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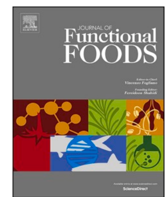

Supplementation with low molecular weight peptides from fish protein hydrolysate reduces acute mild stress-induced corticosterone secretion and modulates stress responsive gene expression in mice

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HPA axis

A B S T R A C T

First evidence started to demonstrate the anxiolytic effects of low molecular weight peptides extracted from natural products, such as fish hydrolysate, but their underlying mechanisms remain to be elucidated. The objective of this study was to evaluate the effect of a chronic administration of fish hydrolysate on stress reactivity and to understand the mechanisms involved. Stress response (corticosterone secretion, expression of stress-responsive genes) was measured in Balb/c mice supplemented with fish hydrolysate (300 mg/kg body weight) or vehicle daily for 7 days before being submitted to an acute mild stress protocol. Our results demonstrated that 30 min after stress induction, fish hydrolysate decreased corticosterone level compared to control mice. Moreover, fish hydrolysate supplementation modulated expression of stress responsive genes involved in hypothalamic pituitary adrenal axis regulation, circadian rhythm and aging process. These findings suggest that fish hydrolysate represents an innovative strategy to prevent stress-induced aversive effects and participate in stress management.

1. Introduction

Since few decades, stress incidence has dramatically increased in occidental society and stress has been called by the World Health Organization as “the health epidemic of the 21st century”. Stress triggers the activation of hypothalamo-pituitary-adrenal (HPA) axis inducing the secretion of glucocorticoids: corticosterone in rodents and cortisol in humans (Pariante & Lightman, 2008). One of the main mechanisms underlying corticosteroid action in the brain is the modification of target gene expression, the so-called genomic action of corticosteroids, through their receptors expressed throughout the brain. These receptors can act as transcription factors and induce the regulation of stress-responsive gene expression (Datson, Morsink, Meijer, & de Kloet, 2008; Hunter et al., 2016; Lupien, McEwen, Gunnar, & Heim, 2009). The genomic actions of corticosteroids can occur within 30 min after the stress and the activation of their receptors (Dong, Poellinger, Gustafsson, & Okret, 1988; Morsink, Joels, et al., 2006). It can affect severals pathways from metabolism, signal transduction, neuronal structure to circadian rhythm or mitochondrial function that participate to the regulation of stress and to the tissues homeostasis return. But if stress becomes persistent and excessive, it can result in health problems such as depression, burnout, anxiety, sleep disorders but also neurological alterations (Milczarek & Gonzales, 2009). Corticosteroids have potentially long-lasting effects on the functioning of brain region. Studies on stress at adulthood in rodents demonstrate that an acute stress inducing a larger and prolonged elevation of corticosteroid level impaired hippocampal functions (Diamond, Bennett, Fleshner, & Rose, 1992). In humans, many studies have reported elevated basal corticosteroid levels in adult with some forms of depression (Burke, Davis, Otte, & Mohr, 2005) or alteration of cognition (Lupien & Lepage, 2001). Moreover, different studies revealed the negative association between stress and sleep quality (Almojali, Almalki, Alothman, Masuadi, & Alaqeel, 2017;

Abbreviations: GR, Glucocorticoid receptor; HPA, hypothalamo-pituitary-adrenal; ACTH, adreno corticotropic hormone; CORT, corticosterone; HC, hippocampus; PFC, prefrontal cortex; OF, open field; EPM, elevated plus maze; EDTA, Ethylenediaminetetraacetic acid.

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Blaxton, Bergeman, Whitehead, Braun, & Payne, 2017).

The use of natural resources and nutritional approaches may be in improving stress management. Recent studies demonstrated the growing interest of bioactive low molecular weight peptides isolated by enzymatic hydrolysis from natural products, like fish hydrolysates. These peptides are described as beneficial for the prevention of different aspects of cerebral ill-being such as stress and anxiety (Gevaert, Veryser, Verbeke, Wynendaele, & De Spiegeleer, 2016; Zamora-Sillero, Ramos, Monserrat, & Prentice, 2018). In humans, a supplementation with yeast hydrolysate in healthy adults revealed an improvement of somatic manifestations of anxiety and an effect of brain mapping pattern (Lee, Jung, & Suh, 2009). In rodents, a recent study from Ohinata's group identified anxiolytic-like peptides from soy and spinach green leaf (Ota et al., 2017) (Kimura et al., 2018). Interestingly, a first study revealed the diazepam-like effect of a fish protein hydrolysate on stress responsiveness (Bernet, Montel, Noel, & Dupouy, 2000). In this study, a high dose of peptides (1200 mg/kg) given to rats by force-feeding decreased adreno corticotropic hormone (ACTH) release after a restraint stress. Moreover, Belhaj *et al.* demonstrated in mice the anxiolytic-like and neuroprotective effects of a phospholipopeptidic complex containing 30% of lipids and 70% of peptides obtained by the enzymatic hydrolysis of salmon heads (Belhaj et al., 2013). Nevertheless, the mechanisms still need to be clarified.

Hence, the aim of this study was to investigate the potential anxiolytic effect of fish hydrolysate, to respond to an acute mild stress in a murine model. Corticosterone secretion and stress-responsive gene expression were determined in the hippocampus (HC), prefrontal cortex (PFC) and amygdala, the main structures implicated in stress management.

2. Material and methods

2.1. Fish hydrolysate

Peptidyss® is a water-soluble powder obtained from a standardized enzymatic hydrolysis of sardine by-products without preservative nor processing aids. Its natural composition is specific with more than 74% of peptides (Table 1) in which 98% have a low molecular weight under 3000 Da (Table 2). Their amino acids repartition is presented in Fig. 1.

2.2. Animals and experimental design

Animal husbandry and experimental procedures were in accordance with the EU Directive 2010/63/EU for animal experiments and approved by the national ethical committee for care and use of animals (approval ID 3136). Every effort were made to minimize suffering and the number of animals used by respecting the principle of the 3Rs (Replacement, Reduction and Refinement).

