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Abstract

Metabolic syndrome represents a major risk factor for severe comorbidities such as cardiovascular diseases or diabetes. It is also associated with an increased prevalence of emotional and cognitive alterations that in turn aggravate the disease and related outcomes. Identifying therapeutic strategies able to improve those alterations is therefore a major socioeconomical and public health challenge. We previously reported that both hippocampal inflammatory processes and neuronal plasticity contribute to the development of emotional and cognitive alterations in *db/db* mice, an experimental model of metabolic syndrome that displays most of the classical features of the syndrome. In that context, nutritional interventions with known impact on those neurobiological processes appear as a promising alternative to limit the development of neurobiological comorbidities of metabolic syndrome. We therefore tested here whether n-3 polyunsaturated fatty acids (n-3 PUFAs) associated with a cocktail of antioxidants can protect against the development of behavioral alterations that accompany the metabolic syndrome. Thus, this study aimed: 1) to evaluate if a diet supplemented with the plant-derived n-3 PUFA α-linolenic acid (ALA) and antioxidants (provided by n-3 PUFAs-rich rapeseed oil fortified with a mix of naturally constituting antioxidant micronutrients, including coenzyme Q10, tocopherol, and the phenolic compound canolol) improved behavioral alterations in *db/db* mice, and 2) to decipher the biological mechanisms underlying this behavioral effect. Although the supplemented diet did not improve anxiety-like behavior and inflammatory abnormalities, it reversed hippocampus-dependent spatial memory deficits displayed by *db/db* mice in a water maze task. It concomitantly changed subunit composition of glutamatergic AMPA and NMDA receptors in the hippocampus that has been shown to modulate synaptic function related to spatial

memory. These data suggest that changes in local neuronal plasticity may underlie cognitive improvements in *db/db* mice fed the supplemented diet. The current findings might therefore provide valuable data for introducing new nutritional strategies for the treatment of behavioral complications associated with MetS.

Key words: n-3 PUFAS, Antioxidants, Metabolic syndrome, Obesity, Inflammation, Anxiety, Memory, Hippocampus, Glutamatergic receptors, *db/db* mice

Highlights:

- n-3 PUFA/AO intake increased brain n-3 PUFA levels in a MetS model, the *db/db* mice
- n-3 PUFA/AO reversed hippocampus-dependent spatial memory deficits in *db/db* mice
- n-3 PUFA/AO modulated subunit composition of glutamate receptors in the hippocampus
- n-3 PUFA/AO may reduce MetS-related memory deficits by changing neuronal plasticity

1. Introduction

The prevalence of the metabolic syndrome (MetS) is steadily increasing worldwide to become a major public health concern. It is not only characterized by metabolic disturbances but also by low-grade inflammation (Mraz and Haluzik, 2014; Wensveen et al., 2015; Cani and Jordan, 2018; Cox et al., 2015). Such a cluster of dysregulations makes the MetS an important risk factor for related severe metabolic and cardiovascular comorbidities (Cani and Jordan, 2018). To worsen the picture, longitudinal, epidemiological and case-control studies also highlight an elevated prevalence of mood symptoms and cognitive alterations in patients with MetS as compared to the general aged-matched population (Agusti et al., 2018; Castanon et al., 2014; Mansur et al., 2015; Philippou et al., 2018). These neuropsychiatric alterations not only impair their quality of life and medical care (Brunault et al., 2012; Hilgendorf et al., 2018), but also represent important risk factors for other MetS-related comorbidities, which in turn compromise the health outcomes (Klakk et al., 2018; Ramirez et al., 2018). This alarming issue promotes heightened interest in identifying new strategies to tackle the development of neuropsychiatric alterations in the context of MetS.

Although pharmacotherapy is a first-line treatment in psychiatry, the remission rates are particularly low in patients with obesity/MetS and they often experience important side effects (Pacher and Kecskemeti, 2004; Rush et al., 2006). This highlights a need to develop further therapeutic strategies for those individuals. As already shown in other conditions associated with neuropsychiatric symptoms, nutritional interventions aiming to guaranty adequate levels of essential nutrients that are crucial for normal body and brain function, but cannot be synthesized *de novo* by mammals, may represent a promising strategy (Husted and

Bouzinova, 2016; Thesing et al., 2018; Vauzour et al., 2015). In that context, nutrients such as n-3 polyunsaturated fatty acids (n-3 PUFAs) and antioxidants (AO) (e.g. phenolic compounds and vitamins), which are insufficiently consumed in western societies, particularly by obese individuals (Simopoulos, 2001), have been shown to alleviate neuropsychiatric symptomatology (Larrieu and Layé, 2018; Miquel et al., 2018; Sanoobar et al., 2016). For example, n-3 PUFAs and vitamin E co-supplementation reduced depressive and anxious symptoms in women with polycystic ovary syndrome (Jamilian et al., 2018). Moreover, supplementation with eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3, the main n-3 PUFA in the brain) decreased stress-induced anxiety in students (Kiecolt-Glaser et al., 2011). Similarly, depressive-like and anxiety-like behaviors are prevented in rodent models of depression by dietary supplementation with vitamin E or EPA/DHA (Larrieu et al., 2014; Manosso et al., 2013). Similarly, supplementation with both n-3 PUFAs and plant-derived AO reduced memory deficits (Bensalem et al., 2018; Bensalem et al., 2016; Delpech et al., 2015a,b; Labrousse et al., 2012; Létondor et al., 2016) and depressive-like behavior in aged rodents (Moranis et al., 2012).

Although the impact of n-3 PUFAs and/or AO supplementations on neuropsychiatric comorbidities of obesity/MetS is still poorly known, mounting evidence already reports beneficial properties regarding metabolic and cardiovascular alterations (for reviews, see Lorente-Cebrian et al., 2013; Mozaffari et al., 2018). Besides, because n-3 PUFAs are sensitive to oxidation due to their unsaturated nature, providing AO together with n-3 PUFAs may improve their beneficial impact on health by reducing their oxidation (Svennevig, 2013; van Meeteren et al., 2005). For example, dietary administration of n-3 PUFA-rich rapeseed oil fortified with naturally constituting AO (i.e. coenzyme Q10, tocopherol and the phenolic

compound canolol) improves obesity-related cardiovascular dysfunction (Leger et al., 2018) and alterations of glucose metabolism in rats (Capel et al., 2018). Combined n-3 PUFAs and AO may similarly alleviate MetS-related neuropsychiatric comorbidities, since they are able to modulate the activity of several neurobiological systems that likely participate to their development (Larrieu and Layé, 2018).

Over the last decades, compelling evidence revealed a key role for dysregulation of peripheral and brain inflammatory processes and resulting alterations of brain function in impairing mood, learning and memory (Capuron and Castanon, 2017; Capuron and Miller, 2011; Dantzer et al., 2008). In animal models of obesity/MetS, increased production of inflammatory cytokines was reported in brain areas important for memory formation and mood, such as the hippocampus (Castanon et al., 2015; Kanoski and Davidson, 2011; Boitard et al., 2014; de Cossio et al., 2017; Dinel et al., 2011, 2014). This was associated with emotional alterations, memory deficits (Dinel et al., 2011, 2014) and synaptic alterations (Dinel et al., 2011, 2014; Erion et al., 2014). Interestingly, n-3 PUFAs and AO can display both immunomodulatory and neuromodulatory properties (Bazinet and Laye, 2014; Fourrier et al., 2017; Laye et al., 2018; Rey et al., 2019; Rey et al., 2016; Vauzour et al., 2015). In the context of MetS, a dietary supplementation with EPA, DHA or their precursor, α-linolenic acid (ALA, 18:3n-3), improved systemic inflammatory alterations both in patients and animal models of the disease (Karlsen et al., 2010; Kolehmainen et al., 2012; Wang and Huang, 2015). Moreover, depressive symptoms are negatively correlated with n-3 PUFAs plasma levels in MetS patients (Chalut-Carpentier et al., 2015). On the other hand, the AO curcumin reduces anxiety in patients with MetS (Esmaily et al., 2015), and dietary supplementation with polyphenols improves spatial memory, neuroinflammation and neuronal plasticity in

murine models of obesity/MetS (Liu et al., 2014; Infante-Garcia et al., 2017). However, the mechanisms underlying the beneficial effects of n-3 PUFAs and AO on MetS-related neuropsychiatric symptoms remain poorly understood. In addition, it is unknown whether their association could increase their respective efficiency.

In this study, we addressed these questions by assessing for the first time if a supplementation with both ALA and a mix of AO (coenzyme Q10, tocopherol and canolol), that may reduce n-3 PUFAs oxidation and engage multiple molecular targets at once (Fairbairn et al., 2019), improves emotional and/or cognitive alterations displayed by a murine model of MetS, the *db/db* mice. In order to identify the mechanisms underlying the expected behavioral improvement, we concomitantly measured the impact of this co-supplementation on the neurobiological systems known to be targeted by n-3 PUFAs and/or AO in other medical conditions with neuropsychiatric comorbidities. The *db/db* mice, which display classical features of MetS (including obesity, insulin resistance, dyslipidemia and dysregulated glucose homeostasis) because of a spontaneous mutation inactivating the leptin receptor, are notably suitable in the context of this study. Indeed, their metabolic dysfunctions have been associated with emotional and cognitive alterations, along with increased hippocampal inflammation (Dinel et al., 2011, 2014; Erion et al., 2014) and decreased local neuronal plasticity (Erion et al., 2014; Stranahan et al., 2008). This innovative experimental design, combining n-3 PUFAs/AO administration with a detailed assessment of MetS-related emotional, cognitive, inflammatory and neurobiological alterations, allowed us to show that this dietary supplementation restored spatial memory performances in *db/db* mice. Moreover, it provided interesting evidence pointing to the involvement of changes in neuronal plasticity-associated protein expression in the reported cognitive improvement.

