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Impact of prebiotics on metabolic and behavioral alterations in a mouse model of metabolic syndrome

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Abstract

Mounting evidence shows that the gut microbiota, an important player within the gut-brain communication axis, can affect metabolism, inflammation, brain function and behavior. Interestingly, gut microbiota composition is known to be altered in patients with metabolic syndrome (MetS), who also often display neuropsychiatric symptoms. The use of prebiotics, which beneficially alters the microbiota, may therefore be a promising way to potentially improve physical and mental health in MetS patients.

This hypothesis was tested in a mouse model of MetS, namely the obese and type-2 diabetic *db/db* mice, which display emotional and cognitive alterations associated with changes in gut microbiota composition and hippocampal inflammation compared to their lean *db/+* littermates. We assessed the impact of chronic administration (8 weeks) of prebiotics (oligofructose) on both metabolic (body weight, food intake, glucose homeostasis) and behavioral (increased anxiety-like behavior and impaired spatial memory) alterations characterizing *db/db* mice, as well as related neurobiological correlates, with particular attention to neuroinflammatory processes.

Prebiotic administration improved excessive food intake and glycemic dysregulations (glucose tolerance and insulin resistance) in *db/db* mice. This was accompanied by an increase of plasma anti-inflammatory cytokine IL-10 levels and hypothalamic mRNA expression of the anorexigenic cytokine IL-1β, whereas unbalanced mRNA expression of hypothalamic orexigenic (NPY) and anorexigenic (CART, POMC) peptides was unchanged. We also detected signs of improved blood-brain-barrier integrity in the hypothalamus of oligofructose-treated *db/db* mice (normalized expression of tight junction proteins ZO-1 and occludin). On the contrary, prebiotic administration did not improve behavioral alterations and associated reduction of hippocampal neurogenesis displayed by *db/db* mice, despite normalization of increased hippocampal IL-6 mRNA expression. Of note, we found a relationship between the effect of treatment on dentate gyrus neurons and spatial memory.

These findings may prove valuable for introducing novel approaches to treat some of the comorbidities associated with MetS.

Keywords: metabolic syndrome; gut microbiota; prebiotics; *db/db* mice; hippocampus; hypothalamus; inflammation; cytokines; anxiety; spatial memory

1. INTRODUCTION

The Metabolic Syndrome (MetS) is a widely accepted concept that clusters together several risk factors for Type 2 Diabetes (T2D) and cardiovascular diseases including: overweight/obesity, hypertension, hyperglycemia, and disturbances of lipid/carbohydrate metabolism. Interestingly, low-grade inflammation originating from the adipose tissue and/or gut microbiota environment increasingly appears as another key component of MetS (Cancello & Clement 2006; Cani et al., 2009; Gregor & Hotamisligil 2011). In addition, clinical data of patients with MetS and experiments conducted in animal models of obesity/MetS also document an augmented risk for neuropsychiatric disorders (Castanon et al 2015; Farooqui et al., 2012). Due to the breadth of complications resulting from the MetS, both the quality of life of affected patients is impaired and the costs to health care systems worldwide have increased. This leads therefore to heightened interest in better understanding the physiopathological mechanisms underlying MetS-associated complications, with the final goal of improving treatment options.

There is now compelling evidence for the participation of the gut microbiota in the systemic low-grade inflammation associated with obesity and related metabolic disorders (for review see Cani et al., 2012; Nakamura & Omaye, 2012). The gut microbiota is involved in high-fat diet-induced endotoxemia leading to the chronic low-grade inflammation observed in obesity, through impaired gut barrier permeability (Cani et al., 2016). Gut microbial population is also altered in genetically obese and diabetic mice (Geurts et al., 2011; Ley et al., 2005), and transferring the microbial flora from genetically obese mice to germ-free lean animals induces the obese phenotype in the latter (Turnbaugh et al., 2006). More recent evidence further suggests that modulating gut microbiota composition through nutritional interventions may have therapeutic potential in the management of MetS and related co-morbidities (Erejuwa et al., 2014; Petra et al., 2015). Interestingly, these interventions may impact both systemic and brain functions, although the nature of the biological pathways

targeted within the so called "gut-brain axis" is still elusive (Cani and Knauf, 2016; Cryan & Dinan, 2012; Moloney et al., 2013). Improvement of metabolic alterations has been shown with the use of probiotics, which are live microorganisms able to confer health benefits to the host when administered in adequate amount (Delzenne et al., 2011). Similarly, prebiotics such as the nondigestible carbohydrate oligofructose, which selectively stimulate the growth of some healthy commensal bacteria, have been shown to improve alterations of glucose homeostasis, plasma lipid profile (Mallappa et al., 2012), and importantly peripheral inflammation associated with MetS and/or obesity (Cani et al., 2007; Cani et al., 2016). Indeed, converging studies strongly suggest that oligofructose's beneficial effects are mediated by the ability of several commensal bacteria to reduce endotoxemia-induced systemic inflammation (Cani et al., 2009; Kootte et al., 2012). Much less is known however about the potential impact on brain inflammation, which is also classically reported in rodent models of MetS/obesity (Dinel et al., 2011; 2014; Guillemot-Legris et al., 2016; Pistell et al., 2010), as well as in other medical conditions sharing systemic inflammation and neuropsychiatric symptoms as common features (Capuron et al., 2016; Capuron & Castanon, 2016).

Accumulating evidence indicates that the gut microbiota can impact the brain (Moloney et al., 2013; Neufeld et al., 2011), with which it communicates through neuronal, hormonal and/or immune pathways (D'Mello et al., 2015; Ochoa-Repáraz & Kasper, 2016). In addition, studies dealing with the pathophysiological consequences of impaired gut-brain communication increasingly report mood and cognitive alterations (Collins et al., 2012; Cryan & Dinan 2012; Savignac et al., 2016). This new research area has recently drawn much attention, but most of the present knowledge on how microbiota can influence brain function and behavior comes from studies primarily using germ-free mice, which display neuroendocrine, neurobiochemical, and behavioral alterations in basal and/or stressful conditions that can be rescued upon gut colonization with normal microbiota (Bercik et al.,

2011; Crumeyrolle-Arias et al., 2014; Neufeld et al., 2011). Similarly, chronic stress exposure has also been reported to induce alterations of both microbiota composition and emotional behavior that can be prevented by prebiotic and/or probiotic treatment (Ait-Belgnaoui et al., 2014; Desbonnet et al., 2010; Gareau, 2014; Tarr et al 2015). Other compelling data come from studies showing that antimicrobial treatments, which change the microbiota profile in mice, induce neurobiochemical changes, as well as emotional and cognitive alterations (Bercik et al., 2011; Desbonnet et al., 2015; Pyndt-Jorgensen et al., 2015), although some contradictory data have also been reported (O'Mahony et al., 2014).

While these studies clearly contribute to better understand the role of the gut-brain axis in those particularly drastic conditions, much less is known regarding MetS-related emotional and cognitive alterations, although recent evidence points to a relationship between high-fat diet-related changes in gut microbiota and behavior (Bruce-Keller et al., 2015; Kang et al., 2014; Magnusson et al., 2015).This issue is however particularly relevant since the MetS emerges as an important risk factor for neuropsychiatric and neurological disorders including depression, anxiety, cognitive impairments, stroke, and Alzheimer's disease (Farooqui et al., 2012). Conversely, these disorders have been shown to significantly aggravate the MetS and related health outcomes, including cardiovascular disease or type 2 diabetes (Capuron et al., 2016; Roberts et al., 2010; Wulsin & Singal, 2003). Understanding the etiology of neuropsychiatric disorders in MetS patients appears therefore as a major public health challenge. Given the recent knowledge on the impact of the gut microbiota on brain and behavior, questions arise as to whether microbiota may play a role in the cognitive and emotional alterations associated with the MetS and whether prebiotics may have potential beneficial effects on such behavioral alterations.

In the present study, we addressed these issues by measuring the consequences of chronic administration of the prebiotic oligofructose on the behavioral alterations displayed by a genetic model of MetS, the *db/db* mouse. In addition, we investigated the potential underlying

mechanisms by measuring the consequences on metabolism and systemic inflammation, but also on brain systems known to be involved in the control of food intake and behavior. The *db/db* model, which displays T2D, obesity, hyperglycemia and insulin-resistance as a consequence of an inactivating mutation in the leptin receptor, is notably suitable in that context since the metabolic alterations associated with this model have been related to changes in microbiota composition (Everard et al., 2014; Geurts et al., 2011). Moreover, previous work from our group further showed that *db/db* mice also exhibit emotional and cognitive alterations that are associated with increased inflammation in the hippocampus, a key area in the regulation of mood and cognition (Castanon et al., 2015; Dinel et al., 2011; Dinel et al., 2014). By using this model that allows simultaneously assessing the effect of microbiota manipulation on behavioral alterations associated with the MetS, and on their main potential systemic and neurobiological correlates, particularly inflammation, we provided here new and important findings helping to decipher the complexity of the gut brain-axis in the context of the MetS.

2. MATERIALS AND METHODS

2.1. Animals and Treatment

All animal care and use were conducted according to the relevant French (Directive 87/148, Ministère de l'Agriculture et de la Pêche) and international (Directive 10/63, European Community) legislation and approved by the Institutional Animal Care and Use Committee (approval ID: 5012047-A). Every effort was made to minimize suffering and the number of animals used. Five week old male *db/+* (C57BLKS/J-leprdb/+; n=28) and *db/db* mice (C57BLKS/J-leprdb/leprdb; n=28) from Charles River Laboratories (France) were housed individually under standardized conditions with a 12-h light/dark cycle. Food (Lab Diet 5k52, Charles River, France) and water were available *ad libitum*. Compared to their healthy control *db/+* mice, *db/db* mice are deficient for functional leptin receptor and

consequently show typical characteristics of MetS, including severe obesity associated with hyperphagia, altered lipid/carbohydrate metabolism, and several indicators of T2D (polydipsia, polyuria, hyperglycemia, hyperinsulinemia, insulin-resistance). On arrival, half of the mice of each genotype was randomly allocated to the control groups receiving tap water or to the prebiotic groups drinking tap water supplemented with the prebiotic oligofructose (OFS) (Orafti®P95 kindly provided by Beneo Orafti, Belgium) in a concentration adequate to reach an intake of 0.6 g/day/mouse. Once a week during the 9 weeks of treatment, mice were weighed, and food and liquid (water or OFS) intake were measured by weighing the wire-tops and the bottles. Mice were individually handled daily for 1 week before behavioral tests were initiated to minimize stress reactions to manipulation.

2.2. Behavioral Measures

Behavioral measures to assess anxiety-like behaviors and spatial memory began after 4 weeks of supplementation. One behavioral experiment was done per week, always in the morning, under conditions of dim light and low noise as follows: Open-Field, Y-maze, Elevated Plus-Maze and Light/Dark box. All behavioral experiments except the Light/Dark box were automatically scored by Smart tracking software (Bioseb, France). The Light/Dark box was scored manually by a trained observer blind to treatment. All testing equipment was thoroughly cleaned between each session.

