SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TIPOLOGIA DI BORSA RICEVUTA: BORSE DI RICERCA PER BREVI PERIODI ALL'ESTERO

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): FINE PERIODO

TITOLO DELLA RELAZIONE: EFFECTS OF A DIET ON FATTY ACID METABOLIC ENZYMES FOLLOWING SOCIAL STRESS: IMPLICATION OF CENTRAL HISTAMINERGIC SYSTEM

RELAZIONE:

Background and aims: Psychosocial stress is a substantial risk factor in the occurrence of mood and anxiety disorders (Kendler et al. 2003). Moreover, recent reports indicate that neuroinflammatory cytokine signalling contributes to the pathophysiology of stress-related psychiatric disorders (Dantzer et al. 2008). Altered dietary intake and/or PUFA metabolism has been reported to be involved in a number of neurological disorders via sustained neuroinflammatory processes (Joffre et al. 2014). Polyunsaturated fatty acids (PUFAs) are generally considered to be essential fatty acids. There are two main families of PUFAs, the ω-6 and ω-3 PUFAs; α-linolenic acid (ALA;18:3n-3) is the dietary-essential shorter chain ω-3 PUFA precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

DHA and EPA effect on neuroinflammatory pathways could be either direct or indirect. Indeed, LC-PUFAs are converted by COX, LOX, and CYP450 into specialized proresolving mediators (SPMs), which display pro or anti-inflammatory activities (Chiang and Serhan 2017), in several tissues including the brain (Orr et al. 2013, Rey et al. 2016, Layé et al. 2018). Eicosanoids, resolvins, protectin and maresin derived from DHA and EPA have anti-inflammatory and pro-resolving activities(Bazan 2009, Serhan et al. 2011). Therefore PUFAs and their biological derivatives raised growing interest to treat inflammation and more specifically neuroinflammation (Joffre 2019).

Another essential nutrient is vitamin A, which through its active metabolite retinoic acid plays a key role in cognitive functions in adult rats (Bonhomme et al. 2014). Recent findings demonstrating

a beneficial synergistic effect of vitamin A and EPA/DHA on behavioural and neurobiological markers in aged rats (Létondor et al. 2016) and the deleterious cognitive decline induced by social instability stress during adolescence, with the amelioration maintained in adulthood (Provensi et al. 2019). Multiple levels of interactions occur between ω3-PUFAs and retinoid signalling, because retinoic acid (the active metabolite of vitamin A) and DHA may bind to common nuclear receptors (RARs, RXR) (de Urquiza et al. 2000) which are known to be activated in several neuronal functions (Tang and Yasuda 2017).

Studies in our laboratory showed that antidepressant molecules, such as SSRI or molecules derived from fatty acids whose antidepressant-like activity has been demonstrated, like Oleoylethanolamide (OEA), require the integrity of the central histaminergic system to perform their effects (Munari et al. 2015, Costa et al. 2018).

Results obtained with my study show that a diet enriched with ω3-PUFA and vitamin A prevents social aversion, cognitive deficits and hippocampal long-term potentiation modifications induced by 10 days of chronic social defeat stress (CSDS), a well-characterized preclinical model of anxiety and depression (Berton et al. 2006, Golden et al. 2011) The novelty of this study consists in the inefficacy of these nutritional interventions in genetically modified mice that are unable to synthesize histamine (histidine decarboxylase (HDC)$^{-/-}$ mice; Figure 1).

In the laboratory of Dr Layé (INRA, Bordeaux) I performed several analyses on sample tissues extracted from experimental animals exposed to stress and EPA/DHA and vitamin A supplemented or control diet in the laboratory of Prof. Passani (UNIFI) using specific approaches to understand 1) the molecular mechanisms underlying ω3-PUFA and vitamin A protective effect of synaptic and behavioral alteration triggered by a chronic stress and 2) brain histamine role in such mechanisms. Experimental groups included stressed mice fed control diet; stressed mice fed the supplemented diet; non-stressed mice fed the control diet. Both HDC$^{+/+}$ and HDC$^{-/-}$ were used for a total of 6 experimental groups.

