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Transgenic Increase in n-3/n-6 Fatty Acid Ratio Protects Against Cognitive Deficits Induced by an Immune Challenge through Decrease of Neuroinflammation

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Polyunsaturated fatty acids (PUFAs) display immunomodulatory properties in the brain, n-3 PUFAs being able to reduce inflammation whereas n-6 PUFAs are more pro-inflammatory. It has been extensively demonstrated that exposure to a peripheral immune challenge leads to the production and release of inflammatory mediators in the brain in association with cognitive deficits. The question arises whether n-3 PUFA supplementation could downregulate the brain inflammatory response and subsequent cognitive alterations. In this study, we used a genetically modified mouse line carrying the *fat-1* gene from the roundworm *Caenorhabditis elegans*, encoding an n-3 PUFA desaturase that catalyzes conversion of n-6 into n-3 PUFA. Consequently, these mice display endogenously elevated n-3 PUFA tissue contents. Fat-1 mice or wild-type (WT) littermates were injected peripherally with lipopolysaccharide (LPS), a bacterial endotoxin, to induce an inflammatory episode. Our results showed that LPS altered differently the phenotype of microglia and the expression of cytokines and chemokines in Fat-1 and WT mice. In Fat-1 mice, pro-inflammatory factors synthesis was lowered compared with WT mice, whereas anti-inflammatory mechanisms were favored 24 h after LPS treatment. Moreover, LPS injection impaired spatial memory in WT mice, whereas interestingly, the Fat-1 mice showed normal cognitive performances. All together, these data suggest that the central n-3 PUFA increase observed in Fat-1 mice modulated the brain innate immune system activity, leading to the protection of animals against LPS-induced pro-inflammatory cytokine production and subsequent spatial memory alteration.
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INTRODUCTION

Increasing attention has been paid to the role of n-3 and n-6 polyunsaturated fatty acids (PUFAs) in the brain and an increasing database attests of their powerful immunomodulatory effects (Calder, 2001; Laye, 2010; Labrousse *et al*, 2012; Orr *et al*, 2013). n-3 PUFAs are precursors of lipid derivatives (neuroprotectins and resolvins) with anti-inflammatory properties, whereas n-6 PUFAs are mostly the precursors of the pro-inflammatory prostaglandins, and stimulate the production and activity of inflammatory cytokines. The dramatic reduction in the dietary supply of n-3 PUFAs in Western societies and the corresponding increase in n-6 PUFAs leads to an imbalanced n-6/n-3 ratio currently estimated at 12–20 in developed countries instead of the recommended ratio of 5 (Simopoulos, 2001). By affecting brain PUFAs composition, this could therefore contribute to the sensitization of the brain to inflammatory cytokines, and thus to the development of neurodegenerative and/or behavioral disorders (Laye, 2010).

Microglia are the resident macrophages of the brain, and constitute the first line of immune defense of the brain (Ransohoff and Cardona, 2010). Once stimulated by an immune challenge, microglia are capable of acquiring diverse and complex phenotypes as well as performing several macrophage-like functions including inflammatory and anti-inflammatory cytokine production (Garden and Moller, 2006; Biber *et al*, 2007; Madore *et al*, 2013). OFF signals (eg CX3CL1) that are released by healthy neurons to keep microglia in a surveillance mode in normal conditions are also downregulated under inflammation (Biber *et al*, 2007). Moreover, exposure to pathogens results in altered memory performances (Yirmiya and Goshen, 2011), as part of the general sickness behavior syndrome (Dantzer *et al*, 2008). More specifically, it has been extensively demonstrated that microglia-derived inflammatory mediators are directly involved in the cognitive disturbances that accompany exposure to endotoxin (Yirmiya and Goshen, 2011). Central nervous system inflammation occurs in a large variety of pathologies including multiple sclerosis, Alzheimer's disease, and Parkinson's disease (Laye, 2010). Thus, identifying inflammatory modulators is a growing area of interest, because it may provide novel targets in disease prevention and treatment.

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In this study, we used a genetically modified mouse line to study the impact of n-3 PUFA supplementation on brain inflammatory response and subsequent cognitive alterations. These mice carry the *fat-1* gene from the roundworm nematode *Caenorhabditis elegans*, encoding an n-3 PUFA desaturase, absent in mammals, that catalyzes the conversion of n-6 into n-3 PUFA (Kang *et al*, 2004). Therefore, these mice have endogenously elevated n-3 PUFA tissue content and exhibit lower n-6/n-3 PUFA ratio compared with their wild-type (WT) littermates. Indeed, Fat-1 mouse displays brain docosahexaenoic acid (DHA) levels that are generally attainable through fish oil feeding (Orr *et al*, 2010) and present lower n-6 PUFA levels in the brain (Boudrault *et al*, 2010).

In the present study, Fat-1 mice or WT littermates were injected peripherally with lipopolysaccharide (LPS), a potent inducer of cerebral inflammation, and we assessed cognitive performances, cytokine production, and microglial phenotype 24 h later. Our results showed that Fat-1 mice were protected against systemic inflammation, in the form of lower production of pro-inflammatory mediators, together with an increased anti-inflammatory profile of microglia. Fat-1 mice also displayed protection against inflammation-induced cognitive impairment.

Table 1 Fatty Acid Composition of the Dietary Lipids (% wt of Total Fatty Acids)

Lipids	Standard diet (A04)
16:0	20.2
18:0	2.2
Other saturated FAs	1.8
Total saturated FAs	24.2
18:1n-9	19.2
18:1n-7	1.5
Other monounsaturated FAs	3.9
Total monounsaturated FAs	24.6
18:2n-6 (LA)	45.6
Other n-6 PUFAs	0.3
Total n-6 PUFAs	45.9
18:3n-3 (ALA)	3.3
20:5 n-3	0.6
22:5 n-3	0.1
22:6 n-3	0.8
Total n-3 PUFAs	4.8
Total PUFAs	50.7

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; FAs, fatty acids; PUFAs, polyunsaturated fatty acids.

MATERIALS AND METHODS

Animals and Treatment

Heterozygous transgenic Fat-1 mice were generated as described previously (Kang *et al*, 2004) and backcrossed onto a C57BL/6J background. The presence of the *fat-1* gene in each mouse was confirmed both by genotyping and brain tissue fatty acid analysis profile (see below). Transgenic and WT animals were kept under pathogen-free conditions in standard cages in temperature- and humidity-controlled conditions with a 12-h light/dark cycle. We used adult male Fat-1 transgenic mice and nontransgenic WT littermates (as controls) all along the study. Studies were carried out according to the Quality Reference System of INRA (http://www.international.inra.fr/content/download/947/11111/file/requirements) and approved by the local ethical committee for care and use of animals (approval ID: A33-063-920).