2. Material and methods

2.1 Animals and Diets

All experiments were conducted according to the relevant French (Directive 87/148, Ministère de l'Agriculture et de la Pêche) and international (Directive 2010/63, European Community) legislation. They adhered to protocols approved by the Animal Care and Use Committee from Bordeaux University (approval ID: 5012047-A). Every effort was made to minimize suffering and the number of animals used. Five weeks old male *db/db* (C57BLKS/J-leprdb/leprdb; n=25) and *db/+* (C57BLKS/Jleprdb/+; n=24) mice (Charles River Laboratories, France) were housed individually in an air-conditioned (21 ± 1°C) animal-keeping room under a normal 12-h light:dark cycle, with food and water available *ad libitum*. On arrival, mice of each genotype were randomly divided into 2 groups fed one of the following diets (12% lipids *w/w*): palm/sunflower oil (Control Diet), and palm/rapeseed oil fortified with a mix of endogenous bioactive nutrients with AO properties that contained alpha-tocopherol (1700 mg/kg oil), CoQ10 (300 mg/kg oil) and canolol (600 mg/kg oil) (n-3 PUFAs/AO Diet) (Capel et al., 2018; Gladine et al., 2013; Leger et al., 2018). The palm/rapeseed diet was rich in the plant-derived n-3 PUFA α-linolenic acid (ALA), which decreases the n-6/n-3 ratio from 68 in the control diet to 2 in the n-3 PUFAs/AO diet (see Table 1 for detailed composition). Body weight, as well as food and water intake were measured once a week over a 12-week period. Mice were handled for a few minutes daily for 3 days before the experiment onset to minimize stress reactions to manipulation. They were 10 to 15 week-old at the time of behavioral assessments. Sacrifice occurred after 13 weeks of diet exposure (Figure 1).

2.2 Behavioral measures

Three complementary tests (light/dark box, elevated plus-maze, open-field) were used to measure anxiety-like behaviors as previously described (de Cossio et al., 2017). Spatial learning and memory were then assessed in a Morris water maze (Bensalem et al., 2016). Experiments were performed in the morning under conditions of dim light and low noise, with at least a one-week interval between tests. All testing sessions were recorded with a Panlab SMART video tracking system (Bioseb, France). Testing equipment was thoroughly cleaned between each session.

2.2.1 Light/dark box (L/D box).

The L/D box test is based on the innate aversion of rodents for illuminated areas and on their spontaneous exploratory behavior in response to mild stressors, namely novel environment and light. The apparatus was composed of two plexiglass chambers: a first one small and dark (14 x 21 x 21 cm) and a second one large and illuminated (30 x 21 x 21 cm). Both chambers were connected by a 7 x 5 cm opening. Each mouse was placed individually in the dark chamber and recordings were made over a 10-min period. A reduction of the time spent in the illuminated chamber was considered as an anxiety-like index.

2.2.2 Elevated plus-maze (EPM).

The EPM apparatus was made of a plus shaped acryl maze with two opposing open arms (30 x 8 x 15 cm) connected by a central platform (8 x 8 cm) and elevated 120 cm above the floor. Each mouse was placed individually in the center of the EPM over a 10-min period and the number of arm entries and the percent of time spent in open arms were recorded. A

reduction of the percent of time spent in the open arms was considered as an anxiety-like index.

2.2.3 Open-field (OF).

The OF apparatus consisted of a square area (40 x 40 cm) from which escape is prevented by surrounding walls (16 cm high). The square area was virtually divided into a central area, considered as an anxiogenic area, and the periphery defined as the rest of the apparatus. Each mouse was placed randomly in one of the corners and allowed to freely explore the OF for 10 min. A reduction of the percent of time spent and number of entries in the central area was used as an anxiety-like index.

2.2.4 Morris water maze (MWM).

As previously described (Bensalem et al., 2016), mice were first submitted to two familiarization days to familiarize with swimming and to avoid jumping from the platform during training. Twenty-four hours after the familiarization phase, they were tested in a cued task to evaluate potential visuo-motor deficits. Seventy-two hours after the cued task, mice were then trained to locate the submerged platform by using distal extra-maze cues with six trials per day for 4 consecutive days. Latency and distance to find the platform were analyzed by Imetronics videotrack system (France). Spatial memory was evaluated 72 h after the last training session, after the platform was removed, by the percentage of time spent swimming in the quadrant were the platform was located during training (target quadrant) using the Panlab SMART system (Bioseb, France). Day 1 path lengths were not included in the analysis because too many mice did not reach the platform before the 90 s cut-off.

2.3 Tissue sampling

After 13 weeks of diet consumption, mice were anesthetized by isoflurane inhalation. Blood samples were immediately collected through cardiac puncture into citrate-treated tubes. After centrifugation (10 min, 1000 *g*, 4°C), aliquots of plasma were stored at -80°C for further analysis. Mice were transcardially perfused with cold PBS (pH 7.4) to remove all traces of blood from tissues. Brains were rapidly extracted from the skull and the two hemispheres were separated to allow different neurobiological measures on the same mouse, and consequently minimize the number of animals used. The right hemisphere was immediately immersed into a 4 % paraformaldehyde fixative solution (prepared in PBS, pH 7.4) for 3 weeks and in a sucrose solution for 1 week before being sliced in 50 µm sections on a vibratome (Leica). Brain slices were then stored in a freezing solution at -20°C for further immunochemistry staining. The left hemisphere was rapidly dissected and the hippocampus and the cerebral cortex were removed and stored at -80°C for further analysis of protein expression and fatty acid composition analyses.

2.4 Cytokine and corticosterone assays

As previously described, plasma TNF-α, IL-6, IL-1β, IL-10 and IFN-γ were measured with the Mouse Cytokine/Chemokine magnetic bead panel multiplex assay (Millipore, France) following manufacturer's instructions (Dinel et al., 2011). Total plasma corticosterone was measured with an in-house radioimmunoassay using a highly specific antibody provided by H. Vaudry (University of Rouen, France) as previously described (Richard et al., 2010). All samples were run in duplicate.

2.5 Brain fatty acid content

Brain fatty acid content was only measured in the cortex, but we recently showed that modulating dietary n-3 PUFAs levels induces similar changes in this structure than in the hippocampus (Joffre et al., 2016). Total lipids from the cortex were extracted according to Folch's method (Folch et al., 1957) and fatty acids transmethylated according to the method of Morrison and Smith (Morrison and Smith, 1964). Briefly, fatty acid methyl esters were analyzed on a FOCUS GC gas chromatograph (Thermo Electron Corporation), separated using a BPX70-fused silica capillary column (SGE, Courtaboeuf, France) and identified by making a comparison with commercial standards as previously described (Fourrier et al., 2017; Lafourcade et al., 2011). Fatty acid composition was expressed as the percentage of total fatty acids.

2.6 RNA and protein extraction

Total RNA was extracted from the hippocampus using Trizol (Invitrogen, Life Technologies). The below phase contained DNA and proteins. After DNA removal, proteins were purified in denaturing conditions.

2.7 RT-qPCR

RNA purity and concentration were determined using a Nanodrop spectrophotometer (Nanodrop, Life Technologies) and reverse-transcribed as previously described (de Cossio et al., 2017). Real-time qPCR was performed using Taqman gene expression assays for sequence-specific primers purchased from Applied Biosytems (Foster City, CA) as previously described (de Cossio et al., 2017; Dinel et al., 2011, 2014). Fluorescence was determined on an ABI PRISM 7500-sequence detection system (Applied Biosystems, Foster,

CA). The calculation of relative expression levels was performed according to the methods of Schmittgen and Livak (2008) and plotted as fold change relative to the appropriate control condition.

2.8 Western Blot analysis

Protein concentration was assessed by bicinchoninic acid assay (Interchim, Montluçon, France) according to the manufacturer's instructions. Equal amounts of protein (25µg) were loaded and separated on SDS-polyacrylamide gels (8% or 10% depending of protein molecular weight) and transferred onto polyvinyl difluoride membranes (Millipore). Membranes were saturated with a blocking solution containing 5% non-fat dried milk and 0.05% Tween-20, and incubated overnight in a solution containing 5% BSA with the primary antibodies (for details, see Supplementary Table S1). After washing, membranes were incubated for 1 h with the appropriate peroxidase-conjugated secondary antibody (1:5000, Jackson Immuno Research laboratories, Westgrove, PA, USA). The blots were developed using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Science, Waltham, MA, USA). Chemiluminescence was captured by a ChemiDoc detection system and quantified by Image Lab software. Between each revelation, membranes were incubated for 15 min in Re-Blot Plus Strong Antibody Stripping Solution (Millipore) according to manufacturer's instructions in order to erase the previous antibody.

2.9 Immunohistochemistry

Adult hippocampal neurogenesis was assessed in the dentate gyrus (DG) of the hippocampus, a brain region critical for memory encoding which continuously generates new

neurons throughout life. Neurogenesis was assessed by counting the number of immature neurons characterized by the marker doublecortin (DCX), a cytoplasmic protein transiently expressed in newborn immature neurons only (Brown et al., 2003). Brain sections were first incubated with an anti-DCX antibody (1:1000) (goat monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by a biotinylated donkey anti-goat secondary antibody (1:200) (Jackson Immuno Research Laboratories Inc, West Grove, PA, USA), and avidin-biotin peroxidase complex (Vector, Burlingame, USA; 1:1000). The presence of peroxidase was then revealed using diaminobenzidine and the number of DCX-positive cells estimated as previously described (de Cossio et al., 2017). The fraction of DCX positive neurons with large vertical dendrites corresponding to a higher level of differentiation was also counted (Touyarot et al., 2013).

2.10 Data analysis

Results are presented as mean ± SEM and were analyzed using a two-way ANOVA with genotype (*db/+ vs. db/db*) and diet (control *vs.* n-3 PUFAs/AO) as between factors, followed by a Bonferroni *post-hoc* test when appropriate. Probe test comparisons of each group with chance level in the MWM have been performed with a one sample t-test. Spatial learning was analyzed using a 3-way ANOVA with repeated measures (genotype x diet x days) followed by a *post-hoc* Fisher PLSD test.

