Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment

Silvia Alboni, Silvia Poggini, Stefano Garofalo, Giampaolo Milior, Hassan El Hajj, Cynthia Lecours, Isabelle Girard, Steven Gagnon, Samuel Boisjoly-Villeneuve, Nicoletta Brunello, David P. Wolfer, Cristina Limatola, Marie-Ève Tremblay, Laura Maggi, Igor Branchi

PII: S0889-1591(16)30349-X
DOI: http://dx.doi.org/10.1016/j.bbi.2016.07.155
Reference: YBRBI 2921

To appear in: *Brain, Behavior, and Immunity*

Received Date: 23 March 2016
Revised Date: 25 July 2016
Accepted Date: 25 July 2016

Please cite this article as: Alboni, S., Poggini, S., Garofalo, S., Milior, G., El Hajj, H., Lecours, C., Girard, I., Gagnon, S., Boisjoly-Villeneuve, S., Brunello, N., Wolfer, D.P., Limatola, C., Tremblay, M-E., Maggi, L., Branchi, I., Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment, *Brain, Behavior, and Immunity* (2016), doi: http://dx.doi.org/10.1016/j.bbi.2016.07.155

Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment

Silvia Alboni[1]*, Silvia Poggini[2]*, Stefano Garofalo[3], Giampaolo Milior[3,4], Hassan El Hajj[5], Cynthia Lecours[5], Isabelle Girard[5], Steven Gagnon[5], Samuel Boisjoly-Villeneuve[5], Nicoletta Brunello[1], David P. Wolfer[6], Cristina Limatola[7,8], Marie-Ève Tremblay[5#], Laura Maggi[3#] and Igor Branchi[2,6#]

[1] *Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy*

[2] *Section of Behavioural Neurosciences, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy*

[3] *Department of Physiology and Pharmacology, Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University of Rome, Italy*

[4] *Inserm U1127, CNRS UMR7225, Sorbonne Universités, UPMC UMR S1127, Institut du Cerveau et de la Moelle épinière, Paris 75013, France*

[5] *Axe Neurosciences, Centre de recherche du CHU de Québec, 2705, boulevard Laurier, Québec, Canada*

[6] *Institute of Anatomy, University of Zurich, Zurich, Switzerland*

[7] *Pasteur Institute Rome-Department of Physiology and Pharmacology, Sapienza University of Rome, Italy*

[8] *IRCCS Neuromed, Pozzilli, IS, Italy*

* these authors equally contributed as first author

# these authors equally contributed as last author

Correspondence to:
Igor Branchi, Ph.D.
Section of Behavioural Neurosciences,
Department of Cell Biology and Neuroscience,
Istituto Superiore di Sanità,
Viale Regina Elena 299,
00161 Roma, Italy

Tel.: +39-06-49902039
Fax: +39-06-4957821
E-mail: igor.branchi@iss.it

Abstract

It has been hypothesized that selective serotonin reuptake inhibitors (SSRIs), the most common treatment for major depression, affect mood through changes in immune function. However, the effects of SSRIs on inflammatory response are contradictory since these drugs act either as anti- or pro-inflammatory. Previous experimental and clinical studies showed that the quality of the living environment moderates the outcome of antidepressant treatment. Therefore, we hypothesized that the interplay between SSRIs and environment may, at least partially, explain the apparent incongruence regarding the effects of SSRI treatment on the inflammatory response. In order to investigate such interplay, we exposed C57BL/6 mice to chronic stress to induce a depression-like phenotype and, subsequently, to fluoxetine treatment or vehicle (21 days) while being exposed to either an enriched or a stressful condition. At the end of treatment, we measured the expression levels of several anti- and pro-inflammatory cytokines and inflammatory mediators in the whole hippocampus and in isolated microglia. We also determined microglial density, distribution, and morphology to investigate their surveillance state. Results show that the effects of fluoxetine treatment on inflammation and microglial function, as compared to vehicle, were dependent on the quality of the living environment. In particular, fluoxetine administered in the enriched condition increased the expression of pro-inflammatory markers compared to vehicle, while treatment in a stressful condition produced anti-inflammatory effects. These findings provide new insights regarding the effects of SSRIs on inflammation, which may be crucial to devise pharmacological strategies aimed at enhancing antidepressant efficacy by means of controlling environmental conditions.

Keywords: antidepressant, SSRI, environment, major depression, plasticity, serotonin, undirected susceptibility to change hypothesis, microglia, inflammation, cytokines

1 Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed drugs for the treatment of major depression (MD), which constitutes an enormous medical, individual, societal and economical challenge and afflicts up to 10-15% of the population worldwide. However, the efficacy of SSRIs is variable and incomplete: 60–70% of patients do not experience remission and 30–40% do not show a significant response (Trivedi et al., 2006). One of the main reasons for such limited efficacy is the poor comprehension of their mechanisms of action at cellular and molecular levels.

In recent decades, the crosstalk between the innate and adaptive immune systems and the brain has been suggested to represent a key factor in antidepressant drug action (Carvalho et al., 2013; Eller et al., 2008; Lanquillon et al., 2000; Tuglu et al., 2003). Indeed, treatment with SSRIs has been shown to decrease MD associated cytokine elevations. In particular, the levels of inflammatory cytokines IL-1β, IL-6 and TNFα have been reported to be reduced following SSRI treatment (Basterzi et al., 2005; Kagaya et al., 2001; Lanquillon et al., 2000; Leo et al., 2006; Tuglu et al., 2003; Yoshimura et al., 2009). In addition, elevated baseline levels of TNFα and IL-6 correlate with treatment failure (Carvalho et al., 2013; Eller et al., 2008; Lanquillon et al., 2000). However, other clinical studies obtained opposite results, demonstrating no or even pro-inflammatory effects of antidepressant treatment (Chen et al., 2010; Haastrup et al., 2012; Hannestad et al., 2011; Jazayeri et al., 2010; Kim et al., 2013; Song et al., 2009). Experimental studies reflect the incongruence of clinical findings. Indeed, though many studies attributed anti-inflammatory effects to antidepressant drugs (Bielecka et al., 2010; Kenis and Maes, 2002; Obuchowicz et al., 2006; Tynan et al., 2012; Xia et al., 1996), pro-inflammatory effects were reported as well (Diamond et al., 2006; Horikawa et al., 2010; Horowitz et al., 2015; Kubera et al., 2005; Tynan et al., 2012). Such discrepancy suggests that SSRIs may not have a univocal effect on inflammatory processes and additional factors may moderate the complex interplay between antidepressants and inflammation (Kraemer et al., 2006).

Recently, a number of preclinical studies have identified the living environment as a key moderator of the outcome of SSRI treatment (Alboni et al., 2016; Branchi, 2011; Branchi et al., 2013). In particular, since the increase in serotonin levels induced by SSRIs enhances neural plasticity, rendering individuals more susceptible to environmental conditions, the outcome of SSRI administration is not univocal but depends on the quality of the environment. This view, named the

undirected susceptibility to change hypothesis, is supported by clinical studies showing that antidepressants are more effective in patients with a good quality of life, while having no or even detrimental consequences in patients experiencing stressful conditions (Cohen et al., 2006; Trivedi et al., 2006). Accordingly, the quality of the environment has been shown to determine the outcome of SSRI treatment on the vulnerability to obesity (Mastronardi et al., 2011; Wong and Licinio, 2001). Though the influence of the living environment in driving SSRI effects on depressive symptomatology starts to be unraveled, no information on its role in moderating SSRI effects on the inflammatory response is yet available.

The aim of the present study was to determine whether fluoxetine treatment, as compared to vehicle, affects the inflammatory response, which notably involves microglial cells within the brain, according to the quality of the living environment. To this purpose, we exposed C57BL/6 mice first to 14 days of stress, in order to induce a depression-like phenotype and, subsequently, to 21 days of either (i) an enriched or (ii) a stressful condition, while receiving fluoxetine or vehicle. We assessed the expression levels of several key inflammatory markers in the hippocampus, a highly plastic brain region that is deeply involved in MD and antidepressant effects (MacQueen and Frodl, 2011). In addition, in order to investigate possible changes in microglial function, we measured the expression levels of several inflammatory markers in freshly isolated hippocampal microglial cells, as well as microglial density, distribution and morphology. Our prediction was that the trajectories of inflammatory and microglial modifications induced by fluoxetine treatment depend on the living environment.

The results concerning the neural and behavioral response to fluoxetine treatment displayed in the different environmental conditions by the experimental subjects used in this study have been published elsewhere (Alboni et al., 2016). These show that the exposure to 14 days of stress before treatment induced a depression-like phenotype and the neurobehavioral profile was affected by treatment according to the quality of the environment.

2 Materials and methods

2.1 Animals and housing conditions

C57BL/6 male mice 12–15 week old were used and kept under 12-hour light-dark cycle at 22–25°C. Animals were housed in the Intellicage system (TSE-system, NewBehavior AG, Zürich, Switzerland), which is an apparatus designed for the automatic monitoring of mouse behavior (Branchi et al., 2013). Food was freely available. Animals were examined for signs of discomfort as indicated by the animal care and use guidelines [National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003)]. All procedures were carried out in accordance with the EC guidelines (EEC Council Directive 2010/63/UE86/609 1987), Italian legislation on animal experimentation (Decreto Legislativo 26/2014).

The animals were gradually habituated to the Intellicage environment during a 14-days period. Five days before being moved to the Intellicage, each animal was injected with a subcutaneous transponder (T-IS 8010 FDX-B; Datamars SA, Switzerland). Three independent experiments were performed. In each experiment, animals were housed in enriched or stressful conditions, and received fluoxetine or vehicle.

Enriched condition: the Intellicage provides an enriched environment because mice are socially housed and exposed to Plexiglas shelters of different colors and shapes (four red transparent Tecniplast plastic nest boxes and four white opaque boxes), and to tissue paper. New paper was provided every 5 days and the plastic shelters were cleaned every week.

Stressful condition: The mice were exposed each day to a different stressor, randomly chosen among social stress and other stressful procedures provided by the Intellicage. Exposing mice to different stressors was used to prevent habituation to each of these. The stressful procedures used are: *Social stress*: moving animals from one Intellicage into another, creating new social groups hence forcing mice to re-establish their social hierarchy; *Short open door*: door to access water or saccharin remains open for only 1.5 seconds; *Open door 25%*: door opens only following 25% of nosepokes; *Air puff*: when the mouse enters the corner, it has a 20% chance of receiving an air puff; *Delayed door*: door opens 2.5 seconds after the first nosepoke. In addition, in the stressful condition, no shelter or tissue paper was provided.

