Statistica 6.0 (StatSoft, Tulsa, USA). Correlation matrix was performed using RStudio software (RStudio Desktop 1.3.1056, Boston, USA).

- A 2-way ANOVA was used to analyze food intake. For the body weight gain, the 4 experimental groups were compared by a one sample *t*-test against the hypothetical value 0 followed by t-tests between groups.
- For the Y-maze test, the 4 experimental groups were compared by a one sample *t*-test against the chance level (33%).
- Concerning the Morris water maze test:
  - Swim speed, spatial learning and spatial strategies over days of training were analyzed using a 3-way ANOVA with repeated measures (age, diet and days of learning).
  - Cued learning and percentage of spatial strategies used each day were analyzed using a 2-way ANOVA (factors: age and diet).
  - Probe test comparisons were performed for each group against chance level (25%) using a one sample *t*-test and a 1-way ANOVA (factor: quadrants) followed by a Dunnett's multiple comparisons *post-hoc* test.
- Time spent in the centre of the open-field, locomotor activity, centre crossings and thigmotaxis were analyzed using a 2-way ANOVA (factors: age and diet) followed by a Fisher's LSD *post-hoc* test when appropriate.
- For corticosterone levels, groups were compared using a 3-way ANOVA with repeated measures (age, diet and time of stress) followed by a Fisher's LSD *post-hoc* test.
- Iba1-positive cells, stress responsive genes as well as pro-inflammatory cytokine and neurotrophin expressions for *in vivo* and *in vitro* experiments were analyzed using a 2-way ANOVA (factors: age and diet or LPS and treatment) followed by a Fisher's LSD *post-hoc* test when appropriate.

All data were expressed as means ± SEM and the differences were considered significant when the p-value was less than 0.05.

3. Results

3.1. Effects of fish hydrolysate supplementation on food intake and weight gain.

Food intake and weight gain were measured all along the 11 weeks of dietary supplementation. Aged mice consumed more food than adult mice as revealed by the 2-way ANOVA on the average of food intake (age effect [$F_{(1,44)} = 6.121$, $p < 0.05$]). Moreover, mice fed the hydrolysate-enriched diet ate more than mice on the control diet (diet effect [$F_{(1,44)} = 175.6$, $p < 0.001$]) but no age × diet interaction was found ($F_{(1,44)} = 3.275$, $p = 0.0772$) (Supplementary Fig. 1A).

Changes in body weight were observed over the 11 weeks of diet. The analysis of weight gain demonstrated that adult control, adult hydrolysate and aged hydrolysate mice significantly gained weight ($t = 3.503$, $p < 0.01$; $t = 8.637$, $p < 0.001$ and $t = 2.87$, $p < 0.05$ respectively) whereas aged control mice lost weight ($t = 6.513$, $p < 0.001$). Interestingly, hydrolysate-enriched diet rescued the loss of weight induced by age (adult hydrolysate vs. aged hydrolysate: $t = 3.593$, $p < 0.01$, aged control vs. aged hydrolysate: $t = 5.237$, $p < 0.001$) (Supplementary Fig. 1B).

3.2. Fish hydrolysate supplementation prevents short-term but not long-term spatial memory deficits in aged mice

The effect of the hydrolysate supplementation on short-term spatial memory was assessed using a Y-maze test with a 1 h ITI. Recognition indexes for control and hydrolysate adult mice were significantly higher than the chance level, indicating that they did not present spatial memory alterations (adult control: $t = 2.566$, $p < 0.05$; adult supplemented: $t = 5.909$, $p < 0.001$) (Fig. 3A). As expected, the recognition index of aged control mice was not different from the chance level ($p = 0.77$), suggesting that these mice did not recognize the new arm. Interestingly, the recognition index of the animals fed hydrolysate supplementation was significantly different from the chance level, meaning that these mice recognized and preferentially explored the novel arm ($t = 2.426$, $p < 0.05$).

Then the effect of the hydrolysate supplementation on spatial learning and long-term memory was assessed in a Morris water maze. To first acquire the procedural aspects of the task such as swimming and climbing onto the platform and evaluate their visuo-motor abilities, mice were trained to find a visible platform in the Morris water maze without distal cues. As aged mice swam slower than adult mice (age effect [$F_{(1,43)} = 14.867$, $p < 0.001$]), the distance travelled to reach the platform was chosen as a more appropriate measure to show the acquisition rate for the spatial learning. All groups had similar visual abilities and did not display any impairment during the cued learning. Indeed, all groups travelled similar distances to reach the visible platform (no effect of age [$F_{(1,29)} = 0.136$, $p = 0.715$] and diet [$F_{(1,29)} = 0.098$, $p = 0.756$] and no age × diet interaction [$F_{(1,29)} = 0.1894$, $p = 0.667$]) (Fig. 3C). Mice were then trained in the Morris water maze to evaluate the effects of age and hydrolysate supplementation on spatial learning and memory. All animals travelled significantly less distance over the four days of training [day effect, $F_{(3,126)} = 23.941$, $p < 0.001$] indicating that all groups learnt the platform location (Fig. 3D). However, aged mice travelled significantly longer distance than adult mice to find the platform,