During my work in Dr. Sophie Layé laboratory I evaluated:

1. Metabolic enzymes of fatty acids in hippocampus and prefrontal cortex
2. retinoic acid receptors (RAR) expression in hippocampus and prefrontal cortex.

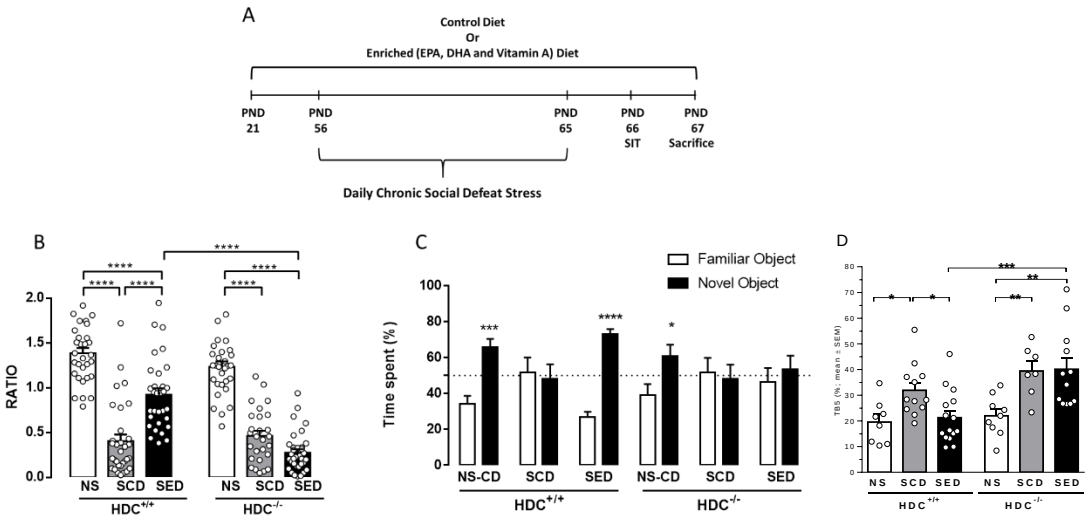

Figure 1 Impact of Chronic Social Defeat Stress on (B) social interaction test, (C) novel object recognition test and (D) electrophysiological experiments . (A) schematic representation of the experimental protocol used in normal (HDC$^{+/+}$) and chronically histamine-depleted (HDC$^{-/-}$) animals. PND: Post-Natal Day; SIT:Social Interaction Test

Materials and Methods:

Real-Time PCR analysis of gene expression in the hippocampus and prefrontal cortex

The hippocampus and prefrontal cortex were rapidly removed and stored at –80°C (see Figure 1A for the timeline). RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies™, Saint-Aubin, France). RNA concentrations were determined using a Nanodrop ND-1000 (Labtech). Using OligodT and random primers (Invitrogen), cDNA was synthesized with SuperScript IV Reverse Transcriptase (Invitrogen, Life Technologies™, Saint-Aubin, France). Briefly, 1 µg of total RNA mixed with RNasin (Invitrogen, Life Technologies™, Saint-Aubin, France) and DNase (Invitrogen, Life Technologies™, Saint-Aubin, France) was incubated at 37°C. Then, OligodT and random primers were added for incubation at 65°C. Then, the SuperScript IV mix was added, and the mixtures were incubated at 23°C for 10 min, followed by 50°C for 10 min and 80°C for 10 min.
To measure microglial markers expression, quantitative PCR 217 was performed using SYBR® assay (Eurogentec, Seraing, Belgium). Real-time PCR was performed using the LightCycler 480 system with a ninety-six-well format (Roche Diagnostics) in a final volume of 10 µl, containing 1×LightCycler 480 SYBR Green I Master solution, 0.5 µM of each primer and 7 µl of cDNA. The following program started with an initial denaturation step for 10 min at 95°C, then an amplification for 45 cycles (10 s denaturation at 95°C, 6 s annealing at 62°C, and 10 s extension at 72°C), finally a melting curve analysis was run.

