Sapienza University of Rome

PhD Program in Behavioral Neuroscience
Curriculum of Psychobiology and Psychopharmacology
XXXI Cycle

The interplay between inflammation and neural plasticity determines serotoninergic antidepressant efficacy

Academic Year 2017/2018

Candidate:
Silvia Poggini

Tutor:
Prof. Stefano Puglisi-Allegra

Supervisor:
Dr. Igor Branchi

ABSTRACT

Major Depressive Disorder (MDD) is a multifaceted disorder that imposes an enormous medical, societal and economic burden. Selective Serotonin Reuptake Inhibitors (SSRIs) are the most commonly prescribed antidepressant drugs. However, their efficacy is variable and incomplete and there is still limited understanding of the factors determining their beneficial action. With the purpose to identify these factors and develop more effective therapeutic strategies for MDD, we carried out preclinical studies exploiting a multidisciplinary strategy, spanning from molecular to cellular and behavioral assessment. We focused on inflammation and neural plasticity because, though these have been widely reported as key factors in determining SSRI outcome, their interplay has been limitedly explored. The results obtained show, on the one hand, that the increased neural plasticity induced by SSRI administration regulates inflammation counterbalancing both the activation and suppression of immune response, and, on the other, that any deviation towards an extreme immune activation or suppression results in reduced neural plasticity. These findings indicate that neural plasticity and inflammation are mutually regulating processes and that inflammatory levels should be kept within a strict physiological range to be permissive for neural plasticity. As further step, we explored a polypharmacological strategy aimed at increasing SSRI efficacy through the add-on treatment with metformin, a drug able to improve metabolic profile, which has been shown to be implicated in antidepressant efficacy. This approach is aimed at producing two concerted effects –increasing neural plasticity (i.e. SSRI) and regulating metabolism (i.e. metformin)—that together should lead to a more effective therapeutic strategy for MDD than the SSRI alone. The results suggest that the combined treatment has an improved efficacy and that multifactorial disorders such as MDD may be more effectively treated with strategies able to targeting several biological processes. Overall, our findings underpin the implementation of the precision medicine paradigm in the psychiatric field. Indeed, information concerning not only the patients' mood but also selected physiological endpoints (e.g. inflammatory levels and metabolic profile) should be considered for an effective antidepressant therapeutic strategy.

GENERAL INTRODUCTION

1. Major depression and current antidepressant drug treatments

1.1 Major depressive disorder

1.1.1 Clinical feature of major depressive disorder

The most common form of depression is the Major Depressive Disorder (MDD), which affects more than 300 million people across the globe, an increase of more than 18% between 2005 and 2015 (WHO, 2017).

According to the criteria established by the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) MDD is diagnosed when the individual experiences, for at least two weeks, feelings of sadness and hopelessness and loses interest in activities once enjoyed. In addition, there are a number of other symptoms causing marked functional impairment, such as sleep disturbance (insomnia or hypersomnia), lack of energy, poor concentration, lack or increase in appetite, inappropriate feelings of self-reproach, recurrent morbid thoughts about death and suicidal ideation (APA, 2013). The diagnosis of MDD requires presence of five out of nine symptoms, consequently is possible for two clinically depressed patients to share only one symptom. Moreover, MDD can manifest itself with increasing degrees of severity: some people have low intensity symptoms, while who suffers the severe form shows higher number, increased intensity and duration of symptoms (Kendler et al., 1998). The several different clinical presentations imply that MDD could be approached as a group of diseases, rather than a single clinical entity.

MDD often carries an unfavorable prognosis, a depressive episode lasts for six months on average (Kruijshaar et al., 2005) and in 20% of the cases it lasts longer than two years (Spijker et al., 2002). It is important to note that MDD is a recurrent disorder, after a first depressive episode only 30% of the patients remain largely

symptom free and there is 85% probability that, after recovery, a new episode occurs in the five years following the index episode (Mueller et al., 1999).

A serious problem in depression is the increased mortality because of suicide, about 60% of all suicides are committed by people who were depressed (Marquet et al., 2005), and mortality rates are higher in people with depression compared to non-depressed subjects (Cuijpers and Smit, 2002). Furthermore, it is important to note that depression is often accompanied by other psychiatric disorders, especially substance use disorder, impulse control disorder, and anxiety disorders. In particular, depressive and anxiety disorders show high comorbidity and substantial symptomatic fluidity with frequent changes of diagnostic subtypes over time (Kessler et al., 2003).

Several risk factors can play a role in MDD, according to the American Psychiatric Association there are four main contributors, (i) biochemistry: differences in certain chemicals in the brain may contribute to symptoms of depression; (ii) genetics: depression can run in families; (iii) personality: people with low self-esteem, who are easily overwhelmed by stress, or who are generally pessimistic appear to be more likely to experience depression; (iv) environmental factors: continuous exposure to violence, neglect, abuse or poverty may make some people more vulnerable to the pathology (Kessler, 2005; APA, 2013).

1.1.2 Socio-epidemiological framework

According to the World Health Organization (WHO), MDD is the leading cause of years lost owing to disability worldwide and the third overall contributor to the burden of disease, projected to be the biggest contributor by 2030 (WHO, 2017). Despite MDD affects people of every age, ethnic background, educational and socio-economic status, the prevalence rates for women are generally twice as high as those for men (Kuehner, 2003). In 2010, the global annual prevalence was 5.1% and 3.6%, respectively in women and in men (Whiteford et al., 2013; Figure 1).

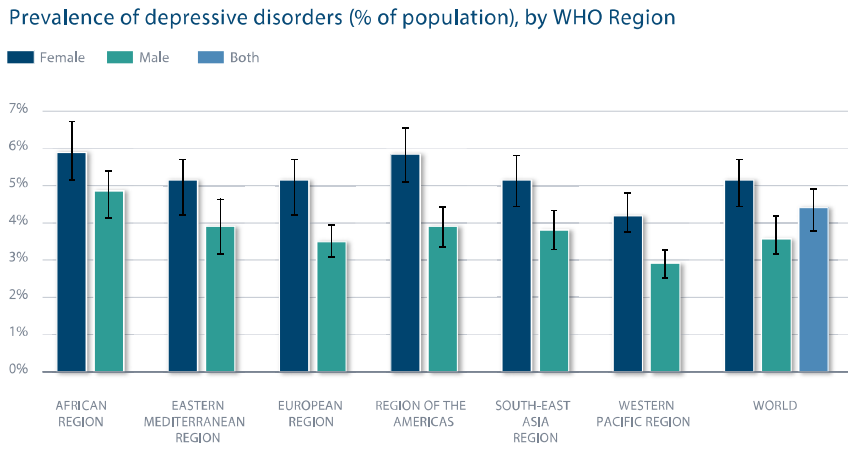

Figure 1. Prevalence of depressive disorders. The proportion of the global population with depression in 2015 is estimated to be 4.4%. Upper and lower uncertainty intervals are represented in the bar graph by the error bars. Depression is more common among females, 5.1%, than males, 3.6% (WHO, 2017).

The average age of onset is 32 years old, but from an epidemiological research results that 3.3% of 13 to 18 year olds have experienced a seriously debilitating depressive disorder. Moreover, like common mental disorders, which are most prevalent among those with a poor standard of living, depression is often associated with unemployment and poverty (Weich and Lewis, 1998).

The economic impact of MDD is alarming considering the health care costs for individuals suffering from depression and other co-morbidities. Patients with depression have significantly higher mean medical costs when compared to non-depressed patients, in every age group and category of medical care. For example, the annual cost of depression in Europe is estimated over 120 billion Euros, which accounts for around 1% of the gross domestic product (Balak and Elmaci, 2007). This number is not surprising since it has been calculated that patients with MDD are unable to perform their normal daily activities on 35 days per year on average (Kessler et al., 2003).

1.2 Antidepressant treatment

The principal therapies to treat MDD are drugs, psychotherapy and Electroconvulsive Therapy (ECT), but the international trends have indicated an increase in the use of antidepressants, particularly after the introduction of selective serotonin reuptake inhibitors (SSRIs) in the early 1990s (Whooley and Simon, 2000).

Currently, SSRIs are the class of compounds typically used as antidepressants in the treatment of MDD and others mood disorders. These drugs increase the extracellular level of the neurotransmitter serotonin by inhibiting its reuptake into the presynaptic cell. Consequently, SSRIs increase serotonin levels in the synaptic cleft available to bind postsynaptic receptors (Hiemke and Hartter, 2000).

The observation that the increase of the synaptic concentrations of monoamines leads to the improvement of the depressive symptoms, on the basis of the *ex iuvantibus* principle, drove to the adoption of the monoamine hypothesis of depression. It posits that decreased function of monoamine, such as norepinephrine and/or serotonin, caused depression and re-establishment of their balance leads to recovery (Belmaker and Agam, 2008; Heninger et al., 1996; Krishnan and Nestler, 2008; Lesch, 2007; Wong and Licinio, 2001). Although monoamine imbalance is certainly involved in the pathogenesis (Lambert et al., 2000; Meyer et al., 2006), MDD affects the brain in a more complex and sophisticated manner and the causes appears to be different from being the decrease of monoamine concentration in the brain. Indeed, it has been reported the absence of changes in monoamine levels in depressed patients, while the depletion of monoamines does not alter mood in healthy controls (Ruhe et al., 2007).

SSRIs are no more efficacious or have rapid onset of action than the older antidepressants drugs, like tricyclic antidepressant (TCA), but their improved safety and tolerability in clinical use has led to be the most widely used of all antidepressants (Beasley et al., 1993; Sternbach, 1991). Moreover, to prevent recurrence of depressive symptoms, antidepressants are taken on a long-term basis and studies have reported no evidence of long-term safety problems with the SSRI treatment (Doogan and Caillard, 1992; Janicak et al., 2001).

1.3 Controversial results of serotoninergic antidepressant treatment

Basing on a large number of studies, we know that pharmacotherapy with SSRIs achieves mixed results, and patient success in achieving remission often depends on several factors. It is becoming increasingly necessary to find more efficient treatment, since it is well documented that depression has the tendency to assume a chronic course, be recurrent, and over time to be associated with increasing disability.

1.3.1 Low SSRIs efficacy

Clinical studies and meta-analyses have shown that 30-50% of patients treated with SSRI do not show a significant response and 60-70% do not achieve remission (Trivedi et al., 2006; Figure 2). Partial remission is characterized by the presence of residual symptoms that are predictors of a relapse. Indeed, patients presenting residual symptoms has up to 6 times higher probability of a relapse than in patients those who experience full remission (Kato and Serretti, 2010; Moncrieff and Kirsch, 2005).

A second unexplained feature of antidepressant drug action is the temporal disconnection between the onset of the serotonin uptake inhibition in the CNS and the onset of the antidepressant activity, given that the former occurs within hours of the first administration while the latter takes at least three weeks. Thus, if the effect alone was sufficient to mediate an antidepressant response, SSRIs should work within hours rather than weeks (Alarcon et al., 2004).

Moreover, antidepressants tend to lose efficacy during treatment even if a number of strategies are used in clinical practice to try to overcome this limits, including switching medication, increasing dose augmentation and combining drugs (Fava, 2002).

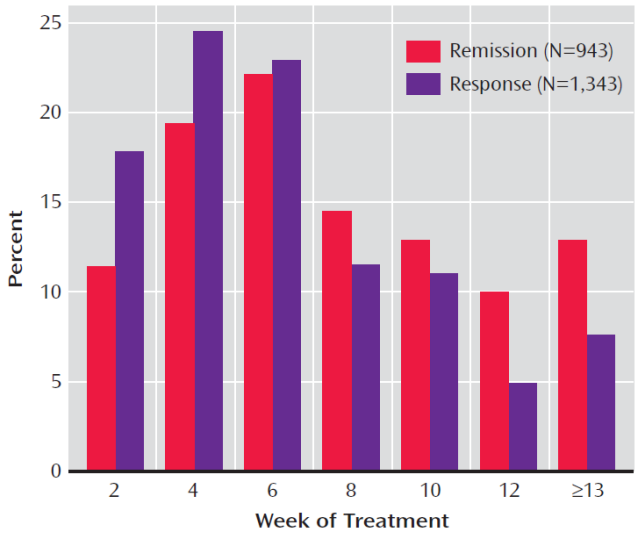

Figure 2. Low remission and response to SSRI citalopram treatment in outpatients with non psychotic major depressive disorder defined by 16-Item Quick Inventory of Depressive Symptomatology-Self-Report (QIDS-SR), scores by week of treatment. Response was defined as improvement of ≥ 50% in QIDS-SR score from baseline. Remission was defined as a QIDS-SR score of ≤ 5 at endpoint. (Trivedi et al., 2006).

1.3.2 High placebo efficacy

In double-blind, randomized controlled trials testing the efficacy of antidepressants, SSRIs demonstrate small yet statistically significant advantages over placebo but do not substantially improve patient symptoms (Figure 3). In particular, placebo response rates range from 30% to 50%; this high percentage occurs partly because some patients show a spontaneous remission from MDD during the 6-8 weeks of the drug trial, and partly because of the power of the placebo effect, which is particularly noticeable in the management of psychiatric illness. Indeed, the efficacy of the placebo is exceptionally large, at least as that attributable to the antidepressants and this means that it is hard to obtain a statistically significant difference between drug and placebo (Fournier et al., 2005; Kirsch et al., 2008). Nevertheless, increasing number of studies suggests that the small effects of antidepressants compared with placebo may be attributable to methodological factors or selective presentation of data from antidepressant trials (Moncrieff and Kirsch,

2005). For this reason, it is advisable conduct further studies using a more universal methods and criteria.

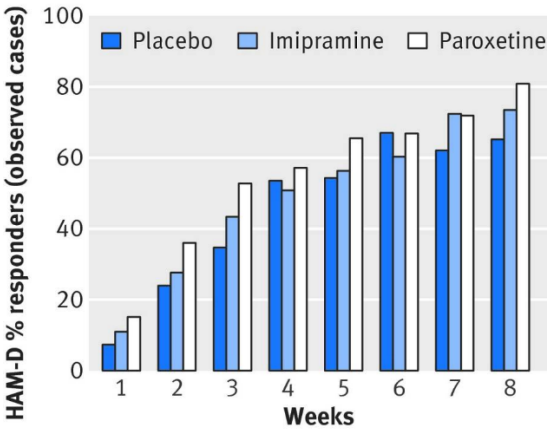

Figure 3. Lack of major differences in the response to placebo, paroxetine or imipramine treatment, measured according to the Hamilton Depression Rating Scale (HAM) in adolescents with major depression. (Le Noury et al., 2015).

1.3.3 Risk of suicides in patient treated with SSRIs

A debate has raged for several years over the supposed association between the treatment with SSRIs and the occurrence of suicidal thoughts and suicide, especially in children and adolescents depressed patients. For instance, the 2004 U.S. Food and Drug Administration (FDA) analysis of clinical trials on children with MDD, found statistically significant increases of the risk of possible suicidal ideation and behavior (80%), and of agitation and hostility (130%) (Hammad et al., 2006).

Although the systematic review conducted by Fergusson and colleagues, did not found a statistically significant increase of fatal suicidal attempts comparing users of SSRIs with users of placebo or other therapeutic interventions, the authors found an almost twofold increase in the odds of fatal and non-fatal suicidal attempts in SSRI treated group (Cipriani et al., 2005; Fergusson et al., 2005).

In addition, the re-analysis of SmithKline Beecham's Study 329, which compared the efficacy, and safety of SSRI paroxetine with placebo in depressed adolescents, showed the significant increases in harms, including suicidal ideation and behavior, in the paroxetine group (Le Noury et al., 2015).

Some studies try to explain this association positing that the ability to react and make decisions may return before the mood improvement. In this phase, it is easier to implement decisions but the depression is still severe and the risk of suicide may increase (Gunnell et al., 2005).

1.4 Serotonin and brain plasticity

Serotonin metabolism has been widely associated with brain plasticity and potentiality for modification. Indeed, serotonin shapes neuronal networks during development and modulates a wide spectrum of essential brain functions ranging from perception and cognitive appraisal to emotional responses in the mature brain (Lesch and Waider, 2012).