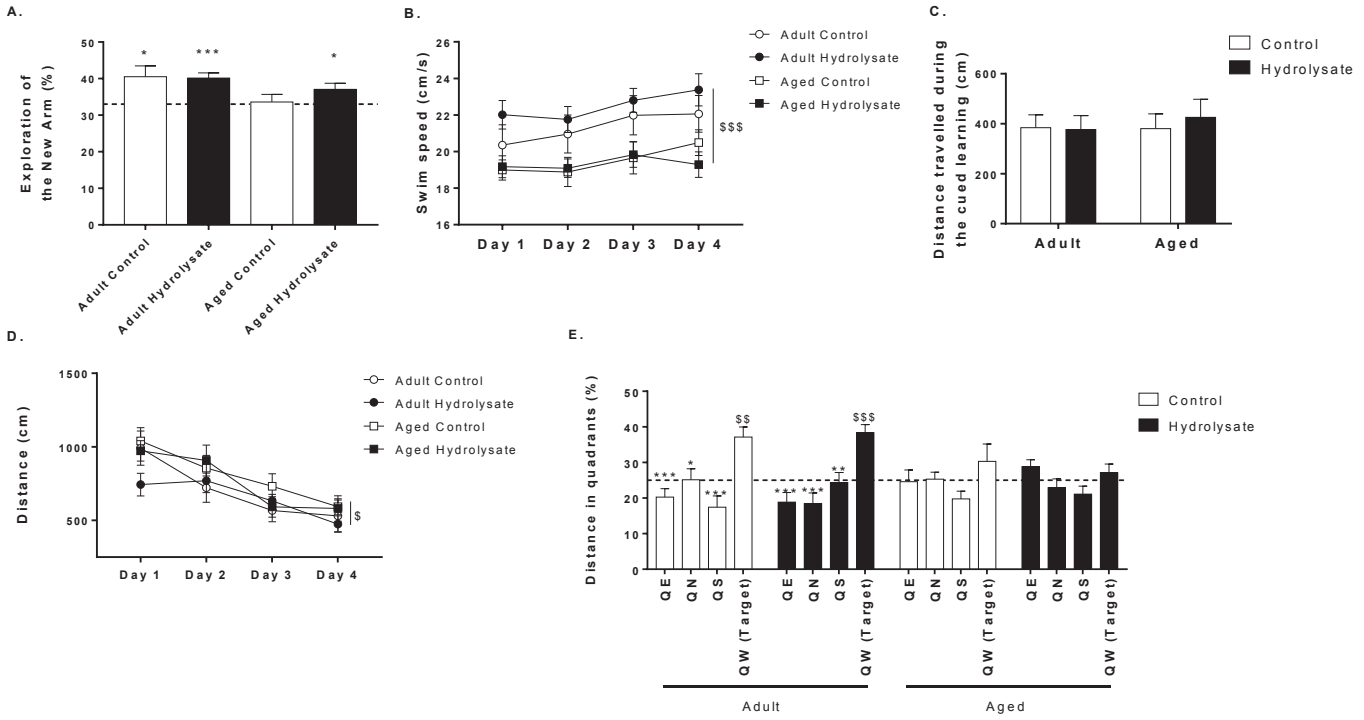

Fig. 3. Effects of the fish hydrolysate supplementation on short-term memory, spatial learning and long-term memory. (A) Recognition indexes of the new arm after a 1 h ITI in adult and aged mice fed the control diet or the hydrolysate-enriched diet. The dotted line corresponds to chance level (33%) (*p < 0.05, ***p < 0.001 vs. chance level by one sample *t*-test). (B) Swim speed during learning (age effect: $$$p < 0.001 by 3-way ANOVA with repeated measures). (C) Distance travelled during the cued learning (D) Distance covered to reach the platform over the 4 consecutive days of spatial learning (day effect p < 0.001; age effect $p < 0.05 by 3-way ANOVA with repeated measures). (E) Percentage of distance travelled in quadrants during the probe test. The dotted line represents chance level (25%) ($ $p < 0.01, $$$p < 0.001 vs. chance level by one sample *t*-test. *p < 0.05, **p < 0.01, ***p < 0.001 compared to QW (Target) by One-way ANOVA and Dunnett's multiple comparison test; n = 11–13 per group). QE: quadrant east, QN: quadrant north; QS: quadrant south; QW: quadrant west.

revealing spatial learning deficits (age effect [$F_{(1,42)}$ = 4.51, p < 0.05]). Mice under the hydrolysate enriched-diet did not show better performance than mice fed a control diet (diet effect [$F_{(1,42)}$ = 0.6544, p = 0.423]).

Spatial memory was assessed during the probe test 72 h after the last day of training. As the swim speed was still affected by age (age effect [$F_{(1,43)}$ = 10.01, p < 0.01]; data not shown), the travelled distance has been chosen as the most appropriate parameter. One sample *t*-test compared to the chance level (25%) showed that adult mice travelled more distance in the target quadrant (adult control: t = 4.197, p < 0.01; adult supplemented: t = 5.701, p < 0.001) (Fig. 3E). This reveals that 72 h after the last day of training mice remembered the location of the platform and did not display memory alterations. Aged control mice did not travel more distance in the target quadrant (t = 1.149, p = 0.277), suggesting that they presented memory impairment since they failed to recognize the target quadrant. This impairment was not prevented by the hydrolysate supplementation (t = 0.9886, p = 0.342) (Fig. 3E).

3.3. Fish hydrolysate supplementation promotes an earlier use of spatial strategies during spatial learning

To go further in the description of age-induced spatial learning deficits and potential beneficial effects of the fish hydrolysate supplementation, we analyzed the navigation path (Fig. 2).

The analysis of the navigational path revealed that all mice from the four groups moved from non-spatial to spatial strategies over the days of learning (Fig. 4A). Indeed, more and more trials were completed with spatial strategies along the training days (day effect: [$F_{(3,126)}$ = 22.28, p < 0.001]). However, aged mice took more time to adopt spatial strategies than adult mice (age effect: [$F_{(1,42)}$ = 4.685, p < 0.05]; Supplementary Fig. 2). To go further, the use of spatial strategies was analyzed for each day of training. Analysis performed on the first day of learning revealed that mice fed with the hydrolysate-enriched diet used more spatial strategies than mice fed the control diet (diet effect [$F_{(1,43)}$ = 4.778, p < 0.05]) suggesting that the fish hydrolysate supplementation promotes an early use of spatial strategies (Fig. 4B). During the second day of training, diet effect was no longer observed [$F_{(1,43)}$ = 0.1275, p = 0.723] and aged mice used less spatial strategies than adult mice as shown in Fig. 4C (age effect [$F_{(1,43)}$ = 4.824, p < 0.05]). From day 3 of training, no differences between groups were found suggesting that all groups used the same percentage of spatial strategies (Fig. 4D and E).

3.4. Fish hydrolysate supplementation decreases basal anxiety-like behaviour and modulates stress response consecutive to restraint stress

Anxiety-like behaviour and locomotion were assessed in the open-field test. First, aged animals showed a significant decrease of their locomotor activity compared to adult animals (age effect [$F_{(1,44)}$ = 15.93, p < 0.001]; Supplementary Fig. 3A). No effect of the hydrolysate supplementation was found ([$F_{(1,44)}$ = 2.341, p = 0.133]; Supplementary Fig. 3A). Aged mice spent less time at the centre than adult mice (age effect [$F_{(1,44)}$ = 6.259, p < 0.05]) (Fig. 5A). There were no effect of the hydrolysate supplementation ([$F_{(1,44)}$ = 0.4071, p = 0.527]). However, the age × diet interaction was significant ([$F_{(1,44)}$ = 4.725, p < 0.05]). Aged control mice spent significantly less time in the central area than adult control mice and adult supplemented mice (Fisher's LSD test: adult control vs. aged control p < 0.01; adult hydrolysate vs. aged control p < 0.05) suggesting anxiety-like behaviour. Interestingly, aged mice fed with the hydrolysate tend to spend more time in the centre than aged control mice (p = 0.0534). Centre crossings were also lower in aged mice compared to adult mice (age effect [$F_{(1,44)}$ = 18.17, p < 0.001]) and tended to be higher in mice supplemented with the hydrolysate (diet effect [$F_{(1,44)}$ = 2.288, p = 0.1375]) (Supplementary Fig. 3B). No