3. Results

3.1 Dietary n-3 PUFAs/AO supplementation increased brain n-3 PUFA levels

Because n-3 PUFAs provided through the diet influence brain n-3 and n-6 PUFA levels (Wainwright, 2002), which in turn modulate neurobiological function, brain fatty acid composition was assessed in the cortex after 13 weeks of exposure to control diet or n-3 PUFAs/AO (Table 2). Statistical analysis of the cortical n-6/n-3 PUFA ratio revealed main effects of genotype ($F_{(20,1)}$=103.04; p<0.001) and diet ($F_{(20,1)}$=441.22; p<0.001), as well as a significant interaction between both factors ($F_{(20,1)}$=99.00; p<0.001). Specifically, this ratio was lower in control diet *db/db* mice than in their *db/+* counterparts ($t_{(10)}$=10.94; p<0.001). Chronic consumption of n-3 PUFAs/AO decreased n-6/n-3 PUFA ratio in supplemented *db/+* ($t_{(10)}$=31.70; p<0.001) and *db/db* mice ($t_{(10)}$=6.33; p<0.001) as compared to their respective controls. As expected, this was associated with an increase in n-3 PUFA levels (diet: $F_{(20,1)}$=104.04; p<0.001) in both genotypes (controls *vs.* supplemented *db/+* and *db/db* mice: $t_{(10)}$=8.57; p<0.001 and $t_{(10)}$=5.53; p<0.001 respectively), although this effect was stronger in *db/+* mice (genotype x diet interaction: $F_{(20,1)}$=19.08; p<0.001). Interestingly, both the genotype ($F_{(1,20)}$=68.86; p<0.001) and n-3 PUFAs/AO (diet: $F_{(1,20)}$=75.98; p<0.001; genotype x diet interaction: $F_{(20,1)}$=16.56; p<0.001) notably impacted levels of DHA (22:6 n-3), the most prominent n-3 PUFA incorporated in the brain. Indeed, its levels were higher in control diet *db/db* mice than *db/+* mice ($t_{(10)}$=7.43; p<0.001), in lines with the hyperlipidemia and alterations of lipid metabolism already reported in the liver of *db/db* mice (Gotoh et al., 2009), and significantly increased in both supplemented *db/+* and *db/db* mice. Conversely, supplemented mice displayed reduced cortical levels of arachidonic acid (AA; 20:4 n-6), the second most prominent PUFA in the brain (diet: $F_{(1,20)}$=47.69; p<0.001). Overall, n-3 PUFAs/AO consumption for 13 weeks was sufficient to increase brain n-3 PUFAs and decrease n-6 PUFAs levels in both *db/+* and *db/db* mice. Of note, it did not change total body

weight gain and food consumption, as well as fasted blood glucose levels that remained higher in *db/db* mice than *db/+* mice, regardless of their diet (data not shown).

3.2 Dietary n-3 PUFAs/AO supplementation improved spatial memory deficits, but not anxiety-like behaviors in *db/db* mice

We and others previously reported increased anxiety-like behavior and altered hippocampus-dependent spatial memory in *db/db* mice (de Cossio et al., 2017; Dinel et al., 2011, 2014; Erion et al., 2014; Stranahan et al., 2008). Previous findings from our laboratory also demonstrated a beneficial effect of n-3 PUFAs or AO supplementation on mood (Larrieu et al., 2014; Moranis et al., 2012) and cognitive alterations (Bensalem et al., 2016; Delpech et al., 2015a; Labrousse et al., 2012; Létondor et al., 2016) associated with aging, stress or an immune challenge. In line with these findings, we tested here if n-3 PUFAs/AO supplementation may similarly improve behavioral alterations displayed by *db/db* mice. Akin to our previous studies, *db/db* mice displayed increased anxiety-like behaviors in comparison to *db/+* mice, since they visited less often (genotype $F_{(1,43)}=77.49$; $p<0.001$; Fig. 2A, left) and spent proportionally less time (genotype $F_{(1,43)}=41.95$; $p<0.001$; Fig. 2A, right) in the anxiogenic central area of the OF. Similarly, when compared to *db/+* mice, all *db/db* mice spent proportionally less time in the open arms of the EPM (genotype $F_{(1,42)}=9.81$; $p<0.01$; Fig. 2B), and in the illuminated chamber of the L/D box (genotype $F_{(1,44)}=47.68$; $p<0.001$; Fig. 2C), which are considered as more anxiogenic than the closed arms or the dark chamber respectively. Importantly, n-3 PUFAs/AO failed to improve anxiety-like behaviors since no significant diet effect was found whatever the behavior assessed, the test used or the

genotype. We then assessed if n-3 PUFAs/AO may in contrast improve spatial learning and memory.

In the MWM task, all mice irrespective of their genotype and diet travelled the same distance to reach the platform in the familiarization step and in the cued learning phase (data not shown), meaning that none of the mice displayed visuomotor deficits. Of note, swimming speed was lower in *db/db* mice than *db/+* mice (genotype $F_{(1,28)}$=307.30; p<0.001; data not shown) regardless of the diet. Hence, path length was used instead of duration of swimming to assess performances during the learning phase. The four groups of mice travelled significantly less distance to reach the platform between the second and the fourth day (time: $F_{(2,56)}$=4.06; p<0.05; Fig. 2D, left), indicating that they all learnt where the platform was located. Across the days, all mice displayed similar spatial learning since path length did not differ between *db/+* and *db/db* mice, and between control diet and n-3 PUFAs/AO. Spatial memory was then assessed 72 h after the end of the training phase. One sample t-test comparing to chance level (25 %) revealed that control diet *db/db* mice displayed spatial memory deficits. Indeed, they swam randomly across the four quadrants ($t_{(7)}$=2.06; p=0.08; Fig. 2D, right), whereas control diet *db/+* mice spent significantly more than 25 % of the time in the target quadrant ($t_{(6)}$=3.01; p<0.05), indicating that they remembered the platform location. Interestingly, n-3 PUFAs/AO *db/db* mice also spent significantly more than 25 % of the time in the target quadrant ($t_{(6)}$=2.69; p<0.05), showing that n-3 PUFAs/AO improved spatial memory deficits displayed by non-supplemented *db/db* mice. However, it is important to note that the n-3 PUFAs/AO restored spatial memory performances in obese mice only. Indeed, it impaired spatial memory performances in *db/+* mice, which displayed normal memory performances when fed the control diet, since this group swam randomly ($t_{(7)}$=1.42;

p=0.20). Altogether, these data showed that in the present conditions n-3 PUFAs/AO improved spatial memory, but not anxiety-like behaviors in *db/db* mice. The next question was then what biological correlates might underlie these behavioral improvements.

3.3 Dietary n-3 PUFAs/AO supplementation reduced neither plasma corticosterone levels nor brain inflammation in *db/db* mice

Increased activity of the hypothalamo-pituitary adrenal (HPA) axis, as well as peripheral and brain inflammatory processes, especially in the hippocampus, have been shown to regulate learning and memory (Bazinet and Laye, 2014; Delpech et al., 2015b; Vauzour et al., 2015), including in *db/db* mice (Erion et al., 2014; Stranahan et al., 2008). Moreover, these processes can be impacted by both n-3 PUFAs and AO (Bazinet and Laye, 2014; Vauzour et al., 2015). We therefore measured the effect of n-3 PUFAs/AO on HPA axis activity (as assessed through plasma corticosterone levels and hippocampal glucocorticoid receptor (GR) activation), plasma cytokine levels, and hippocampal expression of different inflammatory mediators. We focused on the hippocampus, as it is strongly involved in emotional regulation, learning and memory (Bannerman et al. 2002). Moreover, the MWM learning and probe test protocols used here are hippocampus-dependent (Schenk and Morris, 1985; Bensalem et al., 2016). Akin to previously published data (de Cossio et al., 2017; Dinel et al., 2011, 2014), plasma corticosterone levels were higher in *db/db* than *db/+* mice (genotype: $F_{(1,45)}=21.27$; $p<0.001$) regardless of their diet ($p>0.1$; Fig.3A). Consistent with these data, n-3 PUFAs/AO did not change hippocampal GR expression (genotype: $F_{(1,38)}=11.71$; $p<0.01$; Fig. 3B), nor its local activation through its phosphorylation, which remained higher in *db/db* than *db/+* mice (genotype: $F_{(1,38)}=21.17$;

p<0.001; Fig. 3C) regardless of their diet. Memory performances were therefore improved in supplemented *db/db* mice despite persistent overactivation of their HPA axis.

Plasma cytokine concentrations were found to be below the limit of detection in all groups (data not shown). As previously described, hippocampal mRNA expression of the inflammatory cytokine IL-6 (genotype: $F_{(1,43)}$=20.39; p<0.001, Fig. 4A) and TNF-α (genotype: $F_{(1,41)}$=44.20; p<0.001; Fig. 4A) was increased in *db/db* mice when compared to *db/+* mice, irrespective of the diet. On the contrary, n-3 PUFAs/AO brought back IL-1β mRNA expression in *db/db* mice to the level displayed by control *db/+* mice (genotype x diet: $F_{(1,41)}$=5.34; p<0.05; control diet *db/db vs.* n-3 PUFAs/AO *db/db*: $t_{(21)}$=2.31; p<0.05; Fig. 4A). COX-2 mRNA expression, which is induced by inflammatory cytokines, was higher in *db/db* mice than in *db/+* mice (genotype: $F_{(1,43)}$=25.24; p<0.001; Fig. 4A), regardless of the diet. Of note, the expression of another inflammatory marker, namely CD86, was lower in *db/db* mice than *db/+* mice (genotype: $F_{(1,39)}$=8.75; p<0.01; Fig. 4A), independently of the diet. Hippocampal mRNA expression of anti-inflammatory mediators was also affected by genotype and/or diet (Fig. 4B). Although n-3 PUFAs/AO had no impact on CD206 mRNA expression, it tended to increase IL-10 mRNA expression in both control *db/+* and *db/db* mice (diet x genotype: $F_{(1.39)}$=2,28; p=0,14; diet: $F_{(1,39)}$=3.76; p=0.06). In addition, n-3 PUFAs/AO differentially affected hippocampal TGF-β mRNA expression in *db/+* and *db/db* mice (diet x genotype: $F_{(1,42)}$=5.80; p<0.05). Whatever the diet, TGF-β mRNA was elevated in *db/db* mice when compared to *db/+* mice (genotype: $F_{(1,42)}$=73.38; p<0.001). However, n-3 PUFAs/AO increased this expression in *db/+* mice (control diet *vs.* n-3 PUFAs/AO *db/+* mice: $t_{(20)}$=2.16; p<0.05) but not in *db/db* mice.