Of importance and contrary to previous studies on Fat-1 mice, all animals were fed *ad libitum* with a standard diet (A04, SAFE, Augy, France; Table 1), as we recently demonstrated that the long-term consumption of inadequate n-6/n-3 PUFAs ratio triggers spatial memory impairment and depressive and anxiety-like symptoms in mice (Lafourcade *et al*, 2011; Moranis *et al*, 2012). By giving a standard diet to the animals, we prevented any behavioral alterations in WT littermates from occurring. Behavioral experiments were conducted on adult mice (3–5-month old). To induce an inflammatory reaction, a dose of 125 μg/kg of LPS (*Escherichia coli*, 0127:B8, Sigma-Aldrich, Lyon, France), diluted in saline (NaCl 0.9%) was intraperitoneally injected between 9 and 11am. LPS is a component of the cell wall of Gram-negative bacteria known at this dose as a useful model for investigation of changes that accompany inflammation in brain such as spatial memory impairment (Laye *et al*, 1994; Mingam *et al*, 2008). Control mice received an injection of saline solution (0.9%). Food consumption (assessed by weighing the pellets) and body weight were monitored 2 and 24 h after LPS injection as a marker of sickness. For qPCR experiments and lipid measurements, mice were quickly anesthetized by isoflurane inhalation and euthanized by decapitation 2 or 24 h after treatment. Twenty-four hours after the treatment, mice have recovered from the sickness symptoms and present a normal locomotor activity, allowing us to investigate their working memory abilities (O'Connor *et al*, 2009).

Genotyping

DNA was extracted from approximately 2–3 mm of the mouse tail. Tissue was digested in 200 μl of NaOH 0.05 M for 20 min at 100 °C and then 50 μl of Tris HCl 1 M pH8.0 was added to the mix. After 10 min of centrifugation, supernatant containing DNA was removed and left at 4 °C. Two microliters of DNA extracts were used for genotyping by PCR. The primers used for the *fat-1* gene were 5′-TGTTCATGCC TTCTTCTTCTTTTTCC-3′ and 5′-GCGACCATACCTCAAAC TTGG-3′. The 20 μl PCR reaction mix also contained 5 μl of deoxyribonucleotide mix, 10 μl of PCR 2 × mix (Promega, WI, USA), and 0.2 μl of GoTaq DNA Polymerase (Promega). The thermocycler program consisted of a period of 5 min at 94 °C followed by 2 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C, then 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 45 s at 72 °C. The run was completed with 5 min at 72 °C, and then cooled to 4 °C. Amplified PCR products were run through a 2% agarose gel and visualized using Ethidium Bromide on UV table with gel imager (Syngene, Saint Quentin en Yvelines, France).

Fatty Acids Analysis in Brain Phospholipids

We measured the levels of n-3 and n-6 PUFAs in the hippocampus of WT ($n = 4$/saline; $n = 6$/LPS) and Fat-1

mice ($n = 4$/saline; $n = 4$/LPS) as previously described (Lafourcade *et al*, 2011; Labrousse *et al*, 2012; Larrieu *et al*, 2012), as a control of lipid content in mice fed with a standard diet. Fatty acid composition is expressed as the percentage of total fatty acids.

Behavioral Measurement

Y-maze. The Y-maze paradigm was used to assess spatial working memory as previously described by our group (Labrousse *et al*, 2009; Moranis *et al*, 2012). Spatial working memory was assessed in Fat-1 ($n = 9$–10/saline and $n = 8$/LPS) and WT mice ($n = 9$–12/saline and $n = 9$–10/LPS). The apparatus was a Y-shaped maze made of gray plastic. Each arm was 34 cm long, 8 cm wide, and 14 cm high. The floor of the maze was covered with corncob litter, which was mixed between each trial to remove olfactory cues. Visual cues were placed in the testing room and kept constant during the whole test. In the first trial of the test, one arm of the Y-maze was closed with a guillotine door and mice were allowed to visit two arms of the Y-maze for 5 min. After 30-min inter-trial interval, mice were placed back in the start arm and allowed free access to the three arms for 5 min. Spontaneous spatial recognition was also measured using a 5 min inter-trial interval (ITI) between acquisition and retrieval. This ITI was employed to control for potential motivational deficits and to verity that all groups performed more visits to the novel arm when the mnemonic demand was minimal. Start and closed arms were randomly assigned for each mouse. We compared the time spent exploring the novel and the familiar arm during the 5 min of the second trial.

Quantitative Real-Time PCR

Total RNA was extracted from hippocampi using TRIzol (Invitrogen, Life Technologies) (WT: $n = 5$/saline; $n = 5$/LPS; Fat-1: $n = 5$/saline; $n = 5$/LPS). RNA purity and concentration were determined using a Nanodrop spectrophotometer (Nanodrop technologies, Wilmington, DE). One microgram of RNA was reverse transcribed to synthesize cDNA using Superscript III (Invitrogen, Life Technologies) and random hexamers according to the manufacturer's protocol (Labrousse *et al*, 2012).

Quantitative PCR was performed to measure cytokine expression using the Applied Biosystems (California, USA) assay-on demand gene expression protocol as previously described (Mingam *et al*, 2008). In brief, cDNA was amplified by real-time PCR where a target cDNA and a reference cDNA (β2-microglobulin) were amplified simultaneously using an oligonucleotide probe with a 5′ fluorescent reporter dye (6-FAM) and a 3′ quencher dye (NFQ). Fluorescence was determined on an ABI PRISM 7500-sequence detection system (Applied Biosystems). Data were analyzed using the comparative threshold cycle method, results are expressed as relative fold change (Mingam *et al*, 2008) to control target mRNA expression.

Isolation of Microglia

Microglial cells were isolated from whole brain homogenates as previously described (Henry *et al*, 2008; Henry *et al*, 2009; Wynne *et al*, 2010; Wohleb *et al*, 2012; Madore *et al*, 2013). Brains were homogenized in Hanks' Balanced Salt Solution, pH 7.4 passing through a 70 μm nylon cell strainer. Homogenates were centrifuged at 600 g for 6 min. Supernatants were removed and cell pellets were re-suspended in 70% isotonic Percoll (GE-Healthcare). A discontinuous Percoll density gradient was set up as follows: 70, 50, 35, and 0% isotonic Percoll. Gradients were centrifuged at 2000 g for 20 min. Microglia cells were collected at the interface between the 70 and 50% Percoll layers (Frank *et al*, 2006; Nair *et al*, 2007). Cells were washed and counted with a hemacytometer. For each brain extraction, approximately 3×10^5 cells were isolated. Microglial cells were re-suspended in phosphate buffer saline solution/0.1% bovine serum albumin to perform flow cytometric analysis.