The forward- and reverse-primer sequences and the amplicon size for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RAR-α and RXR-α are summarised in Table 1. GAPDH was used as the reference gene, since its expression level was unaffected under our experimental conditions.

Quantification data were analysed using LightCycler 480 Relative Quantification software (version 1.5).

Table 1 Primers used for LightCycler RT-qPCR

Gene name	Nucleotide sequence 5'-3'	Product length (bp)
GAPDH	F: CCAGTGAGCTTCCCGTTCA R: GAACATCATCCCTGCATCCA	78
RAR-α	F: GGCGAACTCCACAGTCTTAATG R: GCTGGGCAAGTACACTACGAAC	118
RXR-α	F: GATTCCGATACGACGACAGT R: CATCACCACTCTCGCCATC	141

To measure fatty acid metabolic enzymes, quantitative PCR was performed using the Applied Biosystems (Foster, CA) assay-on demand gene expression protocol as previously described (Mingam et al., 2008). Briefly, cDNAs for 5-LOX, 12-LOX, COX2, CYP1A1, EPHX2, RXR-β and a housekeeping gene (GAPDH) will be amplified by PCR using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ). PCR program consisted of 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence will be measured using an AB 7500 Real-Time PCR system (Applied Biosystems, Foster city, CA).

Statistical analysis

All values are expressed as means ± SEM, and the number of mice used in each experiment is also indicated. The presence of significant treatment effects was determined by a 2-way ANOVA followed by Bonferroni MCT test, as appropriate. The level of significance was set at $P \leq 0.05$. Statistical analysis was performed using GraphPad Software. The data of real time PCR are expressed as Relative Quantification.

Results:

Effects of ω3-PUFA and Vitamin A supplemented diet on hippocampal and cortical gene expression of enzymes of fatty acid metabolism

To study the fatty acid metabolic pathways, we examined whether the enriched diet modulated the hippocampal and cortical expression of some metabolic enzymes, particularly focusing on lipoxygenases, such as 5-LOX and 12-LOX, cyclooxygenases, such as COX 2, cytochrome p450 (CYP1A1) and Soluble epoxide hydrolases (EPHX2).

Two-way ANOVA revealed no effect of stress or diet on the hippocampal mRNA expression of *5-LOX* (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,23}=1,988$; $F_{(Genotypes)1,23}=0,5971$; $F_{(Conditions)\ 2,23}=1,519$) (Figure 2a); COX-2 (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,22}=1,776$; $F_{(Genotypes)1,22}=0,6114$; $F_{(Conditions)\ 2,22}=0,5747$) (Figure 1c); CYP1A1 (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,21}=1,477$; $F_{(Genotypes)1,21}=1,719$; $F_{(Conditions)\ 2,21}=0,4782$) (Figure 2d) and EPHX2 (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,22}=2,306$; $F_{(Genotypes)1,22}=1,209$; $F_{(Conditions)\ 2,22}=0,9529$) (Figure 1e); indeed, an increase in mRNA *12-LOX* levels was observed in $HDC^{+/+}$ stressed mice fed with enriched diet (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}=1,785$ $p=0,1894$; $F_{(Genotypes)1,24}=0,1961$ $p=0,6618$; $F_{(Conditions)\ 2,24}=3,537$ $p<0.05$) (Figure 2b).
In the prefrontal cortex Two-way ANOVA show a genotype effect on 5-LOX (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}=2,157$ $p=0,1376$; $F_{(Genotypes)1,24}=7,67$ $p<0,05$; $F_{(Conditions)\ 2,24}=0,6399$ $p=0,5361$), COX-2 (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}=0,4020$ $p=0,6734$; $F_{(Genotypes)1,24}=6,874$ $p<0,05$; $F_{(Conditions)\ 2,24}=0,2492$ $p=0,7815$), CYP1A1 (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}=0,3401$ $p=0,7151$; $F_{(Genotypes)1,24}=0,6114$ $p<0,05$; $F_{(Conditions)\ 2,24}=1,296$ $p=0,2922$), and EPHX2 expression (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}=1,084$ $p=0,3542$; $F_{(Genotypes)1,24}=6,097$ $p<0,05$; $F_{(Conditions)2,24}=0,4687$ $p=0,6314$) (Figure 3a, c-e). Moreover, we observed effects of genotype and conditions in 12-LOX expression (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,23}=0,3713$ $p=0,6939$; $F_{(Genotypes)1,23}=10,88$ $p<0,01$; $F_{(Conditions)\ 2,23}=11,22$ $p<0,001$) (figure 3b), in fact, we observed an increase in 12-LOX expression in $HDC^{+/+}$ animals fed an enriched diet compared to $HDC^{-/--}$ animals fed the same type of diet thus indicating the involvement of the central histaminergic system.