To study further the potential impact of the n-3 PUFAs/AO supplementation on hippocampal inflammation, we then assessed the degree of activation of different intracellular signaling pathways known to be induced by cytokines (Fig. 5, Supplementary Table S2). In particular, we measured the activation of the STAT3 pathway, which is activated by both IL-10 and IL-6, and MAPK pathways (including p44/42 and p38 pathways), which are rather targeted by IL-1β and TNF-α. In agreement with increased cytokine expression in the hippocampus, local levels of phosphorylated STAT3 were found to be higher in *db/db* mice than in *db/+* mice (genotype: $F_{(1,41)}=18.52$; $p<0.001$), regardless of the diet. Hippocampal levels of phosphorylated p44/42 (genotype: $F_{(1,39)}=4.23$; $p<0.05$) and p38 proteins (genotype: $F_{(1,37)}=22.38$; $p<0.001$) were also higher in *db/db* than *db/+* mice, whatever the diet. Hence, *db/db* mice displayed increased activation of these signaling pathways in the hippocampus, in agreement with local inflammation, but it was not reduced by n-3 PUFAs/AO.

3.4 Dietary n-3 PUFAs/AO supplementation modulated subunit composition of hippocampal AMPA and NMDA receptors, but did not restore neurogenesis in *db/db* mice

Experimentally changing n-3 PUFAs and/or AO intakes in rodents is known to modulate neurobiological processes underlying memory. These processes include neurogenesis (Shukitt-Hale et al., 2008) and synaptic plasticity (Delpech et al., 2015a; Lafourcade et al., 2011), which have both been shown to be impaired in *db/db* mice (de Cossio et al., 2017; Erion et al., 2014; Stranahan et al., 2008). In agreement with these findings, *db/db* mice also displayed reduced neurogenesis compared to *db/+* mice in the present study (Fig. 6A), as revealed by reduced number of total DCX-positive cells

(genotype: $F_{(1,40)}$=25.52; p<0.001, Fig. 6B). Moreover, among all the DCX-positive cells, the proportion of ramified cells was lower in *db/db* mice than in *db/+* mice (genotype: $F_{(1,40)}$=7.947; p<0.01; Fig. 6C), suggesting that less cells are involved in a process of maturation. However, both the total number of DCX-positive cells and the proportion of these cells with ramifications were unaffected by n-3 PUFAs/AO, suggesting no effect of the supplementation on formation and maturation of newborn neurons.

Hippocampal insulin resistance has been suggested to be an important mediator of impaired synaptic plasticity and cognitive deficits in MetS (Biessels and Reagan, 2015). Moreover, n-3 PUFAs have been shown to modulate the activation of insulin signaling pathway in the hippocampus (Sun et al., 2014). We therefore evaluated whether the cognitive improvement observed in *db/db* mice fed the n-3 PUFAs/AO diet was associated with alleviation of hippocampal insulin resistance by assessing local activation of the insulin signaling pathway. As anticipated, phosphorylation levels of IRS-1 (insulin receptor substrate-1), Akt and GSK3β were increased in *db/db* mice (genotype effect: P-IRS1/IRS: $F_{(1,38)}$=23.31; p<0.001; P-Akt/Akt: $F_{(1,40)}$=6.65; p<0.05; P-GSK3β/GSK3β: $F_{(1,38)}$=23.61; p<0.001), whatever the diet (data not shown). This suggests that the n-3 PUFAs/AO diet did not improve insulin resistance in the hippocampus of *db/db* mice.

In addition to hippocampal neurogenesis and brain insulin resistance, other processes modulating local synaptic plasticity, such as LTP, have also been shown to be impaired in *db/db* mice (Erion et al., 2014; Wosiski-Kuhn et al., 2014). They may therefore be targeted by n-3 PUFAs/AO to improve spatial memory. In the hippocampus, glutamatergic NMDA and AMPA receptors are respectively constituted of the GluN1, GluN2A and GluN2B subunits for NMDA receptors and GluR1 and GluR2 for AMPA receptors. Changing the

subunit composition of these receptors has been shown to modulate synaptic function, especially LTP (Keller et al., 2017). In addition, n-3 PUFAs and AO are known to modulate NMDA and AMPA subunit composition (Calon et al., 2005; Schroeter et al., 2007). We therefore measured if such a modulation may similarly participate to n-3 PUFAs/AO-induced cognitive improvement in *db/db* mice. GluN1 protein expression was similar in all groups, whatever the genotype and the diet (Fig. 7, supplementary Table S2). Interestingly, n-3 PUFAs/AO decreased hippocampal levels of GluN2A (diet: $F_{(1,41)}=6.37$; $p<0.05$) and GluN2B subunits (diet: $F_{(1,41)}=5.07$; $p<0.05$) of the NMDA receptors in both *db/+* and *db/db* mice. Regarding the AMPA receptors, GluR1 expression was similar in all four groups, whereas GluR2 expression analysis revealed a significant interaction between genotype and diet (genotype x diet: $F_{(1,41)}=4.37$; $p<0.05$). Indeed, GluR2 levels were decreased in control diet *db/db* mice in comparison to control diet *db/+* mice ($t_{(14)}=2.35$; $p<0.05$), but n-3 PUFAs/AO normalized this level in *db/db* mice ($t_{(13)}=2.55$; $p<0.05$). In summary, enhanced spatial memory in *db/db* mice fed with n-3 PUFAs/AO was associated with changes in the composition of AMPA and NMDA receptors, which may in turn contribute to modulate synaptic plasticity.

4. Discussion

In the present study, we attempted to measure the impact of combined n-3 PUFAs/AO supplementation on both emotional and cognitive alterations in a model of MetS. Interestingly, we show that this supplementation differentially affected anxiety-like behaviors and memory performances, since it selectively reversed spatial memory deficits displayed by *db/db* mice, but had no effect on anxiety-like behaviors. Moreover, by testing the potential

implication of different neurobiological processes, chosen as candidates because they were known to modulate memory, to be targeted by n-3 PUFAs and/or AO in other conditions associated with memory deficits, and to be impaired in *db/db* mice, we provide evidence that this memory improvement likely involved modulation of neuronal plasticity, through alterations of glutamatergic receptor composition in the hippocampus.

Our results confirm previous findings reporting cognitive alterations in *db/db* mice (Dinel et al., 2011, 2014; Erion et al., 2014), including in the MWM task (Li et al., 2002; Stranahan et al., 2008). Of note, some of these studies show deficits in both the learning and retention phases (Li et al., 2002; Stranahan et al., 2008), whereas *db/db* mice displayed normal learning in the present study. This discrepancy can likely be linked to differences in the experimental protocols respectively used in the different studies. Unlike what was done here, no habituation to the test conditions (i.e. water immersion and swimming) was performed before spatial learning in most studies (Stranahan et al., 2008; Stranahan et al., 2009; Zhao et al., 2011). Testing conditions in the MWM are however highly stressful and likely interfere with spatial learning performances. Supporting this assumption, these performances were shown to be improved when mice are handled or submitted to habituation (Holscher, 1999). Moreover, some of these studies only reported swimming time to reach the platform (Chen et al., 2016), whereas path length is more appropriate since swimming speed is decreased in *db/db* mice (Zhao et al., 2012). An important finding of this study is that n-3 PUFAs/AO supplementation restored spatial memory performances in *db/db* mice. This is consistent with previous studies showing beneficial impact of n-3 PUFAs or AO supplementation on cognitive deficits associated with obesity (Infante-Garcia et al., 2017; Liu et al., 2014) and other conditions, including aging or Alzheimer's disease (Bazinet and Layé,

2014; Vauzour et al., 2015). In agreement with previously published data (Dinel et al., 2011; Sharma et al., 2010; Zhao et al., 2012), *db/db* mice also displayed here increased anxiety-like behaviors, as assessed in several complementary and well-validated behavioral tests that model different core symptoms of anxiety (Cryan and Slattery, 2007). Despite this broad behavioral characterization, anxiety-like behaviors remained higher in *db/db* mice than in *db/+* mice, regardless of their diet. It could be argued that an anxiolytic effect could have been seen after longer exposure to diet supplementation. It is noteworthy however that these findings fit with those reporting a temporal dissociation between the onset of spatial memory alterations and anxiety-like behavior in diet-induced obese models that suggests the involvement of different underlying mechanisms (André et al., 2014). Similarly, our results suggest that MetS-associated anxiety-like behaviors and memory deficits likely rely on independent mechanisms that are differentially impacted by dietary n-3 PUFAs/AO supplementation.

The hippocampus represents a key area for the control of anxiety and memory (Bannerman et al., 2002). Moreover, hippocampal inflammation has been shown to participate in the development of emotional alterations (Capuron and Castanon, 2017; Capuron et al., 2017). In particular, enhancing or blocking brain TNF-α in inflammatory conditions change anxiety-like behavior (Camara et al., 2015; Klaus et al., 2016). Akin to these findings, we recently showed that increased hippocampal TNF-α mediates the development of these behavioral alterations in *db/db* mice (Fourrier et al., 2019). In the present study, we confirmed the association between increased anxiety-like behavior and hippocampal inflammation, in particular increased expression of TNF-α and activation of its intracellular signaling pathways. The lack of impact of the dietary n-3 PUFAs/AO

supplementation on hippocampal TNF-α in *db/db* mice may therefore explain why their anxiety-like behavior remained elevated. Conversely, this suggests that hippocampal TNF-α is unlikely involved in impaired memory performances displayed by *db/db* mice, although it is known, as other cytokines, to modulate local synaptic plasticity (Beattie et al., 2002; Prieto and Cotman, 2017; Stellwagen and Malenka, 2006). These findings also support the assumption that 13 weeks of supplementation are sufficient to improve spatial memory deficits in *db/db* mice, but not to reduce anxiety-like behaviors, even if they were only assessed after 5 to 8 weeks of supplementation. Hippocampal IL-1β has also been shown to play an important role in hippocampal-dependent memory processes (Goshen et al., 2007) and to contribute to spatial memory deficits in *db/db* mice (Erion et al., 2014). Since hippocampal IL-1β expression was normalized by n-3 PUFAs/AO in these mice, it may be assumed that the present dietary supplementation improved spatial memory by targeting hippocampal IL-1β. More studies are however necessary to test this assumption. Although increased IL-1β mRNA expression was already shown to be associated with increased IL-1β protein levels in both *db/+* and *db/db* mice (Kumari et al., 2007), this needs to be confirmed in the present study. This confirmation is particularly relevant since changes in IL-1β mRNA expression were not accompanied by concomitant changes in the activation of the IL-1β-associated p38 and p44/42 signalling pathways. Nonetheless, these findings do not totally exclude a role for IL-1β and/or other inflammatory processes in n-3 PUFAs/AO-induced cognitive improvement in the context of obesity, as previously reported in other inflammatory conditions (Joffre et al., 2014; Labrousse et al., 2012; Miquel et al., 2018). Indeed, n-3 PUFAs and AO may affect other signaling pathways and/or cytokines. They may also target post-transcriptional and/or post-translational mechanisms that modulate production and actions of brain cytokines. Alternatively, these nutrients may act through