Flow Cytometry

Microglial preparations were incubated with anti-CD16/CD32 antibody (eBiosciences) to block Fc receptors for 10 min on ice. Cells were washed and then incubated for 30 min with the appropriate conjugated antibodies: anti-CD11b-APC, anti-CD45-PerCP Cy5.5, anti-MHC-II-FITC (eBio-sciences), and anti-CD36-PE (Biolegend, Saint Quentin Yvelines, France). Cells were washed and then re-suspended in fluorescence-assisted cell sorting buffer (phosphate buffer saline solution/bovine serum albumin 0.1%) for analysis. Non-specific binding was assessed by using non-specific, isotype-matched antibodies. Antigen expression was determined using a Becton-Dickinson LSR Fortessa cytometer. Ten thousand events were recorded for each sample and isotype-matched conjugate. Data were analyzed using FlowJo software (OR, USA) and gating for each antibody was determined based on non-specific binding of appropriate negative isotype-stained controls (Madore *et al*, 2013).

Statistical Analyses

All data are expressed as mean ± SEM. For the analysis of PUFA level, cytokine expression and flow cytometry data, a two-way ANOVA with genotype (WT *vs* Fat-1) and treatment (saline *vs* LPS) as factors was performed. When a significant interaction was reported, ANOVAs were followed by *post hoc* Fisher's LSD test comparisons. For Y-maze analyses, a three-way ANOVA with treatment and genotype as between-subjects factors and arm (novel *vs* familiar) as the within-subjects factor was performed. Specific comparisons between novel and familiar arms were assessed by paired Student's *t*-tests. Statistical significance was set at $p < 0.05$.

RESULTS

N-6/n-3 PUFA Ratio Decreases in the Hippocampus of Fat-1 Mice

We first measured n-3 and n-6 PUFA contents in the hippocampus of Fat-1 mice or WT littermates fed with a standard diet (Table 2). Arachidonic acid (AA, 20:4 n-6) and n-6 docosatetraenoic acid (22:4 n-6) levels were significantly lower in the hippocampus of Fat-1 mice compared with WT littermates ($F(1,13) = 5.174$, $p < 0.05$ and $F(1,13) = 14.31$, $p < 0.01$, respectively). Conversely, DHA (22:6 n-3) level was

Table 2 Fatty Acid Composition of Total Lipids of Adult Hippocampus

Fatty acids mg/100 mg fatty acids	Saline		LPS		Statistical effects		
	WT $N=4$	Fat-1 $N=4$	WT $N=6$	Fat-1 $N=4$	LPS	Genotype	LPS × genotype
18:2 n-6	0.4 ± 0.01	0.5 ± 0.03	0.5 ± 0.02	0.5 ± 0.05	NS	<0.01	NS
20:4 n-6	10.0 ± 0.09	9.5 ± 0.33	9.9 ± 0.12	9.7 ± 0.21	NS	<0.05	NS
22:4 n-6	2.1.0 ± 0.03	1.9 ± 0.08	2.2 ± 0.03	1.8 ± 0.12	N5	<0.01	NS
22:5 n-6	0.2 ± 0.02	0.2 ± 0.05	0.2 ± 0.02	0.2 ± 0.05	NS	NS	NS
20:5 n-3	0.03 ± 0.002	0.08 ± 0.02	0.04 ± 0.01	0.10 ± 0.03	NS	<0.01	NS
22:5 n-3	0.15 ± 0.01	0.22 ± 0.05	0.15 ± 0.01	0.26 ± 0.04	NS	<0.01	NS
22:6 n-3	14.6 ± 0.20	14.5 ± 0.17	14.5 ± 0.21	15.0 ± 0.33	NS	NS	NS
n-6/n-3	0.89 ± 0.02	0.86 ± 0.02	0.90 ± 0.01	0.84 ± 0.04	NS	<0.01	NS

18:2 n-6: linoleic acid; 20:4 n-6: arachidonic acid (AA); 22:4 n-6: docosatetraenoic acid (DTA); 22:5 n-6: docosapentaenoic acid (DPA n-6); 20:5 n-3: eicosaperrtaenoicacid (EPA); 22:5 n-3: docosapentaenoic acid (DPA n-3); 22:6 n-3: docosahexaenoic acid (DHA). Data are mean ± SEM.

unchanged whereas n-3 docosapentaenoic acid (n-3 DPA, 22:5 n-3) and eicosapentaenoic acid (EPA, 20:5 n-3) levels were significantly higher in Fat-1 mice compared with WT littermates (n-3 DPA: $F(1,14)=12.28$, $p<0.01$; EPA: $F(1,14)=13.10$, $p<0.01$). Overall, Fat-1 mice had a lower n-6/n-3 ratio than WT littermates ($F(1,13)=13.59$, $p<0.01$).

We also measured peripherally for the tail fatty acid composition of Fat-1 mice and found more DHA (4.7% in Fat-1 *vs* 2.8% in WT), more EPA (1.6 *vs* 0.6%) and much less AA (0.8 *vs* 8.9%), showing a n-6/n-3 ratio of 1.2 in Fat-1 *vs* 6.0 in WT (data not shown).

LPS Impairs Spatial Memory in WT but not in Fat-1 Mice

LPS treatment has been previously reported to alter memory performance in rats and mice (Aubert *et al*, 1995; Pugh *et al*, 1998; Arai *et al*, 2001; Sparkman *et al*, 2005; Thomson and Sutherland, 2005; Tanaka *et al*, 2006). In the present study, memory performance was assessed 24 h after intraperitoneal LPS injection when most of symptoms of sickness has disappeared and especially motor dysfunctions that may interact with behavioral paradigms (Cunningham and Sanderson, 2008; O'Connor *et al*, 2009; Yirmiya and Goshen, 2011). We used a novelty discrimination task in a Y-shaped maze to assess spatial working memory more specifically (Labrousse *et al*, 2012; Moranis *et al*, 2012).

In a first set of experiments, we used an ITI of 30 min for all experimental groups. Spatial memory performance was significantly impaired only in WT mice injected with LPS (Figure 1a). A three-way ANOVA (Genotype × treatment × arm) revealed a significant effect of arm ($F(1,29)=13.70$, $p<0.001$) and a significant interaction between treatment, genotype, and arm ($F(1,29)=4.30$, $p<0.05$). Further analyses revealed that only WT mice injected with LPS exhibited a random exploration of the two arms (paired-*t*-test, $t(7)=0.23$, $p=0.82$), whereas the other groups significantly distinguished between the novel and the familiar arm (paired-*t*-test, WT/saline: $t(8)=-4.08$, $p<0.01$; Fat-1/saline: $t(8)=-2.58$, $p<0.05$; Fat-1/LPS: $t(6)=-4.33$, $p<0.01$).