Seven-weeks-old male mice (Balb/c mice [highly stress sensitive strain]; Janvier, Le GenestSaint-Isle, France) were housed under normal 12 h light/dark cycle (07 h − 19 h) with food and water available *ad libitum* in a control environment (22 +/- 1 °C, 40% of humidity). Mice were handled daily for 1 week before experiment onset to minimize stress reactions to manipulation. Mice were fed with A04 diet (Safe, Augy, France). They were supplemented by gavage (feeding probe V0105040, ECIMED, Boissy-Saint-Leger, France) with fish hydrolysate (300 mg/kg) or water for control group each morning for 7 days. After one week of supplementation, mice were submitted to an acute mild stress protocol and anxiety-like behavior was evaluated. Blood was collected from the mandibular vein before the beginning of the stress protocol (t0 min), and after 30 and 60 min of stress (t30 min and t60 min). Mice were sacrificed after 90 min of stress (t90 min) to evaluate the effect of stress on gene expression (Dinel et al., 2019). They were euthanized by decapitation after 5% isoflurane inhalation. Brain structures (PCF, HC and amygdala) and plasma were collected and frozen at −80 °C.

Table 1
Total composition of fish hydrolysate supplementation.

Composition	Fish hydrolysate
Total proteins	≥74%
Minerals	≤20%
Lipids	≤2%

Table 2
Composition in amino acids of fish hydrolysate supplementation.

Composition of amino acids	Fish hydrolysate
Essential Amino Acids	43%
Non-essential Amino Acids	56%
Branched-Chain Amino Acids	17%
Sulfur Amino Acids	3%

2.3. Induction of acute mild stress

On the last day of supplementation, half mice (n = 11/group) of each group (supplemented with fish hydrolysate or control) was subjected to an acute mild stress. The stress protocol consisted in submitting mice to an open field (OF) test during 10 min immediately followed by an elevated plus maze (EPM) test during 5 min (see next paragraph for details; Fig. 2). Experiments were performed in the morning, one hour after gavage, under conditions of dim light and low noise. Both tests induce mild stress in animals by placing them in an anxiogenic condition (Dinel et al., 2019).

2.3.1. Open-field (OF) test

Mice were exposed to an unknown square area (40 × 40 cm) from which escape is prevented by surrounding walls (16 cm high). The apparatus was virtually divided into 4 central squares defined as the central area (anxiogenic) and squares along the walls that are defined as the periphery. Each mouse was placed in the central area and allowed to freely explore the OF for 10 min. Parameters recorded to evaluate anxiety-like behavior comprised the number of entries into the central area and the percentage of time spent in this area (Dinel et al., 2019).

2.3.2. Elevated plus maze (EPM) test

The EPM was a plus shaped acryl maze with two opposing open arms (30 × 8 cm) and two opposing closed arms (30 × 8 × 15 cm) connected by a central platform (8 × 8 cm) and elevated 120 cm above the floor. Each mouse was placed in the center of the maze facing an open arm, a situation that is highly anxiogenic. The test was performed over a period of 5 min. The number of arm entries and the percent of time spent in open arms were calculated to evaluate the basal level of anxiety. The behavior was videotaped and scored using "Smart" software (Noldus, Wageningen, Netherlands). An entry was scored as such only when the mouse placed all its four limbs into any given arm. A reduction of the percent of time spent and number of entries into the open arms is considered as an anxiety-like index, independent of locomotor activity (Dinel et al., 2019).

2.4. Biochemical measurements

2.4.1. Measurement of corticosterone

Corticosterone was measured in plasma before and 30 and 60 min after the beginning of stress protocol with a DetectX corticosterone immunoassay kit (Arbor Assays, Strasbourg, France) (Dinel, Joffre, et al., 2014).

2.4.2. RNA expression by Fluidigm microfluidics arrays

One microgram of total RNA was obtained from each brain area as described in Dinel, Andre, et al. (2014) and was reverse transcribed with