Standard condition: mice were housed in a standard laboratory condition, two individuals per cage. Each cage was 33 x 13 x 14 cm Plexiglas box with metal tops and sawdust as bedding. Pellet food and tap water were provided *ad libitum*.

2.2 Treatment

Fluoxetine (Fluoxetine HCl, SantaCruz, USA) was dissolved in water or saccharin solution and delivered ad libitum in the drinking bottles for 3 weeks. Compared to injection, this administration method avoids stress due to the handling. The solutions were prepared according to the mouse average weight and daily water consumption in order to provide an average daily intake of 30 mg/kg. The average amount of fluoxetine administered to each mouse did not differ among the enriched, the stressful, or standard conditions. According to previous studies, such intake allows to reach an effective fluoxetine serum level approximating 150 ng/ml (Dulawa et al., 2004). Bottles were wrapped in tin foil as to protect the substance from light.

Treatment in the enriched condition: mice underwent a 14-days stress period consisting in random exposure to different stressful procedures (see above, Stressful condition). Afterwards, mice were housed for 21 days in the enriched condition while being treated with fluoxetine or vehicle (Fig. 1A).

Treatment in the stressful condition: mice underwent a 14-days stress period (as above described). Afterwards, they were exposed to a second stress period (21 days) while receiving fluoxetine or vehicle (Fig. 1A).

Treatment in the standard condition: mice underwent a 14-days stress period (as above described). Afterwards, they were placed for 21 days in standard laboratory cages, two mice per cage, while receiving fluoxetine or vehicle (Fig. 1B).

2.3 RNA extraction, RT-PCR and Real Time PCR on entire hippocampus

Total RNA was extracted from the hippocampi of 8 animals per group with a GeneEluteTM Mammalian Total RNA Moniprep kit (Sigma, St. Louis, MO. USA) using On-Column DNAse I Digestion Set (Sigma, St. Louis, MO. USA) clean-up step in order to remove genomic contamination. Quantity of total RNA was determined using a ND-1000 Spectrophotometer (Nanodrop), and RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Techonologies) to determinate the ratios of 28S–

18S ribosomal RNA band intensities (RNA integrity number). A cutoff of 8 for RNA integrity number value was applied in order to ensure a high sample quality. Two µg of total RNA were reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Milan) in 20 µL of reaction mix. Real Time PCR was performed in LightCycler® 480 Instrument (Roche, Mannheim, Germany) using Power SYBR Green mix (Applied Biosystems®, Milan) added to the specific primers (see Supplementary Table 1 for primers sequences). The cycling parameters were: 95 °C 10 min and 95 °C 15 s, 60 °C 1 min for 40 cycles. Single PCR products were subjected to a heat dissociation (gradual increase of temperature from 60 °C to 95 °C) and agarose gel separation in order to verify the absence of artifacts, such as primer-dimers or non-specific products. Direct detection of PCR products was monitored by measuring an increase in fluorescence intensity resulting from the binding of SYBR Green dye to neo-formed double strand DNA during the amplification phase. Each sample was normalized to the expression of housekeeping gene GAPDH (NCBI accession number: NM_008084). Cycle threshold (Ct) value was determined by the SDS software 2.2.2 (Applied Biosystems®, Milan) and was utilized to calculate mRNA fold changes using the delta delta ct (ΔΔCt) method (calibrator: average of VEH treated control animals). For an appropriate application of comparative ΔΔCt method, it was demonstrated that amplification efficiency of the target genes and endogenous control gene were approximately equal (Alboni et al., 2011).

2.4 Protein extraction and Western blotting

For protein extraction, hippocampi from 8 animals per group were homogenized by potter (12 stroke at 600 rpm) in lysis buffer containing Hepes 10 mM, EGTA 0.1 mM, sucrose 0.28 M pH 7.4, 1X Complete protease Inhibitor Cocktail (Roche, Mannheim, Germany), NaPP 5 mM, NaF 20 mM, Na3VO4 1 mM. Protein concentration was determined with a standard protocol using Coomassie® reagent (Sigma-Aldrich, Milan, Italy). Western blots were carried out on 30 µg of total or cytoplasm enriched extracts separated by 10-14% SDS-PAGE and transferred onto PVDF (Millipore) membranes. After blocking with 5% non-fat dry milk in TBS-Tween 20 for 1 hr at room temperature, membranes were incubated with specific antibodies overnight at 4°C followed by secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse IgG at appropriate dilutions) for 1 hr at room temperature (see Supplementary Table 2). Antibody binding was detected by using ImmobilonTM Western

Chemiluminescent HRP substrate (Millipore). The levels of protein were calculated by measuring the peak densitometric area of the autoradiography analyzed with an image analyzer. The optical density (OD) for targets signals was normalized according to the OD of β-Tubulin. Ratios were expressed as percentage of relative control ± SEM. Each experiment was performed twice and the mean of the OD ratios (target/internal standard) was analyzed.

2.5 Histology

Animals were sacrificed by an overdose of Pentobarbital (50mg/kg) and perfused transcardially with cold phosphate buffered saline (PBS) followed by cold 1% paraformaldehyde (PFA) with 15% saturated picric acid. Brains were dissected rapidly and the hippocampi removed. Isolated hippocampi were gently straightened and fixed with 4% PFA in grooves (25 x 4 x 8 mm) carved into PVC blocks. Hippocampi were post fixed in this straightened position for 3 hrs, PFA was exchanged every hour.

2.6 Matrix embedding of straightened hippocampi

Randomly selected left or right hippocampi were processed for immunohistochemistry as following: hippocampi were embedded in a gelatin-albumin protein matrix following the protocol developed by Smiley and Bleiwas (Smiley and Bleiwas, 2012). In brief, tissue was first cryoprotected by immersion in glycerol. A base layer of protein composed of gelatine-egg-albumin with the cross-linking reagents glutaraldehyde and lysine was prepared in molds (25 x 20 x 14 mm). Hippocampi were positioned in parallel on the slightly hardened base layer (5-6 hippocampi per mold) and gently pushed below the surface. After 10 min, the entire mold was filled up with freshly prepared protein matrix. Matrix blocks containing the embedded hippocampi were then cryoprotected by immersion into glycerol. Frozen blocks were cut perpendicular to the longitudinal/septotemporal axis of the hippocampi at 40 µm, series of every 10th section were collected and stored in cryoprotection solution until further processing. A reference series was mounted immediately in the correct anatomical order and Giemsa-stained (Giemsa stock solution 1.09204.0500, Merck, Darmstadt, Germany) following the protocol of Iñiguez (Iniguez et al., 1985). One complete series of sections for each animal was processed for IBA1 immunohistochemistry. For this, free-floating sections were washed extensively in Tris buffered saline (TBS) containing 0.05% Triton. For epitope retrieval, sections were treated shortly in the

microwave with citrate buffer (Target Retrieval Solution, DAKO; 1:10, pH 6.0) and endogenous peroxidase activity was blocked with a 15-min incubation in 0.06% peroxidase. After pre-incubation in 2% normal goat serum with 0.25% Triton in TBS for 60 min at RT, the sections were incubated overnight with primary antibodies against IBA1 (rabbit-anti-IBA1, Wako Pure Chemicals, Japan, 1:3000). Incubation in secondary antibody (Goat anti Rabbit, Vectastain, 1:300) was followed by incubation with ABC solution (Vectastain). Finally, sections were stained with 3,3' diaminobenzidine (DAB) and mounted, counterstained with hematoxylin solution, dehydrated and cover-slipped.

2.7 Light microscopy imaging and analysis

Color pictures were acquired in the central hippocampus CA1 stratum radiatum of 7 sections in each of 7 animals per experimental group using an Infinity 2 camera (5 MP; Lumenera), at 10X for the cellular density and spacing analysis, and at 40X for the morphology analysis. All the analysis was performed with the ImageJ software (National Institutes of Health). To determine cellular densities and spacing, the CA1 stratum radiatum was delineated in 10X pictures by using the freehand selection tool, based on the stereotaxic atlas of Paxinos and Franklin (Paxinos and Franklin, 2013), and its area measured in pixels and converted into mm^2. The center of each microglial cell body (area $\geq 20 \mu m^2$) was marked with a dot using the paintbrush tool. The analyze particles function was then used to automatically record cell numbers as well as spatial coordinates, enabling to determine the nearest neighbor distance for each cell by using the nearest neighbor distance plugin. Total cell number was divided by the total area to determine cellular density on a per animal basis. A spacing index was calculated as the square of the average nearest neighbor distance multiplied by microglial density on a per animal basis (Tremblay et al., 2012; Milior et al., 2015).

To analyze morphology, a total of 15 microglial cells in each of 5 animals per experimental group were analyzed at 40X. Only cells whose cell body and proximal processes were perfectly in focus were included in the analysis. Every IBA1-immunopositive microglia in a particular picture was analyzed before moving on to the next picture as to not introduce selection bias (Tremblay et al., 2012; Milior et al., 2015). For each microglia, the soma area was determined by drawing a line around the cell body by using the freehand selection tool. The arborization area was determined with the polygon selection tool to connect the most distal extremities of every process. The soma and arborization

areas were calculated in pixels and converted into micrometers. A morphological index was determined by using the formula: soma area/arborization area. The larger the value, the greater the soma size was in relation to the arborization size (Tremblay et al., 2012). All analyses were performed blind to the experimental condition.

2.8 Isolation of CD11b+ cells, total RNA extraction and Real-time PCR on CD11b+ cells

Mice housed in standard, enriched or stressful environments, receiving fluoxetine or vehicle, were anesthetized and decapitated. Brains were removed, cut into small pieces and single-cell suspension was achieved by enzymatic digestion in trypsin (0.25 mg/ml) solution in Hank's balanced salt solution (HBSS). The tissue was further mechanically dissociated using a wide-tipped and the suspension was applied to a 30 μm cell strainer. Cells were processed immediately for MASC MicroBead separation. The CD11b-positive (+) cells were magnetically labelled with CD11b MicroBeads. The cell suspension was loaded onto a MACS Column (Miltenyi Biotec, Germany) placed into the magnetic field of a MACS Separator. After removing the magnetic field, CD11b+ cells were eluted as positive fraction. Live CD11b+ cells were identified by flow cytometry (FACS) as previously indicated (Garofalo et al., 2015). Upon sorting of the CD11b+ and negative (-) fractions, total RNA was isolated using RNeasy Mini Kit, and processed for real-time PCR. The quality and yield of RNAs were verified with the Ultraspec 2000 UV/Visible (Pharmacia Biotech). Reverse transcription reaction of the CD11b+ and - fractions was performed in a thermocycler (MJ Mini Personal Thermal Cycler; Biorad) using IScript TM Reverse Transcription Supermix (Biorad) according to the manufacturer's protocol, under the following conditions: incubation at 25 °C for 5 min, reverse transcription at 42 °C for 30 min, inactivation at 85 °C for 5 min. Real-time PCR (RT-PCR) was carried out in a I-Cycler IQ Multicolor RT-PCR Detection System (Biorad) using SsoFast EvaGreen Supermix (Biorad) according to the manufacturer's instructions. The PCR protocol consisted of 40 cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 30 s. For quantification analysis the comparative Threshold Cycle (Ct) method was used. The Ct values from each gene were normalized to the Ct value of GAPDH in the same RNA samples. Relative quantification was performed using the $2-\Delta\Delta Ct$ method (Schmittgen and Livak, 2008) and expressed as fold change in arbitrary values.