To evaluate whether the impairment revealed in WT mice injected with LPS was a memory deficit or a performance deficit related to motivational alterations, animals underwent a spatial recognition test with minimal ITI (1 min) between acquisition and retrieval. In this condition, all mice recognized the novel arm (arm effect, $F(1,27)=33.07$, $p<0.0001$) showing a normal response to novelty as well as no abnormal fatigue, anxiety or lack of motivation (Figure 1b).

LPS Alters Differently the Expression of Cytokines and Chemokines in the Hippocampus of WT Compared with Fat-1 Mice

From the behavioral experiments, Fat-1 mice were protected from LPS effects on spatial memory impairment as measured in the Y-maze paradigm. Brain pro-inflammatory cytokines, especially interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, are involved in the memory disturbances that accompany exposure to immune challenge (Yirmiya and Goshen, 2011). Our next objective was thus to quantify the expression level of cytokines mRNA in the hippocampus of Fat-1 mice and WT littermates following LPS injection.

Pro-inflammatory cytokines mRNA expression was thus determined 24 h after LPS injection, the time point we used to conduct behavioral experiments (Figure 2a). Interestingly, although IL-1β mRNA expression was induced 24 h post LPS injection (treatment effect $F(1,14)=124.3$, $p<0.0001$), this increase was significantly smaller in Fat-1 mice than in WT littermates (interaction $F(1,14)=9.045$, $p<0.01$; Fisher's LSD *post hoc* test, $p<0.001$). Moreover, our results showed that TNF-α mRNA expression was significantly increased 24 h post LPS injection for both genotypes (treatment effect $F(1,15)=47.55$, $p<0.0001$) whereas IL-6 mRNA was significantly decreased after LPS treatment in all groups (treatment effect $F(1,13)=65.39$, $p<0.0001$). Regarding anti-inflammatory cytokines, no significant difference was found for IL-10 mRNA expression whatever the genotype or treatment of the animals. On the other hand, TGF-β1 was significantly increased in Fat-1 mice 24 h after LPS treatment compared with all other groups (interaction $F(1,14)=5.124$, $p<0.05$; Fat-1/LPS group

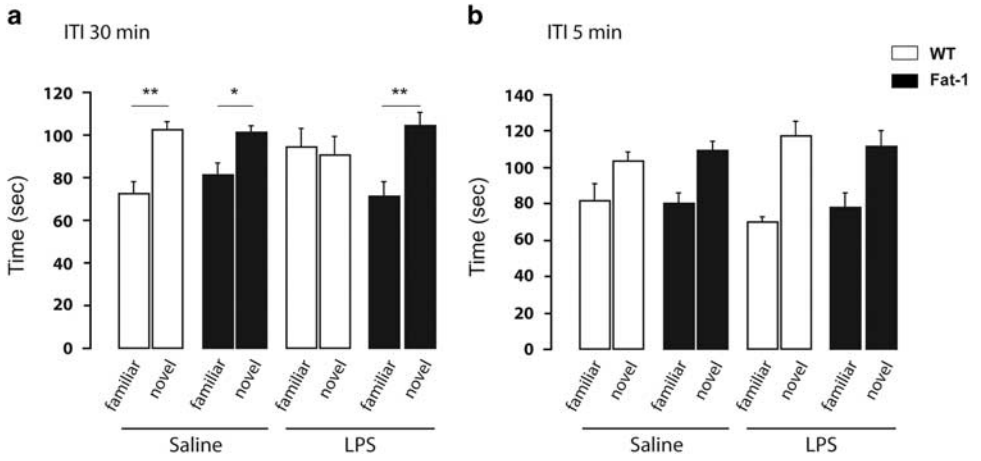

Figure 1 LPS impairs spatial memory in WT but not in Fat-1 mice. Spatial memory was assessed in the Y-maze paradigm for Fat-1 mice and WT littermates. (a) Time spent (in seconds) in the novel or the familiar arm after a 30-min ITI. Only WT mice injected with LPS exhibited a random exploration of the two arms. (b) Time spent (in sec) in the novel or the familiar arm after a 5-min ITI. All the mice spent more time in the novel arm. Data are expressed as mean ± SEM. $*p<0.05$, $**p<0.01$.

different from the other groups, Fisher's LSD *post hoc* test, $p<0.01$).

We also measured cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES1), two enzymes necessary for prostaglandin synthesis. As for TGF-β1, COX-2 and mPGES1 mRNA expression was significantly increased in Fat-1 mice 24 h after LPS treatment compared with all other groups (interaction $F(1,13)=10.15$, $p<0.01$; Fat-1/LPS group different from the other groups, Fisher's LSD *post hoc* test, $p<0.01$).

The mechanisms leading to cytokine dysregulation within the brain and thus risk factors for cognitive disorders remain poorly defined. However, research on ON/OFF signals released by neurons has begun to provide valuable insight into the origin of neuronal factors to regulate the production of cytokines by microglia. CX3CL1, a chemokine produced by neurons, controls the overexpression of IL-1β, TNFα, and IL-6 through its effect on its receptor CX3CR1 (Biber *et al*, 2007). In addition, disruption of CX3CL1/CX3CR1 signaling in rodents leads to increased hippocampal IL-1β and cognitive function (Rogers *et al*, 2011). We thus analyzed CX3CR1 and CX3CL1 mRNA expression in the hippocampus of Fat-1 and WT mice 24 h after LPS treatment. Regarding cytokine mRNA expression, Fat-1 mice showed a striking reduced pro-inflammatory profile compared with WT littermates after LPS injection. CX3CR1 receptor mRNA expression was significantly increased in Fat-1 mice whatever the treatment (Figure 2b) (Genotype effect, $F(1,16)=9.059$, $p<0.01$). CX3CL1 mRNA expression was significantly increased in the hippocampus of Fat-1 but not WT mice after LPS treatment (Interaction $F(1,15)=12.244$, $p<0.01$; Fat-1/LPS group different from Fat-1/saline and WT/LPS groups, Fisher's LSD *post hoc* test, $p<0.05$).

LPS Modulates Microglia Phenotype in the Brain of Fat-1 but not of WT Mice

Once stimulated by an immune challenge, microglia acquire diverse and complex phenotypes that are likely to be the marker for different functions, including phagocytosis, inflammatory and anti-inflammatory cytokine production, or antigen presentation (Garden and Moller, 2006). We thus analyzed microglia phenotype by flow cytometry in the brain of Fat-1 and WT mice 24 h after LPS treatment.

Firstly, the representative bivariate dot plots showed that the majority of the viable cells were CD11b + /CD45^{low}, likely to be microglia, in saline- and LPS-treated mice (Figure 3a). We first verified that the percentage of CD11b + /CD45^{low} cells isolated from saline- and LPS-treated mice was not different (WT/saline: 87.62%; WT/LPS: 87.56%; Fat-1/saline: 85.2% and Fat-1/LPS: 86.53% on average) (Figure 3b).