2.2.1. Open-Field (OF): To assess anxiety-like behavior mice were exposed to an unknown square area (40x40cm) with 16cm high walls without bedding litter and placed in a brightly lit room (60 lux in the center), which constitutes an anxiogenic setup (Dinel et al., 2011). A central area was defined as a virtual 10x10cm square in the middle. Mice were placed in alternate corners and allowed to freely explore the OF for 10 min. Parameters measured to evaluate anxiety-like behavior were the number of entries and the percentage of time spent in the central area.

2.2.2. Y-Maze: Spontaneous spatial recognition in the Y-maze was used as a hippocampal-dependent test as previously described (Dinel et al., 2011; André et al., 2014). The apparatus was a Y-shaped maze with three identical arms (34x8x14cm). Visual cues were placed in the testing room and kept constant during the whole test. Discrimination of novelty *vs.* familiarity was based on the different aspects of the environment that the mouse can perceive from each arm of the Y-maze. In a first trial (acquisition phase) one of the arms was closed with a door and the mouse could only explore two of the arms for 5 min, the "Entrance" arm, which was alternated for each mouse and the "Familiar" arm. In the second trial (retrieval phase), which occurred after a 30 min inter-trial interval, the mouse was placed in its previous "Entrance" arm and allowed free access to all three arms. Soiled corncob litter was used to cover the floor and was mixed before each trial to erase scent cues. The walls of the maze were also wiped clean before each use. Parameters measured included time spent in each arm, from which a Discrimination Index (DI) was calculated according to: time spent in Novel arm/(time spent in Novel + Entrance + Familiar arm) x 100. A DI significantly higher than chance level (33.33%) indicated recognition.

2.2.3. Elevated Plus-Maze (EPM): In order to evaluate anxiety-like behavior, mice were placed in a plus-shaped maze made of four arms (30x8cm), two of which being closed with walls (15cm high) providing shelter (Dinel et al. 2011). Open arms were anxiogenic since the maze was elevated 120cm from the floor. Mice were placed in the center of the maze facing one of the open arms and allowed to freely explore for 5min. 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.

2.2.4. Light/Dark box (L/D box): Anxiety-like behavior was also assessed in the L/D box. The apparatus was composed by a white acryl box with an open-top compartment (30x21x21cm with 205 lux) communicating through an opening (7x5cm) to a black lidded one (14x21x21cm with <5 lux). The communication between compartments was blocked for

5 seconds after inserting the mouse in the lidded compartment and the 10-min test began thereafter. Both compartments were thoroughly cleaned before each use to remove odor cues. The number of transitions between the two chambers and time spent in the light compartment were recorded to assess anxiety-like behavior (Bosch-Bouju et al., 2016).

2.3. Glucose tolerance test

A glucose tolerance test was performed after 9 weeks of treatment (age: 14 weeks), as previously described (Everard et al., 2011). Food was removed before the onset of night cycle and mice were tested for their tolerance to glucose after 13-h fasting period. Mice were weighed and fasting glycemia measured before an intraperitoneal (ip) injection of glucose (2g/kg prepared in NaCl solution), and at 15, 30, 60 and 120 min after. Blood sampling of glucose was made by clipping off the end of the tail to collect a droplet of blood that was immediately analyzed with a Glucometer (Accu-Chek; Roche Diagnostics). Food was returned just after the last blood collection.

2.4. Insulin resistance test

The insulin resistance test was performed after 9 weeks of treatment (age: 14 weeks) in fed animals. Mice were weighed and glycemia measured before an ip injection of human insulin (0.75IU/kg; Insulin Glargine, Lantus,), and at 15, 30, 45, 60 and 120 min after. Blood sampling of glucose was done as above. The insulin solution was prepared in an aqueous vehicle solution (glycerol 0.2 mol/l, m-cresol 0.025 mol/l, ZnCl2 0.2 µmol/l) adjusted to pH 4.0, as previously described (Stammberger et al., 2002).

2.5. Tissue Sampling

After 10 weeks of treatment (age: 15 weeks), overnight fasted mice were anesthetized by isoflurane inhalation within a few seconds after being picked up from their home cage. Blood

samples were immediately collected via cardiac puncture into EDTA (10%)-coated tubes. After centrifugation (10 min, 3000*g*, 4°C), aliquots of plasma were stored at -80°C for further analysis. Seven to eight mice from each experimental group were immediately perfused with chilled PBS via the ascending aorta to remove all traces of blood from tissues. Brains were rapidly extracted from the skulls and carefully dissected. The hypothalamus and hippocampus were immediately collected into labeled-sterile tubes which were frozen with dry ice and then stored at -80°C until assaying. The cecal content was also collected post-mortem in RNA-free tubes, immediately immersed in liquid nitrogen and stored at −80°C until assay for bacteria composition. The remaining mice of each group were transcardially perfused with PBS (pH 7.4) followed by a 4% paraformaldehyde fixative solution (prepared in PBS, pH 7.4). Brains were removed, immediately immersed for 24h in fixative, and then processed as described below (see Immunohistochemistry section).

2.6. Biochemical and histological measures

2.6.1. Plasma Cytokines and Hormones Assay: Plasma cytokines (IL-1β, IL-6, TNF-α and IL-10) and hormones (GLP-1, insulin, resistin, ghrelin and glucagon) were measured with the mouse cytokines and metabolic hormones Milliplex kits (Merk-Millipore, France) following the manufacturer's instructions as previously described (Dinel et al., 2011; 2014). Total plasma corticosterone was measured with an in-house RIA 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.

2.6.2. Reverse Transcription and Real-Time RT-PCR: Total RNA was isolated from extracted structures (hippocampus and hypothalamus) using TRIzol (Invitrogen, Life Technologies™) and reverse-transcribed as previously described (André et al., 2008; Dinel et al., 2011; 2014). Real-time RT-PCR for cytokines (IL-1β, IL-6, TNF-α, IL-10) and tight junction proteins (ZO-1 and Occludin) of the blood brain barrier (BBB) was performed using

Taqman gene expression assays for sequence-specific primers purchased from Applied Biosytems (Foster City, CA). Reactions were performed in duplicate according to manufacturer instructions (André et al., 2008; Dinel et al., 2011; 2014). To measure expression of hypothalamic peptides (CART, POMC, NPY), quantitative PCR was performed using SYBR® Assay (Eurogentec, Seraing, Belgium) following the protocol previously described (Madore et al., 2014). Primers sequences (see Table 1) were designed using Primer Express® Software in order to avoid genomic DNA amplification. mRNA levels of target genes were normalized from β2-microglobulin expression in each sample. 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 & Livak (2008) and plotted as fold change relative to the appropriate control condition.

2.6.3. Bacteria analysis: Bacterial DNA was extracted from the cecal content using a QIAamp-DNA stool minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions. qPCR for total bacteria, *Bifidobacterium* spp., *Lactobacillus* spp., *Roseburia* spp., and *Bacteroides-Prevotella* spp. was performed as previously described (Everard et al., 2011).

2.6.4. Immunohistochemistry and unbiased stereological counting of GFAP and Iba-1 positive cells: After a 24h-period of fixation in a 4% paraformaldehyde solution, brains were sectioned frontally at a width of 40 µm with a cryostat (Leica, Rueil Malmaison, France). Free-floating sections were processed with a standard immunohistochemical procedure. A one-in-six section was treated for immunoreactivity. Astrocytes and microglia were respectively identified in the arcuate nucleus (ArcN) and hippocampus by using a goat polyclonal GFAP antibody (Santa-Cruz Biotechnology, Dallas, USA; 1/1000) followed by a biotinylated rabbit anti-goat antibody (Dako Glostrup, Denmark; 1/2000), and a rabbit

polyclonal Iba-1 antibody (Wako, Neuss, Germany; 1/1000) followed by a biotinylated goat anti-rabbit antibody (Vector, Burlingam, USA; 1/2000).

The unbiased stereological sampling method based on optical dissector stereological probe was used to quantify GFAP and Iba-1 immunoreactive cells in the whole ArcN, hippocampal CA1, CA3 and dentate gyrus (DG) (Madore et al., 2014). Stereological analysis was performed using an Olympus BX51 microscope with a motorized Z and X-Y stage encoders linked to a computer-assisted stereological system (Mercator digital imaging system, Explora Nova, La Rochelle, France). For each animal, ArcN, CA1, CA3 and DG boundaries were delimited at low magnification (x2.5) according to Franklin & Paxinos mouse brain atlas. ArcN, CA1, CA3 and DG boundaries were drawn on every sixth section and the first was randomly chosen. Each structure volume was calculated using the formula "$V_{(XX)} = \Sigma S\ td$"; where "ΣS" is the sum of surface areas (μm^2), "t" the average section thickness and "d" the distance between the sections (Theoret et al., 1999). "t" was estimated to 10µm after immunohistochemistry processing and guard zones of 2µm were used to ensure that section top and bottom were never included in the analysis. Eleven sections were used for each animal. From a random start position, a computer-generated sampling grid placed the counting frames that were used to count the cells/mm^2/animal for each brain region at a 40× magnification. Microglia and astrocytes were counted in the whole slice thickness, only if more than half the cell was inside the two consecutive boundaries taken into account. Split cell counting error was corrected by using the Abercrombie formula (Abercrombie, 1946). To estimate the number of positive immunoreactive cells, we used: $N = V_{(XX)}\ (\Sigma Q^{-}/\Sigma V_{(dis)})$; where N is the estimation of the number of positive immunoreactive cells, V the volume of each brain structure, ΣQ^{-} the number of cells counted in the frames, and $\Sigma V_{(dis)}$ the total volume of the frames (Theoret et al., 1999).

2.6.5. Assessment of microglia morphology: Iba-1 immunoreactive microglia were visualized under the 100X oil lens [numerical aperture (NA), 1,25] using a Leica mcd

microscope. Number of primary processes was assessed in CA1, DG and ArcN, as previously described (Madore et al., 2014). Three sections of a one-in-six series were used for each of these regions. Four microglia cells per section were used to estimate the number of primary process, giving a total of 12 cells/region of interest.

2.6.6. Immunohistochemical detection of doublecortin-positive cells: Neurogenesis was evaluated in the DG region by determining the number of immature neurons characterized by the endogenous marker doublecortin (DCX), a cytoplasmic protein transiently expressed in newborn neurons only (Brown et al., 2003). To assess DCX-immunoreactivity, 40-µm sections were incubated with a rabbit polyclonal antibody (Abcam, Cambridge, UK; 1/800), followed by a secondary biotinylated goat anti-rabbit antibody (Vector, Burlingam, USA; 1/200) and avidin-biotin peroxidase complex (Vector, Burlingam, USA; 1/1000). The presence of peroxidase was then revealed using diaminobenzidine (DAB) as a substrate according to the nickel-enhanced glucose oxidase method giving a black precipitate. The number of DCX-positive cells was estimated by using a modified version of the optical fractionator method with a systematic random sampling of every 6 sections along the rostro-caudal axis of the DG. On each section, immunoreactive cells in the granular and subgranular layers of the DG were counted. All results are expressed as the total number of cells in the whole DG. The fraction of DCX-immunoreactive new neurons with large vertical dendrites (mature new neurons) corresponding to a higher level of differentiation was also counted (Boitard et al., 2014).