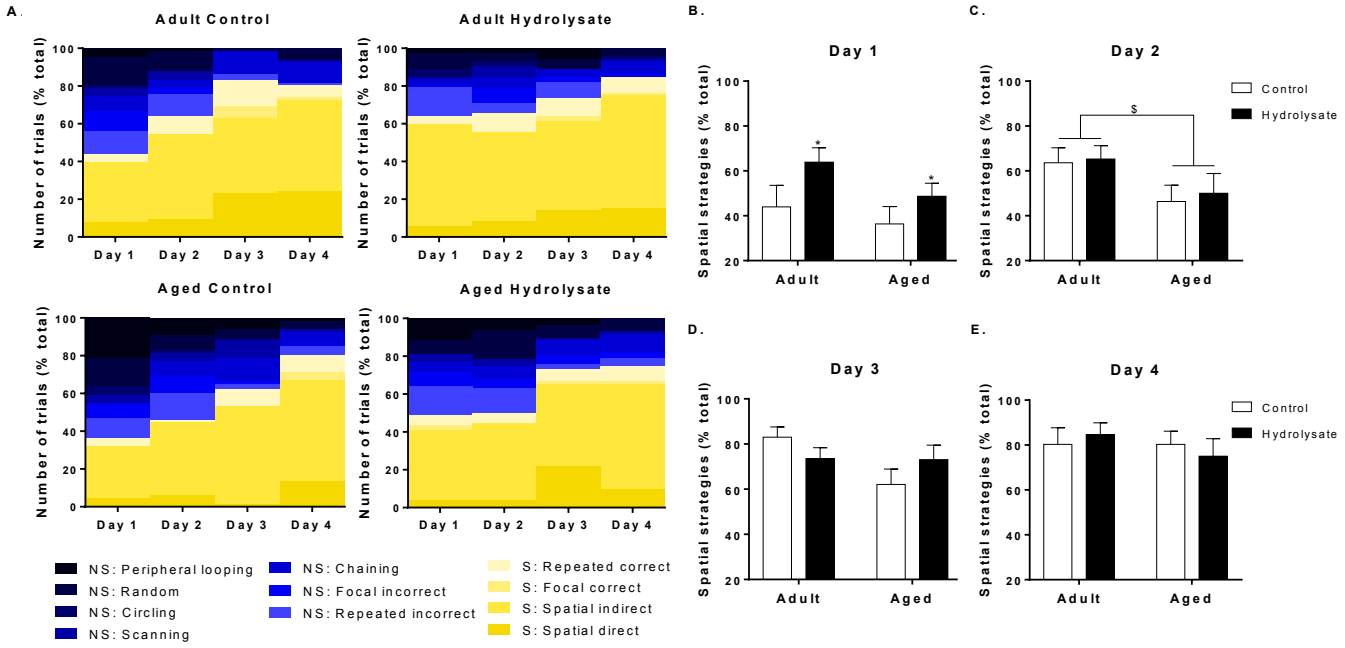

Fig. 4. Effects of the fish hydrolysate supplementation on search strategies during spatial learning in the Morris water maze. (A) Navigational strategies used during spatial learning for each group. (B-E) Percentage of spatial strategies used by each group for each day of spatial learning (diet effect *p < 0.05; age effect $p < 0.05 by 2-way ANOVA; n = 11–13 per group). NS: non spatial strategies; S: spatial strategies.

interaction age × diet was found ($F_{(1,44)}$ = 1.67, p = 0.203) (Supplementary Fig. 3B). An age effect was also found for thigmotaxis, which was higher in aged mice than in adult mice (age effect $F_{(1,44)}$ = 5.239; p < 0.05) (Supplementary Fig. 3C). No significant effect of the diet and no interaction age × diet were found on this parameter (diet effect [$F_{(1,44)}$ = 0.027, p = 0.8705]; interaction [$F_{(1,44)}$ = 0.014, p = 0.9062]) (Supplementary Fig. 3C).

Corticosterone level was measured in plasma prepared from blood samples drawn before the induction of the stress and 30, 60 and 90 min after the start of the restraint protocol. As expected, plasmatic corticosterone levels were increased following the restraint protocol (time effect [$F_{(3,117)}$ = 353.901, p < 0.001]) (Fig. 5B). However, aged mice tended to show a lower corticosterone level compared to adult mice (age effect [$F_{(1,39)}$ = 4.01, p = 0.0522]). Moreover, supplemented mice displayed greater plasmatic corticosterone levels than control mice consecutive to mild-stress protocol (diet effect [$F_{(1,39)}$ = 4.611, p < 0.05]). Interestingly, the interaction age × diet was significant ([$F_{(1,39)}$ = 16.774, p < 0.001]). Aged control mice had lower plasmatic corticosterone levels compared to adult mice, suggesting alterations of their stress response (Fisher's LSD test: adult control vs. aged control p < 0.001; adult hydrolysate vs. aged control p < 0.01). These age-induced alterations were prevented by the hydrolysate supplementation (Fisher's LSD test: aged control vs. aged hydrolysate p < 0.001) To go further, the interaction time × age × diet was significant ($F_{(3,117)}$ = 8.358, p < 0.001). Levels of corticosterone were not different between adult and aged mice fed either the control or the hydrolysate-enriched diet at T0 and at T30 (Fig. 5B). However, at T60 and at T90, aged control mice secreted less corticosterone following the restraint protocol than adult control mice and adult hydrolysate mice (Fisher's LSD test: adult control vs. aged control p < 0.05 (T60) and p < 0.001 (T90); adult hydrolysate vs. aged control p < 0.05 (T60) and p < 0.01 (T90)). This corticosterone level was restored by the hydrolysate supplementation (Fisher's LSD test: aged control vs. aged hydrolysate p < 0.05 (T60) and p < 0.001 (T90)) (Fig. 5B). To go further, genes involved in hypothalamic stress response were quantified by RT-qPCR. As shown in Fig. 5C-E, age didn't impact the mRNA expression of *Crhr1*, *Crhbp* and *Hsd11β1* (age effect [$F_{(1,27)}$ = 2.357, p = 0.1363]; [$F_{(1,27)}$ = 0.023, p = 0.8802] and [$F_{(1,27)}$ = 2.383, p = 0.1343] respectively). Interestingly, the hydrolysate supplementation significantly increased the stress responsive gene expressions of *Crhr1*, *Crhbp* (diet effect [$F_{(1,27)}$ = 6.028, p < 0.05] and [$F_{(1,27)}$ = 9.824, p < 0.01] respectively) and tended to improve the expression of *Hsd11β1* (diet effect [$F_{(1,27)}$ = 3.779, p = 0.0624]), suggesting that mice fed the hydrolysate supplementation had a greater modulation of stress responsive gene expression (Fig. 5C-E). No effects were found on mRNA expression of GR, MR and Crh (Fig. 5F-H).

3.5. Fish hydrolysate supplementation decreases Iba1-positive cell number and CD11b expression after acute mild-stress protocol

As correlations between the incidence of cognitive disorders and the level of inflammatory factors have been shown in the elderly, we analyzed microgliosis markers Iba1 and CD11b as well as the expression of pro-inflammatory cytokines in the hippocampus of mice.