indirect mechanisms to reduce neuroinflammation. For example, changes in glial cell membrane fluidity due to the incorporation of n-3 PUFAs, particularly DHA, have previously been shown to impair the availability of inflammatory receptors for their ligands (De Smedt-Peyrusse et al., 2008; Layé et al., 2018). Moreover, n-3 PUFAs/AO may act on other brain areas than the hippocampus. Supporting this assumption, similar behavioral improvement by long-term dietary supplementation with polyphenols has been associated with reduced microglia activation in the hippocampus and the cortex of *db/db* mice (Infante-Garcia et al., 2017). However, it is worth mentioning that we reported here no reduced expression of the different inflammatory mediators assessed either in the hippocampus (Fig. 4) or prefrontal cortex (Supplementary Fig. S1). Moreover, hippocampal microglia overactivation in *db/db* mice has been related to their increased HPA axis activity (Dey et al., 2016), that was not normalized by the n-3 PUFAs/AO supplementation. This suggests that glucocorticoids unlikely participate to memory improvement reported in supplemented *db/db* mice, and that their microglia likely remained activated. However, more experiments aiming to deeply study in *db/db* mice the impact of the n-3 PUFAs/AO supplementation on brain inflammatory processes, particularly microglial activation, are necessary to understand further the contribution of inflammation in the reported memory improvement.

Hippocampal neurogenesis and brain insulin resistance are also known to play an important role in spatial memory (Biessels and Reagan, 2015; Boitard et al., 2012) and to be targeted by n-3 PUFAs and/or AO (Biessels and Reagan, 2015; Shukitt-Hale et al., 2008; Sun et al., 2014). Although we confirmed that the insulin pathway is impaired in the hippocampus of *db/db* mice (Clodfelder-Miller et al., 2005), we showed that the n-3 PUFAs/AO diet-induced cognitive improvement occurred despite any significant alleviation of brain insulin

resistance. Extending previously published data that showed a reduction of hippocampal neurogenesis in *db/db* mice (de Cossio et al., 2017), we reported here that they specifically displayed a decrease in the number of ramified new-born neurons, meaning that their neurons were less involved in processes of maturation and network integration. It may be therefore assumed that n-3 PUFAs and/or AO may improve memory performances in *db/db* mice by reversing the impairment of their hippocampal neurogenesis. This is however unlikely since these impairments were not reversed in supplemented-*db/db* mice. On the contrary, other hippocampal neurobiological processes also known to modulate learning and memory, and shown to be altered in *db/db* mice, were changed by the n-3 PUFAs/AO supplementation. This particularly concerned synaptic plasticity. Indeed, we demonstrated for the first time that hippocampal levels of the AMPA subunit GluR2 and the NMDA subunit GluN2B were respectively decreased and increased in *db/db* mice in comparison to their *db/+* controls. The subunit composition of AMPA and NDMA receptors is a key regulator of LTP (Luscher and Malenka, 2012; Makino et al., 2011; Paoletti et al., 2013), which is impaired in *db/db* mice (Erion et al., 2014; Li et al., 2002; Stranahan et al., 2008) and represents one of the main neurobiological substrate of spatial memory (Bannerman et al., 2014; Tsien et al., 1996). In particular, GluR2 expression is enhanced in the hippocampus following hippocampal-dependent learning (Whitlock et al., 2006). Interestingly, n-3 PUFAs/AO supplementation normalized the levels of GluR2 in *db/db* mice, and decreased GluN2A and GluN2B in the hippocampus of both *db/+* and *db/db* mice. On the other hand, no detectable impact of this dietary supplementation was detected in the prefrontal cortex (Supplementary Fig. S2), pointing to the hippocampus as a prime target for these nutrients. Both n-3 PUFAs and AO supplementations have already been related to changes in NMDA and AMPA receptor composition in the hippocampus (Calon et al., 2005; Schroeter et al., 2007), but the potential

concomitant impact on cognition and emotional behaviors was not evaluated in these studies. The modulation of hippocampal LTP, that likely occurs as a consequence of these changes in AMPA/NMDA receptor subunit composition, appears therefore as a particularly suitable candidate to explain the impact of n-3 PUFAs/AO supplementation on memory performances. More studies are needed to test this assumption, which is however already supported by previous experiments showing that changing n-3 PUFAs or AO dietary levels beneficially impacts LTP and cognition in other conditions (Delpech et al., 2015b; Wang et al., 2011). A limitation of the present study is that it does not allow to decipher further the mechanisms by which n-3 PUFAs and AO act on those neurobiological processes. Moreover, the respective contribution of each nutrient to the reported behavioral improvement cannot be evaluated, since they were provided together. It is worth mentioning however that their combination has been reported to be actually beneficial (Fairbairn et al., 2019), suggesting that both n-3 PUFAs and AO are likely involved in the overall effect of the supplementation.

In conclusion, we showed here that a dietary supplementation combining n-3 PUFAs and AO improved spatial memory deficits associated with MetS. Moreover, our study provides a detailed description of the neurobiological impact of this supplementation and, by doing so, points to modulation of synaptic plasticity as a likely underlying mechanism of the reported behavioral improvement. It is worth mentioning however that, based on our results, such a dietary supplementation does not seem necessarily appropriate and/or recommended in healthy individuals. In our hands, n-3 PUFAs/AO supplementation indeed impaired memory in lean *db/+* mice, suggesting potential interferences of these nutrients with neurobiological systems underlying normal learning and memory. Supporting this assumption, a few studies previously reported a pro-oxidant effect of AO, such as vitamin C and α-tocopherol,

especially when the dose administered is higher than classical recommendations (Carocho and Ferreira, 2013; Kondakci et al., 2013). Similarly, n-3 PUFA supplementation could make brain DHA more responsive to beta-oxidation (Plourde et al., 2014), which may contribute to the cognitive impairments observed in *db/+* mice (Hennebelle et al., 2014). It is therefore possible that a dietary supplementation with specific nutrients such as the ones used in the present study might be relevant only in subjects who display pre-existing neurobiological and cognitive dysfunctions prior to the supplementation onset. More studies are needed to address this issue, but the present work already highlights the necessity of considering the initial biological profile of people to be supplemented. Meanwhile, by assessing the impact of n-3 PUFAs and AO on a broad panel of neurobiological processes, this study helps understanding how these nutrients may act centrally to modulate behavior, although additional mechanisms not tested here can obviously also participate. Of note, the present findings could be particularly interesting to better understand the mechanisms underlying the positive effects of the Mediterranean diet on aging-related cognitive alterations, since n-3 PUFAs and AO are among the main components of this diet (Capel et al., 2018; Feart et al., 2015; Lourida et al., 2013; Radd-Vagenas et al., 2018). Altogether, this study points to a dietary combination of n-3 PUFAs and AO as a promising nutritional strategy to improve the management of neuropsychiatric symptoms, not only in patients with MetS and/or severe obesity, but also in other conditions associated with cognitive deficits.

DECLARATION OF INTEREST

Declaration of interest: none.

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5. Figure legends

Table 1. Detailed nutritional content of the control and n-3 PUFAs/AO diets.

Table 2. Effect of n-3 PUFAs/AO supplementation on brain fatty acid composition (n=6/group. *p<0.05; **p<0,01 ***p<0.001. When interaction, $^{###}$p<0.001 *vs* control diet same genotype; *p<0.05; ***p<0.001 *vs db/+* same diet). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; AO: antioxidants.

Figure 1. Schematic view of the study protocol.

Figure 2. Effect of n-3 PUFAs/AO supplementation on anxiety-like behaviors and spatial learning and memory performances. A) Number of entries (left) and percent of time spent (right) in the center of the open-field (OF). B) Percent of time spent in the open arms of the elevated plus-maze (EPM). C) Time in the light chamber of the light/dark box (L/D box). D) Spatial learning (left) and memory (right) performances in the Morris Water Maze test. (n=8-13/group **p<0.01; ***p<0.001 genotype effect; for spatial memory, *p<0.05 *vs* chance level)

Figure 3. Effect of n-3 PUFAs/AO supplementation on the hypothalamo-pituitary adrenal (HPA) axis activity. A) Plasma corticosterone levels. B) Hippocampal glucocorticoid receptor (GR) expression (left) and phosphorylation (right). (n=11-13/group. **p<0.01; ***p<0.001 genotype effect)

Figure 4. Effect of n-3 PUFAs/AO supplementation on hippocampal pro- and anti-inflammatory mediators. A) Hippocampal mRNA expression of IL-1β, IL-6, TNF-α, COX-2 and CD86. B) Hippocampal mRNA expression of IL-10, TGF-β and CD206. (n=11-13/group. *p<0.05; ***p<0.001 genotype effect. When interaction, *p<0.05, **p<0.001)

Figure 5. Effect of n-3 PUFAs/AO supplementation on hippocampal cytokine-associated signalling pathways. A) Phosphorylation levels of STAT3, p38 and p44/42. B) Blots of GAPDH, phospho-p44/42, p44/42, phospho-p38, p38, phospho-STAT3 and STAT3. (n=11-13/group. *p<0.05; ***p<0.001 genotype effect)

Figure 6. Effect of n-3 PUFAs/AO supplementation on hippocampal neurogenesis. A) DCX staining in the dentate gyrus. B) Total DCX^+ cells. C) Percentage of DCX^+ cells with ramifications. (n=11-13/group. ***p<0.001 genotype effect)

Figure 7. Effect of n-3 PUFAs/AO supplementation on glutamatergic receptor composition in the hippocampus. A) Hippocampal protein levels of NMDA subunits (GluN1, GluN2A and GluN2B) and AMPA subunits (GluR1 and GluR2). B) Blots of GAPDH, GluN1, GluN2A, GluN2B, GluR1 and GluR2. (n = 11-13/group. *p<0.05 genotype effect; $^{\#}$p<0.05 diet effect. When interaction, *p<0.05)