2.7. Statistical Analysis

Experiments were conducted as a completely randomized design. Repeated Measures ANOVA were performed on data collected across several time points to assess main effects and interactions, and planned contrasts or Fisher's LSD *post-hoc* tests were used for punctual comparisons. A two-way ANOVA was used to evaluate genotype (*db/+ vs. db/db*) and

treatment (water *vs.* OFS) effects and *post-hoc* Student's t tests to look at specific comparisons when interactions between both factors were significant. Correlations and regression analysis were also conducted to look at relationships between variables of interest. All results are graphed as Mean ± SEM. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Prebiotic (OFS) treatment increased gut levels of *Bifidobacterium* spp. in *db/db* mice

No genotype differences were found between untreated *db/+* and *db/db* mice regarding total bacteria number or the abundance of *Lactobacillus* spp., *Roseburia* spp. and *Bacteroides-Prevotella* spp. populations, whatever the treatment (Table 2). On the contrary, both genotype ($F_{(1,44)}= 14.7$; $p<0.001$) and OFS treatment ($F_{(1,44)}= 8.2$; $p<0.001$) affected the abundance of *Bifidobacterium* spp., which was particularly increased in OFS-treated *db/db* mice (genotype x treatment: $F_{(1,44)}= 4.0$; $p=0.05$) compared to their controls (water *vs.*OFS: $p<0.01$) or to *db/+* groups ($p<0.001$). As expected, OFS treatment modified the gut microbiota composition in *db/db* mice, as previously shown in other rodent models of obesity (Cani et al., 2007; Everard et al 2011).

3.2. Prebiotic treatment improved eating behavior and glycemic dysregulations in *db/db* mice

In agreement with their expected phenotype, *db/db* mice weighed significantly more than *db/+* mice during the entire experiment (genotype: $F_{(1,49)}= 207.3$, $p<0.001$; time: $F_{(7,343)}= 54.3$, $p<0.001$). Although no main effect of OFS treatment on body weight was revealed by the statistical analysis, it tended to slightly and progressively reduce body weight gain (as calculated for each time-point by the difference between post-treatment and pre-treatment body weight) in *db/db* mice compared to their untreated counterparts, whereas both *db/+*

groups displayed similar body weight gain overtime (genotype: $F_{(1,343)}$= 12.4, p<0.001; genotype x time: $F_{(7,343)}$= 2.2, p<0.05; data not shown). Consequently, body weight gain was statistically higher overtime in control *db/db* mice than in control *db/+* mice (genotype: $F_{(1,175)}$= 19.6, p<0.001; genotype x time: $F_{(7,175)}$= 3.4, p<0.01), but not anymore after OFS treatment (genotype: $F_{(1,168)}$= 1.2, p>0.3; genotype x time: $F_{(7,168)}$= 17.3, p<0.001). This was associated with a higher food (genotype: $F_{(1,336)}$= 36.4, p<0.001; genotype x time: $F_{(7,336)}$= 2.2, p<0.05; Fig. 1B) and liquid intake (genotype: $F_{(1,343)}$= 68.5, p<0.001; genotype x time: $F_{(7,343)}$= 38.6, p<0.001; Fig. 1C) in control *db/db* mice than in *db/+* mice. Interestingly, OFS treatment progressively reduced both food (treatment: $F_{(1,336)}$= 8.1, p<0.01; genotype x treatment: $F_{(1,336)}$= 14.8, p<0.01) and liquid (treatment: $F_{(1,343)}$= 21.9, p<0.001; genotype x treatment x time: $F_{(7,343)}$= 24.7, p<0.001) intake, in particular in *db/db* mice. Indeed, OFS-treated *db/db* mice consumed significantly less food than their controls (p<0.001), whereas both *db/+* groups were not statistically different (p>0.1). Similarly, *db/db* mice treated with OFS showed a stable, close to normal liquid intake, whereas untreated *db/db* mice were the only ones drastically increasing their drinking over time (treatment x time: $F_{(7,161)}$= 37.6, p<0.001).

As OFS has been previously shown to improve glucose tolerance in several models of obesity and diabetes (Cani et al., 2006; Everard et al., 2011), and it improved excessive eating behavior in *db/db* mice, we measured its impact on their glycemic dysregulations. We therefore assessed basal blood glucose levels in both fasted and fed conditions, and during glucose tolerance and insulin resistance tests. As expected, *db/db* mice had higher basal fasted (genotype: $F_{(1,50)}$= 461.0, p<0.001; Fig. 2A) and fed (genotype: $F_{(1,50)}$= 305.1, p<0.001; Fig. 2C) blood glucose levels than *db/+* mice. OFS treatment significantly reduced these levels in both fasted (treatment: $F_{(1,50)}$= 12.5, p<0.001; genotype x treatment: $F_{(1,50)}$= 17.1, p<0.001; Fig. 2A) and fed *db/db* mice (treatment: $F_{(1,50)}$= 13.2, p<0.001; genotype x treatment: $F_{(1,50)}$= 9.8, p<0.01; Fig. 2C), which displayed in both conditions basal glucose levels still higher than

db/+ groups, but significantly lower than their non-treated counterparts ($p<0.001$). Blood glucose levels rapidly increased after ip glucose injection in all treated mice, although the time-course of such changes depended on both genotype and OFS treatment (genotype: $F_{(1,50)}= 896.9$ $p<0.001$; genotype x treatment x time: $F_{(4,200)}= 8.9$, $p<0.001$; Fig. 2A). Indeed, OFS treatment significantly improved glucose tolerance in *db/db* mice (treatment: $F_{(1,96)}= 7.1$, <0.05), whereas it had no effect in *db/+* mice ($p>0.1$). Of note, this improvement was likely underestimated as most control *db/db* mice displayed blood glucose levels higher than the highest point of the calibration curve (600 mg/ml). Additionally, these levels remained elevated during the entire glucose tolerance test in control *db/db* mice but started reducing 1h after glucose injection in OFS-treated *db/db* mice. Akin with these results, calculation of the area under the curve (AUC) confirmed that supplemented *db/db* mice had significantly higher glucose tolerance compared to their controls (genotype x treatment: $F_{(1,50)}= 4.4$, $p<0.05$; Fig. 2B), while *db/+* mice were the same whether they were treated or not. Interestingly, similar results were obtained regarding insulin resistance that characterized *db/db* mice. All *db/db* mice maintained high blood glucose levels compared to *db/+* mice after being injected with insulin (genotype: $F_{(1,50)}= 305.1$, $p<0.001$; Fig. 2C). Moreover, blood glucose levels slightly decreased in *db/+* mice over the first minutes following insulin injection while they increased in *db/db* mice (genotype x time: $F_{(5,250)}= 15.6$, $p<0.001$). Treatment with OFS selectively reduced blood glucose levels in *db/db* mice but not *db/+* mice (treatment: $F_{(1,50)}=13.2$, $p<0.001$; genotype x treatment: $F_{(1,50)}= 9.8$, $p<0.01$), whatever the time-point post-insulin challenge (no significant time effect or time by treatment interaction). These findings were confirmed by calculation of the AUC, which was significantly reduced after OFS treatment in *db/db* mice only (genotype x treatment: $F_{(1,50)}= 13.4$, $p<0.001$; Fig. 2D). Thus, OFS treatment improved glycemic dysregulations displayed by *db/db* mice, although it did not significantly change plasma levels of insulin (Fig. 2E) and GLP-1 (Fig. 2F), which remained higher in *db/db* than *db/+* mice (genotype: $F_{(1,31)}= 36.0$, $p<0.001$ and $F_{(1,31)}= 5.2$, $p<0.05$ respectively)

regardless of their treatment. On the contrary, resistin levels, which were lower in control *db/db* mice than in all other groups ($p<0.05$; Fig. 2G), were normalized by OFS treatment (treatment: $F_{(1,34)}= 3.7$, $p=0.06$; genotype x treatment: $F_{(1,34)}= 3.9$, $p<0.05$). Lastly, ghrelin and glucagon levels were very low and similar in all groups (data not shown). Improved dysregulated eating behavior and glycemic control in *db/db* mice were therefore associated with increased plasma resistin levels.

3.3. Prebiotic treatment failed to improve anxiety-like behavior, spatial memory deficits and reduced hippocampal neurogenesis displayed by *db/db* mice

We previously reported increased anxiety-like behavior and reduced spatial memory performances in *db/db* mice (Dinel et al., 2011; 2014). Based on mounting evidence illustrating the impact of gut microbiota on brain and behavior (Cryan & Dinan, 2012), we tested if OFS treatment could improve these behavioral alterations. As anticipated, *db/db* mice spent proportionally less time (genotype: $F_{(1,49)}=12.8$, $p<0.001$; data not shown) and visited less often the central area of the OF (genotype: $F_{(1,49)}=185.2$, $p<0.001$; Fig. 3A) or the lighted compartment of the L/D box (genotype: $F_{(1,50)}=42.6$, $p<0.001$; Fig. 3B), which are considered as more anxiogenic than the periphery or the dark compartment respectively. Similarly, *db/db* mice displayed less entries ($F_{(1,48)}= 22.2$, $p<0.001$; data not shown) and reduced time into the open arms of the EPM ($F_{(1,45)}= 16.5$, $p<0.001$; Fig. 3C) compared to *db/+* mice. These consistent data confirmed greater anxiety-like behaviors in *db/db* mice than in *db/+* mice since these behavioral alterations were independent of locomotor impairment, as previously shown (Dinel et al., 2011; 2014). For example, distance covered by *db/db* mice in the OF was reduced in the central area compared to *db/+* mice but increased at the periphery ($p<0.01$; data not shown), suggesting that *db/db* mice avoided the central area. More importantly, chronic OFS treatment failed to reduce anxiety-like behavior as no significant

effect of treatment was found whatever the genotype and the behavioral paradigm used (OF, EPM or L/D box).

Akin with these findings, both OFS-treated and non-treated *db/db* mice randomly explored the different arms of the Y-maze as revealed by an index of discrimination (calculated as the percent of time spent exploring the novel arm) equal to chance level (Fig. 3D). Importantly, these data likely reflect spatial memory deficits rather than motor, sensory or motivational disturbances since *db/db* mice were previously shown to preferentially explore the novel arm compared to familiar arms when tested with a short 2-min inter-trial interval (Dinel et al., 2011). On the contrary, discrimination index was significantly higher than chance level in both water ($t=2.70$, $p<0.05$) and OFS ($t=3.21$, $p<0.01$) *db/+* groups, meaning that they spent more time exploring the novel arm, which they recognized, than the familiar one.

As hippocampal neurogenesis was shown to play an important role in spatial memory (Boitard et al., 2012), we assessed whether it was impaired in *db/db* mice. Neurogenesis was evaluated by determining the number of immature neurons in the DG characterized by the endogenous marker DCX. Interestingly, spatial memory deficits were associated in *db/db* mice with a significant reduction in the number of DCX-positive cells (genotype: $F_{(1,20)}=16.9$, $p<0.001$; Fig. 3E) compared to *db/+* mice. A positive correlation, though marginally significant ($r=0.58$; $p=0.06$), was indeed found in *db/db* mice between this number and the time spent exploring the novel arm of the Y-maze. In agreement with the failure of OFS treatment in improving behavioral alterations displayed by *db/db* mice, no significant treatment effect was found on the number of DCX-positive cells whatever the genotype. However, further exploring how treatment and this number were related to spatial memory performances with a regression analysis, we found that it was the combined effect of treatment and number of DCX-positive cells that predicted performances of *db/db* mice in this task, while treatment alone was not an effective predictor (table 3). Taken together, these results confirmed increased anxiety-like behavior and spatial memory deficits in *db/db* mice,

and although they did not show any detectable beneficial effect of OFS treatment on those measures, they suggest a link between the treatment, the number of DG neurons, and spatial memory performances in *db/db* mice.