Secondly, both TGF-β1 and COX-2 (significantly increased in LPS-treated Fat-1 mice) have previously been shown to favor anti-inflammatory/repair (M2) phenotype (Remington *et al*, 2007; Baitsch *et al*, 2011; Kawahara *et al*, 2012; Bhattacharjee *et al*, 2013; Chhor *et al*, 2013; Hirai *et al*, 2013). We thus determined the surface expression of MHCII and CD36 (two M2 phenotype markers) in the CD11b + /CD45^{low} population (Figure 3c and d). We showed that Fat-1/LPS-treated animals had an increase in CD36 and MHCII surface expression 24 h post injection (CD36: interaction $F(1,11)=8.91$, $p<0.05$; Fat-1/LPS group different from the others, $p<0.001$; MHCII: interaction $F(1,12)=7.21$, $p<0.05$; Fat-1/LPS group different from the others, $p<0.001$).

LPS Decreases Food Intake and Body Weight in both WT and Fat-1 Mice

Overall, our data showed that in Fat-1 mice, pro-inflammatory factor synthesis was lowered compared with WT mice, whereas anti-inflammatory mechanisms were favored 24 h after LPS treatment. Thus, either Fat-1 mice display a lower LPS-induced inflammatory response compared with WT mice or it may be that the recovery from inflammation is faster in these mice. Both situations could explain the anti-inflammatory phenotype we observed 24 h post injection.

To address this question, we measured LPS-induced 'sickness behavior' and cytokine expression in the first phase of inflammation (Dantzer *et al*, 2008). After LPS injection, activated microglia produce pro-inflammatory

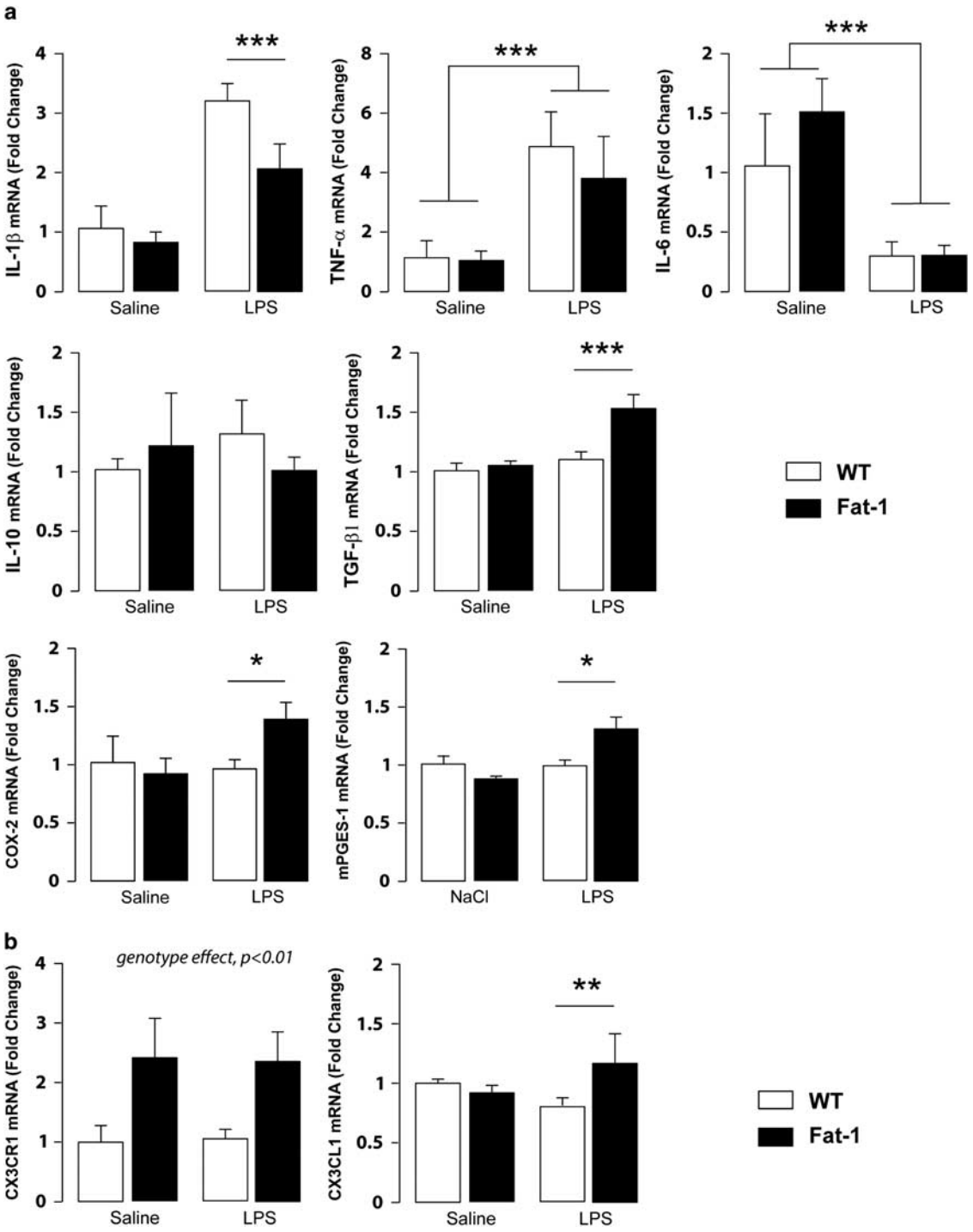

Figure 2 LPS alters differently the expression of cytokines and chemokines in the hippocampus of WT compared with Fat-1 mice. (a) Quantification of cytokines (IL-1β, TNF-α, IL-6, IL-10, TGF-β1), COX-2, and mPGES-1 mRNAs in the hippocampus of Fat-1 and WT littermates. (b) Quantification of the chemokine CX3CL1 (or fractalkine) and its receptor CX3CR1 mRNA expression in hippocampus of Fat-1 and WT littermates. Data are expressed as mean ± SEM. ***$p<0.001$; **$p<0.01$; *$p<0.05$.

cytokines and secondary messengers that elicit a sickness behavior syndrome. Thus, WT and Fat-1 mice were injected with LPS or saline and cytokine expression was evaluated 2 h later in the hippocampus, ie, during the first wave of pro-inflammatory processes in the brain (Figure 4). IL-1β, IL-6, and TNF-α expression levels were increased 2 h post LPS injection at the same extent for both genotypes (IL-1β: treatment effect $F(1,28) = 100.0$, $p<0.0001$; IL-6: treatment effect $F(1,30) = 23.62$, $p<0.0001$; TNF-α: treatment effect $F(1,30) = 197.0$, $p<0.0001$). Only IL-10 expression increase was significantly smaller 2 h post LPS injection in Fat-1 mice in comparison with WT littermates (treatment effect