1.4.1 "Undirected susceptibility to change" hypothesis

To explain the incomplete efficacy of SSRIs, in the 2011 a novel view of the role SSRI action named the "undirected susceptibility to change" hypothesis (USC) has been proposed (Branchi, 2011). According to such view, the relationship between SSRIs administration, the consequent change in serotonin levels, and the recovery from depression is not a direct one. In particular, increasing serotonin levels *per se* does not univocally lead to recover from MDD, but may even worsen the symptomatology. The reason is that high serotonin levels increase brain plasticity and thus enhance biological sensitivity to context, that is, susceptibility to the quality of the environment. Consequently, an individual treated with SSRI is more affected by environmental stimuli, both favorable and adverse, compared to an untreated individual. SSRI treatment is effective because it enhances individual's reactivity to the environment, allowing, in the case of a favorable environment, to be beneficially affected. This hypothesis claims thus that increasing serotonin levels is not the direct cause of recovery from depression but a permissive factor (Figure 4).

The USC hypothesis has been recently demonstrated at preclinical and clinical levels. In particular, it has been showed in a mouse model of depression that the treatment with fluoxetine, one of the most commonly prescribed SSRIs, affects

the molecular and behavioral depression-like phenotype according to the quality of the living environment: when administered in an enriched environment, it led to an improvement, while, when administered in a stressful environment, to a worsening (Branchi, 2013; Alboni, 2017). At clinical level, the study conducted by the same team confirmed that the effects of the SSRI citalopram are dependent on the environment.

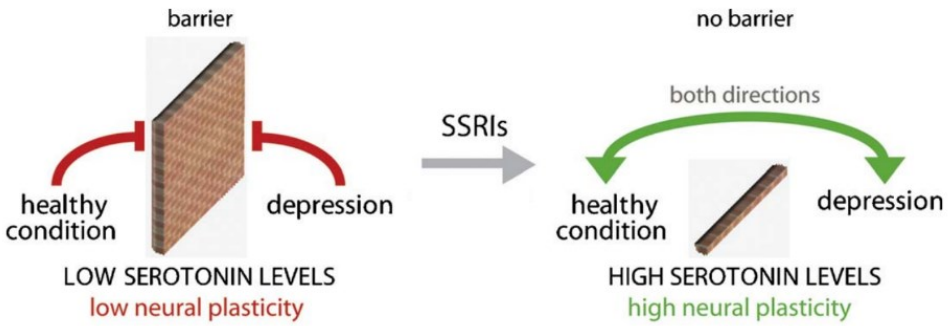

Figure 4. Schematic description of the undirected change susceptibility hypothesis. Serotonin levels determine vulnerability to MDD and capacity to recover from it. Low serotonin levels are associated to reduced neural plasticity. This condition does not allow to shift from an healthy condition to a depression condition (i.e., vulnerability) and vice versa (i.e., capacity to recover). By contrast, when serotonin levels are high, the increased levels of neural plasticity allow to shift from one condition to the other, increasing both vulnerability and capacity to recover. The change in neural plasticity levels can be induced by SSRI administration that increase the extracellular level of serotonin (modified by Branchi, 2011).

1.4.2 Relevance of the environment

The innovative view offered by the USC hypothesis opens new perspectives on how to improve SSRI efficacy by controlling the environment. Indeed, it states that SSRI treatment makes the individual able to change, but does not determine the direction of change. In literature there are several evidences supporting this model. For instance, individuals bearing the s allele of 5-HTTLPR, that leads to higher levels of brain serotonin, if exposed to childhood maltreatment or other stressful life events, show increased neuroticism (Schinka et al., 2004) and incidence of MDD (Caspi et al., 2003). On the contrary, different types of therapy, such as psychological (e.g., cognitive and other) and physical (e.g., light), if associated to a positive environment, exert beneficial effects on depression symptoms and rate of recovery (DeRubeis et

al., 2008; Wirz-Justice et al., 2005). Also in this case, serotonin levels modulate these effects: either cognitive or light therapy when combined with antidepressant administration leads to a greater improvement rate than either treatment alone (Benedetti et al., 1997; Cuijpers et al., 2009; Kaufman et al., 2004; Pampallona et al., 2004; Scott, 2000). Thus, according to the USC model, psychological and drug therapies should not be considered as alternative treatments, but as mutually potentiating tools whose combination is the key factor for the cure of MDD.

1.4.3 USC hypothesis may explains the inconsistent results concerning SSRI efficacy

The USC hypothesis provides a potential explanation of the inconsistent results about the low percentage of depressed patients who benefit from antidepressants and the relatively high percentage who benefit from placebo. Indeed, we can infer that not considering the influence of the environment limits the identification of the conditions permitting beneficial outcome. In addition, mood modifications can occur only because of the environment and in the absence of treatment, though at a slower rate. In particular, the beneficial environment, associated to the expectancy created by placebo, may lead to recovery also without drug administration.

The most serious consequence of depression is suicide. Epidemiological studies have indicated that SSRI treatment, which should reduce the risk of suicide, may cause suicidal feelings, self-harm, and suicide in some susceptible individuals, particularly in adolescents (Barbui et al., 2009; Fergusson et al., 2005; Gunnell et al., 2005; Hall and Lucke, 2006).

2. Inflammation and SSRI action

2.1 Inflammation in the central nervous system

Inflammation consists in a protective immunovascular response to harmful stimuli, such as pathogens, damaged cells, or irritants (Ferrero-Miliani et al., 2007; Medzhitov, 2010). It is the protective attempt by the organism to remove the injurious stimuli and initiate the healing process for the tissue (Chen et al., 2018). A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Inflammation consists in the balance between pro-inflammatory mediators such as cytokines, chemokines and acute phase proteins and anti-inflammatory mediators (Blume et al., 2012; Irwin and Miller, 2007; Zorrilla et al., 2001). If the anti-inflammatory mediators are not able to inhibit the pro- inflammatory ones, for example when the injurious stimuli are persistent, the inflammation could last even for years and become chronic (Kohler et al., 2016).

The central nervous system (CNS) has long been considered an immune-privileged organ largely due to the presence of the blood-brain-barrier (BBB) which protect the brain from been exposed to inflammation and potentially fatal autoimmunity and toxins (Carson et al., 2006). Recently the BBB appeared to be more penetrable than previously assumed. An increasing number of studies showed that several proteins, including cytokines, could pass it and reach the brain (Lorton et al., 2006). The brain releases neurotransmitters and neuropeptides that influence the production and release of hormones from the pituitary to regulate functions such as metabolism, growth and reproduction. For example, the neurotransmitters and neuropeptides released in the lymphoid organs bind to specific receptors on the cells of the immune system and modulate their functions. (ThyagaRajan and Priyanka, 2012). All these evidences, indicating the bidirectional communication between CNS and the periphery, have proven that the isolated view of the CNS is not correct. The CNS' innate immune response involves complex signaling circuitry and cellular networks. The primary cells involved in neuroinflammation are the microglia,

considered the macrophages of the CNS, and the astrocytes, which perform many functions including support of the BBB and maintenance of the extracellular ion balance. These cells regulate both the induction and limitation of neuroinflammatory processes (Carson et al., 2006; Quan and Banks, 2007). Microglia are derived from the yolk sac, and takes up residence in the brain during development, representing up to 12% of the cells of the CNS (Harry and Kraft, 2012). In the mature CNS, microglia are apparently dormant (resting microglia) but are actively monitoring the environment, contributing to the maintenance of neurovascular integrity. They respond to inflammatory stimuli by producing pro-inflammatory cytokines and prostaglandins whose receptors are expressed by both neuronal and non-neuronal brain cells (Galic et al., 2012).

2.2 The role of the immune brain system under quiescent conditions

The immune system is involved not only in processes of surveillance and protection but also in tissue remodelling, which is a continuous process of dynamic alterations in a specific tissue to facilitate morphological and functional adaptations to the changing of the environment (Bajayo et al., 2005; Yirmiya and Bab, 2009). The CNS provides an excellent example of necessity of tissue remodelling to adaptive coping, since neural cells and networks are constantly modified by experience. Indeed, more than 50% of the neurons formed in the brain during development die before birth (Oppenheim, 1991). Moreover, a large percentage of the processes of the developing neurons such as dendrites and axons undergo dynamic and dramatic pruning, leading to the formation of accurate, fine-tuned, and efficiently functioning neural circuits (Luo and O'Leary, 2005). All these processes continue throughout the entire life of the individual, albeit in a less dramatic manner; in particular, dendrites and axons are formed and pruned, synapses and associated structural elements are constantly shaped, retracted and modified, neurons undergo apoptosis and neurogenesis (Luo and O'Leary, 2005). These events, collectively termed neural

plasticity, allow the CNS to adapt to the changes of the environment, and have been shown to be regulated by immune mechanisms. Indeed the remains of cellular corpses and the neuritic debris cannot stay in the tissue because they could interfere with its normal functioning, consequently microglia, astrocytes, and mast cells participate in modulating and sculpting the brain (Yirmiya and Goshen, 2011; Figure 5).

Interestingly, the activation of the immune brain system under quiescent conditions can stimulate two brain-to-body communication pathways, such as the hypothalamus–pituitary–adrenal (HPA) axis and the autonomic nervous system (Besedovsky and Rey, 2007), which in turn influence the central immune responses leading to a feedback loop.

The notion that the immune processes play an important role in normal neural processes is supported by the evidence that cytokines act as neuromodulators in the healthy brain. Indeed, several studies reported that, also under quiescent conditions, neurons produce and respond to inflammatory cytokines and that neuronal activity regulates the production and secretion of cytokines (Vitkovic et al., 2000). An increasing number of studies demonstrated the involvement of cytokines in specific normal neurobehavioral functions such as sleep (Krueger et al., 2001; Opp, 2005) or pain (Wolf et al., 2003).

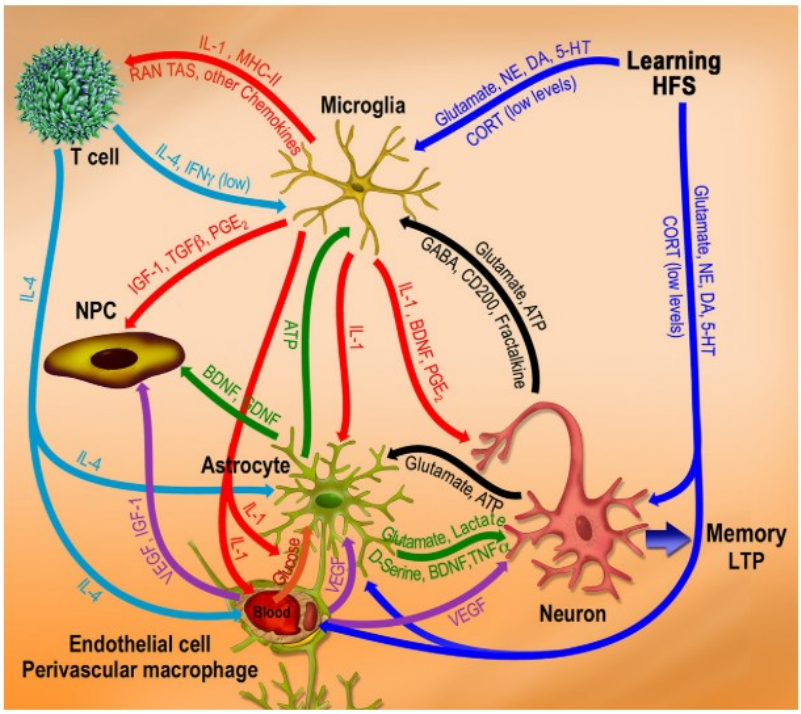

Figure 5. Schematic description of the complex interplay between immune system and brain in regulating neural plasticity and behavioral processes, such as learning and memory, at molecular and cellular level. During learning or high frequency stimulation (HFS) that induces LTP, the external glutamatergic, monoaminergic and adrenocortical input can activate neurons, microglia and astrocytes (blue arrows). Signaling via specific receptors expressed on these glia cells induces the production of various mediators (red arrows). IL-1 can in turn further activate astrocytes, inducing the secretion of several compounds that are critical for memory formation and synaptic plasticity (green arrows). IL-1 has also been shown to facilitate glucose uptake and the production of lactate by astrocytes, which are important for long-term memory consolidation. Microglia and astrocytes also secrete various compounds that directly influence neuronal functioning and neural precursor cells (NPCs). Microglial-derived IL-1 can also directly influence neurons by upregulating NMDA receptor functioning. The production of IL-1 and other glial mediators is tightly regulated by neuronal-derived factors (black arrows). Microglial expression of IL-1, MHC class II and various chemokines can influence T cells, which play an important role in learning and neurogenesis (light blue arrows). Finally, IL-1 can activate endothelial cells, which produce various trophic factors important for memory, neural plasticity and neurogenesis (purple arrows, Yirmiya and Goshen, 2011).

2.3 Inflammation and MDD

Although it is not known whether inflammation causes MDD or vice versa, it has been clearly shown that MDD involves both immune suppression and activation (Blume et al., 2012). The hypothesis mostly corroborated is that the excess of inflammation increases vulnerability to the pathophysiology, supported by, amongst

other things, the increased levels of pro-inflammatory cytokines and acute phase proteins found in depressed patients (Maes et al., 1995; Maes et al., 2009).

Actually, the alterations of cytokines concentrations are associated to several psychiatric disorders. For example, increased levels of Interleukin (IL)-1, IL-6 and Tumor Necrosis Factor (TNF)-α have been reported in bipolar disorder at differing stages of the illness and a recent meta-analysis demonstrated that schizophrenia is associated to increased plasma concentrations of IL-6 and IL-1 (Schmitt et al., 2005).

Focusing on depression, it has long been recognized that there are significant overlaps between sickness behavior, which is the adaptive set of behaviors induced by infection and inflammation, and MDD. Indeed, to combat infection, the body plays out a set of behavioral and affective alterations such as anhedonia, decreased appetite, disturbed sleep, decreased activity and social withdrawal, which are similar to the symptoms present in MDD. The purpose of these behavioral alterations is to cope better with illness or infection. The pro-inflammatory cytokines, produced by the immune system in response to the pathogens and triggering sickness behavior, are IL-1, IL-6 and TNF-α.

Therefore it has been proposed that depression is an evolutionary psychological byproduct of early mechanisms that promoted diversion of energy sources to fight back the infection; in other words depressive symptoms could be the maladaptation of sickness behavior as a result of chronic immune activation and consequential inflammation (Raison and Miller, 2011).

2.3.1 High inflammatory levels in depressed patients

The notion that MDD is associated with raised inflammatory markers was supported by numerous studies reporting the significantly higher concentrations of pro-inflammatory cytokines such as TNF-α, C-reactive protein (CRP) and IL-6 in depressed patients compared with non-depressed patients (Liu et al., 2012). It is worth to note that the increase occurs even in the absence of physical illness or infection and therefore it could be accounted for by depression (Irwin and Miller, 2007).

In a meta-analysis of 22 cross-sectional studies performed by Liu and his colleagues, the concentration of soluble IL-2 receptor, TNF-α, and IL-6 in depressed patients were significantly higher than those of healthy subjects (Liu et al., 2012). This association is supported also by another meta-analytic review indicating significant increased circulating IL-6 levels in MDD. In addition, it has been observed that treatment with pro-inflammatory cytokines can induce MDD in up to 70% of cases (Maes et al., 2009). Other inflammatory markers has been associated to the onset and maintenance of MDD, include acute phase proteins such as CRP, haptoglobin and neopterin. This association is so strong that in the Consensus paper of the WFSBP Task Force on Biological Markers published in the 2017 it has been proposed to consider plasma IL-6 and soluble IL-2 –receptor as biomarker of depression (Schmitt et al., 2017).

The above results indicate an association between inflammatory markers and MDD but not the causal relationship (Dowlati et al., 2010; Liu et al., 2012) and it has not been found yet a correlation between inflammatory levels and the severity of depressive symptoms (Schlatter et al., 2004). Moreover, some studies have found incongruent or even opposite correlations for pro-inflammatory markers and the pathogenesis of depression (Levine et al., 1999; Schlatter et al., 2004; Steptoe et al., 2007).

Interestingly, an increasing number of studies show that the association between inflammatory levels and MDD has been attenuated when the analyses include factors such as body mass index, personality or even gender (Bouhuys et al., 2004).

2.3.2 Association of pro- and anti-inflammatory treatment with antidepressants

A number of pre-clinical studies demonstrated that the administration of cytokines induces a set of behaviors associated to the depressive phenotype. In particular, mice treated with TNF-α shown a depressive-like behavior that is attenuated by administration of antidepressants. Similarity, also in non-human primates, the administration of a large number of pro-inflammatory cytokines lead to the observation of classical depressive-like behaviors.