3.4. Prebiotic treatment reduced corticosterone release and increased plasma levels of the anti-inflammatory cytokine IL-10 in *db/db* mice

Among the main biological factors shown to be altered in *db/db* mice and able to act within the brain to modulate metabolic and/or behavioral reactivity, corticosterone and cytokines are likely candidates (Gregor & Hotamisligil 2011; Marques et al., 2009). We therefore determined whether OFS treatment could normalize over-activation of the hypothalamo-pituitary-adrenal (HPA) axis and/or inflammatory disturbances displayed by *db/db* mice. As previously shown (Dinel et al., 2011; 2014), *db/db* mice displayed higher plasma corticosterone levels than *db/+* mice (genotype: $F_{(1,47)}= 37.3$, $p<0.001$; Fig. 4A). Interestingly, OFS treatment significantly reduced corticosterone concentrations in both genotypes (treatment: $F_{(1,47)}= 6.6$, $p<0.05$) but this effect was particularly marked in *db/db* mice (genotype x treatment: $F_{(1,47)}= 4.5$, $p<0.05$). Consequently, OFS-treated *db/db* mice displayed plasma levels of corticosterone significantly lower than their untreated controls ($p<0.05$).

Regardless of their treatment, *db/db* mice displayed similar plasma levels of IL-1β and TNF-α than *db/+* mice (Fig. 4B,C) and higher IL-6 levels (genotype: $F_{(1,31)}= 7.8$, $p<0.01$; Fig. 4D). Interestingly, OFS markedly increased plasma levels of the anti-inflammatory cytokine IL-10 (treatment: $F_{(1,31)}= 11.8$, $p<0.01$; Fig. 4E), in particular in *db/db* mice (treatment x genotype: $F_{(1,31)}=5.6$, $p<0.05$). Chronic OFS treatment therefore reduced corticosterone release and stimulated anti-inflammatory mechanisms. The next step was then to measure its impact on brain function, in particular in the hypothalamus and hippocampus, two brain areas

respectively involved in the control of energetic metabolism and behavior (Bannerman et al., 2004; Benarroch, 2010).

3.5. Prebiotic treatment altered brain inflammation and blood brain barrier integrity

The hypothalamus is a key player in physiological homeostasis, in particular through the local expression of several key molecules, including orexigenic or anorexigenic peptides or cytokines, some of which already known to be altered in *db/db* mice (Dinel et al., 2011; Reaux-Le Goazigo et al., 2011). In agreement with their increased food intake, *db/db* mice tended to display reduced mRNA expression of the anorexigenic peptide CART (genotype: $F_{(1,25)}$= 4.0, p=0.057; Fig. 5A), this reduction being significant regarding the anorexigenic peptide POMC ($F_{(1,23)}$= 7.6, p<0.05; Fig. 5B). Conversely, the mRNA expression of the orexigenic peptide NPY was significantly increased in *db/db* mice compared to *db/+* mice ($F_{(1,26)}$= 67.5, p<0.001; Fig. 5C). However, OFS treatment did not normalize the expression levels of these peptides. Both *db/+* and *db/db* mice also displayed similar hypothalamic TNF-α, IL-6, and IL-10 mRNA expression levels (Fig. 5E-G), independently of being or not treated with OFS, but they differed regarding the anorexigenic cytokine IL-1β (Fig. 5D). Indeed, reduced IL-1β mRNA expression displayed by control *db/db* mice compared to *db/+* mice (genotype: $F_{(1,20)}$= 20.0, p<0.001) was normalized by OFS treatment ($F_{(1,20)}$= 7.6, p<0.05; water *vs.* OFS *db/db* mice: p<0.05). Interestingly, the profile of cytokine expression was different in the hippocampus, in agreement with previously published data (Dinel et al., 2014). Whereas hippocampus IL-1β mRNA expression was similar in all groups regardless of genotype and treatment (Fig. 6A), TNF-α and IL-6 mRNA expression was found up-regulated in *db/db* mice (genotype: $F_{(1,22)}$= 14.8, p<0.001; $F_{(1,23)}$= 11.1, p<0.01 respectively; Fig. 6B,C). Interestingly, IL-6 but not TNF-α expression was normalized by OFS in *db/db* mice (treatment: $F_{(1,23)}$= 4.2, p=0.05; genotype x treatment: $F_{(1,23)}$= 5.9, p<0.05), whereas it had no effect in *db/+* mice regardless of the cytokine. Lastly, hippocampal expression of the anti-

inflammatory cytokine IL-10 was similar in all groups (Fig. 6D). Chronic OFS treatment therefore partly normalized cytokine expression in the hippocampus and hypothalamus, this effect selectively affecting however some particular cytokines depending on the brain area. Based on the multiple roles displayed by cytokines on selective brain functions, depending on the cytokine considered and the specific brain circuits they modulate (see Quan & Herkerham for review), these results suggest that OFS may, by this way, selectively modulate particular brain functions.

We then determined whether OFS acted by altering the density of microglia, the main cells releasing cytokines within the brain and recently shown to be influenced by the gut microbiota (Dinan & Cryan, 2016; Erny et al., 2015). No significant difference across groups was found in total density of microglia regardless of the brain area (ArcN, CA1, CA3, DG), as revealed by detection of Iba-1 immunoreactivity (Fig. 7). However, a negative correlation was found in the DG of *db/db* mice between microglia density and the number of DCX-positive cells ($r= -0.64$, $p<0.05$), whereas this correlation did not reach significance in *db/+* mice ($r= -0.54$, $p=0.07$). Of note, detailed analysis of microglial morphology assessed by counting the number of primary processes displayed by microglia revealed no significant differences between both genotypes, but an overall significant reduction in microglial ramifications in the hippocampus of OFS-treated mice (treatment: $F_{(1,20)}= 5.6$, $p=0.01$, data not shown). This effect was particularly affecting the CA1 area (treatment: $F_{(1,20)}= 7.8$, $p<0.05$, data not shown). On the contrary, no significant genotype or treatment effect was found regarding microglial morphology in the ArcN. Since astrocytes also release a variety of neuromodulatory molecules, including pro-inflammatory cytokines (Ridet et al., 1997), we measured astrocyte density in the same brain areas as microglia density by assessing GFAP immunoreactivity. Both *db/+* and *db/db* mice displayed similar density of GFAP positive cells whatever their treatment in both the hypothalamus and hippocampus (Fig. 8). Taken

together, these results suggest that OFS-induced alterations of brain cytokine expression were not linked to changes in microglia or astrocytes density.

Lastly, we assessed whether OFS acted at the brain level by improving BBB integrity, as reported regarding the gut barrier (Cani et al., 2009). We therefore measured mRNA expression of the tight junction proteins ZO-1 and occludin, which are required for cellular adhesion between adjacent cerebrovascular endothelial cells, and play therefore a key role to ensure BBB integrity (see for review Wolburg & Lippoldt, 2002). Interestingly, occludin mRNA expression (genotype: $F_{(1,26)}$= 11.2, $p<0.01$; Fig. 5I) was reduced in the hypothalamus of *db/db* mice compared to *db/+* mice, in particular in untreated mice (*db/db vs. db/+* water: $p<0.001$). The same tendency was observed for hypothalamic mRNA ZO-1 expression (genotype: $F_{(1,23)}$=3.4, p=0.07; *db/db vs. db/+* water: $p<0.01$; Fig. 5H). OFS treatment blunted this difference by normalizing both ZO-1 (genotype x treatment: $F_{(1,23)}$= 8.0, $p<0.01$) and occludin (genotype x treatment: $F_{(1,26)}$= 8.2, $p<0.01$) mRNA expression in treated *db/db* mice. Of note, OFS supplementation decreased ZO-1 and occludin mRNA expression in *db/+* mice compared to their non-supplemented controls ($p<0.05$). On the contrary, expression of ZO-1 was slightly higher in the hippocampus of *db/db* mice than *db/+* mice (genotype: $F_{(1,24)}$= 5.3, $p<0.05$; Fig. 6E), independently of treatment, whereas hippocampal occludin mRNA expression was similar in all mice whatever their genotype or treatment (Fig. 6F). Taken together, these results reveal differential expression of ZO-1 and occludin in the hypothalamus and hippocampus of *db/db* mice, which may suggest potential differences in local BBB integrity. Similarly, the impact of OFS on the expression of the tight junction proteins also differed according to the brain area, in line with a recent review reporting a link between the gut microbiota and region-specific regulation of blood-tissue barriers involving the expression of tight junction proteins (Erny et al., 2016).

4. DISCUSSION

Significant lack of knowledge exists regarding the role potentially played by the gut microbiota in neuropsychiatric symptoms associated with obesity/MetS, although a better understanding of their pathophysiological mechanisms has wide ranging therapeutic implications (Castanon et al., 2015; Farooqui et al., 2012). The current study provides valuable findings relevant to this topic by directly assessing, for the first time, the effect of gut microbiota manipulation with a prebiotic not only on metabolic and behavioral alterations associated with the MetS, but also on their systemic and neurobiological correlates. Importantly, the consequences on both peripheral and brain inflammation was investigated as the inflammatory processes, which have been related with neuropsychiatric comorbidity in obesity (Castanon et al., 2014; Capuron et al., 2016), are key players in the gut-brain axis (Dinan & Cryan, 2016). We show that improvement of metabolic alterations following chronic OFS administration in *db/db* mice was associated with selective reduction of peripheral and central inflammation, which is however not accompanied by detectable improvement of anxiety-like behavior or spatial memory deficits.