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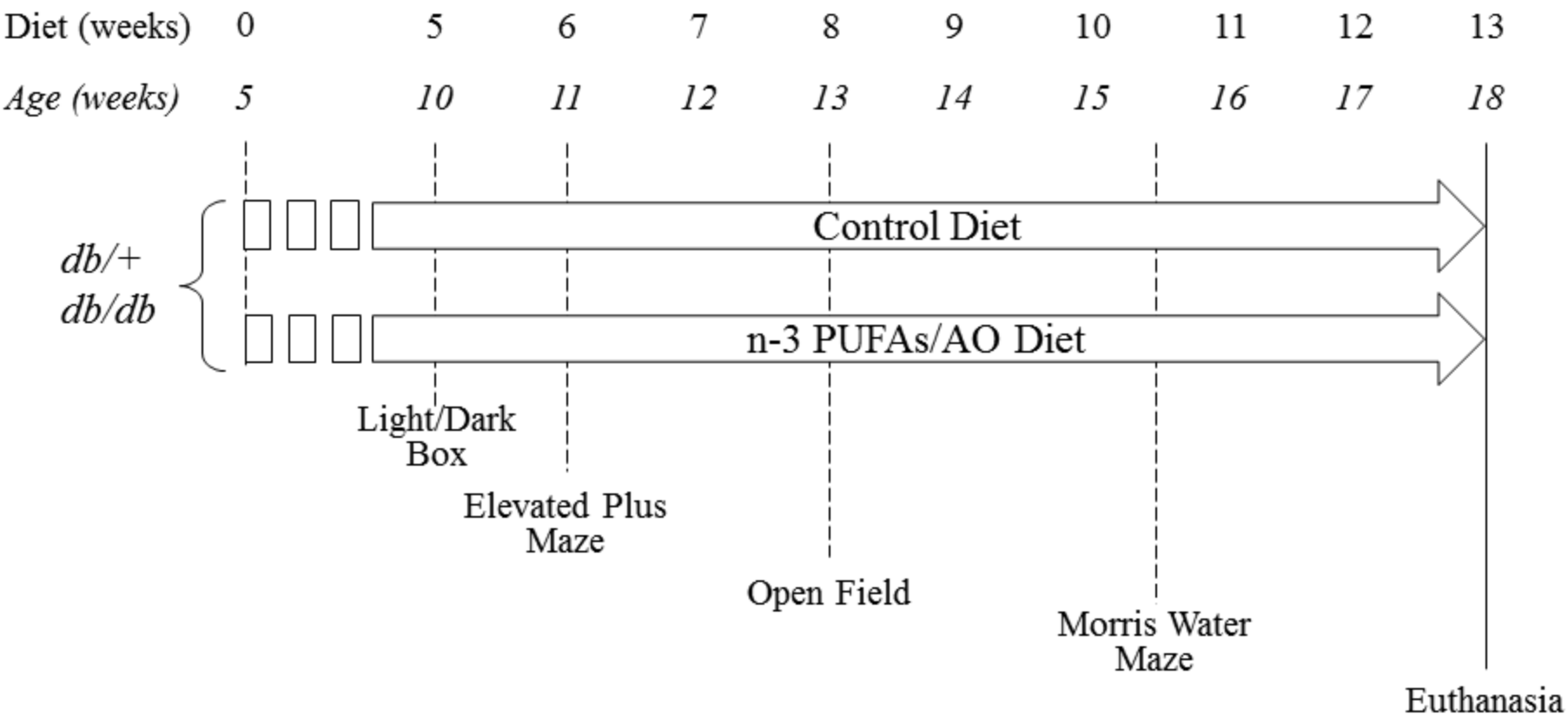
Figure 1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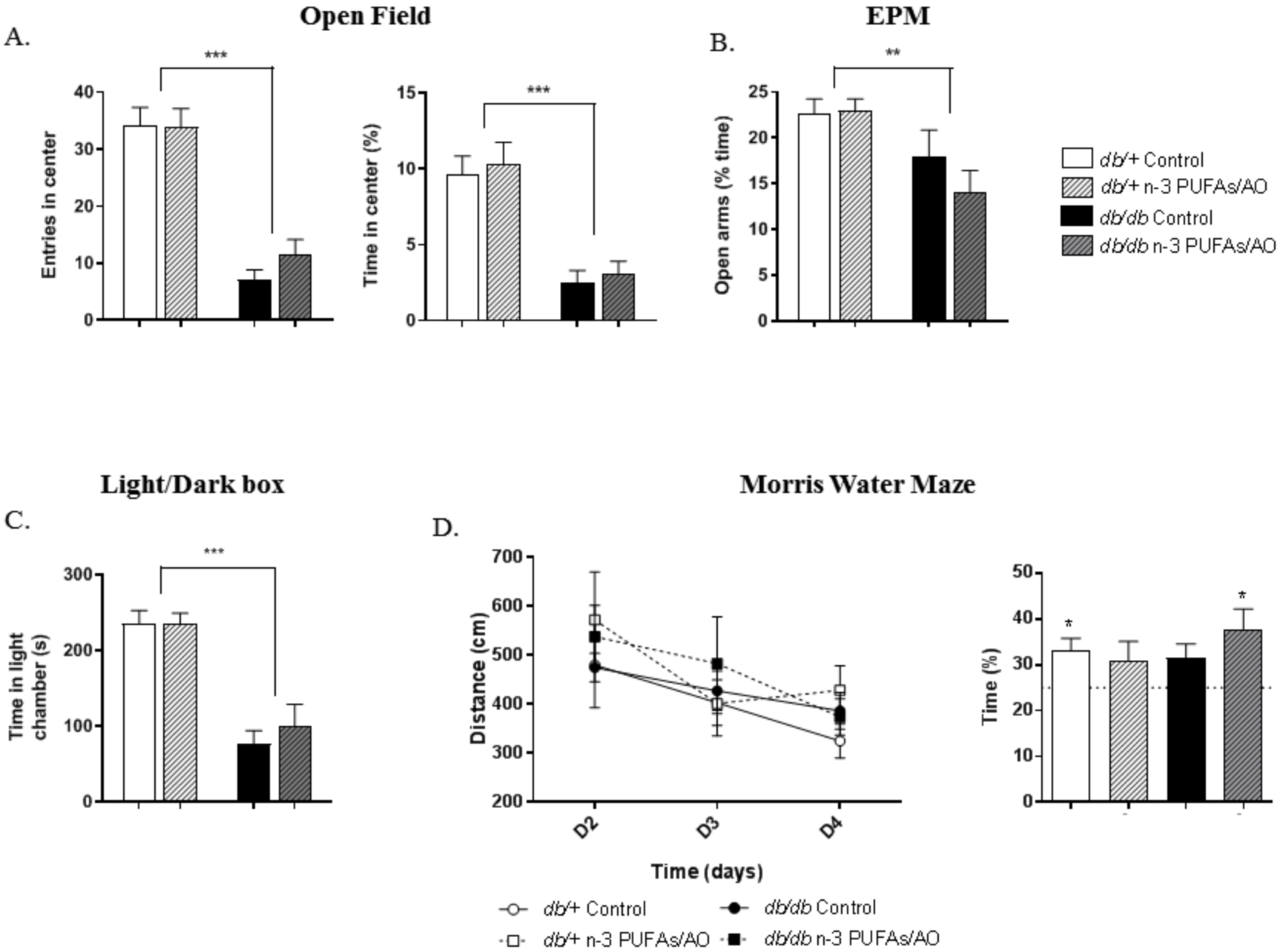
Euthanasia

Figure 2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*

Figure 3

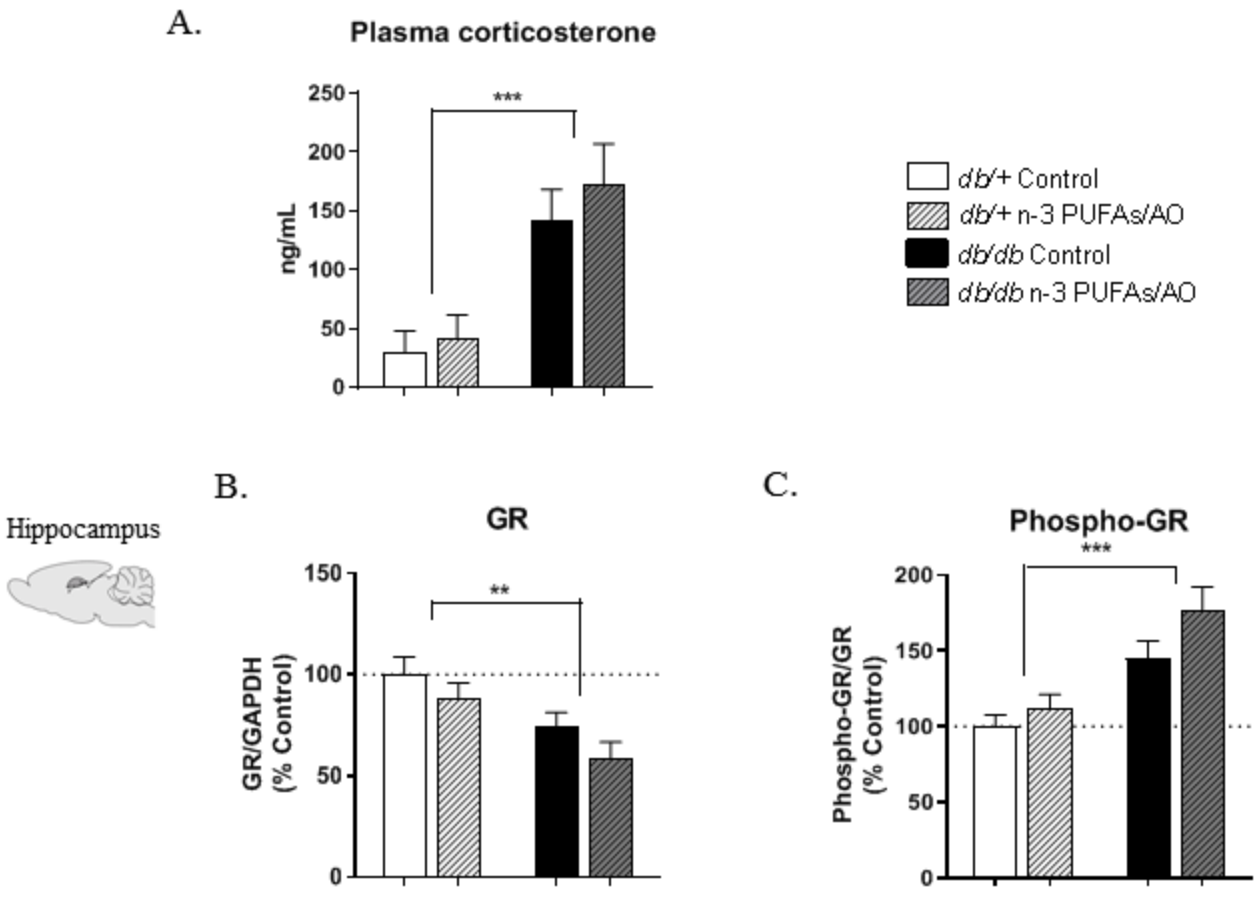

A.
Plasma corticosterone
ng/mL
250
200
150
100
50
0
***
db/+ Control
db/+ n-3 PUFAs/AO
db/db Control
db/db n-3 PUFAs/AO
B.
Hippocampus
GR
GR/GAPDH (% Control)
150
100
50
0
**
C.
Phospho-GR
Phospho-GR/GR (% Control)
200
150
100
50
0
***