Precious data derive from clinical studies concerning the immunotherapy. Immunotherapy consists in the systemic injection of cytokines including INF-α and IL-2 to fight diseases such as hepatitis C and cancer. This kind of therapy induces a strong increase of pro-inflammatory cytokines, especially INF-α and IL-6, which are associated with the onset and maintenance of depressive symptoms. INF-α-induced depression is associated with changes in the metabolism of the serotonin and the alterations of HPA axis (Capuron and Miller, 2011).

A study focusing on patients with hepatitis C treated with interferon (IFN) revealed that 33% of patients developed IFN-α-induced MDD, and that the 85% of these individuals were responsive to antidepressant treatment (Madeeh Hashmi et al., 2013). A different study found that patient receiving IFN-α therapy showed significantly increased scores for MDD during the treatment period compared with the untreated reference group, and that the pre-treatment with the antidepressant Paroxetine was effective to counteract the IFN-α- induced-depression (Kraus et al., 2002). Again, all these evidences show a correlation between MDD, antidepressant treatment and pro-inflammatory compounds administration but they cannot demonstrate the casual relationship between these elements.

There is now a large body of literature proving the antidepressant effects of agents with anti-inflammatory activity. For example, Kohler and colleagues, combining data from 14 randomized clinical trials, reported that depressed patients treated with nonsteroidal anti-inflammatory drugs (NSAIDs) or cytokine inhibitors as primary treatment modalities (Kohler et al., 2014), showed significant reduced

depressive symptoms compare to the placebo control group. The treatment with antagonists of TNF-α, such as Etanercept or Infliximab, for patient with autoimmune disorders has been shown to decrease the depressive symptoms without the combination with antidepressants drugs (Grattendick et al., 2008; Tyring et al., 2006; Wichers et al., 2006). Differently, in the review of Rosenblat and colleagues (Rosenblat et al., 2014), which examined the effect of treatment with NSAIDs, omega-3 fatty acids, n-acetylcysteine, or pioglitazone as adjuncts in the treatment of MDD and bipolar disorder, the results showed that the anti-inflammatory agents reduced the depressive symptoms with an effect size of -0.40 (95% CI -0.65 to -0.14).

Other studies have reported increased efficacy of several antidepressant treatments with the addition of anti-inflammatory agents, for example, adding the NSAID celecoxib to sertraline or fluoxetine and reboxetine, leads to a greater reduction of inflammatory markers and severity of depressive symptoms than either of the antidepressant alone. The combination of fluoxetine and acetylsalicylic acid has been shown to increase the remission rates of MDD probably accelerating the pharmacological action of the SSRI (Brunello et al., 2006).

2.3.3 Antidepressant treatment affects inflammatory levels

The interaction between inflammation and antidepressant drugs is increasingly being recognized. The treatment with antidepressants has been shown to reverse the disequilibrium of the inflammatory markers (Hepgul et al., 2016), in particular fluoxetine treatment can significantly reduce the peripheral concentration of IL-6 in depressed patients (Hannestad et al., 2011). In the same way, antidepressant drugs such as imipramine, clomipramine, venlafaxine, fluoxetine, sertraline and trazodone have been shown to reduce the pro-/anti- inflammatory ratio in vitro human blood samples, linked to an anti-inflammatory effect (Kopschina Feltes et al., 2017). An adjunctive proof is that non-responders patients to SSRIs continue to exhibit raised IL-6 levels, suggesting that response to treatment is linked to a reduction of IL-6 (Kohler et al., 2015).

The administration of tricyclic antidepressants including clomipramine and imipramine significantly decreased the production of nitric oxide and TNF-α in microglia and astrocyte cultures (Wang et al., 2017). This effect was present also at the transcriptomic level, since the same treatment produced the reduction of the mRNA coding these pro-inflammatory cytokines.

Finally, in a meta-analysis comprising clinical trials with depressed patients, the levels of various cytokines, such as IL-1 and IL-6, were significantly reduced by the efficient treatment with SSRIs suggesting that this class of antidepressant, inhibiting the effect of inflammatory cytokines, can relieve depressive symptoms (Hannestad et al., 2011).

3. Current state of the art and purpose of the studies

3.1. Current state of the art and statement of the problem

MDD is an enormous medical, societal and economical challenge. SSRIs, the most prescribed antidepressants, have variable and incomplete efficacy, thus a new and effective pharmacological approach is warranted. The USC hypothesis, positing that SSRI treatment does not affect mood *per se,* but amplifies the influence of the living environment on treatment outcome, may reconcile the apparent inconsistency of the findings reported so far on the SSRI efficacy (Branchi, 2011).

In the study published on Molecular Psychiatry in 2017, we demonstrated the USC hypothesis in a mouse model of depression, showing that the administration of fluoxetine in an enriched condition led to a significant recovery from the depression-like phenotype compared with vehicle, while under stressful condition it led to a worsening of the depression-like phenotype (Table 1, Alboni et al., 2017). Although few clinical studies have attempted to investigate the influence of the environment on antidepressant action, the finding currently available show that living conditions, such as socioeconomic status and lifestyle, modulate the effects of these antidepressants. In particular, these studies suggest that SSRIs are more effective in those individuals who experience a better quality of life (Alboni et al., 2017).

Table 1. Effects of fluoxetine, compared with vehicle, in the enriched and the stressful conditions

	Reduction in liking-type anhedonia	*Reduction in wanting-type anhedonia*	*Cognitive bias*	*Neurogenesis (Ki67)*	*ERK signaling*	*CREB signaling*	*BDNF levels*	*Reduction in CORT levels*	*LTP*
Enriched condition	–	↑	↑	–	–	–	↑	↑	–
Stressful condition	↓	↓	–	↓	↓	↓	–	–	↓

Table 1. The effects of the SSRI fluoxetine are highly dependent on the quality of the living environment. Fluoxetine administration in an enriched condition leads to a significant recovery from the depression-like phenotype compared with vehicle, while fluoxetine treatment under stress led to a worsening of the depression-like phenotype. Such environment-dependent effect is pervasive, as it concerns most of the phenotypic features analysed, from molecular pathways to behavior (Alboni et al., 2017).

To validate the USC hypothesis also at clinical level, Branchi and colleagues exploited the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) data set, which is a study enrolled 4.041 outpatients diagnosed with MDD representing a broad range of ethnic and socioeconomic groups (Trivedi et al., 2006). They found that the sociodemographic characteristics affect citalopram treatment response and that this effect is greater in the patients treated with the higher dose of antidepressant. Therefore, these results confirm that serotoninergic antidepressant treatment amplifies the influence of the living conditions on mood also in depressed patients (Figure 6).

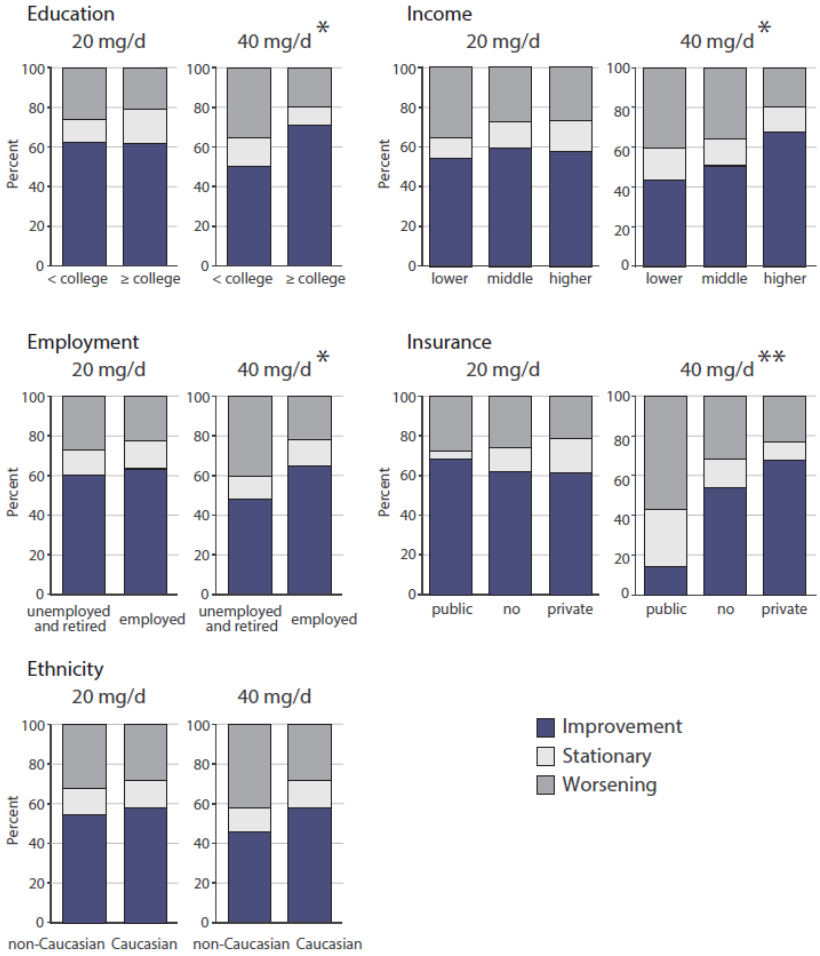

Figure 6. The high dose of SSRI citalopram, compared to the lower, leads to an increase of the percent of improvements in patients living in a favorable environment but also of the percent of worsening in patients living in an unfavorable environment. Percent of

patients showing improvement (i.e., reduction ≤1 in QIDS-SR16 score), percent of stationary patients (i.e. no change in QIDS-SR16 score) and percent of patients showing a worsening (i.e., increase ≥1 in QIDS-SR16 score) were measured between week 4 and 6, according to the dose regimen - either 20 or 40 mg/d dose. Only patients receiving the 40 mg/d dose showed a variation in these percent significantly moderated by the sociodemographic characteristics. Chi-square *p<0.05 and ** p<0.01 (Chiarotti, 2017).

Inflammation is one of the most recent and promising target of therapeutic strategies for psychiatric disorders, such as MDD (Dinan, 2009; Miller and Raison, 2016; Otte et al., 2016). There is an increasing body of literature reporting that inflammatory levels are strongly affected by the quality of the living environment. In turn, immune response affects both vulnerability to MDD and antidepressant efficacy, in particular, up to 40% of depressed patients exhibit increased inflammation, including increased levels of pro-inflammatory cytokines such as, IL-1β, IL-6, TNF-α (Miller et al., 2009; Miller and Raison, 2016; Raison et al., 2013). It has been reported that the activation of inflammatory pathways is associated with a lack of response to antidepressants (Carvalho et al., 2013; Cattaneo et al., 2016; Cattaneo et al., 2013; Eller et al., 2008; Tuglu et al., 2003; Vogelzangs et al., 2014). Furthermore, in 2011 was published and interesting study showing that the administration of anti-inflammatory compounds, such as nonsteroidal anti-inflammatory drugs (NSAIDs), antagonized the behavioral response to SSRI treatment. In particular, the authors demonstrated that depressed patients chronically treated with the SSRI citalopram are significantly less likely to show full remission if they concomitantly take NSAIDs and/or other analgesics (Warner-Schmidt et al., 2011; Figure 7). Basing on all these findings, the control of the inflammatory response in depressed patients may represent a potential approach to modulate the influence of the living environment on SSRI treatment outcome.

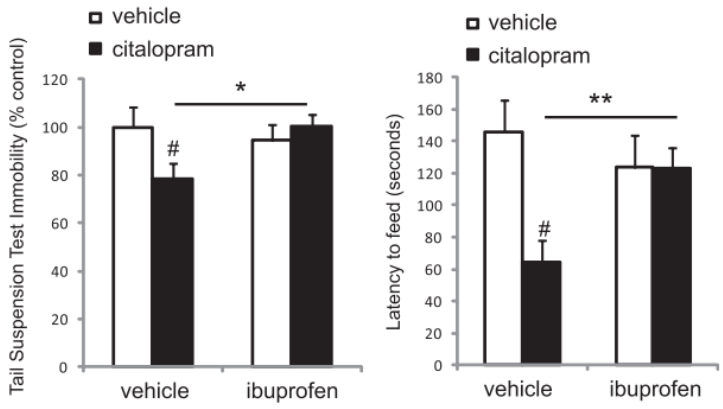

Figure 7. Effects of antidepressants and NSAIDs on behavioral responses. There was no effect of chronic citalopram treatment when ibuprofen was co-administered before testing in the tail suspension and the novelty suppressed feeding test. All data are presented as means ± SEM. Statistically significant effects of antidepressants (#$p < 0.05$) or NSAIDs/analgesics (*$p < 0.05$, **$p < 0.01$) are noted (Warner-Schmidt et al., 2011).

As mentioned before, the view offered by the USC hypothesis leads to new perspectives on how to improve SSRI efficacy through the control the living conditions. However, often it is not possible to act on the quality of the living environment since the patient's life circumstance and his personal history are unchangeable. In these cases, it is advisable modulate, with a pharmacological approach, the factors underlying the link between living environment and SSRI efficacy. In this way, it could be possible to improve the antidepressant outcome even in patients living in non-supportive conditions, which are very common in depressed patients. A large body of literature reports that the quality of the living condition is strongly associated also to the metabolism. For instance, it is well known that having an active physical and social life profoundly modulates the metabolic markers, including hormones and adipokine levels (Corpeleijn et al., 2007; Hafner et al., 2011). On the other hand, the neuroendocrine pathway, which consist in the brain-adipocyte axis, is reportedly involved in the vulnerability to MDD and in antidepressants efficacy. For example, it has been recently shown that metabolic syndrome is associated with MDD and, more in particular, that obesity and metabolic syndrome are involved in the vulnerability of MDD (Anderson et al., 2001; Luppino et al., 2010). In a recent work, it has been demonstrated that the remission from MDD with

antidepressant treatment is associated to the normalization of metabolic markers, while non-remitters patients do not show changes in adipokine levels (Weber-Hamann et al., 2007). Therefore, the modification of metabolism represents a potential approach to modulate the interplay between environment and SSRIs in order to improve antidepressant treatment outcome.

3.2. Purpose of the studies

The general aim of the following studies is to identify the factors that determine the serotoninergic treatment outcome in depressed patients, to improve the SSRI treatment efficacy. According to the USC hypothesis, SSRI outcome are not determined by the drug *per se* but induced by the drugs and driven by different intervening factors. The consequence of such hypothesis is that, controlling the living conditions experienced by the patients treated with SSRIs, it is possible increase the efficacy of the therapy. Therefore it is crucial to find the factors that mediate the influence of the environment on SSRI action and, to this purpose, we focused on neural plasticity and inflammation since they have been widely reported act as key factors in determining SSRI outcome. Indeed microglia and inflammatory system are increasingly emerging as effectors translating the influence of the living environment into changes in brain function. To explore the interplay between neural plasticity and inflammation, first we assessed how the increase in neural plasticity, associated with the fluoxetine treatment, affects the inflammatory levels. Then, we modulate the immune system with two different pharmacological agents, which induced either immune activation or suppression, and we evaluated their effects on neural plasticity at both molecular and cellular level.

As further step, we investigated the effects of the combined administration of fluoxetine and metformin, a drug able to improve metabolic profile, in a mouse model of depression. Our aim was to verify whether the pharmacological intervention on metabolism, which is reported to mimic the quality of the living condition on mood, counteracts the demonstrated limited efficacy of fluoxetine alone, when administered

in stressful conditions. In particular, we wanted identify the metabolic targets in the brain-adipocyte axis that lead to a beneficial effect on depressive symptoms, even when the patient is treated in non-supportive conditions.

3.3. Experimental approach and outline of the thesis

In the **Study 1**, to assess whether the increased neural plasticity induced by SSRI controls inflammation, we chronically administered the SSRI fluoxetine in mice with high levels of inflammation, associated with exposure to a stressful environment, and in mice with low levels of inflammation, associated with exposure to an enriched environment. We measured the expression levels of the principal pro- and anti-inflammatory cytokines in the hippocampus and in the hippocampal isolated microglia. Moreover, we investigate the surveillance state of the microglia determining their density, distribution and morphology

The results we obtained showed that the increased neural plasticity, induced by fluoxetine, leads to opposite effects on inflammation, according to the starting inflammatory conditions of the animals. In particular, when the inflammatory levels were low, as in the enriched environment, neural plasticity increases them, while when the inflammatory levels were high, as in the stressful environment, plasticity decreases them. This suggests that enhanced neural plasticity is associated with an intermediate range of inflammation strictly controlled and that SSRI effects depends on this control.