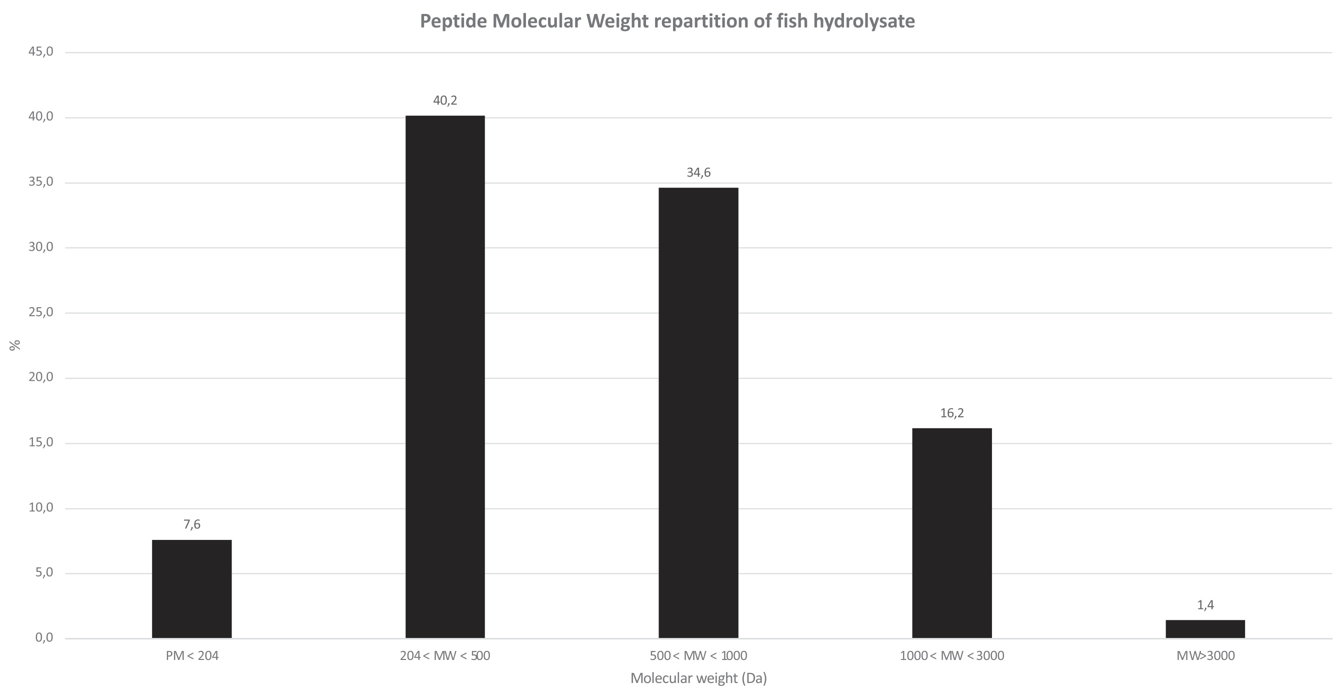

Fig. 1. Peptide molecular weight (MW) repartition of fish hydrolysate, obtained by size-exclusion-chromatography (given as indicative value).

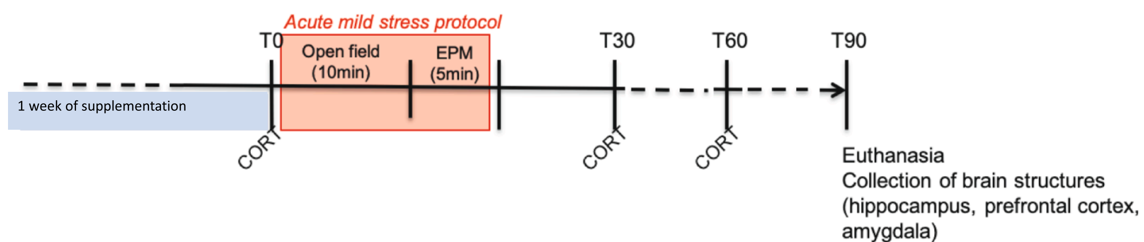

Fig. 2. Experimental protocol in adult Balb/c mice. CORT: corticosterone.

SuperScript III reverse transcriptase (Invitrogen, Cergy- Pontoise, France). Diluted cDNA (2 μL, 5 ng/μL) was added to DNA Binding Dye Sample Loading Reagent (Fluidigm), EvaGreen (Interchim, Montluçon, France) and TE low EDTA to constitute Sample Mix plate. In Assay Mix plate, 10 μL of primer pairs (100 μM) were added to the Assay Loading Reagent (Fluidigm) and TE low EDTA to a final concentration of 5 μM. Following priming the chip in the Integrated Fluidic Circuit Controller, Sample Mix (5 μL) and Assay Mix (5 μL) were loaded into the sample inlet wells. One well was filled with water as a contamination control. To verify specific target amplification and Q-PCR process efficiencies, a sample control (mouse gDNA, Thermo Fisher, Waltham, USA) was treated, preamplified and quantified on assay control (RNaseP TaqMan probe, Thermo Fisher) using the same process in the same plate at the same time. The expected value of cycle quantification was around 13. The chip was placed into the IFC Controller, where 6.3 nL of Sample Mix and 0.7 nL of Assay Mix were mixed. Real-time PCR was performed on the Biomark System (Fluidigm) by GenoToul platform (Toulouse, France) with the following protocol: Thermal Mix at 50 °C, 2 min; 70 °C, 30 min; 25 °C, 10 min, Uracil-DNA N-glycosylase (UNG) at 50 °C, 2 min, Hot Start at 95 °C, 10 min, PCR Cycle of 35 cycles at 95 °C, 15 s; 60 °C, 60 s and Melting curves (from 60 °C to 95 °C). Results were analyzed using the Fluidigm RealTime PCR Analysis software v.4.1.3. (San Francisco, USA) to control specific amplification for each primer. Then the raw data of the qPCR were analyzed using GenEx software (MultiD analyses AB, Freising, Germany) in order to choose the best reference gene to normalize mRNA expression and to measure the relative expression of each 93 genes analyzed between groups. The best reference gene was found to be β-2 microglobulin (B2m) that was thus used for normalization of gene expression.

2.5. Statistical analysis

All data are expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism software. Corticosterone secretion was compared by a 3-way ANOVA test with diet, stress and time as between factors followed by a Fischer's LSD post-hoc. Gene expression were compared using a 2-way ANOVA test (with diet and stress as factors) followed by a Tukey post-hoc test comparison when appropriate. Principal component analysis (PCA) was performed, using RStudio software. PCA summarizes data into principal components (PC) that explain the maximum of the data variance. These PCs are uncorrelated linear combinations of the initial variables which can be interpreted as pattern. PCA provides the correlation between each variable with the PC and a PC score for each individual. The number of components was selected using Cattell criterion. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Fish hydrolysate modulated corticosterone secretion but not anxiety behavior after an acute mild stress protocol

Corticosterone was measured in plasma prepared from blood

samples drawn before the induction of acute mild stress, 30 and 60 min after the start of the stress protocol. A 3-way ANOVA revealed an effect of time ($F(2,60) = 131.3$, $p < 0.0001$) and stress ($F(1,60) = 22.21$, $p < 0.0001$) and an interaction effect between stress and diet ($F(1,53) = 5.540$, $p < 0.05$) (Fig. 3). Nevertheless, at t0, we did not observe any difference in corticosterone level between fish hydrolysate mice (14.589 ng/mL) and control mice (14.38 ng/mL).