Figure 4

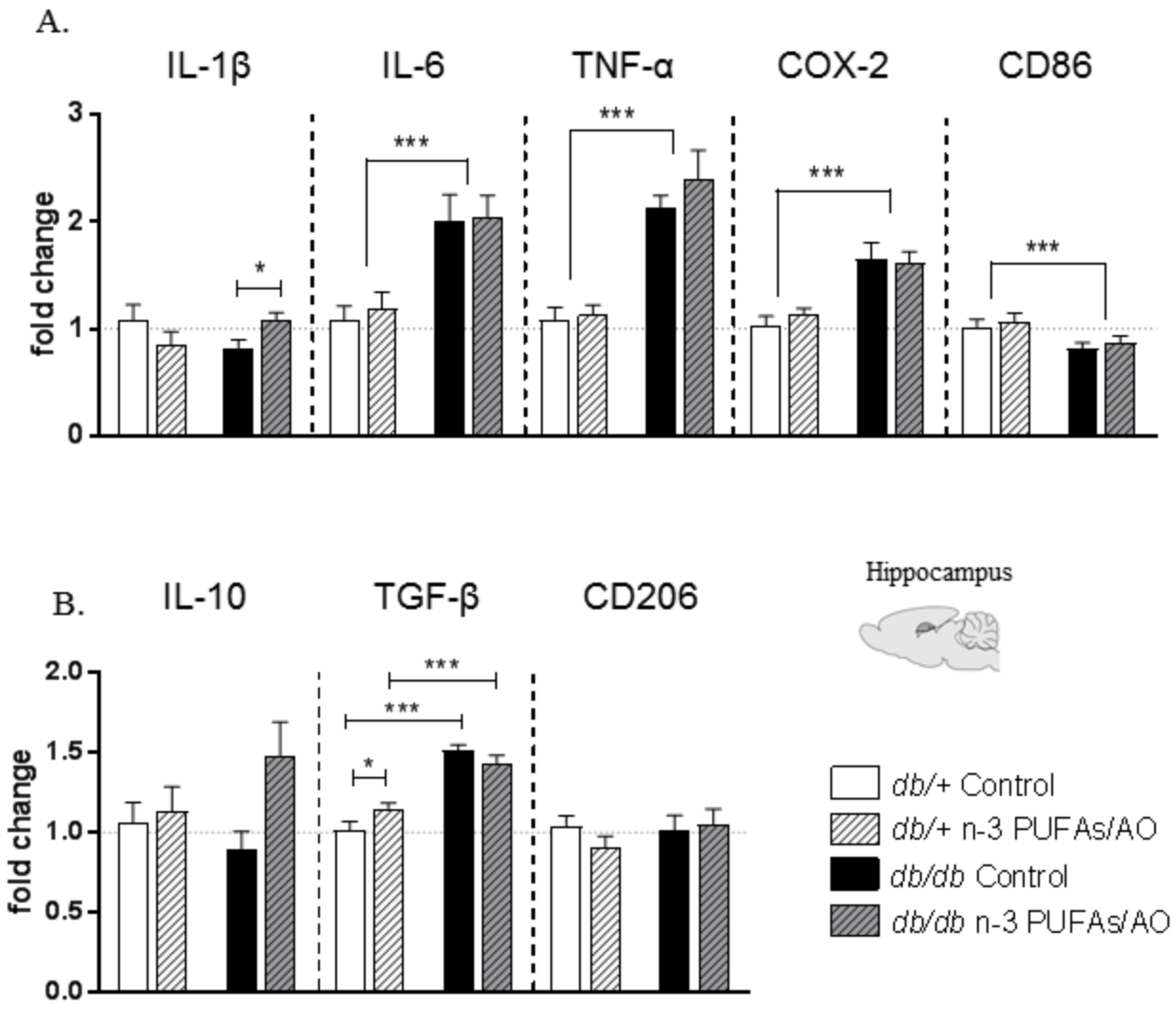
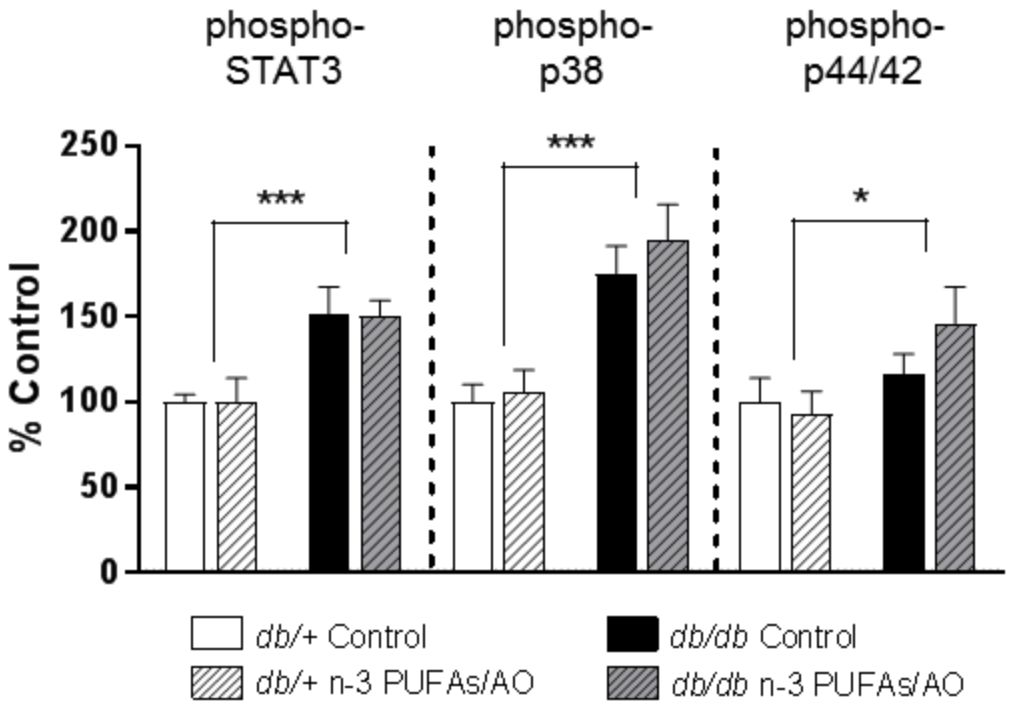

A.
IL-1β
IL-6
TNF-α
COX-2
CD86
fold change
3
2
1
0
*
***
***
***
***
B.
IL-10
TGF-β
CD206
fold change
2.0
1.5
1.0
0.5
0.0
*
***
***
Hippocampus
db/+ Control
db/+ n-3 PUFAs/AO
db/db Control
db/db n-3 PUFAs/AO

Figure 5

A.


phospho-STAT3
phospho-p38
phospho-p44/42
% Control
250
200
150
100
50
0
***
***
*
db/+ Control
db/+ n-3 PUFAs/AO
db/db Control
db/db n-3 PUFAs/AO

B.

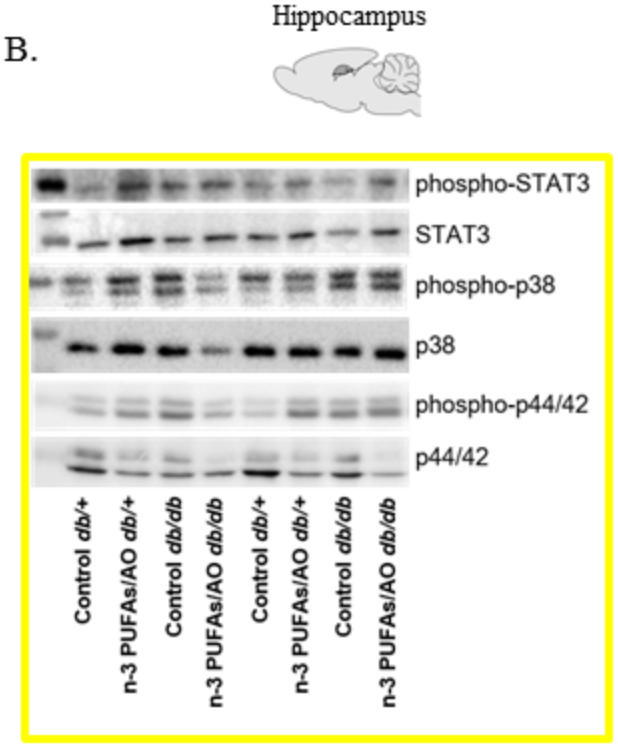

Hippocampus
phospho-STAT3
STAT3
phospho-p38
p38
phospho-p44/42
p44/42
Control db/+
n-3 PUFAs/AO db/+
Control db/db
n-3 PUFAs/AO db/db
Control db/+
n-3 PUFAs/AO db/+
Control db/db
n-3 PUFAs/AO db/db