In the **Study 2**, we investigated the interplay between neural plasticity and inflammation, assessing the level of plasticity following an immune activation or suppression. Indeed it is well known that neural plasticity is influenced by several factors including inflammation (Yirmiya and Goshen, 2011) and we hypothesized that neural plasticity vary in function of inflammatory activity in an inverted U-shape relation. To investigate such interaction, we acutely treated adult male mice with different doses of ibuprofen or lipopolysaccharide (LPS), which respectively decrease and increase inflammatory levels, and we assessed their neural plasticity

both at molecular and cellular level. In particular, we performed functional measures in the hippocampus recording the long-term potentiation (LTP) at Schaffer collateral-CA1 synapses since it is considered a cellular substrate of brain plasticity and we analyzed the hippocampal levels of BDNF, the neurotrophic factor most involved in plasticity process. Our findings showed that the mice treated with the higher doses of ibuprofen and LPS display significant reduced plasticity compared to the mice treated with the lower doses and with the saline. These results demonstrated that the two extreme inflammatory condition lead to an impairment of the brain function, suggesting that the neural plasticity is possible only within a strict range of inflammatory levels and that any deviations from such range results in reduced plasticity.

With the **Study 3,** we explored whether the combined administration of fluoxetine and metformin, an agent drug able to improve metabolic profile, improve the limited efficacy of fluoxetine alone in a mouse model of depression. Indeed metabolism could be an important target to modulate the influence of the environment on the individual and we decide to explore such polypharmacological approach to test whether a multi-target treatment could enhance the already proved limited effectiveness of the treatment with fluoxetine alone when administered in stressful conditions. Adult C57BL/6 male mice were exposed to stressful condition for 6 weeks, the first two aimed at inducing a depression-like phenotype. During the remaining 4 weeks, mice received vehicle, fluoxetine, metformin or a combination of fluoxetine and metformin. We measured behavioral phenotypes of depression and we assessed the expression levels of several genes involved in MDD and antidepressant response in the dorsal and ventral hippocampus, reported to be differently involved in this pathology. Our results showed that the combined treatment was more effective than fluoxetine alone in improving the depressive symptoms, suggesting that this polypharmacological approach could be more effective to treat MDD compared to the currently antidepressant therapy, especially when patients experience stressful living conditions.

References

Alarcon, J.M., Malleret, G., Touzani, K., Vronskaya, S., Ishii, S., Kandel, E.R., Barco, A., 2004. Chromatin acetylation, memory, and LTP are impaired in CBP+/- mice: a model for the cognitive deficit in Rubinstein-Taybi syndrome and its amelioration. Neuron 42, 947-959.

Alboni, S., van Dijk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I., Cirulli, F., Maggi, L., Branchi, I., 2017. Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Molecular psychiatry 22, 552-561.

Anderson, R.J., Freedland, K.E., Clouse, R.E., Lustman, P.J., 2001. The prevalence of comorbid depression in adults with diabetes: a meta-analysis. Diabetes care 24, 1069-1078.

APA, 2013. Diagnostic and Statistical Manual of Mental Disorders, Washington DC.

Bajayo, A., Goshen, I., Feldman, S., Csernus, V., Iverfeldt, K., Shohami, E., Yirmiya, R., Bab, I., 2005. Central IL-1 receptor signaling regulates bone growth and mass. Proceedings of the National Academy of Sciences of the United States of America 102, 12956-12961.

Balak, N., Elmaci, I., 2007. Costs of disorders of the brain in Europe. Eur J Neurol 14, e9.

Barbui, C., Esposito, E., Cipriani, A., 2009. Selective serotonin reuptake inhibitors and risk of suicide: a systematic review of observational studies. Cmaj 180, 291-297.

Beasley, C.M., Jr., Sayler, M.E., Potvin, J.H., 1993. Fluoxetine versus amitriptyline in the treatment of major depression: a multicenter trial. International clinical psychopharmacology 8, 143-149.

Belmaker, R.H., Agam, G., 2008. Major depressive disorder. The New England journal of medicine 358, 55-68.

Benedetti, F., Barbini, B., Lucca, A., Campori, E., Colombo, C., Smeraldi, E., 1997. Sleep deprivation hastens the antidepressant action of fluoxetine. European archives of psychiatry and clinical neuroscience 247, 100-103.

Besedovsky, H.O., Rey, A.D., 2007. Physiology of psychoneuroimmunology: a personal view. Brain, behavior, and immunity 21, 34-44.

Blume, K.E., Soeroes, S., Keppeler, H., Stevanovic, S., Kretschmer, D., Rautenberg, M., Wesselborg, S., Lauber, K., 2012. Cleavage of annexin A1 by ADAM10 during secondary necrosis generates a monocytic "find-me" signal. Journal of immunology 188, 135-145.

Bouhuys, A.L., Flentge, F., Oldehinkel, A.J., van den Berg, M.D., 2004. Potential psychosocial mechanisms linking depression to immune function in elderly subjects. Psychiatry research 127, 237-245.

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., 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.

Capuron, L., Miller, A.H., 2011. Immune system to brain signaling: neuropsychopharmacological implications. Pharmacology & therapeutics 130, 226-238.

Carson, M.J., Doose, J.M., Melchior, B., Schmid, C.D., Ploix, C.C., 2006. CNS immune privilege: hiding in plain sight. Immunological reviews 213, 48-65.

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.

Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A., Poulton, R., 2003. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science (New York, N.Y 301, 386-389.

Cattaneo, A., Ferrari, C., Uher, R., Bocchio-Chiavetto, L., Riva, M.A., Consortium, M.R.C.I., Pariante, C.M., 2016. Absolute Measurements of Macrophage Migration Inhibitory Factor and Interleukin-1-beta mRNA Levels Accurately Predict Treatment Response in Depressed Patients. The international journal of neuropsychopharmacology 19.

Cattaneo, A., Gennarelli, M., Uher, R., Breen, G., Farmer, A., Aitchison, K.J., Craig, I.W., Anacker, C., Zunsztain, P.A., McGuffin, P., Pariante, C.M., 2013. Candidate genes expression profile associated with antidepressants response in the GENDEP study: differentiating between baseline 'predictors' and longitudinal 'targets'. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 38, 377-385.

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., Zhao, L., 2018. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget 9, 7204-7218.

Chiarotti, F., Viglione, A., Giuliani, A., Branchi, I., 2017. Citalopram amplifies the influence of living conditions on mood in depressed patients enrolled in the STAR*D study. Translational psychiatry 7, e1066.

Cipriani, A., Brambilla, P., Furukawa, T., Geddes, J., Gregis, M., Hotopf, M., Malvini, L., Barbui, C., 2005. Fluoxetine versus other types of pharmacotherapy for depression. The Cochrane database of systematic reviews, CD004185.

Corpeleijn, E., Feskens, E.J., Jansen, E.H., Mensink, M., Saris, W.H., Blaak, E.E., 2007. Lifestyle intervention and adipokine levels in subjects at high risk for type 2 diabetes: the Study on Lifestyle intervention and Impaired glucose tolerance Maastricht (SLIM). Diabetes care 30, 3125-3127.

Cuijpers, P., Dekker, J., Hollon, S.D., Andersson, G., 2009. Adding psychotherapy to pharmacotherapy in the treatment of depressive disorders in adults: a meta-analysis. The Journal of clinical psychiatry 70, 1219-1229.

Cuijpers, P., Smit, F., 2002. Excess mortality in depression: a meta-analysis of community studies. Journal of affective disorders 72, 227-236.

DeRubeis, R.J., Siegle, G.J., Hollon, S.D., 2008. Cognitive therapy versus medication for depression: treatment outcomes and neural mechanisms. Nature reviews 9, 788-796.

Dinan, T.G., 2009. Inflammatory markers in depression. Current opinion in psychiatry 22, 32-36.

Doogan, D.P., Caillard, V., 1992. Sertraline in the prevention of depression. Br J Psychiatry 160, 217-222.

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E.K., Lanctot, K.L., 2010. A meta-analysis of cytokines in major depression. Biological psychiatry 67, 446-457.

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.

Fava, G.A., 2002. Long-term treatment with antidepressant drugs: the spectacular achievements of propaganda. Psychotherapy and psychosomatics 71, 127-132.

Fergusson, D., Doucette, S., Glass, K.C., Shapiro, S., Healy, D., Hebert, P., Hutton, B., 2005. Association between suicide attempts and selective serotonin reuptake inhibitors: systematic review of randomised controlled trials. Bmj 330, 396.

Ferrero-Miliani, L., Nielsen, O.H., Andersen, P.S., Girardin, S.E., 2007. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clinical and experimental immunology 147, 227-235.

Fournier, J.C., DeRubeis, R.J., Hollon, S.D., Dimidjian, S., Amsterdam, J.D., Shelton, R.C., Fawcett, J., 2005. Antidepressant drug effects and depression severity: a patient-level meta-analysis. Jama 303, 47-53.

Galic, M.A., Riazi, K., Pittman, Q.J., 2012. Cytokines and brain excitability. Frontiers in neuroendocrinology 33, 116-125.

Grattendick, K.J., Nakashima, J.M., Feng, L., Giri, S.N., Margolin, S.B., 2008. Effects of three anti-TNF-alpha drugs: etanercept, infliximab and pirfenidone on release of TNF-alpha in medium and TNF-alpha associated with the cell in vitro. International immunopharmacology 8, 679-687.

Gunnell, D., Saperia, J., Ashby, D., 2005. Selective serotonin reuptake inhibitors (SSRIs) and suicide in adults: meta-analysis of drug company data from placebo controlled, randomised controlled trials submitted to the MHRA's safety review. Bmj 330, 385.

Hafner, S., Zierer, A., Emeny, R.T., Thorand, B., Herder, C., Koenig, W., Rupprecht, R., Ladwig, K.H., Investigators, K.S., 2011. Social isolation and depressed mood are associated with elevated serum leptin levels in men but not in women. Psychoneuroendocrinology 36, 200-209.

Hall, W.D., Lucke, J., 2006. How have the selective serotonin reuptake inhibitor antidepressants affected suicide mortality? The Australian and New Zealand journal of psychiatry 40, 941-950.

Hammad, T.A., Laughren, T., Racoosin, J., 2006. Suicidality in pediatric patients treated with antidepressant drugs. Archives of general psychiatry 63, 332-339.

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.

Harry, G.J., Kraft, A.D., 2012. Microglia in the developing brain: a potential target with lifetime effects. Neurotoxicology 33, 191-206.

Heninger, G.R., Delgado, P.L., Charney, D.S., 1996. The revised monoamine theory of depression: a modulatory role for monoamines, based on new findings from monoamine depletion experiments in humans. Pharmacopsychiatry 29, 2-11.

Hepgul, N., Cattaneo, A., Agarwal, K., Baraldi, S., Borsini, A., Bufalino, C., Forton, D.M., Mondelli, V., Nikkheslat, N., Lopizzo, N., Riva, M.A., Russell, A., Hotopf, M., Pariante,

C.M., 2016. Transcriptomics in Interferon-alpha-Treated Patients Identifies Inflammation-, Neuroplasticity- and Oxidative Stress-Related Signatures as Predictors and Correlates of Depression. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 41, 2502-2511.

Hiemke, C., Hartter, S., 2000. Pharmacokinetics of selective serotonin reuptake inhibitors. Pharmacology & therapeutics 85, 11-28.

Irwin, M.R., Miller, A.H., 2007. Depressive disorders and immunity: 20 years of progress and discovery. Brain, behavior, and immunity 21, 374-383.

Janicak, P.G., Keck, P.E., Jr., Davis, J.M., Kasckow, J.W., Tugrul, K., Dowd, S.M., Strong, J., Sharma, R.P., Strakowski, S.M., 2001. A double-blind, randomized, prospective evaluation of the efficacy and safety of risperidone versus haloperidol in the treatment of schizoaffective disorder. Journal of clinical psychopharmacology 21, 360-368.

Kato, M., Serretti, A., 2010. Review and meta-analysis of antidepressant pharmacogenetic findings in major depressive disorder. Molecular psychiatry 15, 473-500.

Kaufman, J., Yang, B.Z., Douglas-Palumberi, H., Houshyar, S., Lipschitz, D., Krystal, J.H., Gelernter, J., 2004. Social supports and serotonin transporter gene moderate depression in maltreated children. Proceedings of the National Academy of Sciences of the United States of America 101, 17316-17321.

Kendler, K.S., Karkowski, L.M., Prescott, C.A., 1998. Stressful life events and major depression: risk period, long-term contextual threat, and diagnostic specificity. The Journal of nervous and mental disease 186, 661-669.

Kessler, R.C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K.R., Rush, A.J., Walters, E.E., Wang, P.S., 2003. The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). Jama 289, 3095-3105.

Kirsch, I., Deacon, B.J., Huedo-Medina, T.B., Scoboria, A., Moore, T.J., Johnson, B.T., 2008. Initial severity and antidepressant benefits: a meta-analysis of data submitted to the Food and Drug Administration. PLoS medicine 5, e45.

Kohler, O., Benros, M.E., Nordentoft, M., Farkouh, M.E., Iyengar, R.L., Mors, O., Krogh, J., 2014. Effect of anti-inflammatory treatment on depression, depressive symptoms, and adverse effects: a systematic review and meta-analysis of randomized clinical trials. JAMA psychiatry 71, 1381-1391.

Kohler, O., Krogh, J., Mors, O., Benros, M.E., 2016. Inflammation in Depression and the Potential for Anti-Inflammatory Treatment. Current neuropharmacology 14, 732-742.

Kohler, O., Petersen, L., Mors, O., Gasse, C., 2015. Inflammation and depression: combined use of selective serotonin reuptake inhibitors and NSAIDs or paracetamol and psychiatric outcomes. Brain and behavior 5, e00338.

Kopschina Feltes, P., Doorduin, J., Klein, H.C., Juarez-Orozco, L.E., Dierckx, R.A., Moriguchi-Jeckel, C.M., de Vries, E.F., 2017. Anti-inflammatory treatment for major depressive disorder: implications for patients with an elevated immune profile and non-responders to standard antidepressant therapy. Journal of psychopharmacology 31, 1149-1165.

Kraus, M.R., Schafer, A., Faller, H., Csef, H., Scheurlen, M., 2002. Paroxetine for the treatment of interferon-alpha-induced depression in chronic hepatitis C. Alimentary pharmacology & therapeutics 16, 1091-1099.

Krishnan, V., Nestler, E.J., 2008. The molecular neurobiology of depression. Nature 455, 894-902.

Krueger, M., Nauck, M.S., Sang, S., Hentschel, R., Wieland, H., Berner, R., 2001. Cord blood levels of interleukin-6 and interleukin-8 for the immediate diagnosis of early-onset infection in premature infants. Biology of the neonate 80, 118-123.

Kruijshaar, M.E., Barendregt, J., Vos, T., de Graaf, R., Spijker, J., Andrews, G., 2005. Lifetime prevalence estimates of major depression: an indirect estimation method and a quantification of recall bias. European journal of epidemiology 20, 103-111.

Kuehner, C., 2003. Gender differences in unipolar depression: an update of epidemiological findings and possible explanations. Acta psychiatrica Scandinavica 108, 163-174.

Lambert, G., Johansson, M., Agren, H., Friberg, P., 2000. Reduced brain norepinephrine and dopamine release in treatment-refractory depressive illness: evidence in support of the catecholamine hypothesis of mood disorders. Archives of general psychiatry 57, 787-793.

Le Noury, J., Nardo, J.M., Healy, D., Jureidini, J., Raven, M., Tufanaru, C., Abi-Jaoude, E., 2015. Restoring Study 329: efficacy and harms of paroxetine and imipramine in treatment of major depression in adolescence. Bmj 351, h4320.

Lesch, K.P., 2007. Linking emotion to the social brain. The role of the serotonin transporter in human social behaviour. EMBO reports 8 Spec No, S24-29.

Lesch, K.P., Waider, J., 2012. Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. Neuron 76, 175-191.

Levine, J., Barak, Y., Chengappa, K.N., Rapoport, A., Rebey, M., Barak, V., 1999. Cerebrospinal cytokine levels in patients with acute depression. Neuropsychobiology 40, 171-176.

Liu, Y., Ho, R.C., Mak, A., 2012. Interleukin (IL)-6, tumour necrosis factor alpha (TNF-alpha) and soluble interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: a meta-analysis and meta-regression. Journal of affective disorders 139, 230-239.