2.9 Statistical analysis

One-way ANOVA or Student's t-test were performed with the Statistical software Statview II (Abacus Concepts, CA, USA) to compare (i) vehicle *vs.* fluoxetine-treated groups, independently in the standard, enriched and stressful condition, and (ii) enriched *vs.* stressful *vs.* standard condition, in the vehicle group, to assess the effect of the environment *per se*. All mean differences were considered statistically significant when $p < 0.05$.

3 Results and discussion

3.1 Fluoxetine affects hippocampal inflammatory mediators

In order to explore whether fluoxetine effects on inflammation depend on the quality of the living environment, we analyzed hippocampal expression levels of seven immune mediators reportedly involved in antidepressant drug action (Carvalho et al., 2013; Chen et al., 2010; Eller et al., 2008; Hannestad et al., 2011; Kim et al., 2013; Lanquillon et al., 2000; Song et al., 2009; Tuglu et al., 2003) and affected by environmental stimuli (Chabry et al., 2015; Goshen et al., 2008; Singhal et al., 2014; Wohleb et al., 2011). In particular, we focused on IL-6, TNFα and IL-1β, which are pro-inflammatory cytokines among the most studied in relation to antidepressant effects (Carvalho et al., 2013; Eller et al., 2008; Hannestad et al., 2011). The expression levels of IFNɣ, which has pro-inflammatory effects and pleiotropic activity, and three anti-inflammatory cytokines, IL-10, IL-4 and TGFβ, were analyzed as well.

While hippocampal IL-6 expression was not modified by fluoxetine in both conditions, TNFα and IL-1β levels were affected by treatment in an environment-dependent fashion. In particular, fluoxetine decreased TNFα mRNA expression as compared to vehicle in the stress condition ($t = -3.075$, $p = 0.0082$) but did not affect it in the enriched condition (Fig. 2 A,C). Whereas IL-1β mRNA expression was increased by treatment in enrichment ($t = 4.714$, $p = 0.0008$) but not affected in stress (Fig. 2 A,C). The change in IL-1β mRNA levels was paralleled by concordant modifications of IL-1β precursor and mature protein levels (Fig. 2 B,G), fluoxetine-treated subjects showing a decrease in pro-IL-1β ($t = -2.368$, $p = 0.0281$) and an increase in mature IL-1β ($t = 2.135$, $p = 0.0460$) in comparison with vehicle. No change in protein levels was found in the stressful condition (Fig. 2 D,H). IL-6 and TNFα protein levels were not affected by fluoxetine in both conditions (data not shown). IFNɣ expression levels were modified by fluoxetine treatment as compared to vehicle only in the stressful condition, treated mice showing decreased mRNA levels compared to controls ($t= -3.007$, $p = 0.0109$). No significant effect of fluoxetine on IL-10, IL-4 and TGFβ was found (Fig. 2 E,F). These findings indicate that fluoxetine increases the overall inflammatory milieu in the enriched condition, while exerting an opposite effect in the stressful condition. The latter result is in line with previous studies showing that fluoxetine, when administered to mice exposed to a stressful environment, overall reduces

hippocampal expression of pro-inflammatory cytokines (Cheng et al., 2016; Ji et al., 2014; Jiang et al., 2013; Lu et al., 2014; Tianzhu et al., 2014; Wilson et al., 2014; Xie et al., 2015).

The inflammasome is emerging as a key player in mediating the influence of inflammation on the vulnerability to psychiatric disorders including MD (Alcocer-Gomez and Cordero, 2014; Alcocer-Gomez et al., 2014; Alcocer-Gomez et al., 2015; Miller and Raison, 2015; Pan et al., 2014; Zhang et al., 2015). High expression of NLRP3 inflammasome and caspase 1 in peripheral blood mononuclear cells of depressed patients has been associated to increased blood concentration of IL-1β, which in turn correlates with depression severity (Miller and Raison, 2015). To investigate whether the inflammasome complex responsible for the activation of inflammatory processes is differently affected by fluoxetine in comparison to vehicle when administered in the enriched or stressful condition, we measured the hippocampal expression levels of components of the intracellular NLR inflammasome family. In the stressful condition, mice treated with fluoxetine showed decreased mRNA levels of the adaptor apoptosis-associated speck-like protein (ASC) common to all inflammasomes, as compared to vehicle ($t = -3.391$, $p = 0.0048$). By contrast, mice treated in the enriched condition showed increased caspase-1 mRNA levels ($t = 2.679$, $p = 0.0019$). No significant differences were observed in NLRP3, NLRP1, and IPAF (NLRC4) mRNAs levels in both conditions (Fig. S1). Overall, these results support a dual effect of fluoxetine driven by the quality of the environment and suggest that fluoxetine upregulates inflammasome activity in the enriched condition, while exerting opposite effects under stress.

3.2 Microglial modulation by fluoxetine upon environmental stimulation

Microglial cells represent a potential key player in the brain-immune dialogue since they mediate the influence of the inflammatory milieu on neuronal activity and, thus, on behavioral outcome (Bessis et al., 2007; Branchi et al., 2014). To evaluate whether microglial function is affected by changes in inflammatory markers induced by fluoxetine treatment in the two environmental conditions, we first measured the expression of Toll-like receptors (TLRs). These receptors mediate innate immune responses to exogenous and endogenous threats (Trotta et al., 2014) and can be triggered in the absence of infection (Janova et al., 2015). During inflammatory responses, the membrane-anchored CD14 acts as a co-receptor for TLR4, an important regulator of neuroimmune interactions in the

response to stress and MD (Liu et al., 2014), leading to the downstream release of inflammatory modulators such as TNFα and IL-1β (Zhou et al., 2013). We found that fluoxetine treatment, as compared to vehicle, increases hippocampal levels of both CD14 and TRL4 mRNAs in the enriched condition (t = 3.111, p = 0.0090 and t = 3.204, p = 0.0107, respectively; Fig. S2). Since TLR4 and CD14 are mainly expressed by microglial cells in the brain (Lehnardt et al., 2003), these findings suggest that fluoxetine treatment in an enriched environment makes microglial cells prone to mount an immune response. The expression of these mediators did not differ between fluoxetine treatment and vehicle in the stressful condition.

We next assessed changes in neuron-microglia signaling through the chemokine fractalkine (CX_3CL1) and its unique receptor CX_3CR1 expressed by microglia in the brain, which is known to maintain microglial cells in a surveillance state (Paolicelli et al., 2014). Fractalkine signaling was recently found to play also a prominent role in mediating the influence of environment on brain function, regulating key brain processes such as microglial phagocytosis of synaptic elements and short- and long-term neuronal plasticity (Maggi et al., 2011; Milior et al., 2015). We found that, though CX_3CR1 receptor levels were not affected, hippocampal fractalkine expression was reduced in fluoxetine-treated mice compared to vehicle in the stressful condition (t= -3.519; p= 0.0038), suggesting an "activation" or phenotypic transformation of microglia induced by the drug when administered in mice exposed to stress (Fig. 3 B). No change was found in the enriched condition (Fig. 3 A).

In order to provide a comprehensive picture of the interaction between fluoxetine and environment on inflammatory markers, we investigated the effects of fluoxetine independently from the environment and analyzed the expression levels in the whole hippocampus of mice receiving either vehicle or fluoxetine in the standard condition. This condition consisted in exposing mice for 14 days to a stressful condition in the Intellicage, followed by 21 days of treatment while housed two per cage in standard laboratory cages. We found that fluoxetine in the standard condition produced no effect on three markers of inflammation, CD14, TRL4 and caspase 1, being the expression levels similar in mice receiving treatment or vehicle (Fig. S3).

As further control, we investigated the effects of the environment *per se* on inflammatory markers. In particular, we compared the expression levels of CD14, TRL4, and caspase 1 measured in the

whole hippocampus of mice receiving vehicle and exposed to the enriched or the stressful condition. We found that, though CD14 did not differ in the two conditions, the levels of TRL4 and caspase 1 were reduced in the enriched compared to the stressful condition (t = 2.276, $p = 0.0489$, t = 3.480, $p = 0.0031$, respectively; Fig. S4). These results show that, in line with the literature (Cheng et al., 2016; Goshen et al., 2008; Singhal et al., 2014), the stressful condition increased immune activation compared to the enriched condition.

To investigate the effects of fluoxetine administered in the stressful or enriched condition on microglial surveillance state, we evaluated microglial density, distribution and morphology in the CA1 radiatum through IBA1 immunostaining. This analysis revealed that density was not modified by fluoxetine treatment compared to vehicle in both conditions, suggesting marginal proliferation or brain infiltration by IBA1-positive myeloid cells (Fig. 3 D,F). This finding is concordant with the lack of effects of fluoxetine on IBA1 and CD11b expression in the whole hippocampus (Fig. 3 A). By contrast, microglial spacing was affected by fluoxetine, though only in the stressful condition (Fig. 3 E), where it was significantly increased by the treatment in comparison to vehicle [$F(1, 12) = 6.030$, $p = 0.0303$]. This increased microglial spacing indicates the occurrence of unsampled neuropil areas where a reduced surveillance could compromise the brain response to endogenous and exogenous challenges. Microglial morphology was overall not affected in the enriched condition (Fig. 3 C). However, fluoxetine treatment increased cell body area [$F(1, 148) = 5.062$, $p = 0.0259$] and decreased arborization area [$F(1, 148) = 3.991$, $p = 0.0476$] compared to vehicle in the stressful condition. The morphological index, standardizing the size of microglial cell body to the arborization area, was increased as well [$F(1, 148) = 10.645$, $p = 0.0014$; Fig. 3 I]. These changes suggestive of immune "activation" have been classically associated to a pro-inflammatory profile (reviewed in (Walker et al., 2014) as during aging (Bachstetter et al., 2011; Tremblay et al., 2012). By contrast, here we found that such microglial modifications are associated to an anti-inflammatory profile, prompting to revisit this simplistic association between microglial structure and function, as already suggested (Walker et al., 2014). Such alternative association between microglial morphological changes and an anti-inflammatory function is increasingly reported. For instance, noradrenergic signaling, which is notably enhanced by fluoxetine treatment, has been shown to increase microglial expression of anti-

inflammatory mediators, while inducing microglial process retraction, reducing surveillance and promoting phagocytosis (Gyoneva and Traynelis, 2013; Heneka et al., 2010).