Figure 6

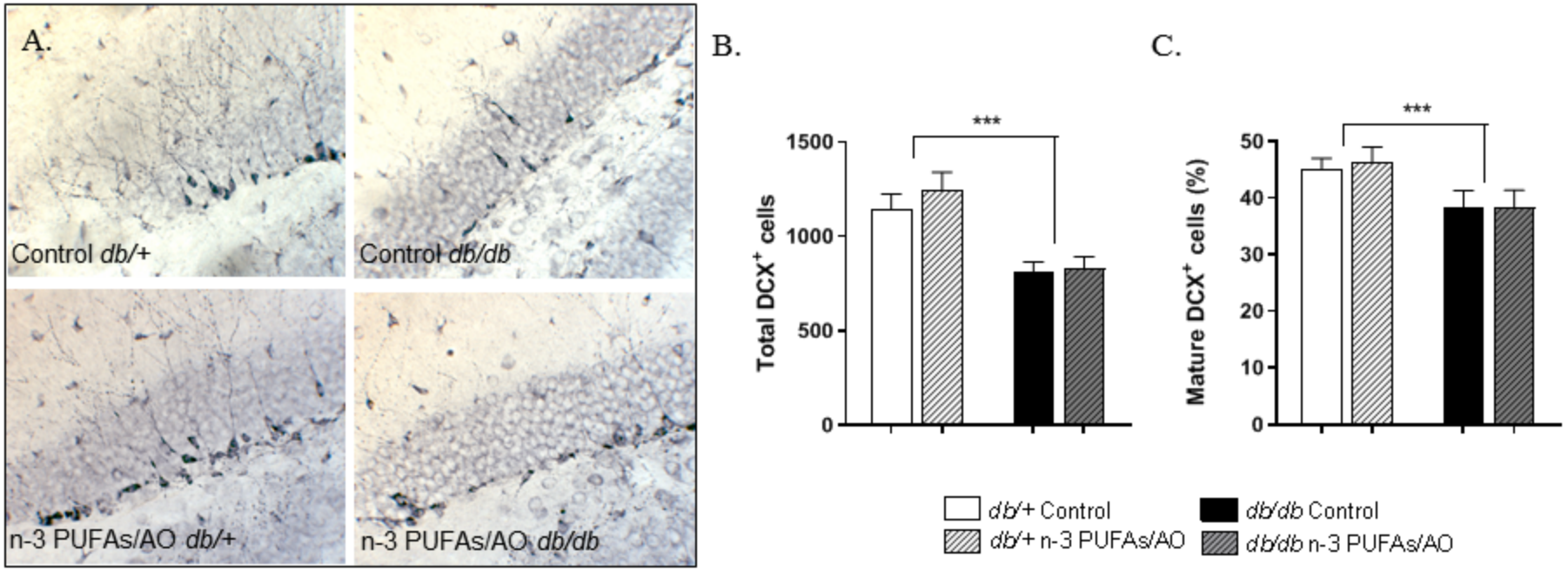
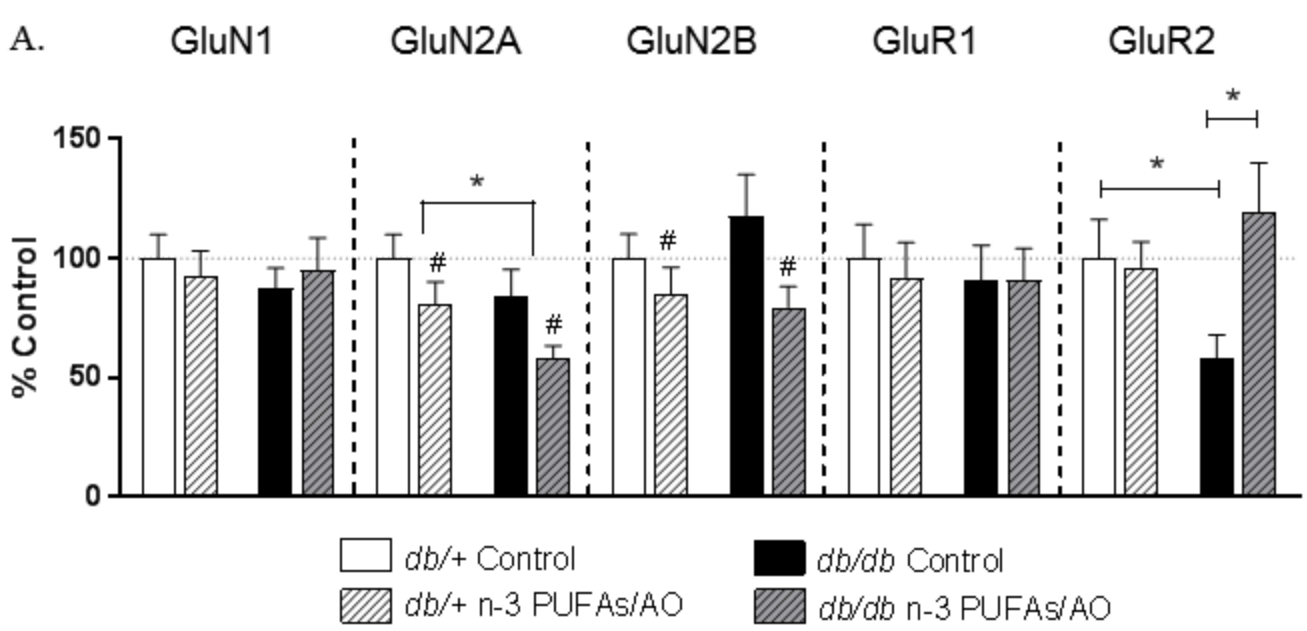

A.
Control db/+
Control db/db
n-3 PUFAs/AO db/+
n-3 PUFAs/AO db/db
B.
Total DCX+ cells
1500
1000
500
0
***
C.
Mature DCX+ cells (%)
50
40
30
20
10
0
***
db/+ Control
db/+ n-3 PUFAs/AO
db/db Control
db/db n-3 PUFAs/AO

Figure 7


A.
GluN1
GluN2A
GluN2B
GluR1
GluR2
% Control
150
100
50
0
*
*
#
#
#
#
db/+ Control
db/+ n-3 PUFAs/AO
db/db Control
db/db n-3 PUFAs/AO

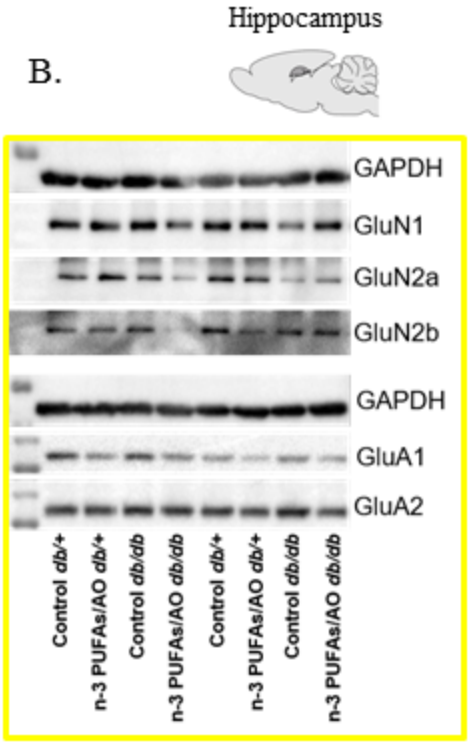

B.
Hippocampus
GAPDH
GluN1
GluN2a
GluN2b
GAPDH
GluA1
GluA2
Control db/+
n-3 PUFAs/AO db/+
Control db/db
n-3 PUFAs/AO db/db
Control db/+
n-3 PUFAs/AO db/+
Control db/db
n-3 PUFAs/AO db/db

Table 1: Detailed nutritional content of the control and n-3 PUFAs/AO diets.

	Control Diet	**n-3 PUFAs/AO diet**
Protein content	20 %	20 %
Carbohydrate content	62 %	62 %
Mineral and vitamins	6 %	6 %
Lipid content	12 %	12 %
Linoleic acid (LA) 18:2 n-6	10 %	14 %
a-linolenic acid (ALA) 18:3 n-3	0.15 %	6 %
LA/ALA	68	2
Total tocopherols	/	1700 mg/kg oil
Coenzyme Q10	/	300 mg/kg oil
Canolol	/	600 mg/kg oil

Table 2: Effect of n-3 PUFAs/AO supplementation on brain fatty acid composition

	db/+		*db/db*		Statistical effect of		
	Control	n-3 PUFAs/AO	Control	n-3 PUFAs/AO	Genotype	Diet	Interaction
	mg/100 mg Fatty acids						
	Cortex						
SFA	44.01± 0.36	43.87± 0.55	44.59± 0.25	44.60± 0.34	***		
MUFA	20.87± 0.65	21.09± 0.36	19.68± 0.41	19.63± 0.34	***		
18:2 n-6	0.34± 0.03	0.43± 0.03	0.60± 0.05	0.72± 0.08	***	***	
20:2 n-6	0.10± 0.00	0.13± 0.00	0.14± 0.01	0.15± 0.02	***	***	
20:3 n-6	0.25± 0.01	0.37± 0.01$^{###}$	0.30± 0.02***	0.38± 0.01***	***	***	***
20:4 n-6	8.46± 0.27	7.80± 0.19	8.50± 0.18	8.05± 0.14		***	
22:4 n-6	2.42± 0.08	1.99± 0.07	2.34± 0.08	2.02± 0.04		***	
22:5 n-6	1.42± 0.11	0.26± 0.02$^{###}$	0.45± 0.04***	0.16± 0.01$^{###***}$	***	***	***
n-6	12.99± 0.41	10.99± 0.29$^{##}$	12.32± 0.30***	11.48± 0.25$^{#***}$		***	***
18:3 n-3	0.03± 0.01	0.04± 0.00	0.04± 0.00	0.04± 0.00			
20:5 n-3	0.07± 0.01	0.07± 0.01	0.06± 0.01	0.05± 0.02	***		
22:5 n-3	0.11± 0.04	0.20± 0.06	0.18± 0.06	0.30± 0.06	**	***	
22:6 n-3	13.20± 0.51	15.13± 0.37$^{###}$	15.07± 0.35***	15.77± 0.17$^{##**}$	***	***	***
n-3	13.41± 0.47	15.45± 0.34$^{###}$	15.35± 0.33***	16.17± 0.14$^{###***}$	***	***	***
PUFA	26.57± 0.85	26.58± 0.61	27.77± 0.38	27.74± 0.35	***		
n-6/n-3	0.97± 0.02	0.71± 0.01$^{###}$	0.80± 0.03***	0.71± 0.01***	***	***	***