Most preclinical studies investigating the contribution of the microbiota to obesity and related comorbidities have used diet-induced obesity models (Bruce-Keller et al., 2015; Cani et al., 2007; Kang et al., 2014; Magnusson et al., 2015; Pyndt-Jorgensen et al., 2014), although high-fat and/or high-sugar diet, which is able to directly influence the gut microbiota composition (Patterson et al., 2014; Turnbaugh et al., 2009), can also alter metabolic health by mechanisms independent from its effect on the microbiota (Lozano et al., 2016; Schwab et al., 2014). Moreover, unlike what has been done in the present study, the impact of the gut microbiota on central regulation of food intake is most of the time poorly considered. Here, we used the *db/db* mice, which progressively developed most of the metabolic alterations characterizing the MetS, including excessive food intake leading to obesity, insulin resistance and hyperglycemia, in agreement with previously published data (Dinel et al 2011; Everard et al 2014). Interestingly, treatment with OFS improved most of these features, as previously

reported in *ob/ob* obese mice (Everard et al., 2011) or in diet-induced obese rats (Cluny et al., 2015). Beyond direct changes in microbiota composition, multiple mechanisms have been proposed to explain this improvement, most of them involving circulating molecules able to regulate feeding behavior and glucose homeostasis, including glucagon, ghrelin, or GLP-1 (Delzenne et al., 2011; Everard et al., 2011). However, the fact that OFS did not affect the production of these molecules in *db/db* mice argues against their significant intervention in the present study. On the contrary, other factors also important for metabolic regulations and for which plasma levels were altered in *db/db* mice, including corticosterone and resistin (Ahima & Flier, 2000; Steppan et al., 2001), were however beneficially affected by OFS. Excess of systemic glucocorticoids has been shown to promote appetite, visceral fat accumulation and insulin resistance (de Guia &Herzig, 2015) whereas reduced glucocorticoid activation, resulting from ablation of the adrenal glands or prebiotic supplementation, is associated with improvement of high-fat diet-induced metabolic alterations (Kim et al., 2013; Perello et al., 2003). Resistin is an adipokine classically associated with insulin resistance and diabetes (Steppan et al., 2001). Akin with previously published data (Dinel et al., 2011; Ye et al., 2013; Xiang et al., 2013), *db/db* mice displayed here reduced plasma resistin levels compared to *db/+* mice. Moreover, OFS-induced improvement of glucose homeostasis and insulin resistance was associated with normalization of those levels. Interestingly, similar association was reported following treatment of *db/db* mice with the antidiabetic drug rosiglitazone (Ye et al., 2013), supporting therefore a potential role for resistin in the metabolic improvement induced by OFS in the present study. Of note, the bacterial lipopolysaccharide (LPS), which is elevated in plasma of obesity/MetS models due to increased gut leakiness (Brun et al., 2007; Cani et al., 2016), has been reported to decrease resistin expression in *db/db* mice (Xiang et al., 2013). Prebiotics are known to reduce gut barrier leakiness, including in *db/db* mice, by acting on expression/distribution of particular tight junction proteins such as ZO-1 and occludin (Cani et al., 2009). The changes in resistin

levels we found could be thus linked to reduced endotoxemia. This should in turn contribute to reduce chronic peripheral inflammation that is classically presented as underlying the metabolic deregulation in obesity (Sanz & Moya-Perez, 2014; Tack et al., 2012). Increased anti-inflammatory cytokine IL-10 production displayed by prebiotic-treated *db/db* mice may similarly contribute to the improvement of their metabolic alterations, in agreement with previously published findings reporting a link between changes in gut microbiota composition, reduced low-grade inflammatory state, and metabolic dysfunctions (Furet et al., 2010; Sokol et al., 2008).

This study shows for the first time in *db/db* mice that beneficial effects of prebiotic treatment also extend to the brain, particularly to the hypothalamus that is involved in central regulation of energy homeostasis. This result fits with the changes in hypothalamic neuronal activation recently reported in prebiotic-treated mice (Anastasovska et al., 2012). Interestingly, by measuring the expression of tight junction proteins that ensure BBB integrity, we report data suggesting that reduced hypothalamic BBB integrity displayed by *db/db* mice was improved by OFS, as previously demonstrated for the gut barrier (Cani et al., 2009; Everard et al., 2011). This assumption, which still needs to be confirmed, is in agreement with a recent study reporting that BBB integrity can be modulated, in a brain area-specific manner, by changes in gut microbiota composition (Braniste et al., 2014). Although no definitive conclusion can be yet drawn about the potential link between the effect of OFS reported here and changes of local cytokines expression, it is worth noting that OFS increased the mRNA expression of the anorexic cytokine IL-1β (Gautron & Layé, 2010), which was reduced in untreated *db/db* mice compared to *db/+* mice. On the contrary, it did not restore normal expression of the different hypothalamic neuropeptides (POMC, NPY) shown to be altered in *db/db* mice, despite its positive effect on food intake. These findings suggest that OFS-induced normalization of hypothalamic IL-1β expression may contribute to beneficially regulate food intake in *db/db* mice, although concomitant detrimental consequences of

increased neuroinflammation cannot be totally excluded. These findings also suggest that besides being likely tightly related, different mechanisms, which need to be deeply studied in the present context, underlie neuropeptides and Il-1β regulation in the hypothalamus. Of note, it has been suggested that the expression of those hypothalamic neuropeptides are regulated by body fat mass (Schele et al., 2013), and this would make sense here since both treated and untreated *db/db* mice displayed almost similar body weight. In addition, an inverse relationship has been reported between plasma corticosterone and IL-1β hypothalamic mRNA expression in a rat model of obesity (Wisse, 2004). Although further studies are required to determine whether OFS-induced normalization of plasma corticosterone levels observed in *db/db* mice is involved in the increased hypothalamic IL-1β expression, this assumption may nicely provide an additional way for corticosterone to participate to the beneficial effects of prebiotics on metabolic health.

Despite having beneficial effects on some of the metabolic and neurobiological alterations displayed by *db/db* mice, OFS did not seem to change their behavioral profile. However, this does not necessarily discard gut microbiota effects on behavior, as it is possible that such improvement may require a higher dosage or longer/sooner implementation of prebiotic treatment. Convincing evidence indeed indicates that the gut microbiota can impact behavior in diet-induced models of obesity (Bruce-Keller et al., 2015; Kang et al., 2014; Magnusson et al., 2015). It could be argued that post-natal interventions in *db/db* mice may not be able to improve behavioral alterations that are linked to the lack of leptin's actions through the developmental period. Although this hypothesis cannot be completely excluded from the present data, it is worth mentioning that normalization of corticosterone circulating levels at adulthood do improve cognitive impairment in *db/db* mice (Stranahan et al., 2008). Here, spatial memory remained impaired in OFS-treated *db/db* mice despite their corticosterone levels being reduced, suggesting that this reduction was insufficient to allow behavioral improvement. Likewise, OFS improved glucose metabolism in *db/db* mice without reducing

anxiety-like behavior, although an increased prevalence of anxiety disorders is classically reported in T2D (Castanon et al., 2015; Farooqui et al., 2012). This may suggest either that this improvement is not sufficient to impact behavior, or that emotional alterations are not directly linked to hyperglycemia in *db/db* mice. In support to this last hypothesis, exercise has been shown to reverse hyperglycemia in *db/db* mice, without reducing anxiety-like behavior (Stranahan et al., 2009).

Supporting further a role for the gut microbiota in the behavioral profile of *db/db* mice, we showed that prebiotic treatment impacted hippocampal neurobiological processes known to modulate behavior, particularly inflammation (Capuron et al., 2016; Castanon et al., 2015). In agreement with the recent literature reporting that the gut microbiota influences microglia maturation, morphology, and immunological function (Dinan & Cryan, 2016; Erny et al., 2015), OFS slightly reduced microglial ramifications in the hippocampus, which suggest modulation of microglial activation (Ransohoff & Perry, 2009). Microglial density in the DG of *db/db* mice was also inversely correlated with the number of DCX-positive cells that, together with OFS treatment, predicted performances in the Y-maze. Altogether, these data point to a link between the gut microbiota, microglial activation, hippocampal neurogenesis and behavioral alterations in *db/db* mice, although the underlying mechanisms still need to be identified. A potential player in this network could be IL-6 since its hippocampal mRNA expression was normalized by OFS in *db/db* mice, and it has been previously associated with hippocampal neurogenesis, mood, and cognition (for review see: Erta et al., 2012). Similar association was found here, although normalizing IL-6 mRNA expression did not result in detectable improvement of neurogenesis or behavior. It could be argued that changes in mRNA expression did not necessarily reflect in changes of functional protein levels, which then contribute to downstream neurobiological and behavioral modulations. This is however unlikely since correlations between brain mRNA expression of cytokines and corresponding protein levels have been already reported (van Dam et al. 1998), notably in *db/db* mice

(Kumari et al. 2007). Moreover, we previously showed that modulating brain cytokine mRNA expression resulted in modulation of the activation of related signaling pathways and behavioral responses (André et al., 2014; Dinel et al., 2011; 2014). Alternatively, improving cognition and emotional behavior in *db/db* mice may have required for OFS normalizing the expression of other factors which are well-known to contribute, together with IL-6, to the development of neurobehavioral alterations and are altered in *db/db* mice, particularly TNF-α (Abbott et al., 2015; Capuron et al., 2016).

In conclusion, although more studies are needed to further decipher the complexity of the different findings reported in the present study, it constitutes a first important step towards a better understanding of the role of the gut-brain axis in the MetS, not only by extending the knowledge regarding metabolic alterations, but more importantly by addressing the issue of its role on associated neurobiological and behavioral alterations. Beyond that, this study also provides new and interesting findings about how prebiotics may act on the brain, particularly on neuroinflammation. Even though the prebiotic treatment did not clearly translate to behavioral improvements, we were still able to parse out some of the likely mediators in the complex interaction between the body's homeostatic super-systems. Notably, OFS was shown to change the microbiota composition in favor of Bifidobacterium spp, which is known to contribute to maintain a healthy gut barrier function, thereby reducing systemic hormonal and inflammatory dysregulations, affecting here in particular corticosterone, resistin and IL-10 plasma levels. These improvements may consequently decrease food intake and diabetes, and may also impact the brain, as recently demonstrated for molecules resulting from microbial fermentation within the gut and able to reach the brain to locally change specific neuronal activity (Frost et al., 2014). Alternatively, we did find evidence for direct effects of prebiotics on neuroinflammation, which were brain-area specific, and were accompanied with changes in the expression of tight junction proteins. All together, these OSF-induced hormonal, neuronal and metabolic changes may explain the improvement of MetS-related alterations.

These findings contribute therefore to make the gut microbiota a promising nutritional and pharmacological target for the management of obesity and obesity-related disorders.

Conflict of interest

The authors declared that no competing interests exist.

Contributors

Conceptualization and experimental design: P.D.C., L.C., S.L., and N.C.; Investigation: L.F. de C., C.F., A.E., J.S., and N.C.; Data analysis: L.F.de C., C.F., J.S., and N.C.; Manuscript writing: L.F.de C., C.F., L.C., S.L., and N.C. All of the authors have read, revised and approved the final manuscript; Funding Acquisition: P.D.C., S.L., N.C.; Supervision: N.C.

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Legends

Figure 1. Follow up weight, food intake and liquid intake (oligofructose: OFS and water). A). Blunted weight gain of supplemented *db/db* mice. B) Progressive decrease in food intake of supplemented *db/db* mice. C) Amount of OFS solution *vs.* water consumption in *db/db* mice and similar quantities of OFS intake by *db/db* and *db/+* groups (n=13-14/group. $^{**}p<0.01$; $^{***}p<0.001$ for comparison between *db/db* Water and *db/db* OFS).

Figure 2. Assessment of glucose homeostasis and plasma hormones. A) Hyperglycemia during two hours after glucose challenge (2 g/kg, ip). B) Comparison of glucose tolerance (AUC) showing milder hyperglycemia for supplemented *db/db* mice. C) Insulin resistance as assessed by measuring glycemia just before and during two hours after insulin injection (0.75IU/kg, ip). D) Comparison of insulin resistance (AUC) showing milder insulin resistance (n=13-14/group. $^{###}p<0.001$ for genotype effect; $^{**}p<0.01$; $^{***}p<0.001$ for comparison between *db/db* Water and *db/db* OFS). E-G) Hormones measured showing genotype effect with *db/db* mice remaining higher in insulin and GLP-1 levels, whereas resistin is normalized by oligofructose (OFS) treatment in *db/db* mice (n=8-9/group. $^{#}p<0.05$; $^{##}p<0.01$ for genotype effects; $^{*}p<0.05$ for comparison between *db/db* Water and *db/db* OFS).