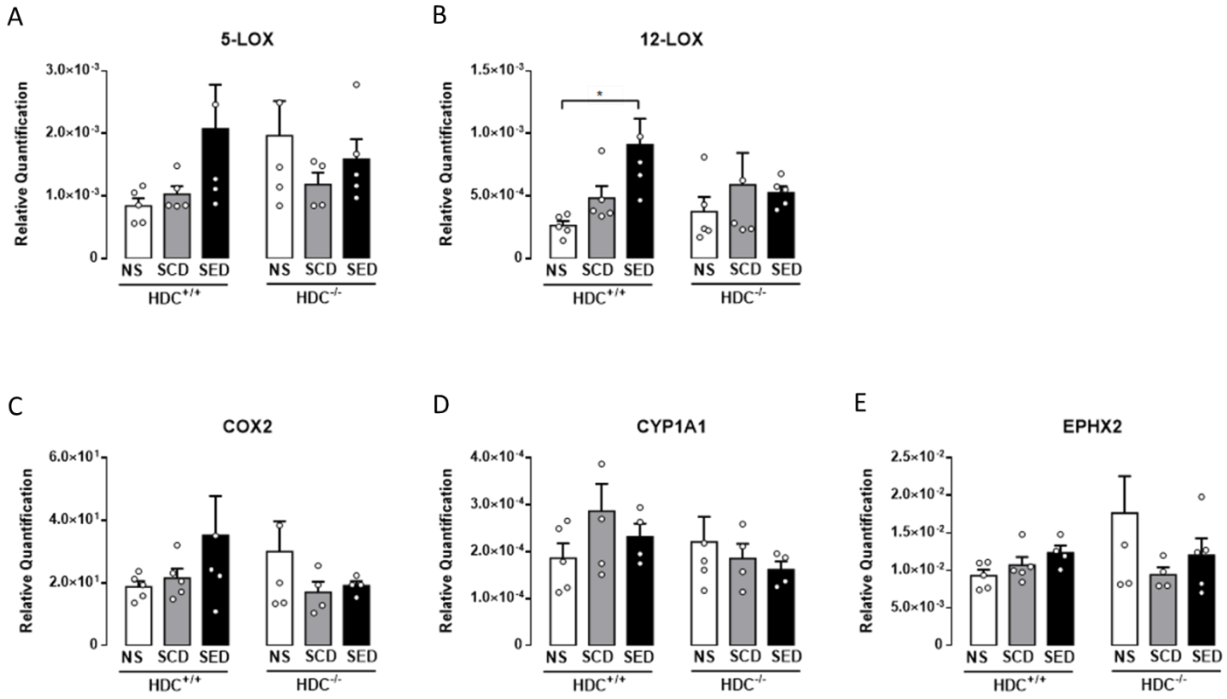

*Figure 2 Effect of enriched diet on hippocampal fatty acid metabolic enzymes. (A) 5-LOX, (B)12-LOX, (C) COX-2 (D) CYP1A1, (E) EPHX2 mRNA expression measured by RT-qPCR. Data are represented as Relative Quantification vs GAPDH. (Two-way ANOVA and Bonferroni's MCT; *p<0.05; ** p<0.01 n=4-5).*