At t30, corticosterone level of control stressed and fish hydrolysate stressed animals were significantly higher compared to non-stressed animals ($p < 0.0001$ and $p < 0.05$ for control and fish hydrolysate groups respectively). We also observed a significant decrease of corticosterone level in supplemented stressed animals compared to control stressed ones ($p < 0.05$). At t60, no effect of stress or diet was found anymore.

This modulation of corticosterone level is not associated with behavioral alteration. As expected, animals spent less time in anxiogenic zone than in safe zone ($F(1, 30) = 76.19$, $p < 0.001$). But no significant effect of the diet was observed (results not shown).

3.2. Fish hydrolysate modulated hippocampal stress-responsive gene expression after an acute mild stress protocol

The expression of 93 genes considered as stress responsive genes by Datson et al. (2008), Datson et al. (2012), Andrus et al. (2012), and Kohrt et al. (2016) was analyzed. All significant genes are presented in Table 3.

In the HC, 10 genes were modulated by stress and/or diet (Fig. 4). Most of these genes are implicated in stress pathway. NGF1a expression was significantly increased by stress ($F(1,28) = 8.307$, $p < 0.05$) and Nur77 tended to increase ($F(1,28) = 3.311$, $p = 0.07$) but the expression of these genes was not affected by supplementation. Interestingly, KIF5C, MAPK1 and SGK1 expression was significantly upregulated by the supplementation ($F(1,28) = 9.316$, $p < 0.01$; $F(1,27) = 6.2$, $p < 0.05$ and $F(1,28) = 6.475$, $p < 0.05$, respectively). The expression of Per2, one of the most important circadian rhythm gene, was increased by stress ($F(1,27) = 11.66$, $p < 0.01$) and by the supplementation ($F(1,27) = 6.461$, $p < 0.05$). The expression of SIRT2, a gene clearly involved in healthy aging, was upregulated by the supplementation ($F(1,28) = 6.046$, $p < 0.05$). Finally, the expression of some mitochondrial genes such as ATP8 was modulated by supplementation ($F(1,28) = 6.1$, $p < 0.05$). An interaction between supplementation and stress was highlighted for ATP8 ($F(1,28) = 4.4$, $p < 0.05$), ATP6 ($F(1,28) = 4.993$, $p < 0.05$) and COX1 ($F(1,27) = 8.1$, $p < 0.01$). Interestingly, fish hydrolysate supplementation significantly decreased or tended to decrease ATP8, ATP6 and COX1 expression in non-stressed animals ($p < 0.05$, $p < 0.05$ and $p = 0.06$ respectively).

We performed a multivariate analysis to evaluate if stress responsive gene expression could be explained by supplementation and stress. The principal component analysis of genes evaluated in HC demonstrated that the expression of genes in stressed animals supplemented with fish

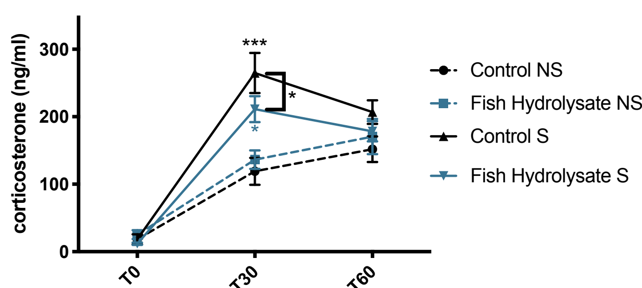

Fig. 3. Kinetic of corticosterone release before (T0) and after (T30 and T60) the induction of acute mild stress. A 3-way ANOVA with diet, stress and time as between factors was realized. Data represent ± means (n = 11/group). *p < 0.05.

Table 3
Stress-responsive genes studied by high-frequency RT-qPCR in the prefrontal cortex, hippocampus and amygdala.

Symbol	Name	Sequence (5′-3′)	References
KIF5C-F KIF5C-R	kinesin family member 5C	ATGTAAAGGGGTGCACCGAGAG ACGTGTCGGTTTGCTTTGCC	Datson et al. (2008)
SGK1-F SGK1-R	serum/ glucocorticoid regulated kinase 1	CGTCAAAGCCGAGGCTGCTCGAAGC GGTTTGGCGTGAGGGTTGGAGGAC	Arteaga et al. (2008)
GABRA4 - F GABRA4 - R	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4	TCCCCAGGACAGAACTCAAAGG AACAGGACCCCCAAATCCAG	Dinel et al. (2019)
MAPK1-F MAPK1-R	mitogen-activated protein kinase 1	AGCTAACGTTCTGCACCGTG TGATCTGGATCTGCAACACGGG	Datson et al. (2008)
PER2-F PER2-R	period circadian clock 2	GCTGGCAACCTTGAAGTATGC TGGTAGTACTCCTCATTAGCCTTCAC	Dinel et al. (2019)
MAOA-F MAOA-R	monoamine oxidase A	TGAGGTATCTGCCCTGTGGTTC CCCCAAGGAGGACCATTATCTG	Datson et al. (2008)
SIRT2-F SIRT2-R	sirtuin 2	TCCACTGGCCTCTATGCAAACC TTGGCAAGGGCAAAGAAGGG	Dinel et al. (2019)
ATP8 - F ATP8 - R	apolipoprotein E	TGCGAAGATGAAGGCTCTGTG GGTTGGTTGCTTTGCCACTC	Dinel et al., 2019
ATP6 - F ATP6 - R	ATP synthase 6, mitochondrial	CGCCTAATCAACAACCGTCTCC CATGTTCGTCCTTTTGGTGTGTG	Hunter et al. (2016)
COX1 - F COX1 - R	ATP synthase 8, mitochondrial	CCACTGGCACCTTCACCAAA** GGGGTAATGAATGAGGCAAA**	Hunter et al. (2016)
Rpl30 - F Rpl30 - R	Ribosomal protein L30	CAGACAAGGCAAAGCGAAGTTG TGATGGACCCCAGTTTTAGCC	Dinel et al. (2019)
ND2 - F ND2 - R	NADH dehydrogenase 2, mitochondrial	TTCATAGGGGCATGAGGAGGAC GTGAGGGATGGGTTGTAAGGAAG	Dinel et al. (2019)

hydrolysate was different from that of the other groups. Indeed, we observed a positive contribution of this group on D1 and D2 components (Fig. 5A). Heatmap representation allowed us to separate stress-responsive genes in two groups and to discriminate one cluster. Genes of this cluster were clearly upregulated (red color) in stressed animals supplemented with fish hydrolysate compared to the other groups. This cluster integrate genes from mitochondria and oxidative stress pathways (Fig. 5B).