Figure 3. Anxiety-like behavior and hippocampal-dependent spatial memory performances. A) Genotype main effect in entries to center of the open-field. B) Genotype main effect in time spent in lighted room of the light/dark box. C) Genotype main effect in time spent in anxiogenic arms of the Plus-maze. D) No better than chance mnesic discrimination for *db/db* mice in the Y-maze (n=13-14/group. $^{\circ}p<0.05$; $^{\circ\circ}p< 0.01$ for difference from chance level; $^{##}p<0.01$; $^{###}p<0.001$ for genotype effects). E) Genotype main effect in DCX-positive cells in

the DG of the hippocampus F) Representative images of DCX staining. (n=6/group; $^{###}p<0.001$ for genotype effects).

Figure 4. Assessment of corticosterone and main plasma cytokines in water or oligofructose (OFS) groups. A) Hypercorticism attenuated in supplemented *db/db* mice (n=13-14/group). B-C) Inflammatory cytokines IL-1β and TNF-α not significantly different across groups. D) Genotype effect on inflammatory cytokine IL-6. E) Anti-inflammatory cytokine IL-10 substantially increased in supplemented *db/db* (n=8-9/group; $^{\#}p<0.05$; $^{\#\#}p<0.01$ for genotype effects; $^{*}p<0.05$; $^{**}p<0.01$ for comparison between *db/db* Water and *db/db* OFS).

Figure 5. Assessment of hypothalamic mRNA expression of orexigenic or anorexigenic peptides, cytokines and tight-junction proteins. A-C) Genotype effect on some known anorexigenic (POMC) and orexigenic (NPY) peptides. D-G) IL-1β is reduced in *db/db* but restored in oligofructose (OFS)-supplemented group, while inflammatory (TNF-α and IL-6) and anti-inflammatory (IL-10) cytokines are not significantly different across groups. H-I) Tight-junction protein occludin is reduced in *db/db,* OFS significantly reduces mRNA expression of both ZO-1 and occludin in *db*/+ mice (n=8-9/group; $^{\#}p<0.05$;$^{\#\#\#}p<0.001$ for genotype effects; $^{*}p<0.05$ for comparisons between Water and OFS within each genotype).

Figure 6. Assessment of hippocampal mRNA expression of cytokines and tight-junction proteins. A-D) Genotype effects on inflammatory cytokines TNF-α and IL-6 but no significant differences across groups in IL-1β or anti-inflammatory cytokine IL-10, oligofructose (OFS) supplementation rescues IL-6 expression. E-F) Tight-junction protein ZO-1 is increased in *db/db* but no differences across groups for occludin (n=8-9/group; $^{\#}p<0.05$;$^{\#\#}p<0.01$;$^{\#\#\#}p<0.001$ for genotype effects; $^{*}p<0.05$ for comparison between *db/db* Water and *db/db* OFS).

Figure 7. Assessment of Iba-1-positive cells in the different areas of the hippocampus and in the arcuate nucleus of the hypothalamus. A) Representative images of Iba-1 staining. B-E) Groups do not present differences in the density of Iba-1-positive cells in any of the regions (n=6/group).

Figure 8. Assessment of GFAP-positive cells in the different areas of the hippocampus and in the arcuate nucleus of the hypothalamus. A) Representative images of GFAP staining. B-E) Groups do not present differences in the density of GFAP-positive cells in any of the regions (n=6/group).

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Table 1. Sybr green primers sequences

Target gene	Forward 5'-3'	Reverse 5'-3'
CART	AAGTCCCCATGTGTGACGCT	TGGCCCCTTTCCTCACTG
NPY	TCCAGCCCTGAGACACTGATT	CACCACATGGAAGGGTCTTCA
POMC	ACGGAGAGCAACCTGCTGG	GCGAGAGGTCGAGTTTGCA

Table 2. Effect of oligofructose supplementation on gut microbiota population

Abundance (log bacteria/g cecal content)	*db/+*		*db/db*	
	Water	**Oligofructose**	**Water**	**Oligofructose**
Total bacteria	11.18 ± 0.15	11.28 ± 0.10	11.36 ± 0.16	11.28 ± 0.14
Lactobocillus spp.	8.12 ± 0.45	7.77 ± 0.43	7.60 ± 0.23	8.01 ± 0.22
Roseburia spp.	7.57 ± 0.56	8.44 ± 0.22	8.55 ± 0.20	8.30 ± 0.33
Bacteroides-Prevotella spp.	9.37 ± 0.18	9.55 ± 0.11	9.57 ± 0.19	9.45 ± 0.32
Bifidobacterium spp.	7.78 ± 0.24	7.94 ± 0.17	8.12 ± 0.17	9.04 ± 0.18$^{**\#\#\#}$

$^{\#\#\#}$: P<0.001 for *db/+ vs. db/db*

**: P<0.01 for *db/db* water *vs. db/db* oligofructose

Table 3. Regression analysis for time spent in the new arm of the Y-maze in *db/db* mice

Model	Unstandardized Coefficients		Standardized Coefficients	t	*p*
	B	Std. Error	Beta		
1 (Constant)	40.273	36.513		1.103	0.299
Treatment	33.947	22.487	0.449	1.51	0.165
2 (Constant)	-51.532	39.918		-1.291	0.233
Treatment	41.357	16.378	0.548	2.525	0.036*
Nb. Neurons DG	0.16	0.052	0.663	3.057	0.016*