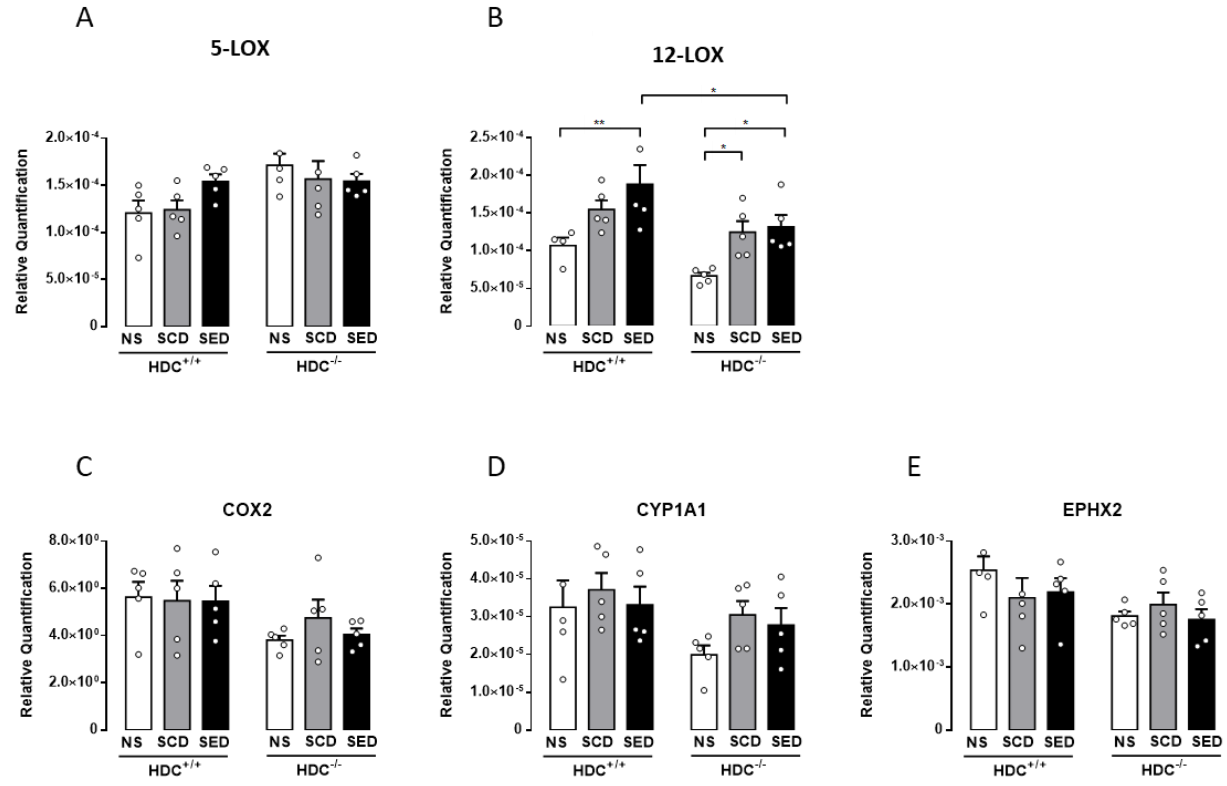

*Figure 3 Effect of enriched diet on cortical fatty acid metabolic enzymes. (A) 5-LOX, (B)12-LOX, (C) COX-2 (D) CYP1A1, (E) EPHX2 mRNA expression measured by RT-qPCR. Data are represented as Relative Quantification vs GAPDH. (Two-way ANOVA and Bonferroni's MCT; *p<0.05; ** p<0.01 n=4-5).*

Effects of ω3-PUFA and Vitamin A supplemented diet on hippocampal and cortical gene expression of Retinoic acid receptors: RAR-α, RXR-α, RXR-β

To study the effect of vitamin A supplementation on stress responses, we examined whether the enriched diet modulated the hippocampal and prefrontal cortex expression of some retinoic acid receptors, such as RAR-α, RXR-α and RXR-β.

Two-way ANOVA analysis showed no differences on RXR-α (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,23}$=0,2759; $F_{(Genotypes)1,23}$=0,002757; $F_{(Conditions)\ 2,23}$=1,447) (Figure 4a) and RXR-β expression (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}$=1,794; $F_{(Genotypes)1,24}$=0,2024; $F_{(Conditions)\ 2,24}$=1,293) (Figure 4b).