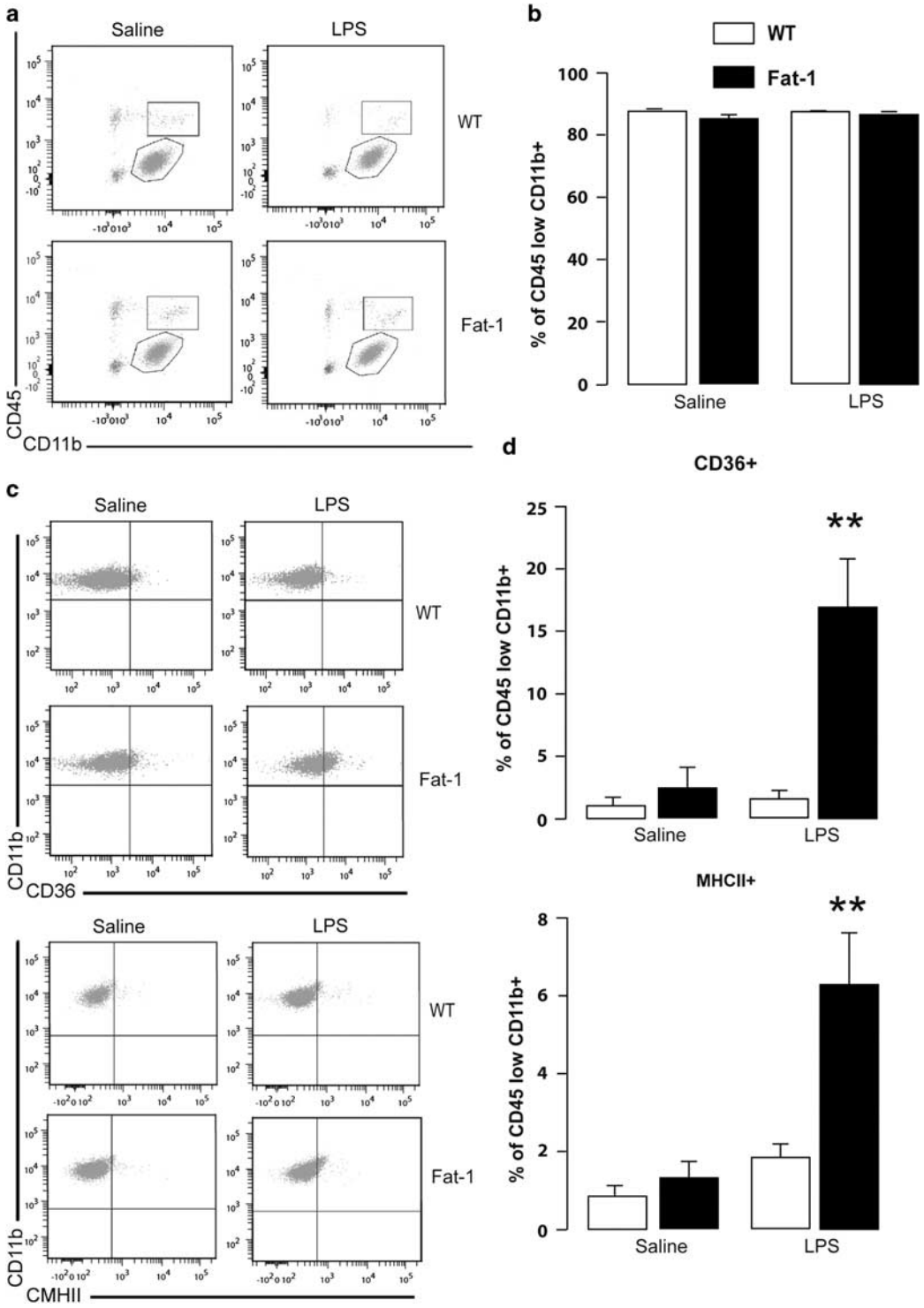

Figure 3 LPS modulates microglia phenotype in the brain of Fat-1 but not of WT mice. (a) Representative bivariate dot plots of Percoll-isolated cells gated on CD11b+/CD45^{low} expression for microglia in saline or LPS-treated animals from both genotypes. (b) Average percentage of events that were CD11b+/CD45^{low} in saline or LPS-treated animals from both genotypes. (c) Representative bivariate dot plots of Percoll-isolated cells gated on CD11b+/CD45^{low} and CD36 or MHCII expression for microglia in saline or LPS-treated animals from both genotypes. (d) Average percentage of microglia that were CD36- or MHCII-positive. Data are expressed as mean ± SEM ($N=4$ independent experiments; $n=2$ brains/N). $^{**}p<0.01$.

$F(1,28)=20.97$, $p<0.0001$; interaction $F(1,28)=4.255$, $p<0.05$; Fisher's LPSD *post hoc* test, $p<0.05$).

Regarding the sickness behavior, we sought to determine the effect of LPS on body weight and food consumption 2 and 24 h after the intraperitoneal injection in Fat-1 and WT mice (Table 3). Our results showed that LPS treatment induced a decrease in body weight in Fat-1 mice and WT littermates at 2 h (LPS effect $F(1,32)=20.75$, $p<0.0001$) and

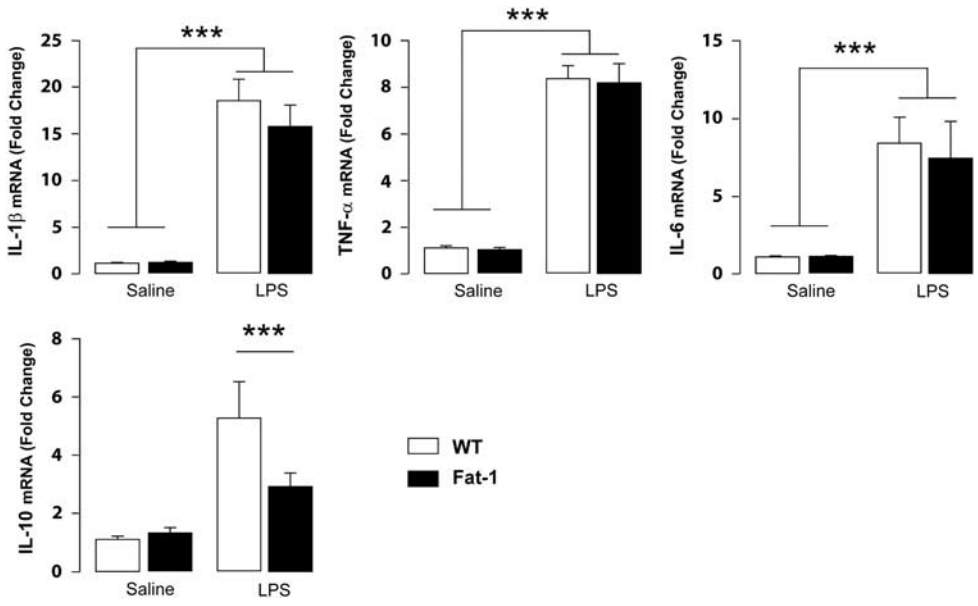

Figure 4 LPS similarly alters the expression of cytokines in the hippocampus of WT and Fat-1 mice 2 h post injection. Quantification of cytokine mRNA expression 2 h after saline or LPS treatment in animals from both genotypes. Data are expressed as mean ± SEM. ***$p < 0.01$.