The analysis showed that the number of Iba1 positive cells and *Cd11b* mRNA expression were significantly higher in the hippocampus of aged mice as compared to adult mice (age effect: [$F_{(1,12)}$ = 10.71, p < 0.01] and [$F_{(1,26)}$ = 10.3, p < 0.01], respectively) (Fig. 6A and B). Interestingly, the hydrolysate supplementation reduced the number of Iba1 positive microglia (diet effect: [$F_{(1,12)}$ = 5.323, p < 0.05]) and significantly decreased mRNA levels of *Cd11b* (diet effect [$F_{(1,26)}$ = 7.12, p < 0.05]).

Gene expression of the pro-inflammatory cytokines *IL-6, IL-1β* and *Tnf-α* was also investigated. The mRNA expression of *IL-6* and *Tnf-α* was significantly higher in the hippocampus of aged mice as compared to adult mice (age effect: IL-6 [$F_{(1,27)}$ = 14.9, p < 0.001] and TNF-α [$F_{(1,28)}$ = 5.479, p < 0.05]) but was not affected by the hydrolysate supplementation (IL-6 [$F_{(1,27)}$ = 0.104, p = 0.75] and TNF-α [$F_{(1,28)}$ = 0.255, p = 0.617) (Fig. 6C and D). No effect was found on *IL-1β* gene expression (Fig. 6E).

3.6. Inflammatory markers are associated with anxiety and cognitive parameters

Correlation analyses demonstrated that the number of Iba1-positive microglia was positively correlated to basal corticosterone level and negatively to the distance travelled in the target quadrant and to the number of spatial strategies used. Moreover, *IL-6* and *Tnf-α* expression

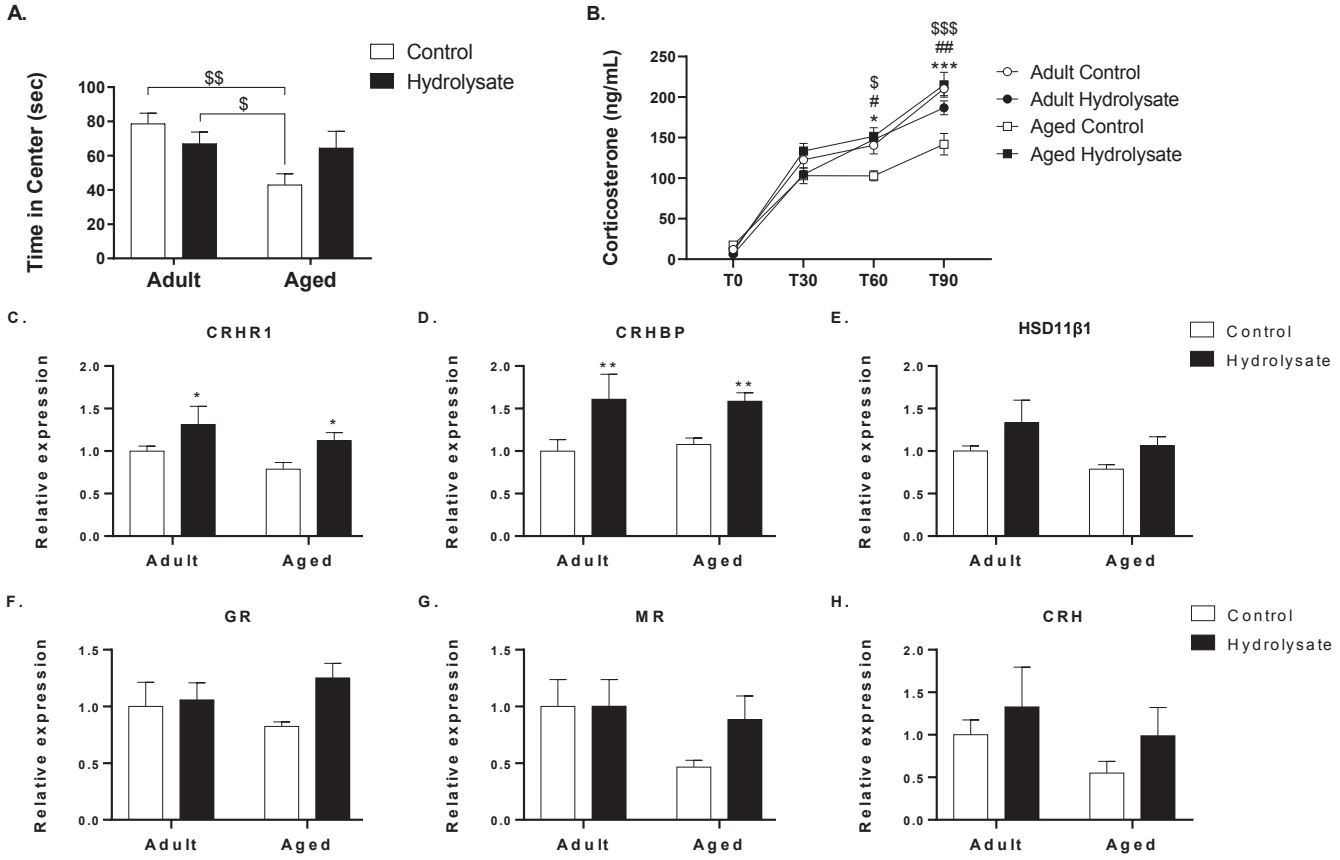

Fig. 5. Effects of the fish hydrolysate supplementation on basal anxiety-like behaviour and stress response after acute mild-stress protocol. (A) Time spent in the central area of the open-field ($p < 0.05, $$p < 0.01 by 2-way ANOVA and Fisher's LSD *post-hoc* test; n = 11–13 per group). (B) Corticosterone level evolution in adult and aged mice fed the control or the hydrolysate-enriched diet before the induction of the stress protocol and at T30min, T60min and T90min after the stress ($p < 0.05, $$$p < 0.001 Adult control vs. Aged control; #p < 0.05, ##p < 0.01 Adult hydrolysate vs. Aged control; *p < 0.05, ***p < 0.001 Aged hydrolysate vs. Aged control by 3-way ANOVA with repeated measures and Fisher's LSD *post-hoc* test; n = 11–13 per group). (C–H) Stress responsive gene expression in the hypothalamus after restraint protocol (diet effect *p < 0.05, **p < 0.01 by 2-way ANOVA; n = 7–9 per group). Data are presented as means ± SEM. CRHR1: Corticotropin releasing hormone receptor 1; CRHBP: Corticotropin releasing hormone binding protein; HSD11β1: 11β-Hydroxysteroid dehydrogenase type 1; GR: Glucocorticoid receptor; MR: Mineralocorticoid receptor; CRH: Corticotropin releasing hormone.

was negatively correlated to the time spent in the centre area of the open field. TNF-α and IL-1β expression was also negatively correlated to the number of spatial strategies used (Fig. 7). Taken together, those correlations highlighted a link between inflammation during aging, anxiety and cognitive ability.

3.7. Fish hydrolysate decreases LPS-induced expression of pro-inflammatory cytokines in vitro

To better characterize the immunomodulatory properties of the hydrolysate, a co-culture model of neuronal and microglial-like cells has been used. Gene expression of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) was measured in BV2 cells.