On the contrary Two-way ANOVA revealed a statistical difference in RAR-α expression (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}$=0,6012; $F_{(Genotypes)1,24}$=0,1648; $F_{(Conditions)\ 2,24}$=3,028) (Figure 4c) in $HDC^{+/+}$ stressed mice compared to non-stressed control group, but no differences were observed in $HDC^{-/-}$ mice; underlining, also in this case, a difference in the gene expression between $HDC^{+/+}$ and $HDC^{-/-}$.

In the prefrontal cortex Two-way ANOVA revealed no differences in RXR-α (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,23}$=2,099; $F_{(Genotypes)1,23}$=1,889; $F_{(Conditions)\ 2,23}$=0,2463), RXR-β α (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}$=0,3707; $F_{(Genotypes)1,24}$=0,4218; $F_{(Conditions)\ 2,24}$=1,58) and RAR-α expression (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,23}$=1,324; $F_{(Genotypes)1,23}$=1,979; $F_{(Conditions)\ 2,23}$=0,5793) (Figure 5).

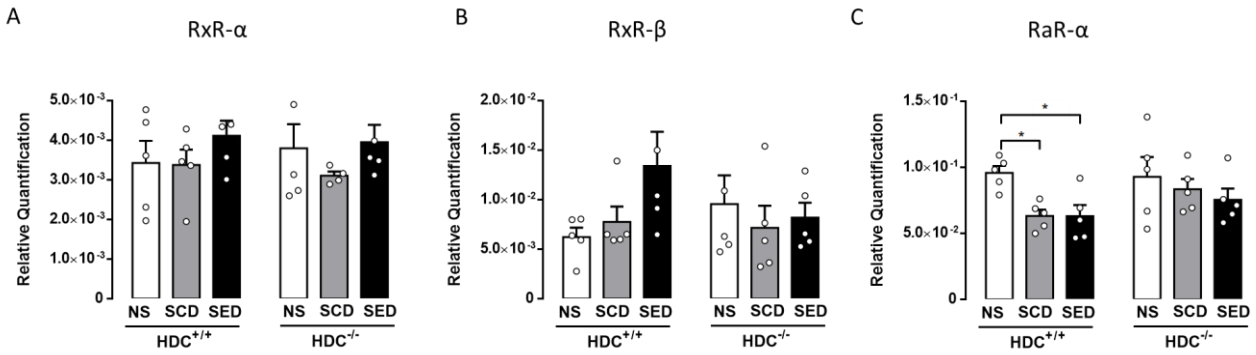

*Figure 4 Effect of enriched diet on hippocampal Retinoic acid receptors. (A) RXR-α, (B) RXR-β, (C) RAR-α mRNA expression measured by RT-qPCR. Data are represented as Relative Quantification vs GAPDH. (Two-way ANOVA and Bonferroni's MCT; *P<0.05; n=4-5).*