Lorton, D., Lubahn, C.L., Estus, C., Millar, B.A., Carter, J.L., Wood, C.A., Bellinger, D.L., 2006. Bidirectional communication between the brain and the immune system: implications for physiological sleep and disorders with disrupted sleep. Neuroimmunomodulation 13, 357-374.

Luo, L., O'Leary, D.D., 2005. Axon retraction and degeneration in development and disease. Annual review of neuroscience 28, 127-156.

Luppino, F.S., de Wit, L.M., Bouvy, P.F., Stijnen, T., Cuijpers, P., Penninx, B.W., Zitman, F.G., 2010. Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. Archives of general psychiatry 67, 220-229.

Madeeh Hashmi A, Awais Aftab M, Mazhar N, Umair M, Butt Z. The fiery landscape of depression: A review of the inflammatory hypothesis. Pak J Med Sci. 2013;29(3):877-84.

Maes, M., Smith, R., Scharpe, S., 1995. The monocyte-T-lymphocyte hypothesis of major depression. Psychoneuroendocrinology 20, 111-116.

Maes, M., Yirmyia, R., Noraberg, J., Brene, S., Hibbeln, J., Perini, G., Kubera, M., Bob, P., Lerer, B., Maj, M., 2009. The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression. Metabolic brain disease 24, 27-53.

Marquet, R.L., Bartelds, A.I., Kerkhof, A.J., Schellevis, F.G., van der Zee, J., 2005. The epidemiology of suicide and attempted suicide in Dutch General Practice 1983-2003. BMC family practice 6, 45.

Medzhitov, R., 2010. Inflammation 2010: new adventures of an old flame. Cell 140, 771-776.

Meyer, J.H., Ginovart, N., Boovariwala, A., Sagrati, S., Hussey, D., Garcia, A., Young, T., Praschak-Rieder, N., Wilson, A.A., Houle, S., 2006. Elevated monoamine oxidase a levels in the brain: an explanation for the monoamine imbalance of major depression. Archives of general psychiatry 63, 1209-1216.

Miller, A.H., Maletic, V., Raison, C.L., 2009. Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biological psychiatry 65, 732-741.

Miller, A.H., Raison, C.L., 2016. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nature reviews. Immunology 16, 22-34.

Moncrieff, J., Kirsch, I., 2005. Efficacy of antidepressants in adults. Bmj 331, 155-157.

Mueller, T.I., Leon, A.C., Keller, M.B., Solomon, D.A., Endicott, J., Coryell, W., Warshaw, M., Maser, J.D., 1999. Recurrence after recovery from major depressive disorder during 15 years of observational follow-up. The American journal of psychiatry 156, 1000-1006.

Opp, M.R., 2005. Cytokines and sleep. Sleep medicine reviews 9, 355-364.

Oppenheim, R.W., 1991. Cell death during development of the nervous system. Annual review of neuroscience 14, 453-501.

Otte, C., Gold, S.M., Penninx, B.W., Pariante, C.M., Etkin, A., Fava, M., Mohr, D.C., Schatzberg, A.F., 2016. Major depressive disorder. Nature reviews. Disease primers 2, 16065.

Pampallona, S., Bollini, P., Tibaldi, G., Kupelnick, B., Munizza, C., 2004. Combined pharmacotherapy and psychological treatment for depression: a systematic review. Archives of general psychiatry 61, 714-719.

Quan, N., Banks, W.A., 2007. Brain-immune communication pathways. Brain, behavior, and immunity 21, 727-735.

Raison, C.L., Miller, A.H., 2011. Is depression an inflammatory disorder? Current psychiatry reports 13, 467-475.

Raison, C.L., Rutherford, R.E., Woolwine, B.J., Shuo, C., Schettler, P., Drake, D.F., Haroon, E., Miller, A.H., 2013. A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. JAMA psychiatry 70, 31-41.

Rosenblat, J.D., Cha, D.S., Mansur, R.B., McIntyre, R.S., 2014. Inflamed moods: a review of the interactions between inflammation and mood disorders. Progress in neuro-psychopharmacology & biological psychiatry 53, 23-34.

Ruhe, H.G., Mason, N.S., Schene, A.H., 2007. Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: a meta-analysis of monoamine depletion studies. Molecular psychiatry 12, 331-359.

Schinka, J.A., Busch, R.M., Robichaux-Keene, N., 2004. A meta-analysis of the association between the serotonin transporter gene polymorphism (5-HTTLPR) and trait anxiety. Molecular psychiatry 9, 197-202.

Schlatter, J., Ortuno, F., Cervera-Enguix, S., 2004. Monocytic parameters in patients with dysthymia versus major depression. Journal of affective disorders 78, 243-247.

Schmitt, A., Bertsch, T., Tost, H., Bergmann, A., Henning, U., Klimke, A., Falkai, P., 2005. Increased serum interleukin-1beta and interleukin-6 in elderly, chronic schizophrenic patients on stable antipsychotic medication. Neuropsychiatric disease and treatment 1, 171-177.

Schmitt, A., Martins-de-Souza, D., Akbarian, S., Cassoli, J.S., Ehrenreich, H., Fischer, A., Fonteh, A., Gattaz, W.F., Gawlik, M., Gerlach, M., Grunblatt, E., Halene, T., Hasan, A., Hashimoto, K., Kim, Y.K., Kirchner, S.K., Kornhuber, J., Kraus, T.F.J., Malchow, B., Nascimento, J.M., Rossner, M., Schwarz, M., Steiner, J., Talib, L., Thibaut, F., Riederer, P., Falkai, P., Members of the, W.T.F.o.B.M., 2017. Consensus paper of the WFSBP Task Force on Biological Markers: Criteria for biomarkers and endophenotypes of schizophrenia, part III: Molecular mechanisms. The world journal of biological psychiatry: the official journal of the World Federation of Societies of Biological Psychiatry 18, 330-356.

Scott, J., 2000. Treatment of chronic depression. The New England journal of medicine 342, 1518-1520.

Spijker, J., de Graaf, R., Bijl, R.V., Beekman, A.T., Ormel, J., Nolen, W.A., 2002. Duration of major depressive episodes in the general population: results from The Netherlands Mental Health Survey and Incidence Study (NEMESIS). Br J Psychiatry 181, 208-213.

Steptoe, A., Hamer, M., Chida, Y., 2007. The effects of acute psychological stress on circulating inflammatory factors in humans: a review and meta-analysis. Brain, behavior, and immunity 21, 901-912.

Sternbach, H., 1991. The serotonin syndrome. The American journal of psychiatry 148, 705-713.

ThyagaRajan, S., Priyanka, H.P., 2012. Bidirectional communication between the neuroendocrine system and the immune system: relevance to health and diseases. Annals of neurosciences 19, 40-46.

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. The American journal of psychiatry 163, 28-40.

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.

Tyring, S., Gottlieb, A., Papp, K., Gordon, K., Leonardi, C., Wang, A., Lalla, D., Woolley, M., Jahreis, A., Zitnik, R., Cella, D., Krishnan, R., 2006. Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. Lancet 367, 29-35.

Vitkovic, L., Bockaert, J., Jacque, C., 2000. "Inflammatory" cytokines: neuromodulators in normal brain? Journal of neurochemistry 74, 457-471.

Vogelzangs, N., Beekman, A.T., van Reedt Dortland, A.K., Schoevers, R.A., Giltay, E.J., de Jonge, P., Penninx, B.W., 2014. Inflammatory and metabolic dysregulation and the 2-year course of depressive disorders in antidepressant users. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 39, 1624-1634.

Wang, K., Ye, L., Lu, H., Chen, H., Zhang, Y., Huang, Y., Zheng, J.C., 2017. TNF-alpha promotes extracellular vesicle release in mouse astrocytes through glutaminase. Journal of neuroinflammation 14, 87.

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.

Weber-Hamann, B., Kratzsch, J., Kopf, D., Lederbogen, F., Gilles, M., Heuser, I., Deuschle, M., 2007. Resistin and adiponectin in major depression: the association with free cortisol and effects of antidepressant treatment. Journal of psychiatric research 41, 344-350.

Weich, S., Lewis, G., 1998. Poverty, unemployment, and common mental disorders: population based cohort study. Bmj 317, 115-119.

Whiteford, H.A., Degenhardt, L., Rehm, J., Baxter, A.J., Ferrari, A.J., Erskine, H.E., Charlson, F.J., Norman, R.E., Flaxman, A.D., Johns, N., Burstein, R., Murray, C.J., Vos, T., 2013. Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. Lancet 382, 1575-1586.

WHO, W.H.O., 2017. Depression: let's talk" says WHO, as depression tops list of causes of ill health.

Whooley, M.A., Simon, G.E., 2000. Managing depression in medical outpatients. The New England journal of medicine 343, 1942-1950.

Wichers, M.C., Kenis, G., Leue, C., Koek, G., Robaeys, G., Maes, M., 2006. Baseline immune activation as a risk factor for the onset of depression during interferon-alpha treatment. Biological psychiatry 60, 77-79.

Wirz-Justice, A., Benedetti, F., Berger, M., Lam, R.W., Martiny, K., Terman, M., Wu, J.C., 2005. Chronotherapeutics (light and wake therapy) in affective disorders. Psychological medicine 35, 939-944.

Wolf, M., Clark-Lewis, I., Buri, C., Langen, H., Lis, M., Mazzucchelli, L., 2003. Cathepsin D specifically cleaves the chemokines macrophage inflammatory protein-1 alpha,

macrophage inflammatory protein-1 beta, and SLC that are expressed in human breast cancer. The American journal of pathology 162, 1183-1190.

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

Yirmiya, R., Bab, I., 2009. Major depression is a risk factor for low bone mineral density: a meta-analysis. Biological psychiatry 66, 423-432.

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

Zorrilla, E.P., Luborsky, L., McKay, J.R., Rosenthal, R., Houldin, A., Tax, A., McCorkle, R., Seligman, D.A., Schmidt, K., 2001. The relationship of depression and stressors to immunological assays: a meta-analytic review. Brain, behavior, and immunity 15, 199-226.

EXPERIMENTAL SECTION

Study 1. Brain plasticity inducted by SSRI depends on the quality of the environment

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 as a key factor contributing to 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 TNF-α 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 TNF-α 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., in press; 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 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 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.

Material and methods

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.

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. 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.

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. 1).

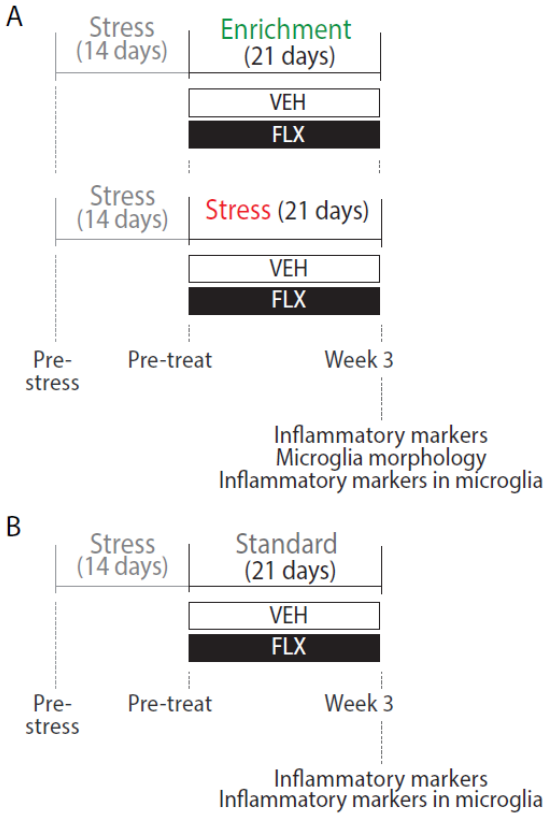

Figure 1. Experimental design. Fluoxetine treatment in (A) the enriched and stressful and (B) the standard condition. In all conditions, before treatment, mice are exposed to a 14-days period of stress to induce a depression-like phenotype.

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

Total RNA was extracted from the hippocampi of 8 animals per group by using 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; RIN). A cutoff of 8 of RIN value was applied in order to ensure a high samples 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° 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).

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 were 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) were analyzed.

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.

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 serie 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 Tris-buffered saline (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.

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.

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

Mice housed in 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.

Statistical analysis

One-way ANOVA, Student's t-test or non-parametric analyses (when data were non-normally distributed) were performed with the Statistical software Statview II (Abacus Concepts, CA, USA) to compare vehicle vs. fluoxetine-treated groups, independently in the enriched or stressful conditions. All mean differences were considered statistically significant when $p < 0.05$.

Results and discussion

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 six 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, TNF-α 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 IFN-γ, which has pro-inflammatory effects and pleiotropic activity, and two anti-inflammatory cytokines, IL-4 and TGF-β, were analyzed as well.

While hippocampal IL-6 expression was not modified by fluoxetine in both conditions, TNF-α and IL-1β levels were affected by treatment in an environment-dependent fashion. In particular, fluoxetine decreased TNF-α 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), 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). IL-6 and TNF-α protein levels were not affected by fluoxetine in both conditions (data not shown). IFN- γ 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-4 and TGF-β 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).

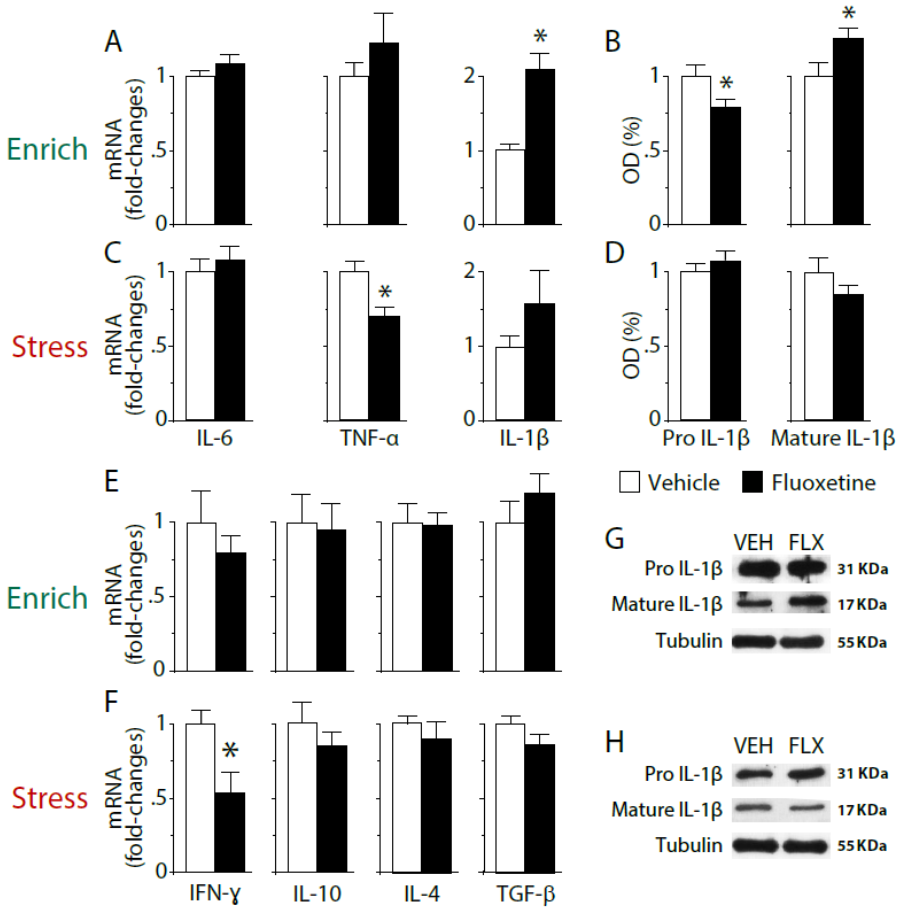

Figure 2. Fluoxetine effects on inflammatory markers in the whole hippocampus. In the enriched condition, we found (A) increased levels of IL-1β mRNA, (B) decreased protein levels of pro IL-1β and increased levels of mature IL-1β. (C) TNF-α expression was reduced by fluoxetine treatment in mice treated 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 TGF-β 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.

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 A). Overall, these results support a dual effect of fluoxetine driven by the quality of the environment and suggest that fluoxetine downregulates inflammasome activity in the enriched condition, while exerting opposite effects under stress.

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 TNF-α 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).

To investigate 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 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).