As shown in Fig. 8, LPS increased significantly the pro-inflammatory cytokine expression (LPS effect: IL-6 [$F_{(1,38)} = 263.8$, $p < 0.001$]; IL-1β [$F_{(1,38)} = 104.8$, $p < 0.001$]; TNF-α [$F_{(1,38)} = 40.84$, $p < 0.001$]). The hydrolysate treatment decreased IL-6 and IL-1β mRNA expression but did not affect TNF-α mRNA expression (treatment effect: IL-6 [$F_{(1,38)} = 17.12$, $p < 0.001$]; IL-1β [$F_{(1,38)} = 6.404$, $p < 0.05$]; TNF-α [$F_{(1,38)} = 3.088$, $p = 0.087$]). The 2-way ANOVA analysis also revealed a LPS × treatment interaction for IL-6 and IL-1β (IL-6 [$F_{(1,38)} = 21.8$, $p < 0.001$]; IL-1β [$F_{(1,38)} = 7.51$, $p < 0.01$]). Indeed, the hydrolysate treatment significantly reduced the LPS-induced expression of IL-6 and IL-1β (Fisher's LSD test: IL-6: $p < 0.001$; IL-1β: $p < 0.01$).

3.8. Fish hydrolysate treatment increases the expression of neurotrophins in both inflammatory and basal conditions in vitro

Similarly, the effects of the hydrolysate on the neurotrophic factors (BDNF and NGF) were assessed in the co-culture model. In microglial cells, LPS and the hydrolysate treatment increased significantly BDNF mRNA expression (LPS effect [$F_{(1,38)} = 5.537$, $p < 0.05$] ; treatment effect : [$F_{(1,38)} = 5.48$, $p < 0.05$]) (Fig. 9A). Moreover, we observed an LPS × treatment interaction ($F_{(1,38)} = 4.61$, $p < 0.05$; Fisher's LSD test: $p < 0.05$) suggesting that microglial cells treated with the hydrolysate showed a significant increase of BDNF mRNA expression under inflammatory conditions.

In neuronal cells, LPS had no effect on the mRNA expression of BDNF and NGF (Fig. 9B). Interestingly, the hydrolysate treatment significantly increased NGF expression (treatment effect: [$F_{(1,40)} = 6.993$, $p < 0.05$]) and tended to increase BDNF expression (treatment effect [$F_{(1,39)} = 4.029$, $p = 0.0517$]) in both saline and inflammatory conditions.

4. Discussion

This work provides evidence of a beneficial effect of the fish-derived hydrolysate on age-induced spatial memory deficits. Indeed, this hydrolysate ameliorated short-term memory and promoted the earlier use of spatial strategies. Moreover, it presented anti-inflammatory, anxiolytic and neuroprotective properties.

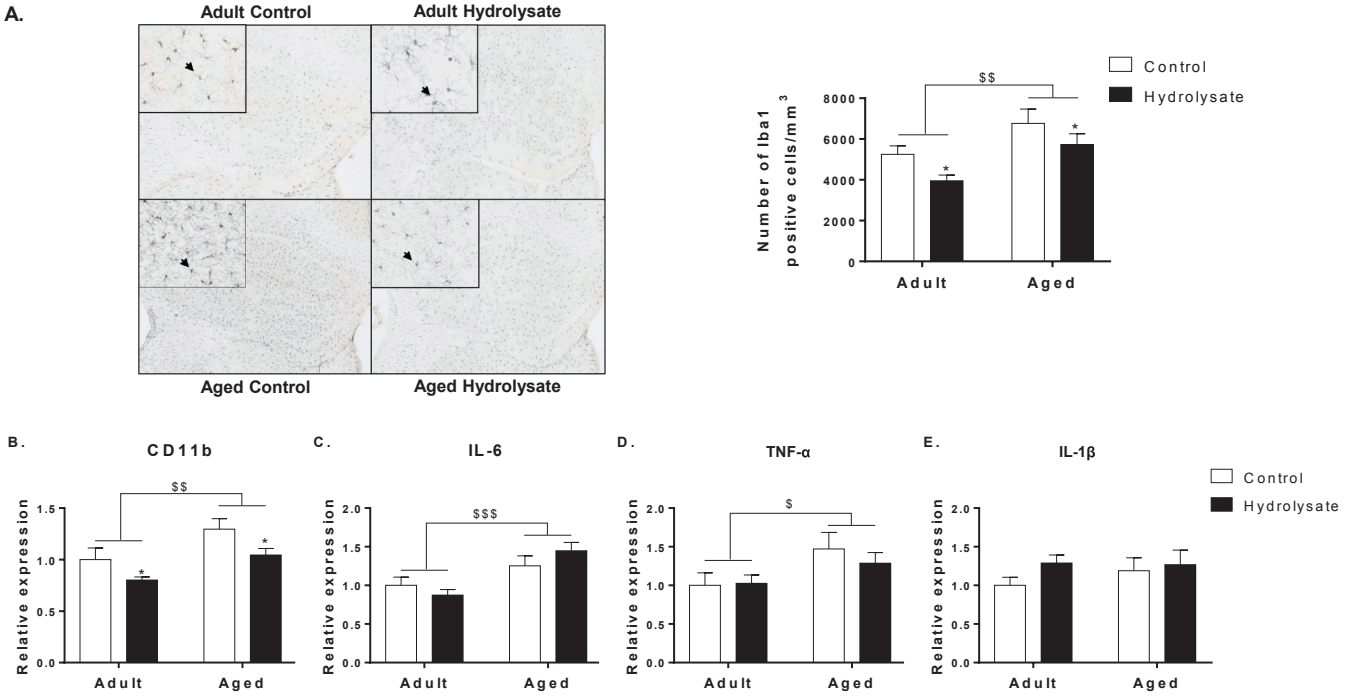

Fig. 6. Effect of the fish hydrolysate supplementation on Iba1-positive cell number and CD11b and pro-inflammatory cytokines expression in the hippocampus after acute mild-stress protocol. (A) Representative images of Iba1-positive microglia (scale bars: 200 μM for the hippocampus and 100 μM for zooms) and analysis of the number of Iba1-positive cells in the hippocampus (age effect $$p < 0.01; diet effect *p < 0.05 by 2-way ANOVA; n = 4 per group). (B-E) CD11b and pro-inflammatory cytokines IL-6, TNF-α and IL-1β mRNA expressions in the hippocampus of adult and aged mice fed the control diet or the hydrolysate-enriched diet for 6 weeks (age effect $p < 0.05, $$p < 0.01, $$$p < 0.001; diet effect *p < 0.05 by 2-way ANOVA; n = 7–9 per group). Data are presented as means ± SEM.