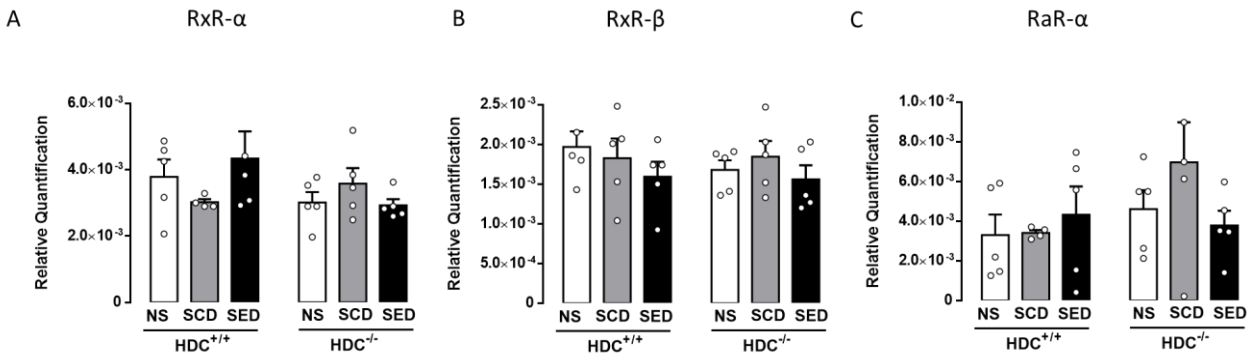

*Figure 5 Effect of enriched diet on cortical Retinoic acid receptors. (A) RXR-α, (B) RXR-β, (C) RAR-α mRNA expression measured by RT-qPCR. Data are represented as Relative Quantification vs GAPDH. (Two-way ANOVA and Bonferroni's MCT; *P<0.05; n=4-5).*

Conclusion

Psychosocial stressors contribute to the pathophysiology of affective disorders and variations of cytokine functioning have been implicated in this process (Audet, Mangano and Anisman 2010).
ω3-PUFAs, DHA, and EPA and their derivatives are well-known regulators of the inflammatory response (Serhan 2014, Serhan 2017).
In this part of the study we investigated the impact of stressful aggressive encounters on fatty acid metabolic enzymes mRNA expression within the hippocampus and prefrontal cortex (PFC) in $HDC^{+/+}$ and $HDC^{-/-}$ mice fed a control diet or a diet supplemental ω3-PUFA and vitamin A.
Our results show that in the hippocampus the supplemented diet significantly increased 12-LOX expression and induced a trend in the increase of 5-LOX and COX-2 expression in $HDC^{+/+}$ mice compared to non-stressed control group. This effect is not shown in $HDC^{-/-}$ mice fed with supplemented diet indicating a loss of this action in histamine-depleted animals.
In the PFC we observed an increase in 12-LOX expression in $HDC^{+/+}$ and $HDC^{-/-}$ stressed animals fed with supplemented diet compared to non-stressed control group.
The increase in mRNA expression of the 12-LOX enzyme produces an incremented synthesis of 12-HETE which promote the activation of PPARγ that is neuroprotective through its anti-inflammatory properties (Shalini et al. 2018).
Recently, it was reported that intracerebroventricular treatment with resolvin D1, D2, E1, E2 and E3, which are derived from ω3-PUFA through the 5-LOX and 12-LOX enzymes, and infusion of these lipids to the PFC and hippocampus ameliorates depressive-like behaviours induced by bacterial endotoxin (Deyama et al. 2017, Deyama et al. 2018b, Deyama et al. 2018a). The beneficial effects of resolvin D1 and D2 were also demonstrated in a mouse model of chronic mild stress (Ishikawa et al. 2017).
Our results show also that EPA/DHA and vitamin A supplemented diet produce an increase in the 12-LOX expression in the prefrontal cortex of $HDC^{+/+}$ mice fed a supplemented diet compared to $HDC^{-/-}$ animal fed with the same diet. This indicates that the histaminergic system is necessary for the enriched diet to exert its effects in the hippocampus and cortex by increasing the gene expression of an enzyme (12-LOX) which leads to the production of anti-inflammatory molecules.

Multiple levels of interactions occur between ω-3 PUFAs and retinoid signaling, because retinoic acid (the active metabolite of vitamin A) and DHA may bind to common nuclear receptors (de Urquiza et al. 2000), for this reason we investigated the retinoic acid receptors expression in the hippocampus and PFC. RXR-α and RXR-β receptors expression was not different among experimental groups, but we found that $HDC^{+/+}$ stressed mice had a decreased expression of RAR-α expression independently of the diet. This effect is not observed in $HDC^{-/-}$ indicating a role of brain histamine in the stress induced modulation of this receptor.

Our present study provides preclinical evidence suggesting that supplementation with ω-3 PUFAs and vitamin A prevents the changes induced by chronic social defeat stress on hippocampal and cortical expression of the enzymes that metabolize DHA and EPA in pro and anti-inflammatory compound. This effect is lost in histamine depleted animals indicating the essential role of brain histamine in the effects of this diet.

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