3.3. Pre-frontal cortex and amygdala structures were less sensitive to stress-responsive genes modulation induced by the fish hydrolysate supplementation than hippocampus

In PFC, 4 genes were modulated by stress and/or diet (Fig. 6A). The expression of Rpl30 was significantly decreased by stress without any effect of diet ($F(1,27) = 8.497$, $p < 0.01$). An interaction effect between diet and stress was significantly observed for MAOA, SIRT2 and ATP6 expression ($F(1,27) = 4.2$, $p < 0.05$; $F(1,27) = 11.08$, $p < 0.01$ and $F(1,28) = 4.640$, $p < 0.05$ respectively). Interestingly, fish hydrolysate significantly reduced SIRT2 expression in stressed animals ($p < 0.05$) whereas this expression was significantly increased by stress in control group ($p < 0.05$).

In amygdala, stress induced an increase in COX1 and ND2 expression ($F(1,27) = 9.140$, $p < 0.01$ and $F(1,26) = 8.153$, $p < 0.01$ respectively) (Fig. 7A). Moreover GABRA4 gene expression was significantly downregulated by the supplementation ($F(1,28) = 5.221$, $p = 0.0301$) (Fig. 7A). MAPK1 gene tended to be modulated by the interaction between diet and stress ($F(1,28) = 3.5$, $p = 0.0704$). The principal component analysis and the heatmap did not discriminate differences between groups in both PFC and amygdala structures (Fig. 6 B-C, Fig. 7

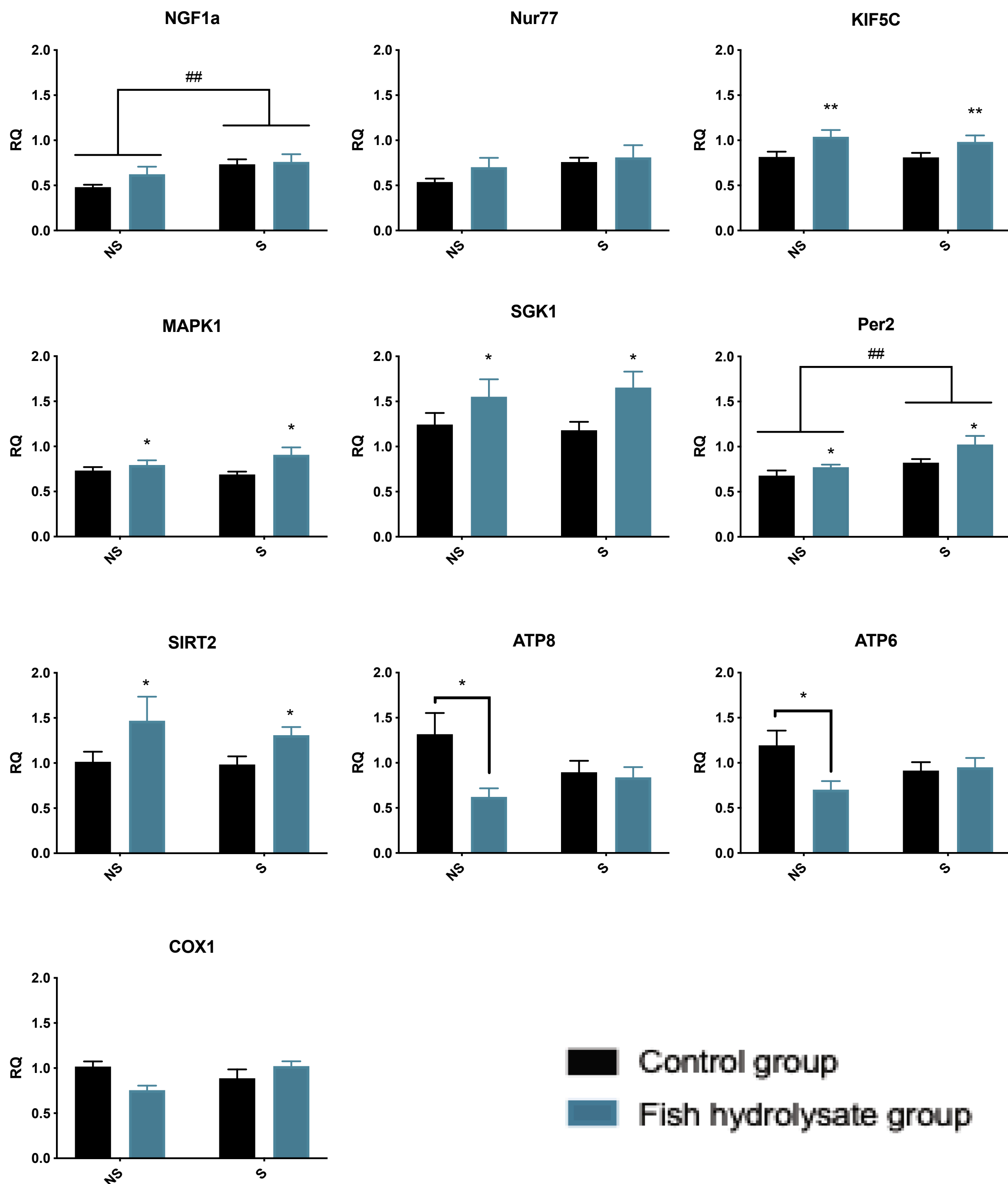

Fig. 4. Evaluation of stress-responsive gene expression measured by RT-PCR in hippocampus. Relative fold changes in the levels of stress responsive genes 90 min after the induction of acute mild stress. Data represent means ± SEM (n = 8/group). *p < 0.05, **p < 0.01 for supplementation effect. ##p < 0.01 for stress effect. RQ: relative quantity.