Table 3 Body Weight and Food Consumption of WT and Fat-1 Mice 2 and 24 h Post Treatment

	Time after injection	Saline		LPS		Statistical effects		
		WT $N = 10/12$	Fat-1 $N = 8/10$	WT $N = 9$	Fat-1 $N = 8/9$	LPS	Genotype	LPS × genotype
Body weight loss	2 h	+0.12 ± 0.1	+0.09 ± 0.1	+0.80 ± 0.1	+0.40 ± 0.1	<0.0001	NS	NS
Food consumption	2 h	0.49.0 ± 0.1	0.53 ± 0.1	0.16 ± 0.04	0.23 ± 0.1	<0.0001	NS	NS
Body weight loss	24 h	−0.18 ± 0.2	+0.07 ± 0.4	+1.42 ± 0.1	+1.67 ± 0.2	<0.0001	NS	NS
Food consumption	24 h	5.18.0 ± 0.2	4.75 ± 0.2	3.22 ± 0.2	2.68 ± 0.3	<0.0001	<0.05	NS

Data are means ± SEM.

24 h post-injection (LPS effect $F(1,35) = 82.761$, $p < 0.001$). Additionally, LPS injection also decreased food consumption in both groups at 2 h (LPS effect $F(1,32) = 18.78$, $p < 0.0001$) and 24 h post treatment (LPS effect $F(1,35) = 78.337$, $p < 0.0001$).

DISCUSSION

This study is the first to report that Fat-1 mice that fed a standard diet had a higher level of n-3 PUFA content in the brain compared with WT animals. We also showed that LPS altered differently the phenotype of microglia and the expression of cytokines and chemokines in Fat-1 and WT mice. In Fat-1 mice, pro-inflammatory factor synthesis was lowered compared with WT mice, whereas anti-inflammatory mechanisms were favored 24 h after LPS treatment. It is likely that the recovery from inflammation was faster in Fat-1 mice explaining for such differences. Finally, we showed that LPS injection impaired spatial memory in WT mice, whereas interestingly, the Fat-1 mice showed normal cognitive performances. All together, these data suggested that the decrease of the central n-6/n-3 ratio observed in Fat-1 mice modulated the brain innate immune system activity, leading to the protection of animals against LPS-induced pro-inflammatory cytokine production and subsequent spatial memory alteration.

Dietary supplementation with n-3 PUFAs (mainly fish oil) is usually performed for enriching tissues with long-chain n-3 PUFAs such as EPA or DHA. This approach allows evaluating the effects of n-3 PUFAs *in vivo*. However, feeding animals with fish oil may induce confounding effects on the results because of other compounds provided in the fish oil such as vitamins. Thus, the use of the Fat-1 mice, a transgenic mouse line which expresses the *C. elegans fat-1* gene and is capable of producing n-3 PUFAs from the n-6 type, is a model allowing the elimination of confounding factors provided by the diet. Moreover, we and others

have previously demonstrated that a lifelong n-3 PUFA-deficient diet is related to increased depressive-like symptoms and spatial memory disturbance (Lafourcade *et al*, 2011; Moranis *et al*, 2012). Thus, both WT and Fat-1 mice were fed with a standard diet to avoid cognitive and emotional alterations in WT littermates. To verify that Fat-1 mice exposed to a standard diet indeed displayed a cerebral enrichment in n-3 PUFAs, we first measured hippocampal lipid fatty acid composition in both genotypes and showed that Fat-1 mice had a significant decrease of the n-6/n-3 ratio in the brain compared with WT littermates, even if exposed to a standard diet. However, contrary to what was demonstrated previously by Kang *et al* (2004), we could not find any effect of *fat-1* gene on DHA levels in the hippocampus. This difference may be attributable to the lower levels of n-6 PUFA available in our standard chow compared with the deficient diet used by Kang *et al* (2004). It is thus likely that the *fat-1*-dependent n-6 to n-3 PUFA conversion is less effective in our experimental conditions, accounting for the lack of DHA increase. Of note, we found that tail fatty acid composition of Fat-1 mice contain more DHA (4.7% in Fat-1 *vs* 2.8% in WT), more EPA (1.6 *vs* 0.6%), and much less AA (0.8 *vs* 8.9%), showing a n-6/n-3 ratio of 1.2 in Fat-1 *vs* 6.0 in WT. Thus, Fat-1 mice displayed a classical and expected phenotype peripherally, ruling out a genetic drift of our colony. Consequently, the results that are presented in this manuscript are likely to be specific to the brain and/or are due to exposure of the mice to a standard diet.

However, Fat-1 mice interestingly displayed an increase of n-3 EPA and DPA levels in the hippocampus compared with WT littermates and a decrease in AA and docosatetraenoic acid levels, whereas amount of n-6 DPA did not change. These changes are in agreement with the effect of the *fat-1* gene which can transform AA into EPA and docosatetraenoic acid into n-3 DPA (Kang, 2007). A previous study conducted on Fat-1 mice fed with an enriched n-6 PUFA diet could not find any difference in n-3 EPA and DPA levels measured after a whole brain lipid extraction (Orr *et al*, 2010). Here, we analyzed total fatty acid composition in the isolated hippocampus. It is thus likely that the slight but significant differences we found represented a hippocampus-specific effect.