Short-term memory was evaluated in the Y maze test, which has been shown to be impaired during aging (Labrousse et al., 2012; Moranis et al., 2012). Supplementation with the fish hydrolysate prevented the spatial short-term memory deficits occurring during aging. This effect could be attributed to the low molecular weight peptides and/or to the n-3 LC-PUFAs from the fish hydrolysate. Several papers have reported the benefits of n-3 LC-PUFAs on cognition. Indeed, observational and interventional studies have demonstrated that a supplementation with n-3 LC-PUFAs could improve cognitive function in healthy adults (Kalmijn et al., 2004; Muldoon et al., 2010; Stonehouse et al., 2013) and in the elderly (Vakhapova et al., 2010; Witte et al., 2014; Yurko-Mauro et al., 2010). Similar effects have been shown in adult (Moriguchi and Salem, 2003; Pan et al., 2011) and in aged rodents (Cutuli et al., 2014; Hashimoto et al., 2015; Labrousse et al., 2012). To our knowledge, only one study has highlighted beneficial effects of a supplementation with fish protein hydrolysate enriched in DHA on short-term memory in men and women between 45 and 65 years old (Le Poncin-Séac'h and Le Poncin-Lafitte, 2010). As the fish hydrolysate used contained 10 to 100 times less n-3 LC-PUFAs than the efficient doses reported in the literature, we hypothesized that either the effects of n-3 LC-PUFAs were potentiated by the low molecular weight peptides and/or peptides alone had an effect on memory deficits. This point needs to be further explored.

Spatial learning and long-term memory were tested in Morris water maze test which is particularly sensitive to detect age-related deficits (de Fiebre et al., 2006; Frick et al., 1995; Lindner, 1997). Indeed, during aging, spatial learning and long-term memory are affected as shown here in this study, in rodents (Bensalem et al., 2016; de Fiebre et al., 2006; Frick et al., 1995; Lindner, 1997; Markowska et al., 1989), in monkeys and in humans (Gazova et al., 2013; Grady et al., 2003; Lai et al., 1995; Rapp et al., 1997; Wilkniss et al., 1997). The fish hydrolysate supplementation did not improve spatial learning but influenced spatial navigation strategies. Spatial learning strategies are classified according to the involvement or not of the body position in the environment. Adult animals first adopted non-spatial strategies that are egocentric navigation strategies body-centred and dependent on the individual or animal's position and also on the start location. They do not require the use of spatial information (Maguire et al., 1998; White and McDonald, 2002). In this case, previously learned movements were repeated to reach the platform, which is often assimilated to a cued-guided response (Packard and Knowlton, 2002; White and McDonald, 2002). Then, adult animals moved on intermediate strategies, "focal incorrect" (intensive search of the platform in a small and wrong portion of the pool) and "repeated incorrect" strategies (swimming in a precise direction that does not correspond to the platform location and that is repeated several times) suggesting that distal cues may be used in a wrong manner and that individual or animal's cognitive map is not fully acquired. Finally, they used spatial strategies that are allocentric navigation strategies, world-centred and relied on external cues independently of the individual or animal's position (Astur et al., 2002; Brody and Holtzman, 2006; Cain et al., 1997; Chapillon, 1999; Garthe et al., 2009; Janus, 2004; Matthews and Best, 1997; Ruediger et al., 2012). In our study, aged mice took more time to adopt spatial strategies to solve the task. This has been already suggested in several studies reporting that aging affects allocentric strategies, which are hippocampus-dependent, promotes the use of egocentric strategies, more striatum-dependent, and delays the evolution from non-spatial to spatial strategies in mice at 12 months of age and over (Bensalem et al., 2016; Fouquet et al., 2011; Gil-Mohapel et al., 2013; Kim et al., 2001; Martel et al., 2007). This has been also observed in an adapted version of the Morris water maze for humans in which allocentric spatial navigation learning, but not egocentric navigation, is particularly altered over 70 (Gazova et al., 2013). We show for the first time that a supplementation with the fish hydrolysate promotes an earlier use of spatial strategies in adult and aged mice but had no effect on long-term memory. However, supplementation with n-3 LC-PUFAs enhances memory performances in aged mice and rats (Cutuli et al., 2014; Kelly et al., 2011; Létondor et al., 2016). Also, supplementation with low molecular weight peptides (milk, marine-derived as well as walnut-derived peptides) improves memory in models of AD or D-galactose-induced aging in mice (Chai et al., 2016; Chen et al., 2015; Feng et al., 2018; Min et al., 2017; Pei et al., 2010). The differences we observed in our conditions, could be attributed to the length of the spatial learning sessions which was too short (4 days) to allow memory consolidation and to