B-C).

4. Discussion

Bioactive peptides play a critical role in regulating various biological processes and start to be considered as a “hidden treasure” of drug candidates (Liu et al., 2017). Their high digestibility and bioavailability with low side effects make them very interesting compounds for human health (Chakrabarti, Guha, & Majumder, 2018).

By the way, investigating the potential anxiolytic activity of those nutritional molecules appears as an innovative therapy for anxiety disorders. Our results demonstrated that a supplementation with fish hydrolysate blunted corticosterone secretion 30 min after an acute mild stress and modulated the expression of stress-responsive genes in HC, PFC and amygdala.

In this study, we submitted highly stress sensitive Balb/c mice to an OF test and an EPM test consecutively (Moloney, Dinan, & Cryan, 2015). This protocol was previously described to generate an acute mild stress by placing the animals in an anxiogenic environment, and to induce a release of corticosterone 30 min after the induction of the stress (Dinel

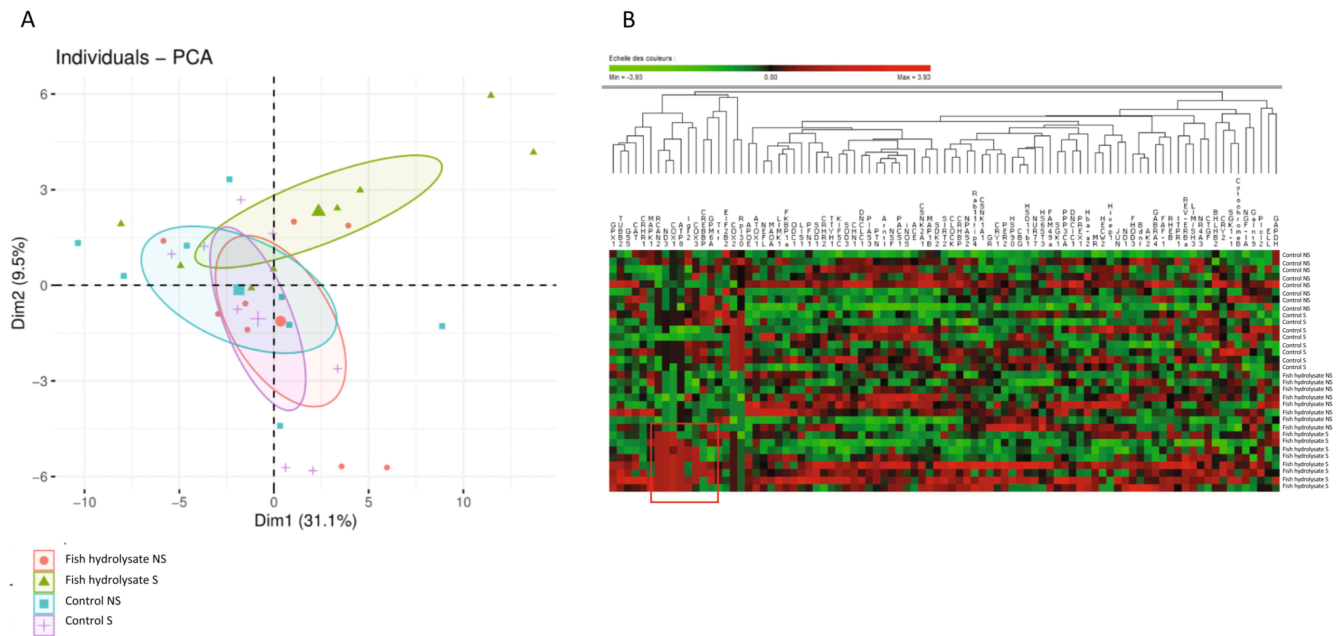

Fig. 5. Evaluation of stress-responsive gene expression measured by RT-PCR in hippocampus. A) Graphic representation defined by the first two principal components (D1 and D2) of the principal component analysis (PCA) of gene expression. B) Phylogenic relationship based on Pearson's correlation in the hippocampus.