ANOVA for Model 1: $F(1,9)= 2.28$ $p=0.17$, $R^2=0.202$

ANOVA for Model 2: $F(2,8)= 6.87$ $p=0.018$*, $R^2=0.632$

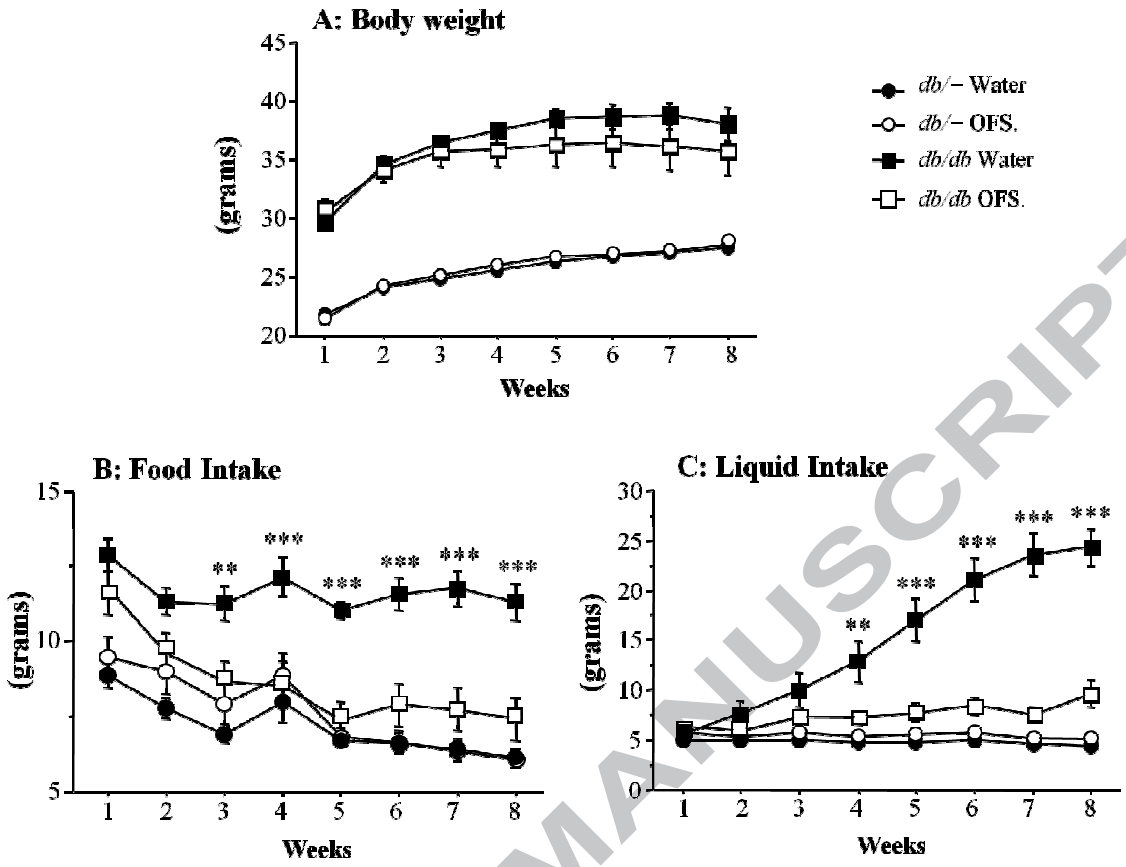

Figure 1

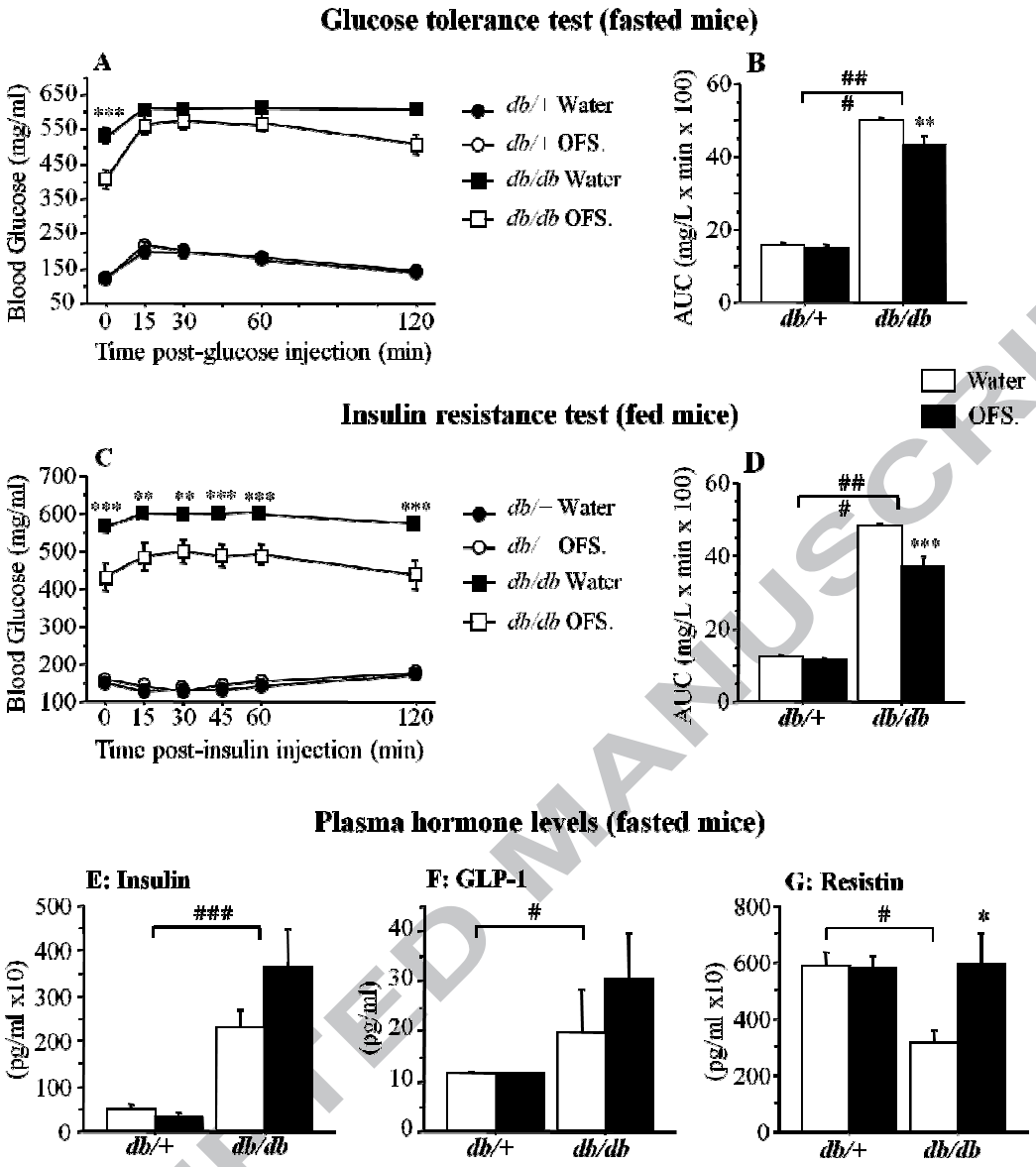

Figure 2

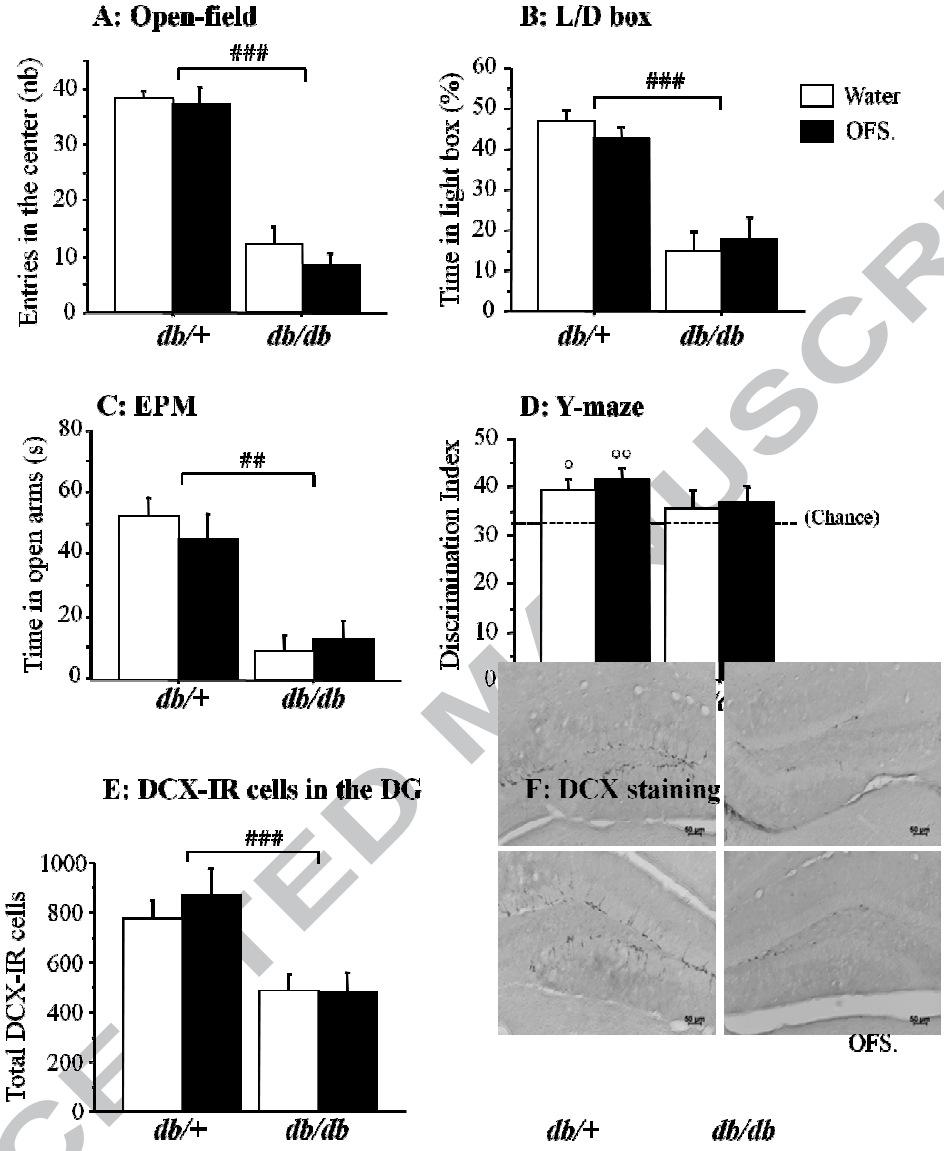

Figure 3

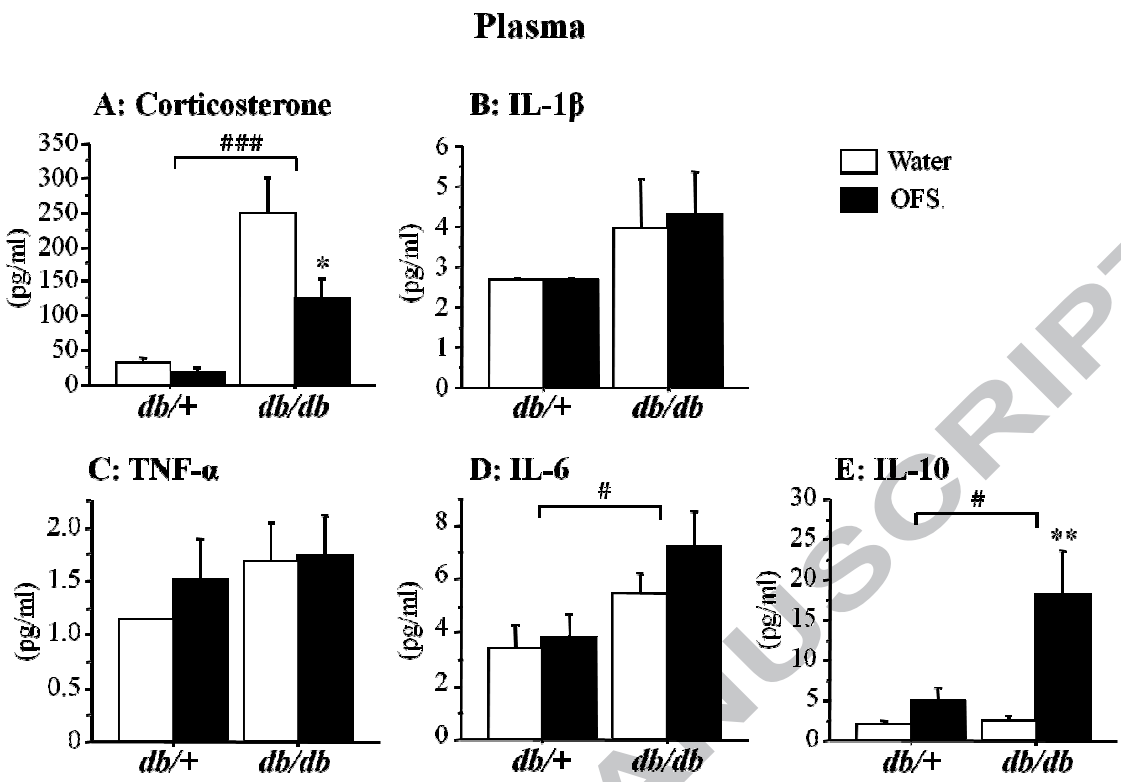

Figure 4

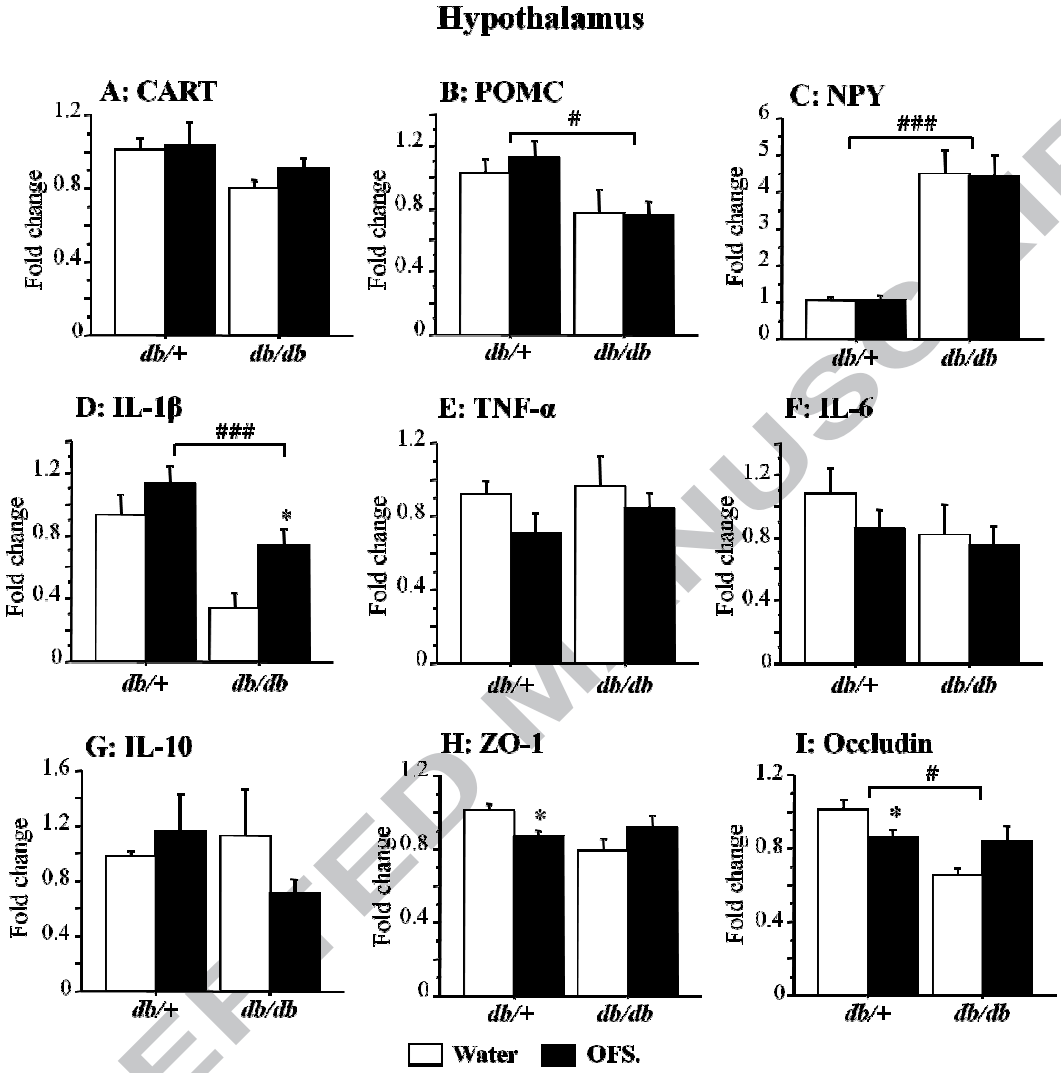

Figure 5