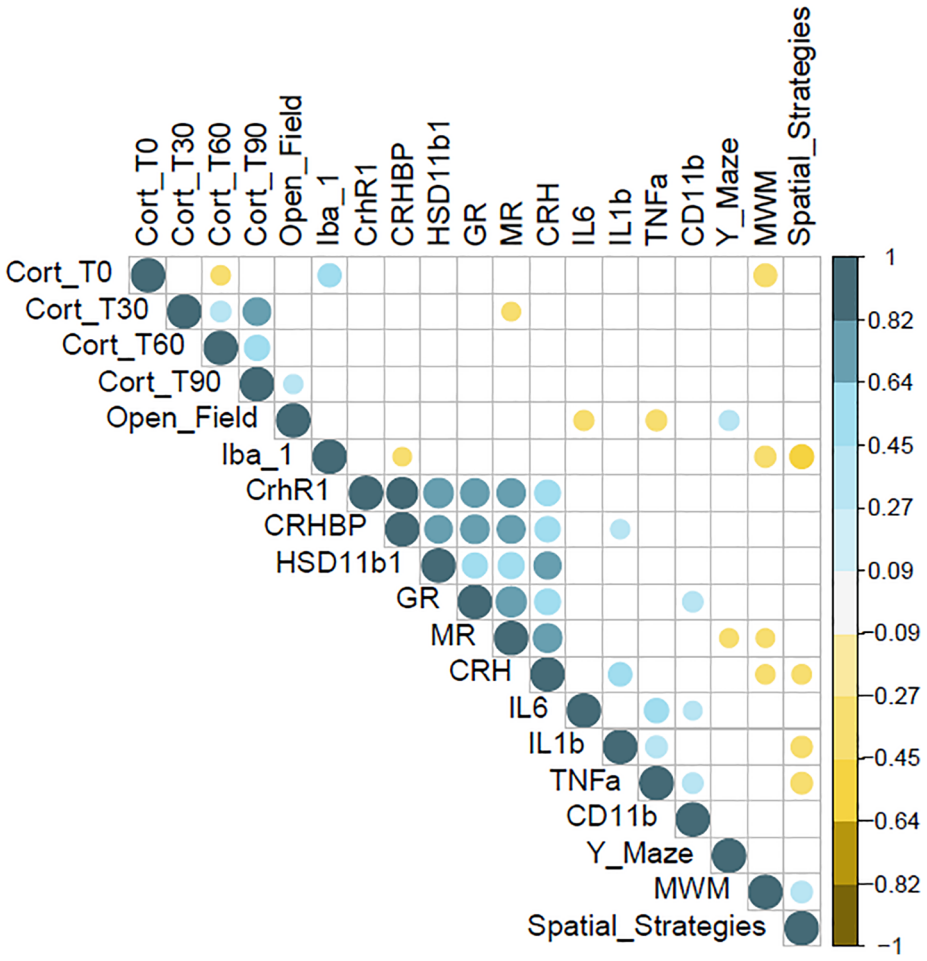

Fig. 7. Correlation matrix of anxiety-like behaviour measures, cognitive parameters, inflammatory markers. Positive correlations are represented in blue, negative correlations in yellow. No significant correlations are represented by a blank. Cort_T0: corticosterone level at T0; Cort_T30: corticosterone level at T30; Cort_T60: corticosterone level at T60; Cort_T90: corticosterone level at T90; Open_Field: time spent in the centre; Y_Maze: percentage of exploration of the new arm; MWM: percentage of exploration of the target quadrant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a longer time of the ITI (72 h), parameters that were defined in the study of Bensalem et al (2016). Indeed, other protocols present a number of spatial training sessions comprised between 4 and 16 days and with an ITI of 24 h (Chai et al., 2016; Cutuli et al., 2014; Feng et al., 2018; Létondor et al., 2016; Min et al., 2017; Pei et al., 2010; Weitzner et al., 2015). Another hypothesis is that the fish hydrolysate supplementation could not balance long-term memory deficits in this challenging protocol due to low amounts of n-3 LC-PUFAs and peptides it contained. Indeed, other studies used 22 to 100 times more n-3 LC-PUFAs (Cutuli et al., 2014; Kelly et al., 2011; Létondor et al., 2016) and 2 to 5 times more peptides (Chai et al., 2016; Chen et al., 2015; Feng et al., 2018; Min et al., 2017).

Memory deficits observed in the present study can also be accentuated by anxiety disorders. Indeed, the works of Knight and Durbin (2015) and Puigoriol-Illamola et al (2018) have established a relationship between anxiety and memory capacity. They have shown that anxious people have an increased risk of developing memory deficits, and that inhibition of the glucocorticoid synthesis enzyme prevents cognitive impairment (Knight and Durbin, 2015; Puigoriol-Illamola et al., 2018). In the present study, aged mice displayed higher anxiety-like behaviour compared to their adult counterparts in the open-field test, associated with an increase of basal plasmatic corticosterone level. Furthermore, following a restraint protocol, their stress response was altered. Although plasmatic levels of corticosterone were high as a result of successive mandibular vein withdrawals, aged mice displayed lower level of corticosterone in response to stress compared to adult control mice, reinforcing the fact that their stress response is insufficient and suggesting an impaired HPA axis activity. These observations corroborate the results of Tronche et al (2010) reporting that the mechanisms of return to homeostasis are disrupted in

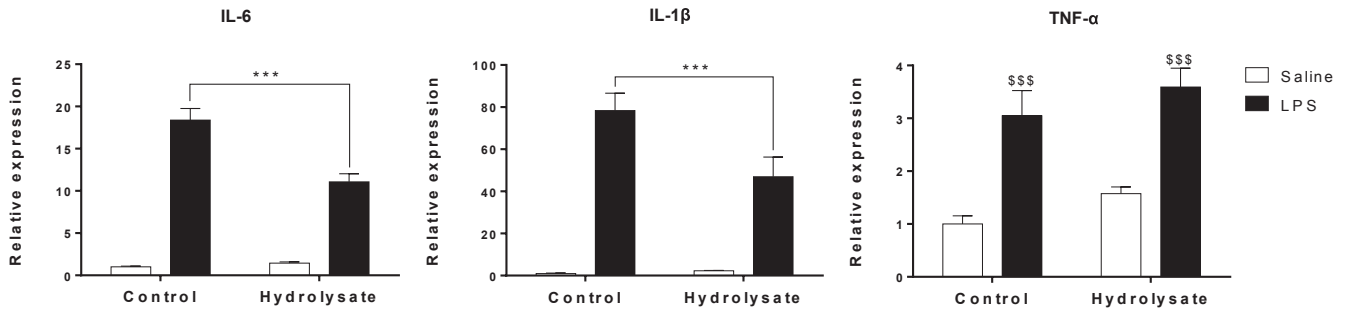

Fig. 8. Pro-inflammatory cytokine expression in microglia co-cultured with neurons. IL-6, IL-1β and TNF-α mRNA expression in BV2 microglial cells pre-treated with the hydrolysate or not and incubated with LPS or not (LPS effect $$$p < 0.001. ***p < 0.001 Control LPS vs. Hydrolysate LPS by 2-way ANOVA and Fisher's LSD *post-hoc* test; n = 11 per group). Data are presented as means ± SEM. LPS: lipopolysaccharide.

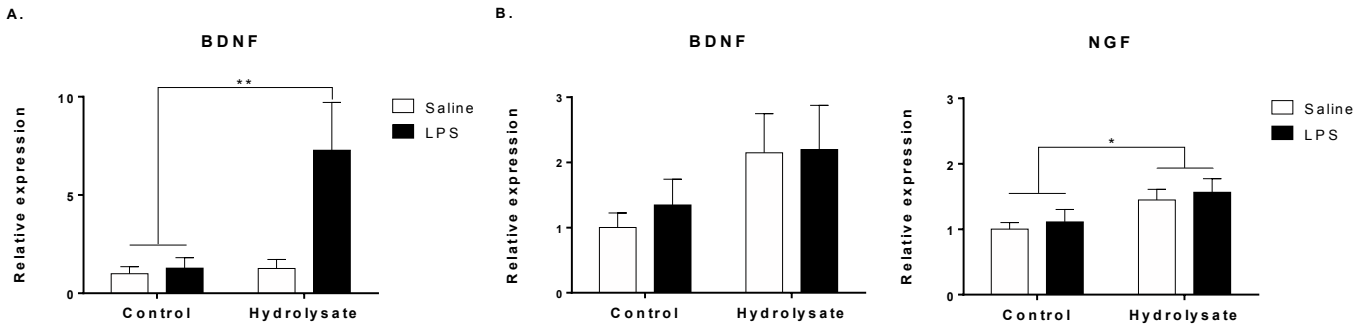

Fig. 9. Neurotrophin expression in co-cultured microglial and neuronal cells. BDNF and NGF mRNA expression in BV2 microglial cells and HT22 neuronal cells pre-treated with the hydrolysate or not and incubated with LPS or not. (A) BDNF expression in BV2 cells (**p < 0.05 by 2-way ANOVA and Fisher's LSD *post-hoc* test; n = 11 per group). (B) BDNF and NGF expression in HT22 cells (treatment effect *p < 0.05 by 2-way ANOVA; n = 11 per group). Data are presented as means ± SEM. LPS: lipopolysaccharide.