3.3 Fluoxetine modulates microglial phenotype in an environment dependent manner

Different activation states of microglia are characterized by a wide variety of phenotypic markers, including cytokines, chemokines, surface receptors and metabolic enzymes that lead to a pro or anti-inflammatory action (Franco and Fernandez-Suarez, 2015). In order to investigate how the interplay between the environment and fluoxetine treatment affects microglial phenotype and to assess whether resident immune cells contribute to the inflammatory changes measured in the whole hippocampus, we isolated and analyzed CD11b+ cells. Considering that peripheral immune cells infiltration appears to be marginal in our model, the CD11b+ population should mainly comprise microglia. RT-PCR analysis in CD11b+ cells revealed that, compared to vehicle, fluoxetine treatment administered in the enriched condition led to a pro-inflammatory profile, increasing pro-inflammatory and decreasing anti-inflammatory-related genes expression (Fig. 4). In particular, iNOS ($t = -2.965$, $p = 0.041$), cd86 ($t = -6.399$, $p < 0.001$), IL-15 ($t = -3.925$, $p = 0.008$), IL-1β ($t = -2.675$, $p = 0.038$) and IL-23 ($t = -7.343$, $p < 0.001$) mRNA levels were increased, while arg-1 ($t = -3.059$, $p = 0.022$), ym-1 ($t = -5.547$, $p = 0.005$), IL-10 ($t = -5.201$, $p = 0.002$), IL-1ra ($t = -3.151$, $p = 0.025$) were reduced compared to vehicle (Fig. 4 A,B). Interestingly, an opposite effect was found when fluoxetine treatment was administered in the stressful condition, which produced a clear shift towards an anti-inflammatory profile. Specifically, Arg-1 ($t = -4.042$, $p = 0.007$), cd206 ($t = -2.783$, $p = 0.032$), ym-1 ($t = -2.961$, $p = 0.042$), TGFβ ($t = -2.605$, $p = 0.040$), socs3 ($t = -2.963$, $p = 0.031$), IL-10 ($t = -4.117$, $p = 0.006$), IL-1ra ($t = -3.752$, $p = 0.009$), fizz-1 ($t = -3.051$, $p = 0.022$) mRNA levels were increased, while iNOS ($t = -5.535$, $p = 0.005$), TNFα ($t = -3.217$, $p = 0.018$), IL-1β ($t = -3.917$ $p = 0.008$), IL-6 ($t = -2.934$, $p = 0.026$) and IL-23 ($t = -3.366$, $p = 0.015$) levels were decreased compared to vehicle (Fig. 4 D,E).

TNFα and IL-1β were modulated by fluoxetine in the whole hippocampus and in isolated microglial cells in a similar fashion, suggesting that microglia participate in setting the hippocampal levels of these cytokines. Moreover, in the stress condition, the fluoxetine-induced reduction in the whole hippocampus levels of IFNɣ, a main cytokine leading to pro-inflammatory microglial response, was

concordant with the increased expression of anti-inflammatory markers in CD11b+ cells. Among these, iNOS, which is deeply involved and upregulated in the inflammatory response (Ghosh et al., 2016), was markedly reduced upon fluoxetine treatment. In the enriched condition, the fluoxetine-induced increase of TLR4 and CD14 levels in the whole hippocampus is in line with the expression of pro-inflammatory-related genes in CD11b+ cells. It is worth noting that in the stressful condition, microglial IL-6 expression is affected by treatment in an opposite fashion as compared to the expression of other pro-inflammatory-related genes. Previous studies have already found unique IL-6 expression profile compared to that of other pro-inflammatory cytokines in response to stressful (Audet et al., 2010) or immunogenic stimuli (Skelly et al., 2013). In addition, IL-6 has been reported to produce unexpected effects when used to modulate a variety of physiological processes, such as memory function (Arnold et al., 2002; Yirmiya and Goshen, 2011). Therefore, the functional role of IL-6 modulation by fluoxetine warrants further investigation.

We measured the effects of fluoxetine independently from the environment by analyzing the expression of pro- and anti-inflammatory-related genes in hippocampal CD11b+ cells from mice receiving either vehicle or fluoxetine in the standard condition. The results show that, contrary to subjects exposed to the enriched condition, those exposed to the standard condition display a microglial expression profile overlapping with that shown by mice exposed to the stressful condition, suggesting that, when administered to individuals first exposed to stress and afterwards treated in standard laboratory cages, fluoxetine produces effects overlapping with those produced in individuals kept in a stressful condition before and during treatment (Fig. 4 C,F). In particular, Arg-1 ($t = 10.000$, $p = 0.029$), cd206 ($t = -7.085$, $p = 0.001$), ym-1 ($t = -6.929$, $p = 0.001$), TGFβ ($t = 10.000$, $p = 0.029$), IL-10 ($t = -2.588$, $p = 0.041$), IL-1ra ($t = -7.173$, $p < 0.001$) and fizz-1 ($t = -10.435$, $p < 0.001$) mRNA levels were increased by treatment, while TNFα ($t = 3.301$, $p = 0.021$), IL-1β ($t = 4.151$, $p = 0.009$), IL-6 ($t = 26.000$, $p = 0.029$) and IL-15 ($t = 12.680$, $p < 0.001$) levels were decreased compared to vehicle. The only exception concerned socs3, an anti-inflammatory marker, which was increased by fluoxetine treatment ($t = 7.655$, $p < 0.001$).

In order to assess whether, independently from treatment, the environment affected inflammation in microglia, we measured the expression levels of pro- and anti-inflammatory related genes in hippocampal CD11b+ cells of mice exposed to the standard, enriched or stressful condition. We found

that, in line with previous studies (Chabry et al., 2015; Wohleb et al., 2011), enrichment led to an anti-inflammatory while stress led to a pro-inflammatory profile compared to the standard condition (Fig. 5). In particular, mice housed in the enriched condition showed reduced levels of IL-6, IL-23 and TGFβ (t = 37.494, $p < 0.0001$, t = 14.051, $p < 0.0001$, t = 43.000, $p < 0.0001$, respectively) accompanied by increased levels of IL-1Ra and IL-10 (t -8.014, $p = 0.0002$, t = -5.123, $p = 0.0022$, respectively) compared to mice housed in the standard condition. In addition, mice housed in the enriched condition showed reduced levels of IL-1β, IL-6, IL-23 and TGFβ (t = -3.091, $p = 0.0214$, t = -3.802, $p = 0.0191$, t = -5.044, $p = 0.0023$, t = -5.044, $p < 0.0001$, respectively) and increased levels of IL-1Ra and IL-10 (t = 8.271, $p = 0.0002$, t = 5.28, $p = 0.0019$, respectively) compared to mice housed in the standard condition. Finally, mice housed in the stressful condition showed increased levels of IL-1β (t = -3.842, $p = 0.0085$), and reduced levels of IL-1Ra (t = 22.093, $p < 0.0001$) compared to mice housed in the standard condition.

Overall, these results support the undirected susceptibility to change hypothesis and show that fluoxetine induces an anti- or pro-inflammatory profile depending on the quality of the environment.

4 Conclusion

The main finding of the present study is the divergent effect of fluoxetine treatment, as compared to vehicle, in the enriched and stressful conditions on the expression levels of inflammatory markers in the whole hippocampus and isolated microglial cells, accompanied by changes in microglial surveillance. In particular, in line with the literature and our hypothesis, treatment in a stressful condition overall led to an anti-inflammatory response (Cheng et al., 2016; Ji et al., 2014; Jiang et al., 2013; Lu et al., 2014; Tianzhu et al., 2014; Wilson et al., 2014; Xie et al., 2015). By contrast, fluoxetine treatment in an enriched condition led to an increased expression of inflammatory genes. To our knowledge, this is the first study investigating the effects on the inflammatory response of antidepressants administered in an enriched environment. It is worth noting that, in order to assess the effects of treatment in individuals showing a depression-like phenotype, we administered fluoxetine to mice previously exposed to chronic stress. Such pre-exposure activates the hypothalamic-pituitary-adrenal axis, increasing corticosterone levels (Alboni et al., 2016; Milior et al., 2015), and likely affects the interaction among the drug, the brain and immune system, making the results not applicable to different circumstances. However, this procedure has been chosen because it reproduces the clinical condition of patients who, before receiving the treatment, already show depressive symptoms and have high levels of cortisol (Juruena et al., 2009). The results concerning the neural and behavioral response of the same subjects tested in the present study have been published elsewhere (Alboni et al., 2016) and show that the effect of treatment depends on the quality of the environment.

Preclinical (Alboni et al., 2016; Branchi et al., 2013) and clinical (Cohen et al., 2006; Trivedi et al., 2006) studies have already shown that the effects of SSRIs are dependent on the quality of the living environment. In particular, it has been shown that SSRI treatment amplifies the influence of the environment on depressive symptomatology. Therefore, fluoxetine administration in a favorable environment promotes the reduction of symptoms, while in a stressful environment it may even lead to a worsened prognosis (Branchi, 2011). The key role of the environment in SSRI action has been shown also for other endpoints, such as vulnerability to obesity (Mastronardi et al., 2011; Wong and Licinio, 2001). Therefore, the environment acts as a moderator (Kraemer et al., 2006), driving treatment outcome.

The dual effect of fluoxetine on inflammatory markers here reported may explain the apparent discrepancy present in the literature. Indeed, though many studies showed that antidepressants have anti-inflammatory effects (Basterzi et al., 2005; Kagaya et al., 2001; Lanquillon et al., 2000; Leo et al., 2006; Tuglu et al., 2003; Yoshimura et al., 2009), an increasing number of studies are reporting a pro-inflammatory action (Chen et al., 2010; Haastrup et al., 2012; Hannestad et al., 2011; Jazayeri et al., 2010; Kim et al., 2013; Song et al., 2009). In addition, the bi-directional crosstalk between SSRI administration and inflammation has been described also by studies investigating the effects of anti-inflammatory drugs on antidepressant efficacy. Indeed, experimental and clinical studies have shown that anti-inflammatory drugs, such as celecoxib or aspirin, can either improve (Akhondzadeh et al., 2009; Andrade, 2014; Brunello et al., 2006) or impair treatment outcome (Warner-Schmidt et al., 2011). Though we found that fluoxetine effects on inflammation are driven by the quality of the environment, we often did not observe a match in the markers modified by treatment in the enriched and stressful conditions. For instance, IL-1β levels in the whole hippocampus were increased in the enriched, but not affected in the stressful condition. By contrast, other parameters, such as microglial surveillance state, were modified only when the drug was administered in the stressful environment. This suggests that the mechanisms underlying the anti- or pro-inflammatory action of SSRIs may differ. It should also be noted that studies describing antidepressant effects on inflammation, though showing similar overall pro- or anti-inflammatory effects, often report differences in the nature of the individual cytokines affected by treatment. For instance, some studies report a decrease of IL-1β expression following fluoxetine administration in mice exposed to stress (Cheng et al., 2016; Lu et al., 2014), while others do not (Wilson et al., 2014). The variable SSRI effects on cytokine levels have also been described in the peripheral inflammatory markers of depressed patients (Jazayeri et al., 2010; Song et al., 2009).