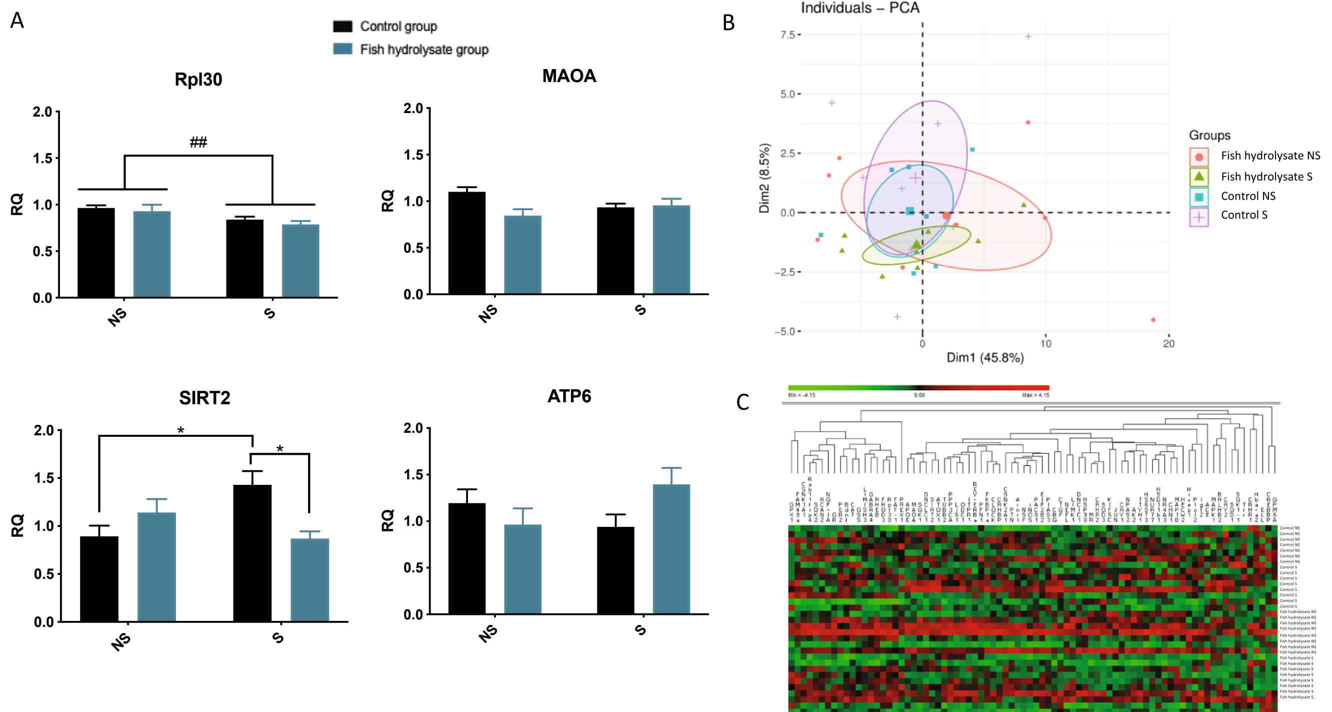

Fig. 6. Evaluation of stress-responsive gene expression measured by RT-PCR in pre-frontal cortex. A) Relative fold changes in the levels of stress responsive genes 90 min after the induction of acute mild stress. Data represent means ± SEM (n = 8/group). *p < 0.05 for supplementation effect. ##p < 0.01 for stress effect. RQ: relative quantity B) Graphic representation, defined by the first two principal components (D1 and D2) of the principal component analysis (PCA) of gene expression. C) Phylogenic relationship based on Pearson's correlation in the pre-frontal cortex.

et al., 2019). That's what we observed, at T30, control mice presented a significant increase of corticosterone secretion. Interestingly, fish hydrolysate supplementation significantly attenuated this corticosterone spike. At 60 min, corticosterone level was comparable between stressed and no-stressed animals but different from basal level (T0). The return to basal level is classically described between 60 and 90 min after stress induction (McClennen, Cortright, & Seasholtz, 1998). We hypothesize that the return to the homeostatic state was masked by consecutive samplings realized in mandibular vein to evaluate corticosterone kinetic.

The anxiolytic effect of the fish hydrolysate observed 30 min after stress induction is consistent with previous studies reporting that peptides extracted from fish reduced anxiety-like behavior in rats and mice (Belhaj et al., 2013; Bernet et al., 2000). Gabolysat® PC60 (1200 mg/

Fig. 7. Evaluation of stress-responsive gene expression measured by RT-PCR in amygdala. A) Relative fold changes in the levels of stress responsive genes 90 min after the induction of acute mild stress. Data represent means ± SEM (n = 8/group). *p < 0.05 for supplementation effect. ##p < 0.01 for stress effect. RQ: relative quantity B) Graphic representation, defined by the first two principal components (D1 and D2) of the principal component analysis (PCA) of gene expression. C) Phylogenic relationship based on Pearson's correlation in the amygdala.

kg), a fish protein hydrolysate extracted from cod and mackerel, reduces ACTH secretion induced by restraint stress after a 5-days oral feeding but did not affect corticosterone release in rats (Bernet et al., 2000). Another study demonstrated that a two weeks supplementation with a dose (246 mg/kg) of phospholipopeptidic complex extracted from salmon hydrolysate induced a decrease of anxiety-like behaviors in OF and EPM tests in mice (Belhaj et al., 2013). But the lipidic fraction representing 30% of this salmon hydrolysate extract explains a part of the results obtained. Here, we first demonstrated in this study that a short supplementation (7 days) with a low dose of low molecular weight peptides (300 mg/kg) extracted from marine products had an anxiolytic-like activity in mice by limiting the stress-induced corticosterone release. Several studies in rodents and humans have demonstrated that acute or chronic stress has negative effects on cognition, sleep quality and depression (Almojali et al., 2017; McEwen & Sapolsky, 1995; Park et al., 2019). It has been notably demonstrated in human that elevated corticosteroids are associated with memory performance decline (de Souza-Talarico, Marin, Sindi, & Lupien, 2011). Limiting the corticosteroid release without blocking stress response appears an interesting strategy to prevent the negative effects of acute stress occurring in daily life.