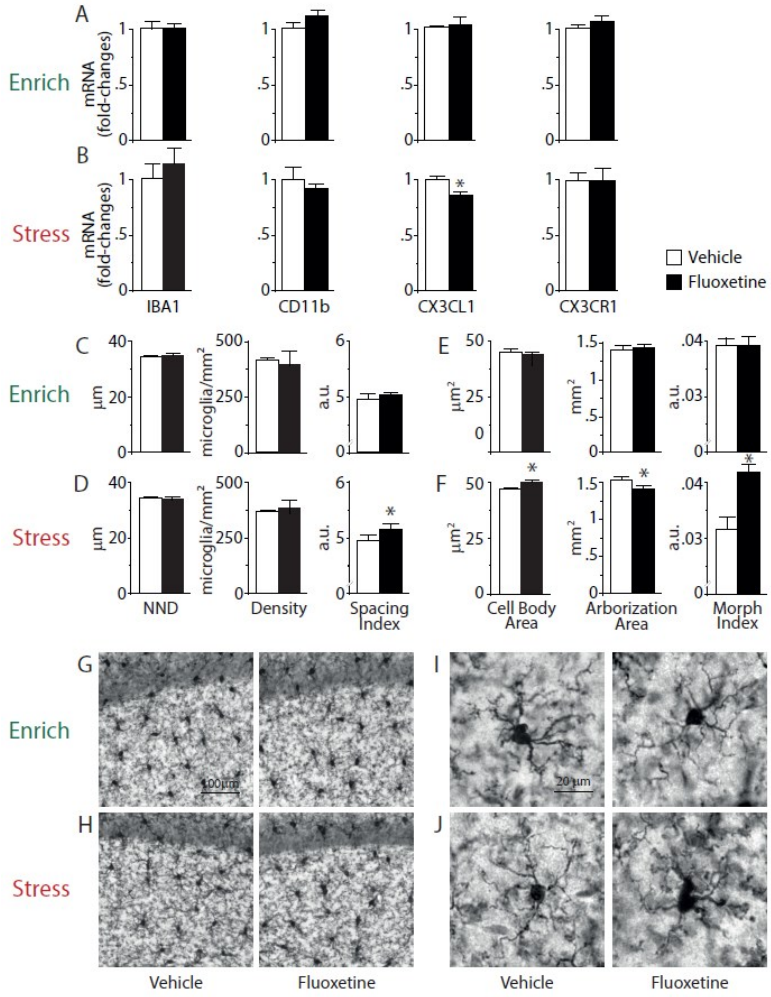

Figure 3. Microglial surveillance state in the hippocampus. (A) In the enriched condition, IBA1, CD11b, CX_3CR1 and CX_3CL1 mRNAs levels were not modified by fluoxetine treatment. However, (B) in the stressful condition, the expression of CX_3CL1 was significantly reduced in fluoxetine treated mice with 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).

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-1b ($t = 2.675$, $p = 0.038$) 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. 4A, D). 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 ($t = 2.961$, $p = 0.042$), TGF-β ($t = 2.605$, $p = 0.040$), 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 ($t = 5.535$, $p = 0.005$), TNF-α ($t = 3.217$, $p = 0.018$), IL-1b ($t = 3.917$ $p = 0.008$), IL-6 ($t = 2.934$, $p = 0.026$) and IL-23 ($t = 3.366$, $p = 0.015$) levels were decreased compared to vehicle (Fig. 4B, E). TNF-α and IL-1b 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 IFN-γ, 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 similar to 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 partially overlapping with those produced in individuals kept in a stressful condition before and during treatment (Fig. 4C, F). In particular, arg-1 ($t = 10.000$, $p = 0.029$), cd206 ($t = 7.085$, $p = 0.001$), ym-1 ($t = 6.929$, $p = 0.001$), TGF-β ($t = 10.000$, $p = 0.029$), 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 TNF-α ($t = 3.301$, $p = 0.021$), IL-1b ($t = 4.151$, $p = 0.009$), 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 ($t = 7.655$, $p<0.001$).

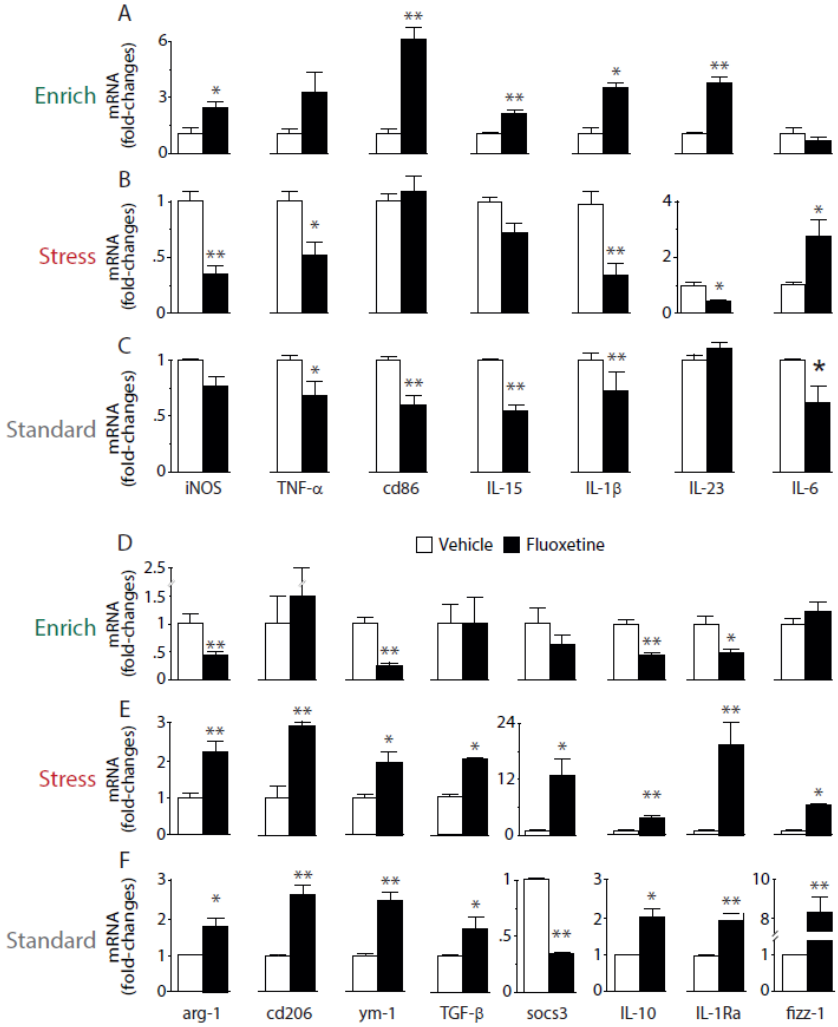

Figure 4. Microglial phenotypic change induced by fluoxetine treatment. Expression of pro- and anti-inflammatory-related genes in CD11b+ cells isolated from the hippocampus of mice exposed to the enriched or the stressful condition, treated with fluoxetine or vehicle. Results of RT-PCR analysis are shown 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 * $p<0.05$ ** $p<0.01$.

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 TGF-β ($t = 37.494$, $p < 0.0001$, $t = 14.051$, $p < 0.0001$, $t = 43.000$, $p < 0.0001$, 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-1b, IL-6, IL-23 and TGF-β ($t = 3.091$, $p = 0.0214$, $t = 3.802$, $p = 0.0191$, $t = 5.044$, $p = 0.0023$, $t = 5.044$, $p < 0.0001$, 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-1b ($t = 3.842$, $p = 0.0085$), 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.

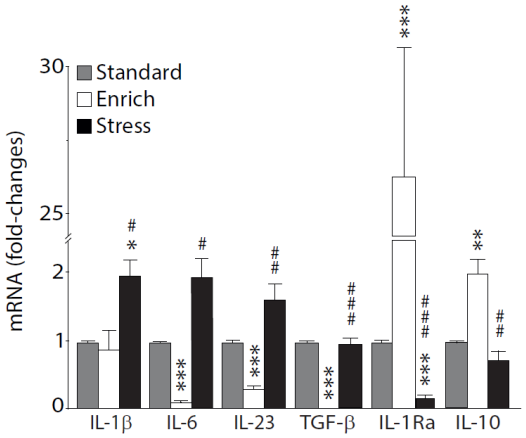

Figure 5. Inflammatory markers in hippocampal CD11b+ cells are affected by the environment. Expression of pro- and anti-inflammatory-related genes in CD11b+ cells isolated from the hippocampus of mice exposed to the standard, enriched or stressful conditions. Results are shown as fold changes relative 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.

Conclusions

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 led to an overall 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 investigate 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., in press; Milior et al., 2015), and likely affects the interaction among the drug, the brain and immune system, making the results not replicable in 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).

Preclinical (Alboni et al., in press; 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 microglia 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 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).

In addition to confirming the environment-dependent outcome of antidepressant treatment, here we show that fluoxetine administration affects the inflammatory response counteracting the influence of the environment instead of

amplifying it as for its effect on endophenotypes of MD (Alboni et al., in press). 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.

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.

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., in press. Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Mol Psychiatry.

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.

Supplementary materials

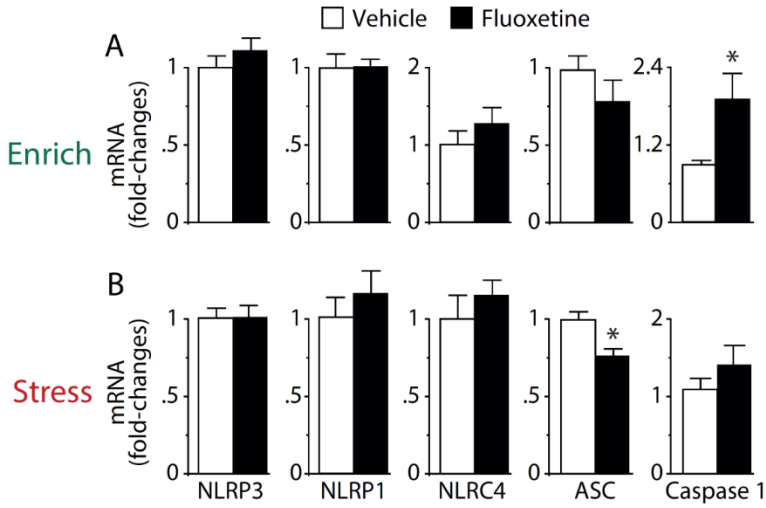

Figure S1. Fluoxetine effects on the expression of inflammasome components in the hippocampus. (A) In the enriched condition, fluoxetine treatment increased caspase 1 mRNA levels but did not affect the expression of other inflammasome components. (B) In the stressful condition, ASC mRNA levels were lower in fluoxetine treated mice compared to the relative control. No other transcriptional effects were observed. Data are expressed as mean + S.E.M. (n = 8 per treatment group). * $p<0.05$ *vs.* relative vehicle treated group.

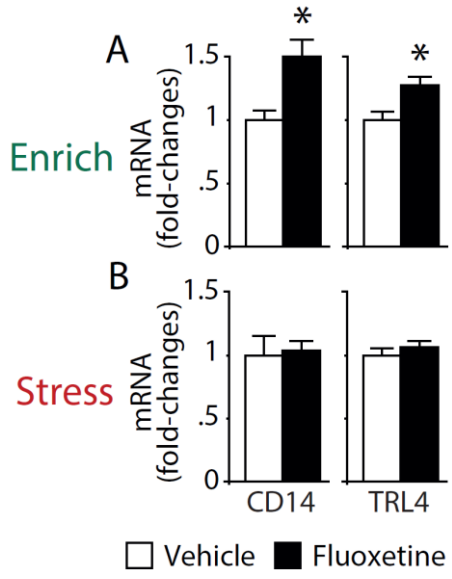

Figure S2. Fluoxetine affects expression of CD14 and TRL4. (A) In the enriched condition, increased mRNA levels of CD14 and TRL4 were found in fluoxetine treated mice compared to vehicle. (B) In the stressful condition, no significant effect was observed. Data are expressed as mean ± S.E.M. (n = 8 per treatment group). * $p<0.05$ *vs.* relative vehicle treated group.

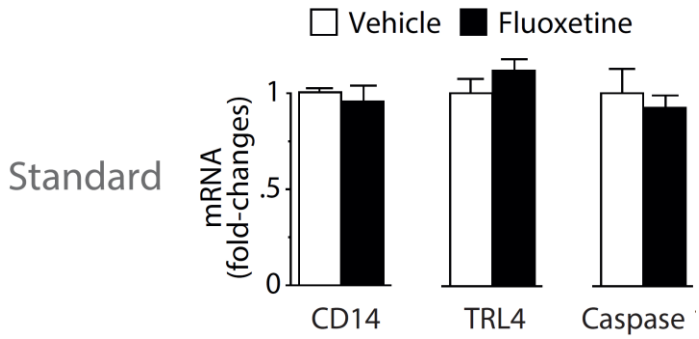

Figure S3. Modifications in inflammatory markers in the whole hippocampus of mice receiving either vehicle or fluoxetine in the standard condition. Treatment did not affect CD14, TRL4, and Caspase 1 levels, compared to vehicle. Results are shown as fold changes relative to vehicle. Data are expressed as mean + S.E.M. (n=4 per group). * $p<0.05$ *vs.* relative vehicle treated group.

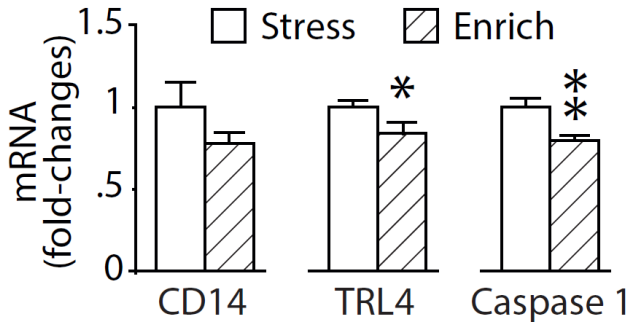

Figure S4. Effects of the environment on the expression of inflammatory markers. Though CD14 levels did not differ between the two conditions, the expression levels of mRNAs encoding TRL4and Caspase 1 were reduced in mice housed in the enriched condition. Results are shown as fold changes relative to the stress condition. Data are expressed as mean + S.E.M. (n=6 per group). * $p<0.05$, ** $p<0.01$ *vs.* mice housed in the stressful condition.

Supplementary table 1

Primers used for Real Time RT-PCR analysis.