Here we show that the effects of the antidepressant treatment on inflammatory markers depend on the environment. However, at variance with the endophenotypes of MD, where treatment amplifies the influence of the environment, fluoxetine affects the inflammatory response counteracting the effects of the environment (Alboni et al., 2016). Indeed, fluoxetine administration, increasing and decreasing inflammatory markers respectively in the enriched and stressful condition, produced an effect opposite to that caused by enrichment and stress *per se* (Chabry et al., 2015; Goshen et al.,

2008; Singhal et al., 2014; Wohleb et al., 2011). The analysis of the interplay among SSRI, plasticity and inflammation provides a potential explanation for such unexpected effect of fluoxetine treatment. Indeed, neural plasticity requires inflammatory responses to be kept within a tightly controlled range. Brain plasticity processes, such as learning and memory, neurogenesis, neuronal excitability and synaptic scaling are dependent on the highly regulated interaction among microglia, cytokine production and neurons (Yirmiya and Goshen, 2011). Consequently, any deviation from such range, caused either by immune activation or suppression, results in plasticity impairment (Hewett et al., 2012; Santello and Volterra, 2012; Yirmiya and Goshen, 2011). Therefore, it can be hypothesized that since fluoxetine enhances neural plasticity (Branchi, 2011; Ruiz-Perera et al., 2015; Wang et al., 2008), fluoxetine keeps inflammation within a range that is permissive for plasticity. It is important to highlight that present results comprise mainly mRNA transcript levels. Therefore, further analyses devoted to measure protein levels are warranted to provide a comprehensive picture of the effects of the environment in driving the effects of SSRI on inflammation.

In conclusion, the present findings further confirm the complexity of the crosstalk among antidepressant effects, inflammatory processes and microglial activity. This is moderated by quality of the living environment, suggesting a possible explanation for the inter-individual differences in SSRI action and effects. The increased understanding of the molecular mechanisms underlying this interplay may allow for more effective personalization of antidepressant treatment strategies based on the quality of the living environment of the depressed patient.

Acknowledgements

We warmly thank Irmgard Amrein, Stefanie Engler, Roloef Maarten van Dijk, Giovanna Rigillo, Audray-Maude Baril-Lecompte and Stella Falsini for technical support. Funding for this study was provided by the Italian Ministry of Health, Ricerca Finalizzata RF-2011-02349921 to IB, Olga Mayenfisch foundation to Irmgard Amrein. IB was partially supported by EU, Marie Curie IEF fellowship for Experienced Researcher n: 302881 –Acronym: MODELMOOD.

References

Akhondzadeh, S., Jafari, S., Raisi, F., Nasehi, A.A., Ghoreishi, A., Salehi, B., Mohebbi-Rasa, S., Raznahan, M., Kamalipour, A., 2009. Clinical trial of adjunctive celecoxib treatment in patients with major depression: a double blind and placebo controlled trial. Depression and anxiety 26, 607-611.

Alboni, S., Montanari, C., Benatti, C., Blom, J.M., Simone, M.L., Brunello, N., Caggia, F., Guidotti, G., Marcondes, M.C., Sanchez-Alavez, M., Conti, B., Tascedda, F., 2011. Constitutive and LPS-regulated expression of interleukin-18 receptor beta variants in the mouse brain. Brain, behavior, and immunity 25, 483-493.

Alboni, S., van Dijk, R., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I., Cirulli, F., Maggi, L., Branchi, I., 2016. Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Mol Psychiatry in press.

Alcocer-Gomez, E., Cordero, M.D., 2014. NLRP3 inflammasome: a new target in major depressive disorder. CNS neuroscience & therapeutics 20, 294-295.

Alcocer-Gomez, E., de Miguel, M., Casas-Barquero, N., Nunez-Vasco, J., Sanchez-Alcazar, J.A., Fernandez-Rodriguez, A., Cordero, M.D., 2014. NLRP3 inflammasome is activated in mononuclear blood cells from patients with major depressive disorder. Brain, behavior, and immunity 36, 111-117.

Alcocer-Gomez, E., Ulecia-Moron, C., Marin-Aguilar, F., Rybkina, T., Casas-Barquero, N., Ruiz-Cabello, J., Ryffel, B., Apetoh, L., Ghiringhelli, F., Bullon, P., Sanchez-Alcazar, J.A., Carrion, A.M., Cordero, M.D., 2015. Stress-Induced Depressive Behaviors Require a Functional NLRP3 Inflammasome. Molecular neurobiology.

Andrade, C., 2014. Antidepressant augmentation with anti-inflammatory agents. The Journal of clinical psychiatry 75, 975-977.

Arnold, M.C., Papanicolaou, D.A., O'Grady, J.A., Lotsikas, A., Dale, J.K., Straus, S.E., Grafman, J., 2002. Using an interleukin-6 challenge to evaluate neuropsychological performance in chronic fatigue syndrome. Psychological medicine 32, 1075-1089.

Audet, M.C., Mangano, E.N., Anisman, H., 2010. Behavior and pro-inflammatory cytokine variations among submissive and dominant mice engaged in aggressive encounters: moderation by corticosterone reactivity. Frontiers in behavioral neuroscience 4.

Bachstetter, A.D., Morganti, J.M., Jernberg, J., Schlunk, A., Mitchell, S.H., Brewster, K.W., Hudson, C.E., Cole, M.J., Harrison, J.K., Bickford, P.C., Gemma, C., 2011. Fractalkine and CX 3 CR1 regulate hippocampal neurogenesis in adult and aged rats. Neurobiology of aging 32, 2030-2044.

Basterzi, A.D., Aydemir, C., Kisa, C., Aksaray, S., Tuzer, V., Yazici, K., Goka, E., 2005. IL-6 levels decrease with SSRI treatment in patients with major depression. Human psychopharmacology 20, 473-476.

Bessis, A., Bechade, C., Bernard, D., Roumier, A., 2007. Microglial control of neuronal death and synaptic properties. Glia 55, 233-238.

Bielecka, A.M., Paul-Samojedny, M., Obuchowicz, E., 2010. Moclobemide exerts anti-inflammatory effect in lipopolysaccharide-activated primary mixed glial cell culture. Naunyn-Schmiedeberg's archives of pharmacology 382, 409-417.

Branchi, I., 2011. The double edged sword of neural plasticity: Increasing serotonin levels leads to both greater vulnerability to depression and improved capacity to recover. Psychoneuroendocrinology 36, 339-351.

Branchi, I., Alboni, S., Maggi, L., 2014. The role of microglia in mediating the effect of the environment in brain plasticity and behavior. Front Cell Neurosci 8, 390.

Branchi, I., Santarelli, S., Capoccia, S., Poggini, S., D'Andrea, I., Cirulli, F., Alleva, E., 2013. Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice. PLoS One 8, e62226.

Brunello, N., Alboni, S., Capone, G., Benatti, C., Blom, J.M., Tascedda, F., Kriwin, P., Mendlewicz, J., 2006. Acetylsalicylic acid accelerates the antidepressant effect of fluoxetine in the chronic escape deficit model of depression. International clinical psychopharmacology 21, 219-225.

Carvalho, L.A., Torre, J.P., Papadopoulos, A.S., Poon, L., Juruena, M.F., Markopoulou, K., Cleare, A.J., Pariante, C.M., 2013. Lack of clinical therapeutic benefit of antidepressants is associated overall activation of the inflammatory system. Journal of affective disorders 148, 136-140.

Chabry, J., Nicolas, S., Cazareth, J., Murris, E., Guyon, A., Glaichenhaus, N., Heurteaux, C., Petit-Paitel, A., 2015. Enriched environment decreases microglia and brain macrophages inflammatory phenotypes through adiponectin-dependent mechanisms: Relevance to depressive-like behavior. Brain, behavior, and immunity 50, 275-287.

Chen, Y.C., Lin, W.W., Chen, Y.J., Mao, W.C., Hung, Y.J., 2010. Antidepressant effects on insulin sensitivity and proinflammatory cytokines in the depressed males. Mediators of inflammation 2010, 573594.

Cheng, Y., Pardo, M., Armini, R.S., Martinez, A., Mouhsine, H., Zagury, J.F., Jope, R.S., Beurel, E., 2016. Stress-induced neuroinflammation is mediated by GSK3-dependent TLR4 signaling that promotes susceptibility to depression-like behavior. Brain, behavior, and immunity.

Cohen, A., Houck, P.R., Szanto, K., Dew, M.A., Gilman, S.E., Reynolds, C.F., 3rd, 2006. Social inequalities in response to antidepressant treatment in older adults. Arch Gen Psychiatry 63, 50-56.

Diamond, M., Kelly, J.P., Connor, T.J., 2006. Antidepressants suppress production of the Th1 cytokine interferon-gamma, independent of monoamine transporter blockade. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 16, 481-490.

Dulawa, S.C., Holick, K.A., Gundersen, B., Hen, R., 2004. Effects of chronic fluoxetine in animal models of anxiety and depression. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 29, 1321-1330.

Eller, T., Vasar, V., Shlik, J., Maron, E., 2008. Pro-inflammatory cytokines and treatment response to escitalopram in major depressive disorder. Progress in neuro-psychopharmacology & biological psychiatry 32, 445-450.

Franco, R., Fernandez-Suarez, D., 2015. Alternatively activated microglia and macrophages in the central nervous system. Progress in neurobiology 131, 65-86.

Ghosh, M., Xu, Y., Pearse, D.D., 2016. Cyclic AMP is a key regulator of M1 to M2a phenotypic conversion of microglia in the presence of Th2 cytokines. Journal of neuroinflammation 13, 9.

Goshen, I., Kreisel, T., Ben-Menachem-Zidon, O., Licht, T., Weidenfeld, J., Ben-Hur, T., Yirmiya, R., 2008. Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. Mol Psychiatry 13, 717-728.

Gyoneva, S., Traynelis, S.F., 2013. Norepinephrine modulates the motility of resting and activated microglia via different adrenergic receptors. The Journal of biological chemistry 288, 15291-15302.