One of the main mechanisms of action of corticosteroids in the brain is their genomic effect, resulting in modification of transcription of target genes called stress-responsive genes (Datson et al., 2008, 2012; Hunter et al., 2016; Kohrt et al., 2016). Morsink and collaborators investigated this corticosteroid-mediated transcriptional changes resulting in a highly dynamic transcriptional response to corticosteroid receptor activation throughout a specific time window (Morsink, Steenbergen, et al., 2006). Here, we evaluated the effect of the fish hydrolysate on the expression of a pattern of stress-responsive genes previously described by Datson and Hunter (Datson et al., 2008, 2012; Hunter et al., 2016). We specially focused on PFC and amygdala, structures involved in the regulation of stress and on HC, implicated in the formation of memory and highly susceptible to stress. Fish hydrolysate modulated stress-responsive gene expression in a structure-dependent manner. Most of modulated genes were in HC. First, the principal component analysis clearly demonstrated that the fish hydrolysate modulated differentially stress-responsive genes in stressed animals compared to non-stressed animals or to control mice. Specifically, fish hydrolysate supplementation upregulated SGK1, MAPK1 and KIF5C. It has been demonstrated in human that SGK1 potentiated and maintained GR activation in the presence of cortisol, and even after cortisol withdrawal, by increasing GR phosphorylation and GR nuclear translocation. SGK1 was described as a mediator for the effects of cortisol on neurogenesis and GR function, with relevance to stress and depression (Anacker et al., 2013). In the same way, MAPKs are also involved in synaptic plasticity and remodeling in the brain, occurring during e.g. induction of long-term potentiation, learning and memory consolidation and have been shown to be activated after stress and to be involved in corticosteroid signal transduction in rodents (Meller et al., 2003; Revest et al., 2005; Sananbenesi, Fischer, Schrick, Spiess, & Radulovic, 2003). KIF5C is a motor protein that affect GR translocation itself, thereby affecting GR signaling (Pfister et al., 2005). Interestingly, there are indications that GR signaling may additionally be modulated by changes in gene expression which affect receptor levels and receptor binding affinity (Datson et al., 2008). In a matter of fact, the upregulation of these specific genes can act as a primer of the corticosteroid signaling. Fish hydrolysate supplementation could modulate GR translocation and signaling and modify GR trafficking (Harrell et al., 2004) and by the way, limiting the release of corticosterone.

Moreover, fish hydrolysate supplementation also upregulated two interesting genes: PER2 and SIRT2. PER2 is one of the core genes of the circadian clock, it generates the circadian rhythms in the central nervous system and peripheral organs (Arjona & Sarkar, 2006a, 2006b; Sujino, Nagano, Fujioka, Shigeyoshi, & Inouye, 2007). It plays a critical role in sleep, especially in familial advanced sleep phase syndrome (Kim, de la Pena, Cheong, & Kim, 2018). Moreover some studies demonstrated a link between PER2 expression and depression: high serotonine levels

induced by serotonine reuptake inhibitors during nighttime induce - PER2 expression (Cuesta, Clesse, Pevet, & Challet, 2009). Conversely, mice exposed to unpredictable chronic stress show depressive-like behaviors and decreased Per2 expression (Jiang et al., 2011; Logan et al., 2015). The upregulation of PER2 by fish hydrolysate supplementation could participate to the anxiolytic effect of the hydrolysate and should be investigated in further studies.

SIRT2 plays diverse roles in cellular metabolism and aging and the connection between SIRT2 and neurological disorders is now well established. The potential of SIRT2 as a therapeutic target in neurodegenerative diseases started to be studied (Fourcade, Outeiro, & Pujol, 2018). The upregulation of SIRT2 by the fish hydrolysate in basal and stress conditions could be of great interest to prevent neurodegenerative alterations. Recent studies demonstrated that *Sirt2-/-* mice display an alteration of mitochondrial content and morphology, which results in energy failure and redox dyshomeostasis in the central nervous system (Fourcade et al., 2017; Gomes, Fleming Outeiro, & Cavadas, 2015). This is in accordance with the effect of the fish hydrolysate on mitochondrial genes (ATP8, ATP6 and Cox1) expression observed in this study (cluster of mitochondrial genes, Fig. 5B). Indeed, an emerging concept proposes that mitochondria sense, integrate, and transduce psychosocial and behavioral factors into cellular and molecular modifications. Mitochondrial signaling might in turn contribute to the biological embedding of psychological states like stress (Picard & McEwen, 2018a, 2018b). Nevertheless, the link between stress, mitochondrial gene expression and supplementation should be further investigated.

Finally, even if PFC and amygdala seemed to be less sensitive to gene expression modulation, the effects observed were in accordance with those described in HC. Indeed, most of the regulated genes were mitochondrial genes as Rpl30 and ATP6 in PFC or COX1 and ND2 in amygdala. Interestingly, SIRT2 was clearly upregulated by stress in PFC of control mice but this increase was prevented by fish hydrolysate supplementation. This result confirms the potential impact of fish hydrolysate supplementation on aging process. In amygdala, fish hydrolysate supplementation clearly induced a decrease of GABAR4 expression, the receptor of GABA neurotransmitter. This result is of interest since Bernet et al demonstrated that Gabolysat® PC60 showed some diazepam-like effects by increasing GABA release (Bernet et al., 2000). Fish hydrolysate supplementation could present anxiolytic effect by reducing the corticosterone release, increasing GABA release and modulating expression of target genes. The concentration of GABA should be investigated to confirm this hypothesis.

5. Conclusion

To conclude, in this model of acute mild stress, fish hydrolysate decreased the corticosterone level and modulated the expression of stress responsive genes, especially in HC. Most of genes regulated are implicated in HPA axis regulation or in mitochondrial activity, that are essential for stress management. Moreover, fish hydrolysate might modulate circadian rhythm and aging process. Complementary studies are needed to reinforce these preliminary data and to investigate the role of fish hydrolysate in the prevention of neurodegenerative process. Nevertheless, fish hydrolysate appears as an innovative strategy to regulate and manage stress.

Ethical Statements

Animal husbandry and experimental procedures were 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 A13169).

CRediT authorship contribution statement

Anne-Laure Dinel: Conceptualization, Methodology, Software, Validation, Formal analysis, Resources, Data curation, Writing - review & editing, Visualization, Project administration, Funding acquisition. **Céline Lucas:** Conceptualization, Investigation, Resources. **Julie Le Faouder:** Writing - review & editing. **Elodie Bouvret:** Writing - review & editing. **Véronique Pallet:** Validation. **Sophie Layé:** Validation. **Corinne Joffre:** Validation, Writing - original draft, Supervision.

Declaration of Competing Interest

ALD, CL, SL, VP and CJ report no disclosures. Abyss Ingredients funds JLF and EB.

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Author contributions

All the authors contributed equally to this work.

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