Gene (NCBI reference sequence)	Primer (5' 3')➢	
Entire hippocampus		
IL-1β (*NM_008361*)	Sense:	TGAAAGCTCTCCACCTCAATG
	Antisense:	CCAAGGCCACAGGTATTTTG
IL-6 (*NM_031168*)	Sense:	CTTCACAAGTCGGAGGCTTA
	Antisense:	CAAGTGCATCATCGTTGTTC
TNF-α (*NM_013693.2*)	Sense:	GGCCTCCCTCTCATCAGTTC
	Antisense:	CACTTGGTGGTTTGCTACGA
IL-4 (*NM_021283.1*)	Sense:	CCAAGGTGCTTCGCATATTT
	Antisense:	ATCGAAAAGCCCGAAAGAGT
IFN-γ (*NM_008337.3*)	Sense:	TCTTCAGCAACAGCAAGGCGAA
	Antisense:	ACAGCTGGTGGACCACTCGGA
TGF-β (*NM_011577.2*)	Sense:	CCTTGCCCTCTACAACCAAC
	Antisense:	CTTGCGACCCACGTAGTAGAC
IL-18 (*NM_008360.1*)	Sense:	TGAAGAAAATGGAGACCTGGA
	Antisense:	GGCTGTCTTTTGTCAACGAAG
IL-18BP (*NM_010531*)	Sense:	TCCCCTACTTCAGCATCCTC
	Antisense:	GAGCTGTCTTCAACCCATCC
CX3CL1 (*NM_009142.3*)	Sense:	CTTCCATTTGTGTACTCTGCTGC
	Antisense:	GACTCCTGGTTTAGCTGATAGCG
CX3CR1 (*NM_009987.3*)	Sense:	TCAGCAGAATCGTCATACTCAAA
	Antisense:	CGTGAGACTGGGTGAGTGACT
NLRP3 (*NM_145827.4*)	Sense:	AGAAGAGTGGATGGGTTTGCT
	Antisense:	GCGTTCCTGTCCTTGATAGAG
NLRP1 (*NM_001004142.2*)	Sense:	TGACAAGGGCAGTGACAATC
	Antisense:	GCCACAAAATGAGGGAGGTA
IPAF (*NM_001033367.3*)	Sense:	GCTCCTGAACATACCCGACT
	Antisense:	GTGGTGGTGGTGACAATGAC
ASC (*NM_023258*)	Sense:	CAAACGACTAAAGAAGAGTCTG
	Antisense:	AGAGCTTCCTCATCTTGTCT

CASPASE-1 (*NM_009807.2*)	Sense:	CCGTGGAGAGAAACAAGGAG
	Antisense:	AATGAAAAGTGAGCCCCTGA
Iba1 (*NM_019467.2*)	Sense:	GTCCTTGAAGCGAATGCTGG
	Antisense:	CATTCTCAAGATGGCAGATC
CD11b (*NM_001082960.1*)	Sense:	ACGCCATCTACATGATTGTCAC
	Antisense:	AAGACTACACTGACAGGGAGGC
CD14 (*NM_009841.3*)	Sense:	AGATGTGGAATTGTACGGCG
	Antisense:	CGTAAGCCGCTTTAAGGACA
TLR4 (*NM_021297.3*)	Sense:	ACTGGGTGAGAAATGAGCTGG
	Antisense:	AGGATTCGAGGCTTTTCCAT
GAPDH (*NM_008084*)	Sense:	TTCGCAAAACAAGTTCACCA
	Antisense:	TCGTTGTGGTTGTAAATGGAA

CD11b+ hippocampal cells

GAPDH (*NM_008084*)	Sense:	TCGTCCCGTAGACAAAATGG
	Antisense:	TTGAGGTCAATGAAGGGGTC
TNF-α (*NM_013693.2*)	Sense:	GTGGAACTGGCAGAAGAG
	Antisense:	CCATAGAACTGATGAGAGG
CD86 (*NM_019388.3*)	Sense:	AGAACTTACGGAAGCACCCA
	Antisense:	GGCAGATATGCAGTCCCATT
IL-15 (*NM_ 0012547.1*)	Sense:	CATCCATCTCGTGCTACTTGTGTT
	Antisense:	CATCTATCCAGTTGGCCTCTGTTT
IL-18(*NM_008360)*	Sense:	GCCTCAAACCTTCCAAATCA
	Antisense:	TGGATCCATTTCCTCAAAGG
IL-1β (*NM_008361*)	Sense:	CAACCAACAAGTGATATTCTCCATG
	Antisense:	GATCCACACTTCTCCAGCTGCA
IL-1Ra (*NM_031167.5*)	Sense:	AAGCCTTCAGAATCTGGGATAC
	Antisense:	TCATCTCCAGACTTGGCACA
Arg1 (*NM_007482.3*)	Sense:	CTCCAAGCCAAAGTCCTTAGAG
	Antisense:	AGGAGCTGTCATTAGGGACATC
Fizz1 (*NM_020509.3*)	Sense:	CCAATCCAGCTAACTATCCCTCC
	Antisense:	ACCCAGTAGCAGTCATCCCA
IL-23 (*NM_031252.2*)	Sense:	AGCGGGACATATGAATCTACTAAGAGA
	Antisense:	GTCCTAGTAGGGAGGTGTGAAGTTG

Socs3 (*NM_007707.3*)	Sense:	GCTCCAAAAGCGAGTACCAGC
	Antisense:	AGTAGAATCCGCTCTCCCTGCAG
CD206 (NM_008625.2)	Sense:	CAAGGAAGGTTGGCATTTGT
	Antisense:	CCTTTCAGTCCTTTGCAAGT
Ym1 (NM_009892)	Sense:	CAGGTCTGGCAATTCTTCTGAA
	Antisense:	GTCTTGCTCATGTGTGTAAGTGA
IL-6(NM_031168)	Sense:	GATGGATGCTACCAAACTGGA
	Antisense:	TCTGAAGGACTCTGGCTTTG
IL-10(NM_010548.2)	Sense:	GAAGACCCTCAGGATGCG
	Antisense:	CCAAGGAGTTGTTTCCGTTA
iNOS (NM_001313922.1)	Sense:	ACATCGACCCGTCCACAGTAT
	Antisense:	CAGAGGGGTAGGCTTGTCTC
TGF-β (NM_011577.2)	Sense:	GGAGAGCCCTGGATACCAAC
	Antisense:	GTGTCCAGGCTCCAAATGTAGG

Supplementary table 2

Antibodies used in Western blot analysis.

Antibody	Manufacturer	Catalog Number
Anti-IL-β	Santa Cruz	SC-7884
Anti-IL-6	Abcam	ab6672
Anti-TNF-α	Abcam	ab6671
Anti-β-Tubulin	Santa Cruz	SC-9104
Anti-IBA1	Wako Pure Chemicals	#019-19741
Anti-Rabbit IgG (HRP)	CST	#7074S
Anti-Rabbit IgG (Biotinylated)	Vectastain	BA-1000

Abcam (Abcam, Massachusetts, USA); CST (Cell Signaling Technologies, Massachusetts, USA); Santa Cruz (Santa Cruz Biotechnologies, Texas, USA).

Study 2. Interplay between inflammation and neural plasticity: both immune activation and suppression impair LTP and BDNF expression

Introduction

Inflammation consists in the protective response to harmful stimuli, such as pathogens, damaged cells, or irritants, and leads to the healing process for the tissue (Ferrero-Miliani et al., 2007; Medzhitov, 2010). To orchestrate this process, the immune cells produce a wide range of soluble mediators such as cytokines (Chertov et al., 2000). These mediators are able to cross the blood–brain barrier and modulate the function of central nervous system (CNS), since they could directly interact with both neurons and glia, including microglia, which are the tissue-resident macrophages of the brain (Branchi et al., 2014; Paolicelli et al., 2011; Tay et al., 2017; Tremblay et al., 2011).

The literature of last decades focused on the detrimental consequences of activation of the immune system in the brain, reporting that increased inflammation is associated to a number of pathological conditions (Chen et al., 2016; Hotamisligil and Erbay, 2008; Libby, 2006; Wyss-Coray and Mucke, 2002). In particular, mediators involved in the promotion of inflammation, are associated with the onset and progression of major depression in vulnerable individuals, and with neurodegenerative disorders such as amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, and Huntington's disease (Chen et al., 2016; Hurley and Tizabi, 2013). The detrimental impact of inflammation on brain function has been hypothesized to be mediated by its effect on neural plasticity, i.e. the capability of the CNS for structural and functional changes. Indeed, high levels of inflammatory markers have been reported to decrease a wide number of markers of neural plasticity such as long-term potentiation (LTP), Brain Derived Neurotrophic Factor (BDNF) levels, and neurogenesis (Raison et al., 2006).

Recently, a new framework is emerging pointing out that inflammation is not a detrimental per se, but it is deeply implicated in the physiological functioning of

the healthy brain. In particular, this new view posits that inflammation is critical in regulating neural plasticity that includes processes such as growing and pruning of dendrites and axons, shaping of synapses and associated structures, apoptosis and neurogenesis of neurons (Yirmiya and Goshen, 2011). These events produce neuritic remains that are removed by the immune system to allow brain tissue remodelling, keeping neuronal homeostasis. Therefore, albeit high levels of inflammation are reported to be detrimental, inflammation in a physiological range is beneficial and plays a critical physiological role in neural plasticity. Indeed, it is increasingly reported that the microglial cells, interacting with neurons, promote a range of processes such as learning and memory consolidation (Yirmiya and Goshen, 2011).

Basing on this view, we hypothesized that inflammation should be in a specific physiological range to be permissive for neural plasticity so that either an immune activation or suppression impairs such capability of the brain. To assess the potential U-shaped regulation of neural plasticity by inflammation, we administered in a mouse model either the endotoxin lipopolysaccharide (LPS) or the non-steroidal anti-inflammatory drug (NSAIDs) ibuprofen (IBU). LPS is commonly used to stimulate the innate immune system (Martin et al., 2013) and promotes brain pro-inflammatory cytokine production, such as Interleukin (IL)-1β, IL-6 or Tumor Necrosis Factor (TNF)-α. (Kubera et al., 2013; Trotta et al., 2014). IBU has prominent analgesic and antipyretic effects, which are due to its inhibitory action on cyclooxygenases (COX) and, consequently, on the synthesis of prostaglandins (PG) (Rainsford, 2013; Shaw et al., 2005). First, we assessed the changes in physiological indices following the administration to confirm the effectiveness of the treatments. Then, we assessed both molecular and cellular endpoints, including LTP at Schaffer collateral-CA1 synapses since it is the most commonly used paradigm of synaptic plasticity (Bliss and Collingridge, 2013; McEachern and Shaw, 1999), and the expression levels of a number of inflammatory and plasticity markers, including BDNF, which is deeply involved in the neural plasticity (Lu et al., 2014).

Methods

Animals

Fifty C57BL/6 male mice 12–15 week old were used and kept under 12-hour light-dark cycle at 22–25°C. Mice were purchased at Envigo Italia (Udine, Italy). 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). In addition, animals were examined for signs of discomfort as indicated by the animal care and use National Academy of Sciences guidelines. (National Research Council 2003).

Housing condition

For the entire duration of the experiment, animals were housed in standard laboratory condition, four to five individuals per cage. Each cage was a Plexiglas box (48 x 37.5 x 21 cm) with metal tops and sawdust as bedding. Pellet food and tap water were provided ad libitum. The day before the treatment, mice were moved in smaller cage (33.1 x 15.9 x 13.2 cm) to individually monitor them.

Treatment

Mice were acutely treated through intraperitoneal (ip) injection of LPS (serotype 0127:B8; RBI/Sigma) diluted in saline at two different doses: 0.33 and 0.83 mg/kg, IBU (Ibuprofen sodium salt, Sigma Aldrich) diluted in saline at two different doses: 30 and 70 mg/kg or saline (0.9% NaCl) 100 mL/100 g. LPS and IBU solutions were prepared daily.

Physiological indexes

The day before the treatment, immediately before and three hours after the injection, rectal temperature (MicroTherma 2T Hand Held, Thermometer) and body weight were measured. At the same time points, we assessed the amount food and liquid consumed by each animal.

Electrophysiological analysis

In order to perform electrophysiological experiments, acute hippocampal slices were prepared from 2 months old male mice. Three hours after treatment, animals were anesthetized by inhalation of halothane (Sigma-Aldrich S.r.l., Milan, Italy) and decapitated. The brain was rapidly removed from the skull and immersed in ice-cold artificial cerebrospinal fluid (ACSF) solution composed of (in mM): NaCl 125, KCl 4, CaCl2 2.5, MgSO4 1.5, NaHPO4 1, NaHCO3 26 and glucose 10). ACSF was continuously oxygenated with 95% O2 + 5% CO2 to maintain the proper pH (7.4).

One of the two hemispheres isolated were conserved for molecular analysis. Following removal, one hemisphere were blocked on the stage of a vibrating microtome (Thermo Scientific, USA) and 350 μm thick slices were cut in ice-cold ACSF. The slices were then transferred to an incubation chamber containing oxygenated ACSF, where they were allowed to recover for 1 h at 30 °C prior to electrophysiological recording.

For field recordings, individual slices were transferred to the interface slice-recording chamber (BSC1, Scientific System Design Inc) to perform experiments within 1–6 hours after slice preparation. Slices were maintained at 30 to 32 °C and constantly superfused with ACSF at the rate of 2 ml/min. Solutions were applied to the slices by a peristaltic pump. A concentric bipolar stimulating electrode (SNE-100 × 50 mm long, Elektronik–Harvard Apparatus GmbH) was placed in the stratum radiatum to stimulate Schaffer collateral fibers. Stimuli consisted of 100 μs constant current pulses of variable intensity, applied at 0.05 Hz. A glass micropipette (0.5–1 MΩ) filled with ACSF was placed in the CA1 hippocampal region, at 200–600 μm from the stimulating electrode, in order to measure orthodromically-evoked field extracellular postsynaptic potentials (fEPSP). Stimulus intensity was adjusted to evoke fEPSPs of amplitude about 50% of maximal amplitude with minimal contamination by a population spike. Evoked responses were monitored online and stable baseline responses were recorded for at least 10 min. Only the slices that showed stable fEPSP amplitudes were included in the experiments. LTP was induced

by high-frequency stimulation (2 train of stimuli at 100 Hz, of 1 s duration and separated by 3 s). To analyze the time course of fEPSP slope, the recorded fEPSP was routinely averaged over 1 min (n = 3). fEPSP slope changes following the LTP induction protocol were calculated with respect to the baseline.

RNA extraction and RT-RTqPCR

Three hours after treatments animals were sacrificed by decapitation, the brains removed, hippocampi dissected, rapidly frozen and then stored at -80 °C for further molecular analyses. Total RNA was prepared combining extraction whit TRI Reagent® and GenElute™ Mammalian Total RNA Miniprep Kit and (Sigma Aldrich ®, Milan, Italy) as previously described (Alboni et al., 2017). Two μg of total RNA were reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA USA) in a final reaction volume of 20 μL (Alboni et al., 2013). The cDNA was stored at -20 °C until Real Time PCR that was performed in Roche LightCycler® 480 (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) using Power SYBR Green mix (Life Technologies Corporation, Carlsbad, CA, USA). Specific forward and reverse primers, at a final concentration of 150 nM, were used to measure mRNA levels were as follows: for ionized calcium-binding adapter molecule 1 (Iba1) F5'-GTCCTTGAAGCGAATGCTGG-3' and R5'-CATTCTCAAGATGGCAGATC-3'; for Tryptophan 2,3-Dioxygenase (TDO2) F5'-TGTGGTCCTGAGACACTTCAGT-3' and R5'-ACACCAGTTTGAGCTCTGTCTTC-3'; for prostaglandin-endoperoxide synthase 2 COX-2 F5'-TTCTACGGAGAGAGTTCATC-3' and R5'-CAGTTTATGTTGTCTGTCCA-3'; for interleukin 1 beta (IL-1β) F5'-TGAAAGCTCTCCACCTCAATG-3' and R5'-CCAAGGCCACAGGTATTTTG-3'; for transforming growth factor (TGF)-β F5'-CCTTGCCCTCTACAACCAAC-3' and R5'-CTTGCGACCCACGTAGTAGAC-3'; for interleukin 1 receptor antagonist IL-1 Ra F5'-AAGCCTTCAGAATCTGGGATAC-3' and R5'-TCATCTCCAGACTTGGCACA-3'; for CCAAT Enhancer Binding Protein Delta (CEBPD) F5'-GCGGCCTTCTACGAG-3' and R5'-GCCATGGAGTCAATGTAG-

3’; for total Brain Derived Neurotrophic Factor (BDNF) F5’-CCATAAGGACGCGGACTTGTAC-3’ and R5’-AGACATGTTTGCGGCATCCAGG-3’ and for Glyceraldehyde 3-phosphate dehydrogenase (GADPH) F5’-TTCGCAAAACAAGTTCACCA-3’ and R5’-TCGTTGTGGTTGTAAATGGAA-3’ as a house-keeping gene. The cycling parameters were: 95°C 10 min and 95 °C 15 s, 60° 1 min for 40 cycles. Melt curve analysis and agarose gel separations were performed at the end of every RTqPCR to confirm formation of a single PCR product. Ct (cycle threshold) value was determined by the LightCycler® 480 Software (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) and mRNA expression was calculated with the ΔΔCt method with GAPDH as endogenous control as previously described (Benatti et al., 2011). It was demonstrated that amplification efficiency of the target genes and endogenous control gene were approximately equal. Relative expression of the genes of interest was performed by using as calibrator (RQ value=1) expression levels in the hippocampus of saline treated animals.

Statistical analyses

All data were analyzed with one-way ANOVA or Student’s t-test with the Statistical software Statview II (Abacus Concepts, CA, USA), comparing LPS high and low dose, IBU high and low doses and saline treated mice. When a significant main effect was found, selected pairwise comparisons were made using Tukey post-hoc analysis. The animals were assigned to various treatments in order to obtain balanced body temperature and weight across the experimental groups.

Results

We examined the effect of immune activation and suppression on neural plasticity administering different doses of, respectively, LPS and IBU. In particular, 3 hours after the ip injection with these agents, we evaluated (i) the effectiveness of the treatments by measuring changes in body temperature and weight, the amount of food and liquid consumed, (ii) the CA1 hippocampal plasticity by field potential

recordings in acute slices, and (iii) molecular changes of targets related to inflammation and plasticity processes.