Haastrup, E., Knorr, U., Erikstrup, C., Kessing, L.V., Ullum, H., 2012. No evidence for an anti-inflammatory effect of escitalopram intervention in healthy individuals with a family history of depression. Journal of neuroimmunology 243, 69-72.

Hannestad, J., DellaGioia, N., Bloch, M., 2011. The effect of antidepressant medication treatment on serum levels of inflammatory cytokines: a meta-analysis. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 36, 2452-2459.

Heneka, M.T., Nadrigny, F., Regen, T., Martinez-Hernandez, A., Dumitrescu-Ozimek, L., Terwel, D., Jardanhazi-Kurutz, D., Walter, J., Kirchhoff, F., Hanisch, U.K., Kummer, M.P., 2010. Locus ceruleus controls Alzheimer's disease pathology by modulating microglial functions through norepinephrine. Proceedings of the National Academy of Sciences of the United States of America 107, 6058-6063.

Hewett, S.J., Jackman, N.A., Claycomb, R.J., 2012. Interleukin-1beta in Central Nervous System Injury and Repair. European journal of neurodegenerative disease 1, 195-211.

Horikawa, H., Kato, T.A., Mizoguchi, Y., Monji, A., Seki, Y., Ohkuri, T., Gotoh, L., Yonaha, M., Ueda, T., Hashioka, S., Kanba, S., 2010. Inhibitory effects of SSRIs on IFN-gamma induced microglial activation through the regulation of intracellular calcium. Progress in neuro-psychopharmacology & biological psychiatry 34, 1306-1316.

Horowitz, M.A., Wertz, J., Zhu, D., Cattaneo, A., Musaelyan, K., Nikkheslat, N., Thuret, S., Pariante, C.M., Zunszain, P.A., 2015. Antidepressant compounds can be both pro- and anti-inflammatory in human hippocampal cells. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 18.

Iniguez, C., Gayoso, M.J., Carreres, J., 1985. A versatile and simple method for staining nervous tissue using Giemsa dye. Journal of neuroscience methods 13, 77-86.

Janova, H., Bottcher, C., Holtman, I.R., Regen, T., van Rossum, D., Gotz, A., Ernst, A.S., Fritsche, C., Gertig, U., Saiepour, N., Gronke, K., Wrzos, C., Ribes, S., Rolfes, S., Weinstein, J., Ehrenreich, H., Pukrop, T., Kopatz, J., Stadelmann, C., Salinas-Riester, G., Weber, M.S., Prinz, M., Bruck, W., Eggen, B.J., Boddeke, H.W., Priller, J., Hanisch, U.K., 2015. CD14 is a key organizer of microglial responses to CNS infection and injury. Glia.

Jazayeri, S., Keshavarz, S.A., Tehrani-Doost, M., Djalali, M., Hosseini, M., Amini, H., Chamari, M., Djazayery, A., 2010. Effects of eicosapentaenoic acid and fluoxetine on plasma cortisol, serum interleukin-1beta and interleukin-6 concentrations in patients with major depressive disorder. Psychiatry research 178, 112-115.

Ji, W.W., Li, R.P., Li, M., Wang, S.Y., Zhang, X., Niu, X.X., Li, W., Yan, L., Wang, Y., Fu, Q., Ma, S.P., 2014. Antidepressant-like effect of essential oil of Perilla frutescens in a chronic, unpredictable, mild stress-induced depression model mice. Chinese journal of natural medicines 12, 753-759.

Jiang, H., Wang, Z., Wang, Y., Xie, K., Zhang, Q., Luan, Q., Chen, W., Liu, D., 2013. Antidepressant-like effects of curcumin in chronic mild stress of rats: involvement of its anti-inflammatory action. Progress in neuro-psychopharmacology & biological psychiatry 47, 33-39.

Juruena, M.F., Pariante, C.M., Papadopoulos, A.S., Poon, L., Lightman, S., Cleare, A.J., 2009. Prednisolone suppression test in depression: prospective study of the role of HPA axis dysfunction in treatment resistance. The British journal of psychiatry : the journal of mental science 194, 342-349.

Kagaya, A., Kugaya, A., Takebayashi, M., Fukue-Saeki, M., Saeki, T., Yamawaki, S., Uchitomi, Y., 2001. Plasma concentrations of interleukin-1beta, interleukin-6, soluble interleukin-2 receptor and tumor necrosis factor alpha of depressed patients in Japan. Neuropsychobiology 43, 59-62.

Kenis, G., Maes, M., 2002. Effects of antidepressants on the production of cytokines. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 5, 401-412.

Kim, J.W., Kim, Y.K., Hwang, J.A., Yoon, H.K., Ko, Y.H., Han, C., Lee, H.J., Ham, B.J., Lee, H.S., 2013. Plasma Levels of IL-23 and IL-17 before and after Antidepressant Treatment in Patients with Major Depressive Disorder. Psychiatry investigation 10, 294-299.

Kraemer, H.C., Frank, E., Kupfer, D.J., 2006. Moderators of treatment outcomes: clinical, research, and policy importance. Jama 296, 1286-1289.

Kubera, M., Maes, M., Kenis, G., Kim, Y.K., Lason, W., 2005. Effects of serotonin and serotonergic agonists and antagonists on the production of tumor necrosis factor alpha and interleukin-6. Psychiatry research 134, 251-258.

Lanquillon, S., Krieg, J.C., Bening-Abu-Shach, U., Vedder, H., 2000. Cytokine production and treatment response in major depressive disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 22, 370-379.

Lehnardt, S., Massillon, L., Follett, P., Jensen, F.E., Ratan, R., Rosenberg, P.A., Volpe, J.J., Vartanian, T., 2003. Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proceedings of the National Academy of Sciences of the United States of America 100, 8514-8519.

Leo, R., Di Lorenzo, G., Tesauro, M., Razzini, C., Forleo, G.B., Chiricolo, G., Cola, C., Zanasi, M., Troisi, A., Siracusano, A., Lauro, R., Romeo, F., 2006. Association between enhanced soluble CD40 ligand and proinflammatory and prothrombotic states in major depressive disorder: pilot observations on the effects of selective serotonin reuptake inhibitor therapy. The Journal of clinical psychiatry 67, 1760-1766.

Liu, J., Buisman-Pijlman, F., Hutchinson, M.R., 2014. Toll-like receptor 4: innate immune regulator of neuroimmune and neuroendocrine interactions in stress and major depressive disorder. Frontiers in neuroscience 8, 309.

Lu, M., Yang, J.Z., Geng, F., Ding, J.H., Hu, G., 2014. Iptakalim confers an antidepressant effect in a chronic mild stress model of depression through regulating neuro-inflammation and neurogenesis. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 17, 1501-1510.

MacQueen, G., Frodl, T., 2011. The hippocampus in major depression: evidence for the convergence of the bench and bedside in psychiatric research? Mol Psychiatry 16, 252-264.

Maggi, L., Scianni, M., Branchi, I., D'Andrea, I., Lauro, C., Limatola, C., 2011. CX(3)CR1 deficiency alters hippocampal-dependent plasticity phenomena blunting the effects of enriched environment. Front Cell Neurosci 5, 22.

Mastronardi, C., Paz-Filho, G.J., Valdez, E., Maestre-Mesa, J., Licinio, J., Wong, M.L., 2011. Long-term body weight outcomes of antidepressant-environment interactions. Mol Psychiatry 16, 265-272.

Milior, G., Lecours, C., Samson, L., Bisht, K., Poggini, S., Pagani, F., Deflorio, C., Lauro, C., Alboni, S., Limatola, C., Branchi, I., Tremblay, M.E., Maggi, L., 2015. Fractalkine receptor deficiency impairs microglial and neuronal responsiveness to chronic stress. Brain, behavior, and immunity.

Miller, A.H., Raison, C.L., 2015. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nature reviews. Immunology 16, 22-34.

Obuchowicz, E., Kowalski, J., Labuzek, K., Krysiak, R., Pendzich, J., Herman, Z.S., 2006. Amitriptyline and nortriptyline inhibit interleukin-1 release by rat mixed glial and microglial cell cultures. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 9, 27-35.

Pan, Y., Chen, X.Y., Zhang, Q.Y., Kong, L.D., 2014. Microglial NLRP3 inflammasome activation mediates IL-1beta-related inflammation in prefrontal cortex of depressive rats. Brain, behavior, and immunity 41, 90-100.

Paolicelli, R.C., Bisht, K., Tremblay, M.E., 2014. Fractalkine regulation of microglial physiology and consequences on the brain and behavior. Front Cell Neurosci 8, 129.

Ruiz-Perera, L., Muniz, M., Vierci, G., Bornia, N., Baroncelli, L., Sale, A., Rossi, F.M., 2015. Fluoxetine increases plasticity and modulates the proteomic profile in the adult mouse visual cortex. Scientific reports 5, 12517.

Santello, M., Volterra, A., 2012. TNFalpha in synaptic function: switching gears. Trends in neurosciences 35, 638-647.

Singhal, G., Jaehne, E.J., Corrigan, F., Toben, C., Baune, B.T., 2014. Inflammasomes in neuroinflammation and changes in brain function: a focused review. Frontiers in neuroscience 8, 315.

Skelly, D.T., Hennessy, E., Dansereau, M.A., Cunningham, C., 2013. A systematic analysis of the peripheral and CNS effects of systemic LPS, IL-1beta, [corrected] TNF-alpha and IL-6 challenges in C57BL/6 mice. PLoS One 8, e69123.

Smiley, J.F., Bleiwas, C., 2012. Embedding matrix for simultaneous processing of multiple histological samples. Journal of neuroscience methods 209, 195-198.

Song, C., Halbreich, U., Han, C., Leonard, B.E., Luo, H., 2009. Imbalance between pro- and anti-inflammatory cytokines, and between Th1 and Th2 cytokines in depressed patients: the effect of electroacupuncture or fluoxetine treatment. Pharmacopsychiatry 42, 182-188.

Tianzhu, Z., Shihai, Y., Juan, D., 2014. Antidepressant-like effects of cordycepin in a mice model of chronic unpredictable mild stress. Evidence-based complementary and alternative medicine : eCAM 2014, 438506.

Tremblay, M.E., Zettel, M.L., Ison, J.R., Allen, P.D., Majewska, A.K., 2012. Effects of aging and sensory loss on glial cells in mouse visual and auditory cortices. Glia 60, 541-558.

Trivedi, M.H., Rush, A.J., Wisniewski, S.R., Nierenberg, A.A., Warden, D., Ritz, L., Norquist, G., Howland, R.H., Lebowitz, B., McGrath, P.J., Shores-Wilson, K., Biggs, M.M., Balasubramani, G.K., Fava, M., 2006. Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice. Am J Psychiatry 163, 28-40.