older mice (Tronche et al., 2010). Fish hydrolysate supplementation prevented age-induced anxiety-like behaviour and restored basal corticosterone levels in aged mice. This is consistent with previous works reporting the individual effects of peptides or n-3 PUFAs. Indeed, peptides extracted from cod and mackerel (Gabolysat® PC60) or salmon hydrolysate both reduce anxiety-like behaviour in rats and mice (Belhaj et al., 2013; Bernet et al., 2000). Furthermore, several studies have highlighted that blood and brain levels of n-3 LC-PUFAs are decreased in humans presenting emotional disorders, including anxiety (Green et al., 2006; Liu et al., 2013; Tiemeier et al., 2003) as well as in mice (Lafourcade et al., 2011; Larrieu et al., 2014). These disorders are reduced after a supplementation in n-3 LC-PUFAs in humans (Kiecolt-Glaser et al., 2011; Yehuda et al., 2005) and in rodents (Ferraz et al., 2011; Larrieu et al., 2014). In addition, the fish hydrolysate supplementation enhances the hypothalamic expression of genes involved in the stress response (Datson et al., 2012). This is particularly interesting since a reduction in stress reactivity is associated with adaptation difficulties in all circumstances of daily life in the elderly (Wurm et al., 2004). In this study, only male mice have been included but it can be assumed that sex differences can be observed. Indeed, basal and stimulated HPA activities are increased in females compared to male rodents and have been linked to hormones (Minni et al., 2014; Rhodes and Rubin, 1999).

We hypothesized that the alterations of memory and anxiety observed in aged animals could be due to impairments in hippocampus, one of the main brain structures involved in cognition, as supported by the correlation analyses. The major roles of hippocampus include consolidation of episodic memory and context-dependent spatial learning *via* the formation, the conservation and the use of spatial cognitive maps of the environment (El-Falougy and Benuska, 2006; Morris et al., 1986; O'Keefe and Conway, 1978). In Humans, episodic memory comprises encoding, storage, and retrieval processes allowing the recall of personal past events (Thakral et al., 2017). In animals, episodic memory is modelled by the study of the ability to perform spatial learning and memory tasks, both hippocampal-dependents (Sharma et al., 2010). Hippocampus is strongly impaired during aging, as demonstrated in humans (Gazova et al., 2013; Grady et al., 2003), in monkeys (Gallagher and Rapp, 1997; Lai et al., 1995) and in rodents (Barnes, 1979; Markowska et al., 1989). Indeed, many studies have identified age-related deficits in different hippocampal-dependent tasks assessing spatial learning and memory in humans (Driscoll et al., 2003; Nemmi et al., 2017; Persson et al., 2012) and in rodents (Drapeau et al., 2003; Rosenzweig and Barnes, 2003). Experimental data have shown in older rodents that age-related memory deficits, including spatial learning deficits, are similar to those induced by hippocampal lesions (Stoelzel et al., 2002; Wang et al., 2006). The aged-induced impairments of hippocampus consisted in a set-up of a chronic low-grade inflammation characterized by overexpression of pro-inflammatory cytokines, an increase in Iba1-positive cell number and CD11b gene expression, which have both been linked to the development of cognitive impairments (Barrientos et al., 2009; Labrousse et al., 2012; Rafnsson et al., 2007; Weaver et al., 2002). Microgliosis, which is characterized by an increased microglial number and, assessed by an increase in Iba-1 positive cells, could be the reflect of an overactivation of microglial cells. This overactivation is characterized by microglia releasing more pro-inflammatory cytokines and chemokines and also expressing more CD11b which could lead to more complement-mediated phagocytosis of synapses. Thus, it would enhance inflammation and amplify neuronal damage (Block and Hong, 2005; Njie et al., 2012; Teismann et al., 2003). These results are consistent with the literature since one of the hallmarks of age-dependent microglia changes is their overactivation (Griffin et al., 2006; Harry, 2013; Wong et al., 2005) and the increase in their number (Mouton et al., 2002). This aged-induced microgliosis was reversed by the fish hydrolysate supplementation. Previous studies have only reported the effect of endogenous n-3 LC-PUFAs in Fat-1 mice or dietary n-3 LC-PUFAs supplementation in an IL-1β administration mouse model (Dong et al., 2018; Gu et al., 2018). We are the first to report an effect of n-3 LC-PUFAs and low molecular weight peptides on microgliosis. However, fish hydrolysate had no effect on the expression of hippocampal pro-inflammatory cytokines whereas peptides and n-3 LC-PUFAs have both individual beneficial effect on inflammation. Indeed, milk peptides suppress the expression of inflammatory factors such as TNF-α, MCP-1 or iNOS in the hippocampus of AD mice (Min et al., 2017). Moreover, n-3 LC-PUFA supplementation in aged mice decreases the hippocampal inflammatory IL-6, IL-1β, TNF-α markers (Labrousse et al., 2012). We hypothesized that the amount of low molecular weight peptides and n-3 LC-PUFAs in the diet was sufficient to reduce microgliosis but was too low to reduce hippocampal brain pro-inflammatory cytokines. Indeed, the fish hydrolysate treatment decreased the LPS-induced expression of pro-inflammatory cytokines IL-6 and IL-1β *in vitro*, suggesting a potential anti-inflammatory activity of the hydrolysate. These results are consistent with data obtained *in vitro* on the effect of DHA on microglial cells (Ajmone-Cat et al., 2012; De Smedt-Peyrusse et al., 2008; Inoue et al., 2017; Lu et al., 2010), on the effect of fish-derived peptides on macrophage cell line (Ahn et al., 2015) and of food-derived low-molecular weight peptides (other than fish-derived) in various models of cell lines (Majumder et al., 2013; Nakamura et al., 2013; Zhang et al., 2015). In addition, the fish hydrolysate treatment increased the production of neurotrophic factors NGF and BDNF. This is particularly interesting since an intracerebral infusion of NGF increases neurogenesis and improves spatial memory, and an intracerebral infusion of BDNF decreases neuroinflammation, *via* the inhibition of the NF-κB pathway (Xu et al., 2017; Zhang et al., 2013). Therefore, fish hydrolysate containing n-3 LC-PUFAs and low molecular weight peptides is promising in terms of protection against inflammation and in neuroprotection.

5. Conclusion

The present study is the first to demonstrate a beneficial effect of a fish hydrolysate containing n-3 LC-PUFAs and low molecular weight

peptides on cognitive functions, anxiety-like behaviour and stress response in aged mice. This fish hydrolysate obtained through the valorisation of marine by-products in the context of sustainable development remains an innovative and good candidate for the prevention of age-related cognitive decline. Further investigations are still needed to better determine the action mechanisms at the cerebral level and to characterize the bioactive peptides contained in this hydrolysate.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors Contribution

VP, SL, EB, AM, ALD and CJ devised the project, the main conceptual ideas and proof outline. MC, VP, SL, ALD and CJ conceived and designed experiments. MC, PM and CL conducted research, analysed data and performed statistical analysis. MC wrote the manuscript with support of ALD and CJ. All authors critically reviewed the manuscript and approved the final version of the manuscript.

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Appendix A. Supplementary data

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