An increasing number of publications attest of the powerful immunomodulatory effects of PUFAs in the brain (Calder, 2001; Laye, 2010; Orr *et al*, 2013) with n-3 PUFAs showing anti-inflammatory properties, whereas n-6 PUFAs stimulate the production and activity of inflammatory mediators. We thus hypothesized that LPS-induced cerebral immune system activation was limited in Fat-1 mice compared with WT littermates, accounting for a role of n-3 PUFAs on the basal activity of microglia. We thus quantified the expression levels of pro- and anti-inflammatory cytokines after LPS injection. Our data showed that IL-1β expression was much smaller in Fat-1 mice than in WT littermates 24 h after LPS injection. This confirms a previous report showing a significantly lower expression of IL-1β in the brain of Fat-1 mice, 24 h after an intracerebroventricular injection of LPS compared with WT littermates (Orr *et al*, 2013). Recently, Heerwagen *et al* (2013) also demonstrated that maternal expression of the *fat-1* transgene protects mothers from increase in obesity-associated IL-1β overexpression in plasma. The reduced expression of IL-1β we observed in Fat-1 mice is of particular interest because this cytokine has been shown to specifically interfere with spatial learning which depends on normal hippocampal functioning (Gibertini *et al*, 1995; Song and Horrobin, 2004; Yirmiya and Goshen, 2011). Accordingly, using the Y-maze paradigm, we were able to show that 24 h after LPS injection, WT littermates spent the same amount of time in the novel and familiar arm whereas Fat-1 animals behaved like saline-injected animals, spending more time in the novel arm. This confirms our previous results demonstrating that during aging, a model of low grade inflammation, mice showed impaired spatial working memory when tested in the Y-maze, associated with an increase of IL-1β expression in the hippocampus (Labrousse *et al*, 2012). Short-term exposure to an EPA- and DHA-enriched diet was able to reduce IL-1β expression in the hippocampus and reverse spatial working memory deficits found in aged mice (Labrousse *et al*, 2012).Thus, reduced IL-1β expression may be one of the main mechanisms by which Fat-1 mice are protected against an immune challenge.

EPA and/or n-3 DPA may be good candidates to explain the modulation of IL-1β expression observed in Fat-1 mice. Interestingly, previous works have shown that specialized pro-resolving mediators derived from n-3 PUFAs, particularly from EPA, are anti-inflammatory and pro-resolving (Serhan *et al*, 2002; Serhan *et al*, 2008). Thus, a higher production of these specialized pro-resolving mediators may explain the anticipated recovery from inflammation we observed in Fat-1 mice. Indeed, we found almost no difference in the first wave of inflammatory factors induction (2 h post treatment), whereas Fat-1 mice displayed a faster recovery from inflammation (24 h post treatment). Moreover, it has been shown that EPA and/or EPA derivates are able to downregulate microglial activation (Kelly *et al*, 2011).

To further characterize the impact of the *fat-1* gene on microglial physiology, we looked at microglial markers by flow cytometry. Notably, cell-sorting experiments revealed a higher proportion of CD36- and MHCII-positive microglial cells in Fat-1 mice treated with LPS, these two phenotypic markers being described as more anti-inflammatory and/or neuroprotective (Kawahara *et al*, 2012). All together, our results tend to show that the *fat-1* gene conferred protection against an inflammatory challenge to the mice, characterized by a lower expression of pro-inflammatory markers and a switch of microglial phenotype towards a more anti-inflammatory/neuroprotective profile.

The increased expression of TGF-β1 that we observed in Fat-1 mice may explain, at least in part, the reduced inflammatory episode observed after an immune challenge as well as phenotypic modifications of microglia. Indeed, previous works conducted *in vitro* have shown that TGF-β1 decreases microglial activation over the course of an inflammatory challenge (Merrill and Zimmerman, 1991). Exposure to TGFβ is also known to induce a specific phenotype in macrophages, termed M2c. This phenotype is characterized by high levels of anti-inflammatory cytokines and low levels of pro-inflammatory cytokines production (Mantovani *et al*, 2005). Of note, we found that COX-2 and mPGES1 mRNA expression was significantly increased in Fat-1 mice 24 h after LPS treatment. Although these enzymes are known as markers of the M2c phenotype, they may also display a

pro-inflammatory action in certain conditions (Matsumura *et al*, 1998; Quan *et al*, 1998; Nadjar *et al*, 2010). However, a recent *in vivo* study also suggested a role of PGE2, a well-described prostaglandin produced in the brain as a product of COX-2/mPGES1 activity, in resolution of neuroinflammation through EP2 activation (Brenneis *et al*, 2011). Moreover, not only AA but also EPA is metabolized by COX-2. As a result, EPA-derived eicosanoids (3-series prostaglandins, PGE3) are generated. EPA-derived 3-series prostanoids have much less pro-inflammatory activity than AA-derived eicosanoids (reviewed in (Farooqui *et al*, 2007). Indeed, increasing omega-3 content of membrane phospholipid of macrophages results in a decrease in PGE2 synthesis *in vitro* (Bagga *et al*, 2003). *In vivo*, the ratio of PGE2/PGE3 is greatest in n-3 PUFA-fed animals and lowest in those fed with linseed oil (Henderson *et al*, 1996). In our work, Fat-1 mice display increased COX-2 mRNA expression in the hippocampus 24 h after a LPS treatment. In addition, Fat-1 mice fed with a regular chow display decreased AA and increased EPA level in the brain. However, whether decreased AA/EPA ratio in the brain of Fat-1 lead to decreased PGE2/PGE3 production in the brain of LPS-treated mice remains to be determined.

Moreover, as another plausible mechanism for lower pro-inflammatory cytokine production and microglia phenotype modification, we found that expression levels of CX3CR1 and its ligand CX3CL1 (or fractalkine) were increased in Fat-1 mice. In the central nervous system, the fractalkine is expressed by neurons whereas its receptor is exclusively expressed by microglia. In this regard, CX3CR1-CX3CL1 signaling represents one communication pathway between microglia and neurons and has shown to control the overproduction of several pro-inflammatory cytokines (Biber *et al*, 2007). Evaluation of several pathological processes showed that signaling through the fractalkine receptor reduced neuronal damage (Cardona *et al*, 2006; Ransohoff, 2009). CX3CR1 signaling has also been shown to modulate microglial activation and protects against cognitive deficits in a mouse model of Alzheimer's disease (Cho *et al*, 2011). In addition, genetic disruption of CX3CL1 signaling increases the level of IL-1β in the hippocampus of mice, which is involved in the spatial memory deficit observed in these mice (Rogers *et al*, 2011). Thus, increased expression of the fractalkine system observed in Fat-1 mice may lead to neuroprotection and subsequent decreased action of inflammatory processes on neuronal activity. This remains to be demonstrated in the context of LPS injection.

The present study looks at the protective effect of n-3 PUFAs on the effect of inflammation on both brain microglia activity and spatial memory using a model that excludes dietary confounding factors. Our results indicate that a greater hippocampal n-3/n-6 PUFA ratio may be responsible for reduced LPS-induced neuroinflammatory activation in Fat-1 mice. Specifically, the production of IL-1β was reduced in these mice under inflammatory conditions in addition to a shift of microglia towards a more neuroprotective phenotype. The results of this study not only provide insight into how n-3 PUFAs can influence microglia activity but also suggest a role for EPA and DPA in the prevention of spatial memory deficit linked to inflammation.

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The authors declare no conflict of interest.

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