Trotta, T., Porro, C., Calvello, R., Panaro, M.A., 2014. Biological role of Toll-like receptor-4 in the brain. Journal of neuroimmunology 268, 1-12.

Tuglu, C., Kara, S.H., Caliyurt, O., Vardar, E., Abay, E., 2003. Increased serum tumor necrosis factor-alpha levels and treatment response in major depressive disorder. Psychopharmacology 170, 429-433.

Tynan, R.J., Weidenhofer, J., Hinwood, M., Cairns, M.J., Day, T.A., Walker, F.R., 2012. A comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia. Brain, behavior, and immunity 26, 469-479.

Walker, F.R., Beynon, S.B., Jones, K.A., Zhao, Z., Kongsui, R., Cairns, M., Nilsson, M., 2014. Dynamic structural remodelling of microglia in health and disease: a review of the models, the signals and the mechanisms. Brain, behavior, and immunity 37, 1-14.

Wang, J.W., David, D.J., Monckton, J.E., Battaglia, F., Hen, R., 2008. Chronic fluoxetine stimulates maturation and synaptic plasticity of adult-born hippocampal granule cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 28, 1374-1384.

Warner-Schmidt, J.L., Vanover, K.E., Chen, E.Y., Marshall, J.J., Greengard, P., 2011. Antidepressant effects of selective serotonin reuptake inhibitors (SSRIs) are attenuated by antiinflammatory drugs in mice and humans. Proceedings of the National Academy of Sciences of the United States of America 108, 9262-9267.

Wilson, C.B., McLaughlin, L.D., Ebenezer, P.J., Nair, A.R., Dange, R., Harre, J.G., Shaak, T.L., Diamond, D.M., Francis, J., 2014. Differential effects of sertraline in a predator exposure animal model of post-traumatic stress disorder. Frontiers in behavioral neuroscience 8, 256.

Wohleb, E.S., Hanke, M.L., Corona, A.W., Powell, N.D., Stiner, L.M., Bailey, M.T., Nelson, R.J., Godbout, J.P., Sheridan, J.F., 2011. beta-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. The Journal of neuroscience : the official journal of the Society for Neuroscience 31, 6277-6288.

Wong, M.L., Licinio, J., 2001. Research and treatment approaches to depression. Nature reviews. Neuroscience 2, 343-351.

Xia, Z., DePierre, J.W., Nassberger, L., 1996. Tricyclic antidepressants inhibit IL-6, IL-1 beta and TNF-alpha release in human blood monocytes and IL-2 and interferon-gamma in T cells. Immunopharmacology 34, 27-37.

Xie, H., Jin, D., Kang, Y., Shi, X., Liu, H., Shen, H., Chen, J., Yan, M., Liu, J., Pan, S., 2015. The effect of Piper laetispicum extract (EAE-P) during chronic unpredictable mild stress based on interrelationship of inflammatory cytokines, apoptosis cytokines and neurotrophin in the hippocampus. BMC complementary and alternative medicine 15, 240.

Yirmiya, R., Goshen, I., 2011. Immune modulation of learning, memory, neural plasticity and neurogenesis. Brain, behavior, and immunity 25, 181-213.

Yoshimura, R., Hori, H., Ikenouchi-Sugita, A., Umene-Nakano, W., Ueda, N., Nakamura, J., 2009. Higher plasma interleukin-6 (IL-6) level is associated with SSRI- or SNRI-refractory depression. Progress in neuro-psychopharmacology & biological psychiatry 33, 722-726.

Zhang, Y., Liu, L., Liu, Y.Z., Shen, X.L., Wu, T.Y., Zhang, T., Wang, W., Wang, Y.X., Jiang, C.L., 2015. NLRP3 Inflammasome Mediates Chronic Mild Stress-Induced Depression in Mice via Neuroinflammation. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 18.

Zhou, M., Wang, C.M., Yang, W.L., Wang, P., 2013. Microglial CD14 activated by iNOS contributes to neuroinflammation in cerebral ischemia. Brain research 1506, 105-114.

Figure legends

Figure 1. Experimental design. Fluoxetine treatment in (**A**) the enriched and stressful and (**B**) the standard condition. In all conditions, before treatment, mice are exposed to a 14-days period of stress to induce a depression-like phenotype.

Figure 2. Fluoxetine effects on inflammatory markers in the whole hippocampus. In the enriched condition, we found (**A**) increased levels of IL-1β mRNA, (**B**) decreased protein levels of pro IL-1β and increased levels of mature IL-1β. (**C**) TNF-α expression was reduced by fluoxetine treatment in mice treated in the stressful condition. (**D**) IL-1β protein level was not affected in the stressful condition. (**E**) No significant effect was found for IFN-ɣ, IL-10, IL-4 and TGFβ mRNA levels in the enriched condition. (**F**) By contrast, IFN-ɣ mRNA levels were lower in mice treated in the stressful condition. Representative blots of pro- and mature- forms of IL-1β, and their relative β-tubulin bands, in (**G**) the enriched or (**H**) the stressful condition. Results are shown as fold increases relative to vehicle. Data are expressed as mean + S.E.M. (n = 8 per group). * $p<0.05$ *vs.* relative vehicle group.

Figure 3. Microglial surveillance state in the hippocampus. (**A**) In the enriched condition, IBA1, CD11b, CX_3CR1 and CX_3CL1 mRNAs levels were not modified by fluoxetine treatment. However, (**B**) in the stressful condition, the expression of CX_3CL1 was significantly reduced in fluoxetine treated mice with respect to their control. Results of RT-PCR analysis are shown as fold increases relative to vehicle. Gene expression data are expressed as mean + S.E.M. (n = 8 per group). * $p<0.05$ *vs.* relative vehicle group. (**C**, **E**) In the enriched condition, the nearest neighbor distance (NND), density and spacing index (square of the average NND multiplied by microglial density), cell body area, arborization area and morphological index (cell body area over arborization area) were not altered by fluoxetine treatment. (**D, F**) By contrast, in the stressful condition, the spacing index and cell body areas were increased, the arborization area was decreased and the morphological index was increased in fluoxetine treated mice. Representative images of IBA1-stained microglia from the four experimental groups, captured at low (10X) or high magnification (40X), are respectively shown in (**G**, **H**) and (**I**, **J**).

Figure 4. Microglial phenotypic change induced by fluoxetine treatment. Expression of pro- and anti-inflammatory-related genes in CD11b+ cells isolated from the hippocampus of mice exposed to the enriched or the stressful condition, treated with fluoxetine or vehicle. Results of RT-PCR analysis are shown as fold increases *vs.* vehicle. mRNA levels of pro-inflammatory-related genes measured in the (**A**) enriched, (**B**) stressful and (**C**) standard condition. mRNA levels of the anti-inflammatory-related genes measured in the (**D**) enriched, (**E**) stressful and (**F**) standard condition. Results are shown as fold increases relative to vehicle. Data are expressed as mean + S.E.M. (n=4 per group) Student *t*-test * $p<0.05$ ** $p<0.01$.

Figure 5. Inflammatory markers in hippocampal CD11b+ cells are affected by the environment. Expression of pro- and anti-inflammatory-related genes in CD11b+ cells isolated from the hippocampus of mice exposed to the standard, enriched or stressful conditions. Results are shown as fold changes relative to mice housed in the standard condition. Data are expressed as mean + S.E.M. (n=4 per group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* mice from the standard condition, # $p<0.05$, ## $p<0.01$, ### $p<0.001$ *vs.* mice from the enriched condition.