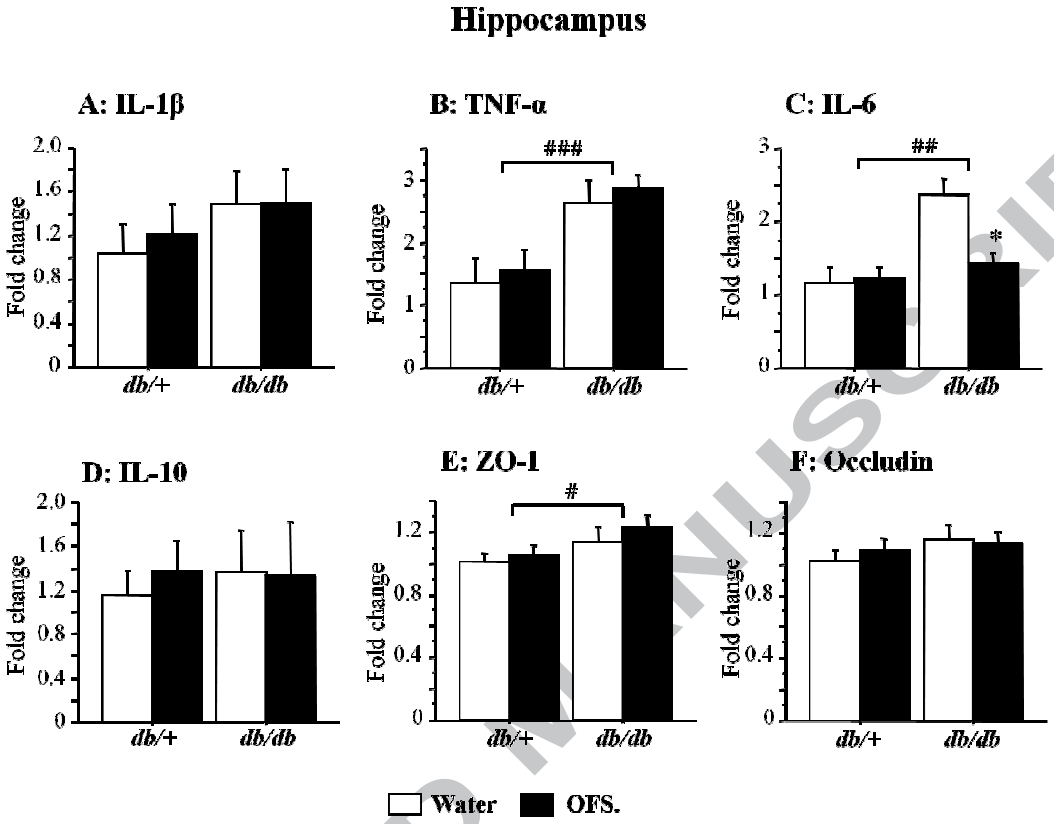

Figure 6

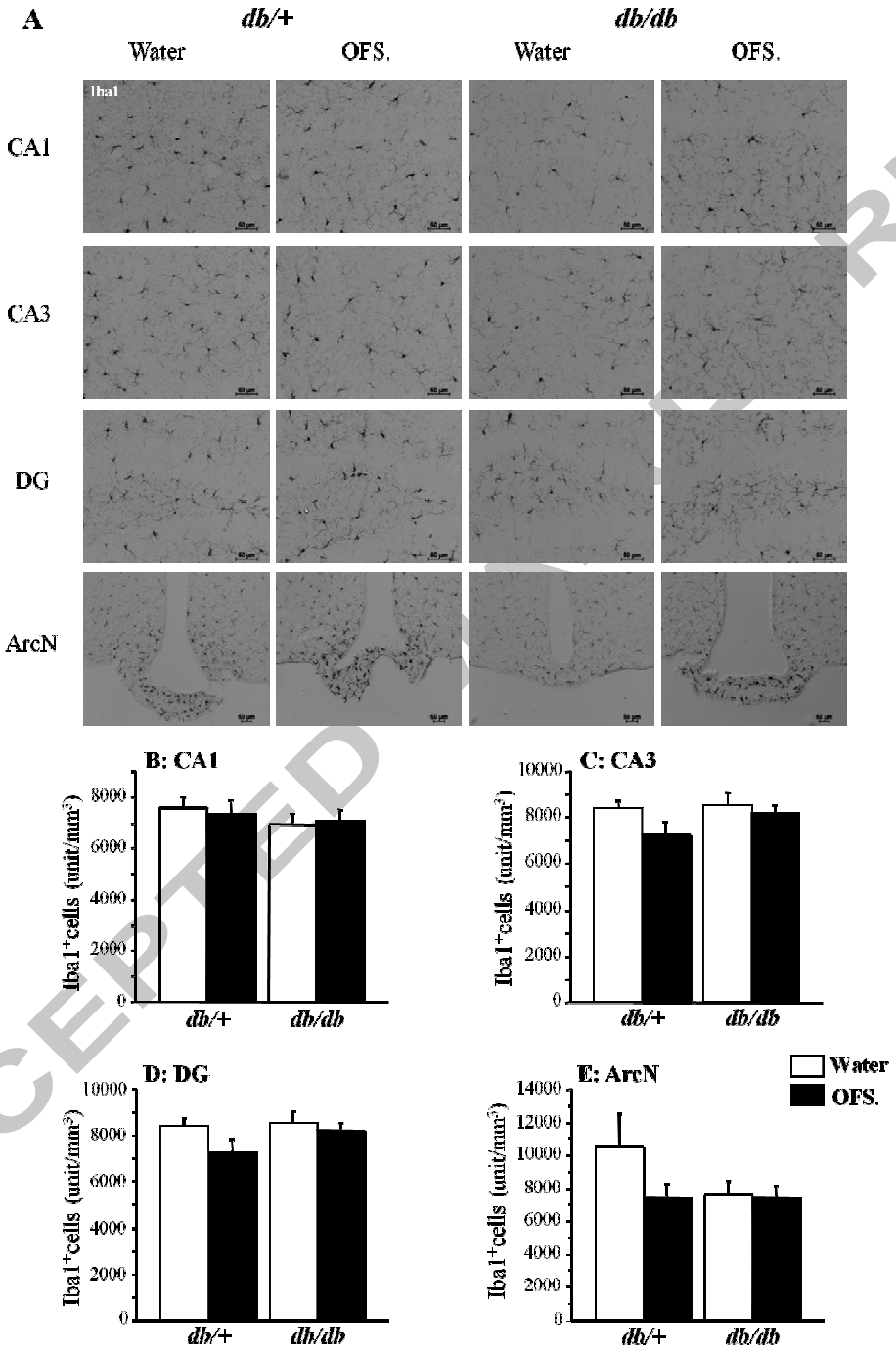

Figure 7

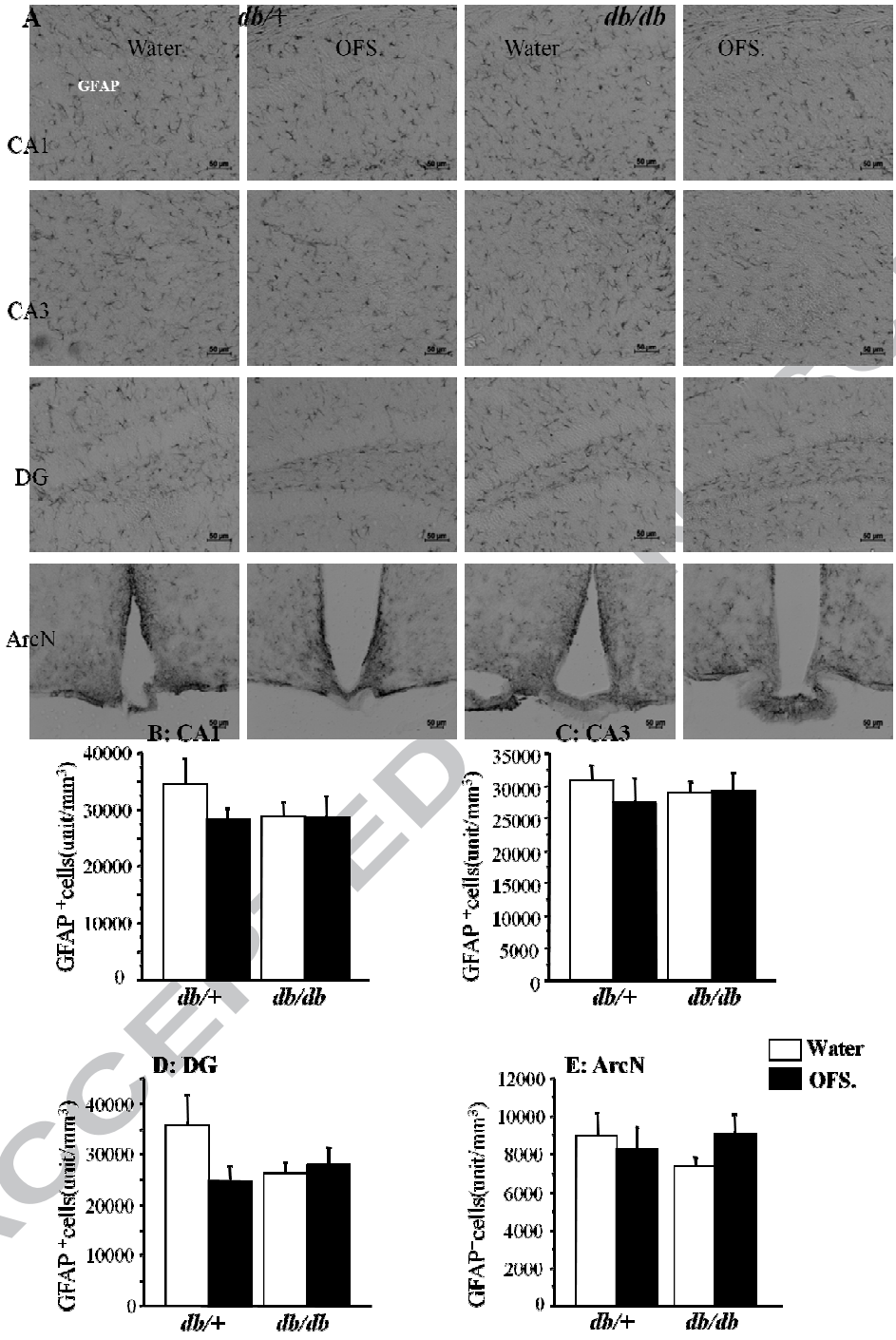

Figure 8

Highlights

- The gut microbiota influences metabolic syndrome-associated alterations
- Prebiotics reduces peripheral and brain inflammation in obese *db/db* mice
- Link between prebiotics, hippocampal neurogenesis and spatial memory