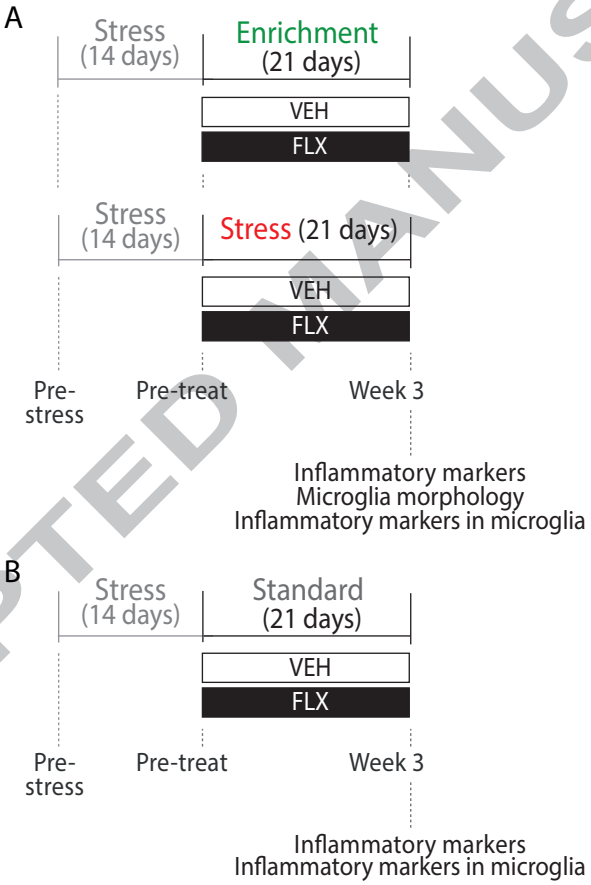

Figure 1

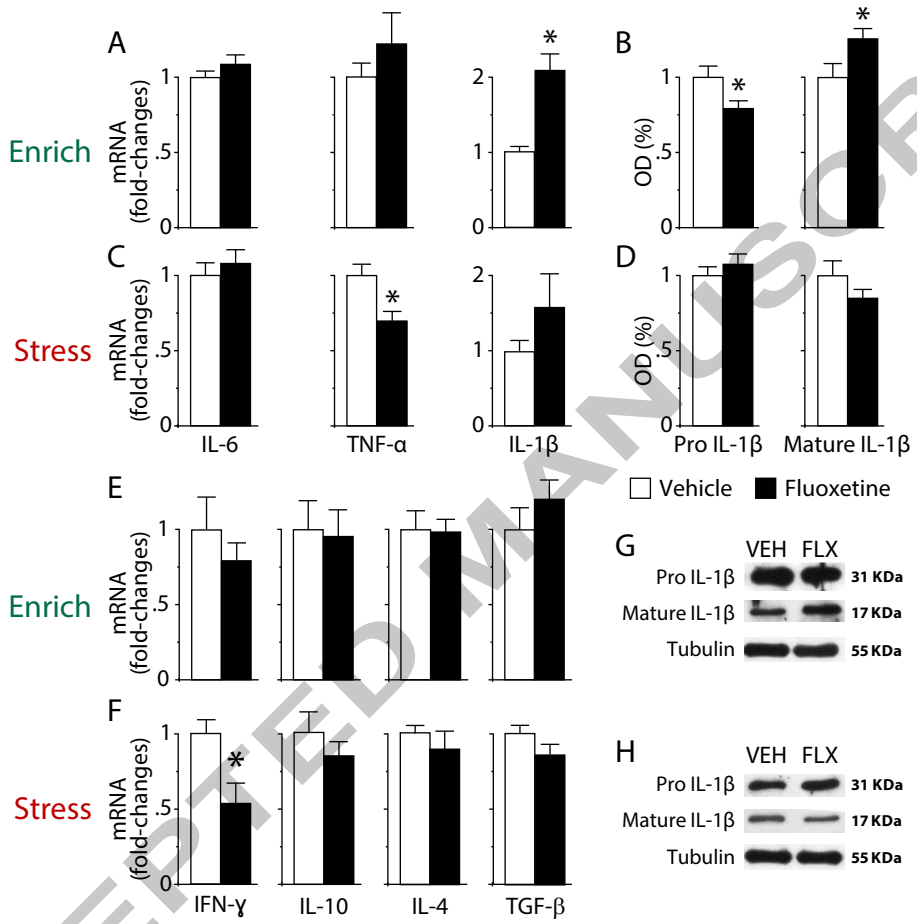

Figure 2

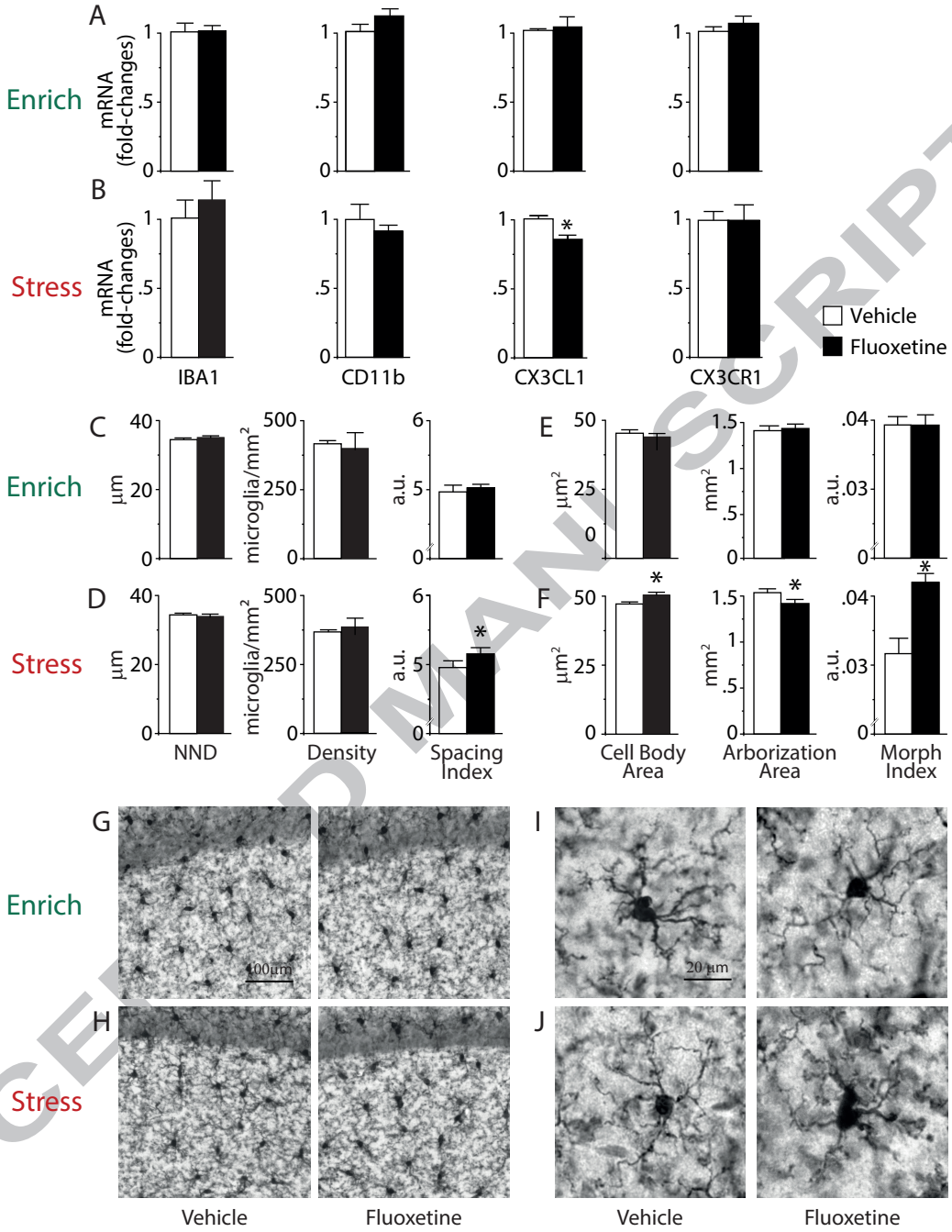

Figure 3

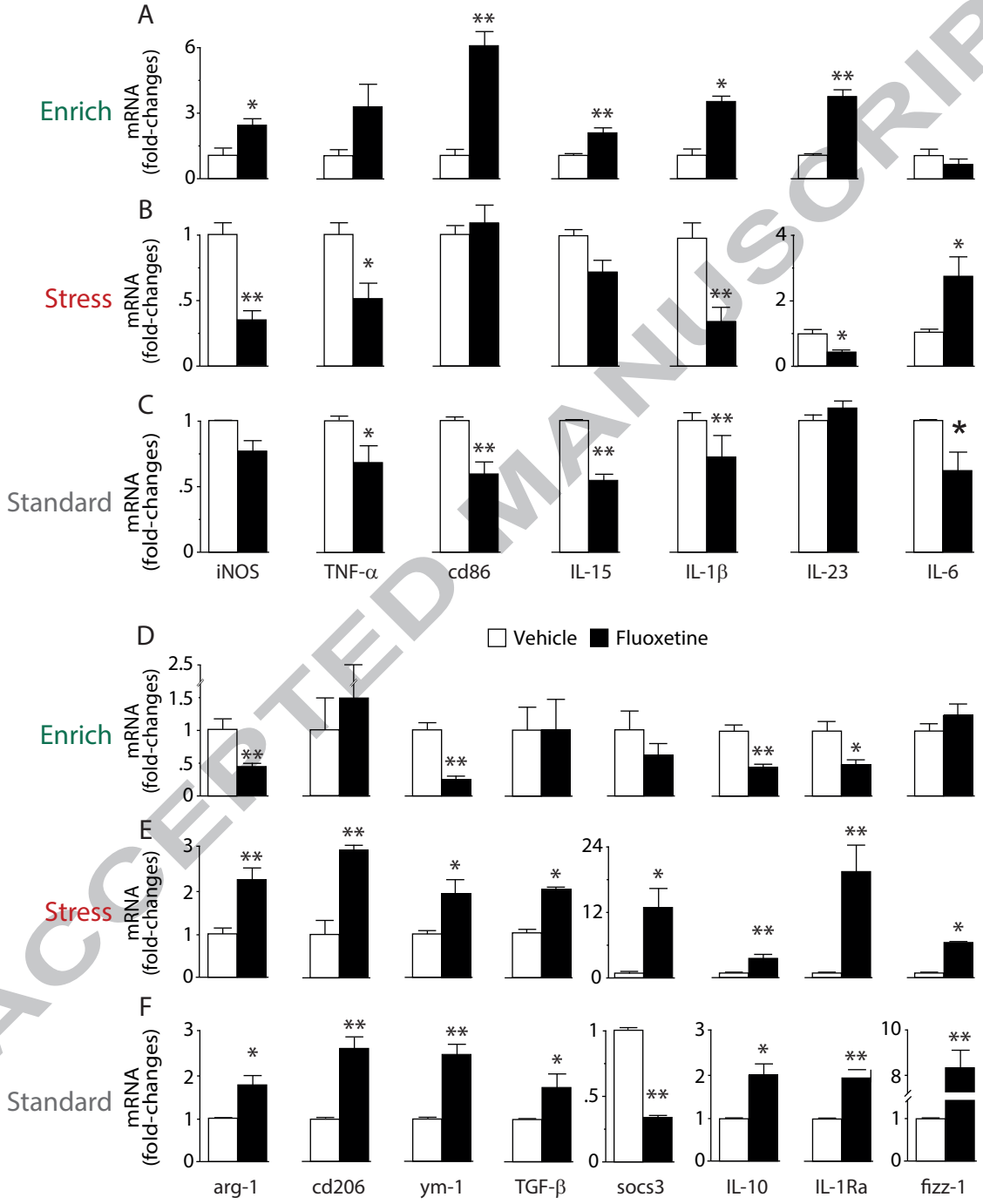

Figure 5

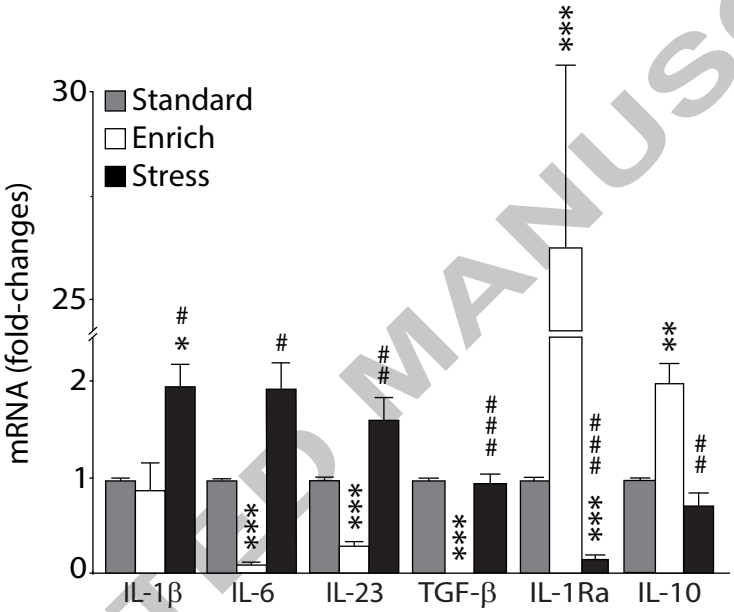

Figure 6

Highlights

- contradictory results concerning SSRI effects on inflammation were reported

- the effects of fluoxetine on inflammation are dependent on the environment

- in the enriched condition, fluoxetine is pro-inflammatory compared to vehicle

- in the stressful condition, fluoxetine is anti-inflammatory compared to vehicle