Physiological assessment of treatment's effectiveness

It has been largely demonstrated that LPS administration produces sickness behavior (Yirmiya, 1996; Yirmiya et al., 2001), while the treatment with NSAIDs, such as IBU is effective in improving symptoms of inflammation (Pecchi et al., 2009). To verify the effectiveness of the treatment we used, we assessed a number of endpoints, including body weight and temperature, food and liquid intake, associated with the sickness response. We measured all these parameters the day before the treatment and no difference between the experimental groups has been emerged (data not shown).

The difference of body temperature before and 3 hours after the injection showed a main effect of the treatment [$F(4,45)=9.304$; $p<0.0001$]. Post hoc comparison revealed that both high and low doses of LPS significantly decreased the body temperature compared to the other groups ($p<0.05$) (Fig. 1A). As for the temperature, also the body weight (Fig. 1B) was affected by the treatment [$F(4,45)=18.925$, $p<0.0001$], with post-hoc analysis revealing that LPS treated mice displayed a significant reduction of weight compared to saline, high and low dose of IBU. Accordingly, we observed the effect of treatment on the food intake [$F=(4,36)=7.161$, $p=0.0002$], which was decreased by the LPS treatment (Fig. 1C). Especially, post-hoc analysis revealed that mice treated with both doses of LPS consumed significant lower amount of food compared to IBU 70 mg/kg treated mice ($p<0.05$). Finally, the amount of liquid drank was affected by the treatment [$F(4,44)=2.483$, $p=0.0574$)], the mice treated with the higher dose of LPS displayed a significant reduced consumption of liquid compared to the control group (Fig. 1D). Taken globally, these results confirmed the effectiveness of the treatment, especially regarding the injection with LPS.

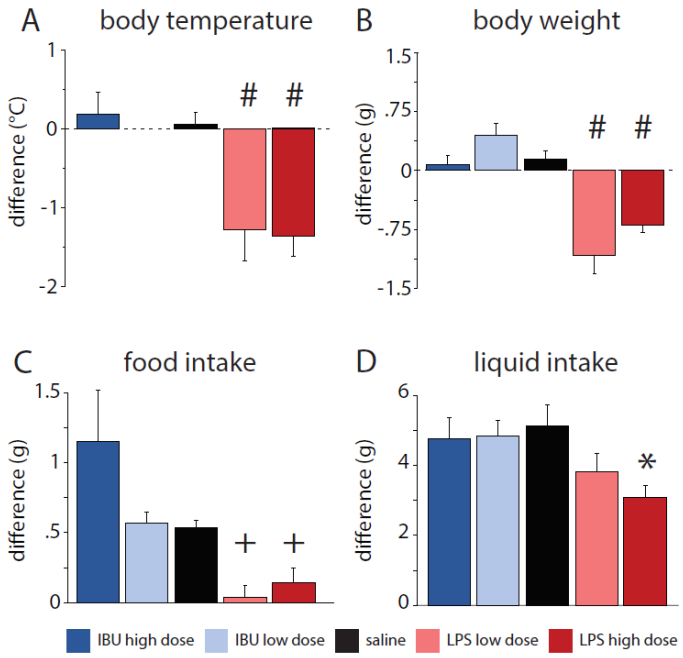

Figure 1. Physiological and behavioral parameters affected by immune alterations. Histogram representing the variations in body temperature (A) and weight (B), in food (C) and liquid (D) intake. Treatments as indicated in the legend, n = 7-15 mice per group. * $p<0.05$ vs saline, # $p<0.05$ vs IBU high dose and. $ $p<0.05$ vs saline, IBU low dose and IBU high dose. Data are shown as mean±s.e.m.

Expression levels of inflammatory markers are affected by the LPS treatment

We assessed the effect of treatment at molecular level measuring the expression levels of selected markers reported to be involved in inflammatory processes. In particular, we analyzed the hippocampal levels of IL-1β, IL-1Ra, CEBPB, TGF-β and COX-2. Regarding the IL-1β, we obtained the main effect of the treatment [$F(4,24)=6.259$, $p=0.001$] with the post-hoc analysis revealing that mice treated with both high and low dose of LPS had significant increased levels of IL-1β compared to the saline mice (respectively, $p=0.025$ and $p=0.007$). In addition, mice receiving the lower dose of LPS displayed significantly increased level of IL-1β compared to mice treated with the two doses of IBU (Fig. 2A). When we assessed the level of IL-1Ra, we obtained the main effect of treatment [$F(4,22)=4.333$,

p=0.010] and the post-hoc analysis revealed that the group treated with the lower dose of LPS had significant increased level of IL-1Ra compared to the mice receiving IBU (high and low dose), higher dose of LPS, and saline (Fig. 2B). We found the main effect of treatment also on CEBPD levels [F(4,23)=6.416, p=0.001]. Post-hoc analysis highlighted that the animals treated with the lower dose of LPS displayed significant increased levels compare to the saline mice (p=0.006). In addition, they were significant higher compared to both doses of IBU (Fig. 2C). The analyses of TGF-β and COX-2 levels did not show a significant effect of the treatment on these two markers (Fig. 2D,E).

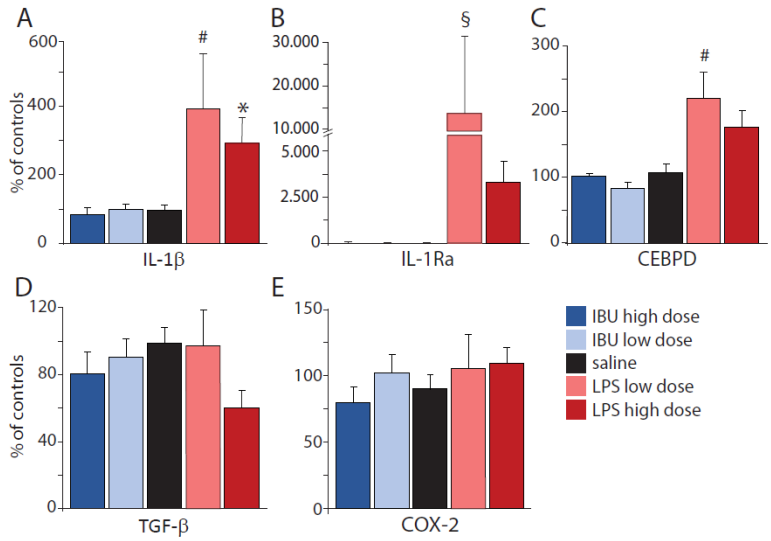

Figure 2. Expression level of inflammatory markers are affected by the LPS treatment. (A) Higher and lower dose of LPS significantly increase the expression level of IL-1β compared to saline mice. IL-1Ra **(B)** and CEBPD **(C)** levels are significantly increased by the lower dose of LPS compared to saline mice. **(D, E)** TGF-β and COX-2 levels are not affected by the treatment. Treatments as indicated in the legend, n =5-10 mice per group. Data are shown as mean±s.e.m.

Neuronal Plasticity is similarly affected by treatments

We evaluated the long-term CA1 plasticity inducing LTP by a robust stimulation (two 100 Hz bursts, separated by a 3 s interval) of Shaffer collateral inputs. We observed that the treatments, with both high doses of LPS and IBU, significantly reduced the amplitude of LTP (Fig. 3). In particular, in mice treated with saline we measured a robust LTP amplitude (1.323 ± 0.149, n/N = 25/11), that was decreased of about 40% following IBU (1.156 ± 0.0794; n/N = 13/5; t = 3.133; p = 0.010; vs saline) or LPS treatment (1.200 ± 0.136, n/N = 15/6; t = 2.578; p=0.041 vs saline; t = 0.751; p= 0.001 vs IBU). In contrast, low doses of LPS and IBU did not affect the amplitude of LTP (1.346 ± 0.0883, n/N = 8/4 and 1.327 ± 0.161, n/N = 8/4, respectively). Overall, these results demonstrate that IBU and LPS treatments have a detrimental effect on long-term plasticity mainly affecting the postsynaptic LTP expression.

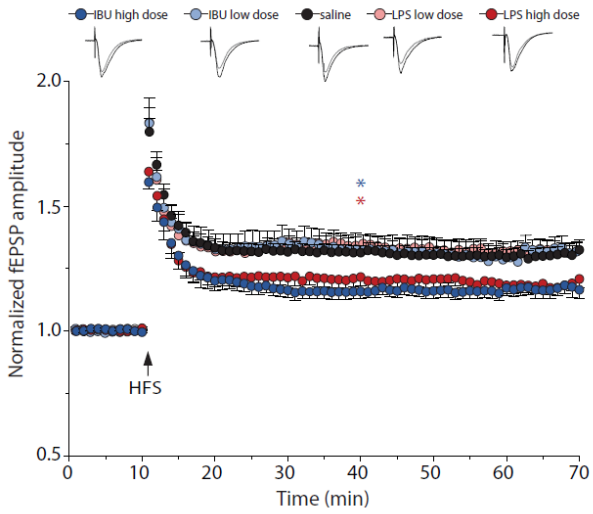

Figure 3. High doses of IBU and LPS treatments decrease LTP amplitude in the CA1 hippocampal area. IBU and LPS treatments, at different doses as indicated in the legend, decreased LTP amplitude, denoted by a lower increase of fEPSP amplitude after LTP induction (two 100 Hz bursts, separated by a 3s interval), compared to saline, 30 min after stimulation (min 41-45). fEPSP, field excitatory postsynaptic potential. Treatments as indicated in the legend. N/n refers to the number of slices on total number of mice analyzed (N=8-21, n=4-9). Data are shown as mean±s.e.m.

Expression level of BDNF is differently affected by the treatment with IBU and LPS

To assess the effect of immune activation and suppression on neural plasticity at molecular level, we investigate whether the expression levels of BDNF were differently affected by the treatment with IBU and LPS. The measure of hippocampal level of mature BDNF showed the main effect of the treatment [$F(4,23)= 4.772$, $p=0.006$). The post-hoc analysis revealed that the higher doses of IBU and LPS were associated with significant reduced level of this neurotrophin compared to control mice (respectively, $p=0.047$ and $p=0.006$; Fig. 4).

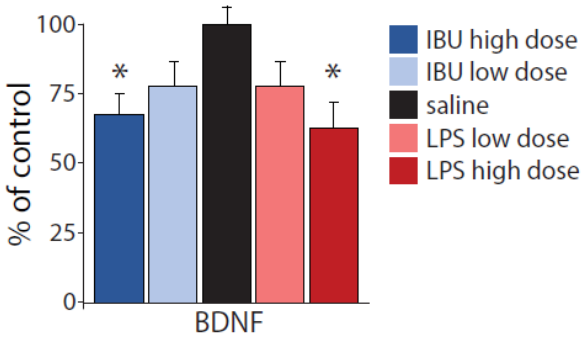

Figure 4. Expression level of plasticity markers are differently affected by the treatments. Higher doses of both IBU and LPS significantly decrease the levels of BDNF compared to saline mice. Treatments as indicated in the legend, n =5-10 mice per group. Data are shown as mean±s.e.m.

Discussion

The present results show that both pro- and anti-inflammatory treatments, respectively LPS and IBU, impair neural plasticity measured at both molecular and cellular levels. Such effects appear to be dose-dependent since only the higher, but not the lower, dose of both compounds leads to a plasticity impairment.

In line with the literature about the well-characterized pro- and anti-inflammatory actions of LPS and IBU, the two compounds produced different physiological effects. As expected, LPS induced the sickness response, a generalized defense response to an acute infection or an immune challenge comprising physiological, endocrinological, and behavioral changes (Hart et al., 1988; Kent et al., 1992; Pecchi et al., 2009; Wieseler-Frank et al., 2005; Yirmiya, 1996; Yirmiya et al., 2001). In particular, LPS significantly reduced body temperature and weight compared to all the other treatment groups and decreased food intake compared to the high IBU dose group. By contrast, IBU produced no effects on sickness behavior when compared to saline. This result is apparently discordant with previous studies showing that NSAIDs rescue the sickness response. However, such effect has been found in animal models of inflammation while, to our knowledge, no previous study investigated the effects of IBU in naïve animals. Overall, these results point out that the two compounds trigger different biological processes leading to different physiological responses.

The different action exerted by LPS and IBU is evident also when evaluating the expression levels of a number of inflammatory markers. We found a significant IL-1β increased induced by both LPS doses compared to all the other groups. This is in line with the literature describing IL-1β as a key mediator of the inflammatory response (Lopez-Castejon and Brough, 2011) and main contributor to the pathogenesis of LPS-induced acute inflammation (Ulich et al., 1991). It is worth noting that, in line with our results on physiological parameters described above, both LPS and IL-1β have been reported to trigger the set of physiological responses typical of sickness behavior (Dantzer, 2004). The lower dose of LPS significantly increased the level of both IL-1Ra and CEPBD compared to both IBU doses and saline. IL-1Ra

is a natural inhibitor of the pro-inflammatory effect of IL-1β since it blocks further IL-1β activity and terminates the inflammatory response (Arend et al., 1998). Previous studies have largely demonstrated that the IL-1Ra expression can be induced in monocytes and macrophages through treatment with LPS (Jenkins and Arend, 1993). On the other hand, CEBPD is a transcription factor involved in the regulation of genes associated with activation and/or differentiation of macrophages, and consequently, with an important regulatory role of immune responses. CEBPD is expressed at a relatively low level under normal physiological conditions and is upregulated by a variety of pro-inflammatory challenges, such as IL-6, IL-1β and LPS (Chang et al., 2012). Contrary to the low, the high LPS dose missed to reach a statistically significant increase of IL-1Ra and CEBPD, possibly because the pro-inflammatory pathways triggered by different LPS doses do not overlap, as previously reported (Deng et al., 2013; Dudele et al., 2015; Morris et al., 2014). The expression level of TGF-β and COX-2 did not show significant effect, though we obtained a trend towards of a reduction of COX-2 levels in the high IBU dose group in line the literature indicating that IBU inhibits COX enzymes (Hawkey, 1999; Mitchell et al., 1993). Overall, these results confirm the pro-inflammatory action of LPS. Whereas IBU did not produce marked changes compared to saline, probably because the treatment in naïve animals had a ceiling effect.

Though LPS and IBU produced significantly different effects in physiological endpoints and expression of inflammatory markers, they led to overlapping effects on plasticity at cellular level: both LPS and IBU, at high doses, significantly reduced LTP amplitude by around 40%. According to the literature, LPS affected LTP most likely through an increase in pro-inflammatory cytokines and, among these, IL-1β that has notably been demonstrated to have detrimental effects on LTP induction and maintenance (Bellinger et al., 1993; Ikegaya et al., 2003). Therefore, the reduced LTP we observed is in line with our molecular measures of IL-1β expression level, which was significantly increased by the treatment. By contrast, IBU could have affected LTP through the modulation of COX-2 activity, which is a regulator of synaptic plasticity and LTP (Chen et al., 2002; Shaw et al., 2003; Yamagata et al., 1993).

Indeed, IBU broadly inhibits COX activity (Rainsford, 2013) inducing a decrease in PG concentrations that impairs LTP. Moreover, COX-2 activity and the consequent production of PGs in postsynaptic dendritic spines (Kaufmann et al., 1996) are upregulated after a high frequency stimulation associated with LTP induction (Yamagata et al., 1993). In order to confirm such view, the assessment of COX-2 activity levels and of PGs, in addition to COX-2 expression levels, is warranted.

In line with the results concerning LTP, the assessment of BDNF levels confirms that both LPS and IBU, at high doses, similarly affect neural plasticity, significantly reducing the expression of this neurotrophin. In line with our data on inflammatory marker expression, these effects are likely to be mediated by an increase in IL-1β that reportedly reduces BDNF levels (Tong et al., 2008). In addition, lower BDNF levels have been found to be associated to a reduction of LTP, as we here observed. With regard to the mechanisms linking IBU administration with reduced BDNF expression levels in naïve animals, a large body of literature indicates that this could be the broad-spectrum COX inhibition produced by IBU, which in turn decreases the levels of mature BDNF (Hein and O'Banion, 2009; Shaw et al., 2003). It is important to highlight that present molecular data comprise mRNA transcript levels and the assessment of protein levels will provide a more comprehensive picture of the interaction between inflammatory markers and neural plasticity.

Overall, the present findings suggest that neural plasticity requires inflammatory responses to be kept within a tightly controlled range and any deviation from such range, caused by either immune activation or suppression, results in a plasticity impairment. The impact such deviations appears to be dose-dependent because higher is the dose of either LPS or IBU, larger is the plasticity impairment. This is in line with previous studies and in particular the seminal review paper by Yirmiya and Goshen (2011) suggesting that both anti- and pro-inflammatory processes are required for brain functioning since unbalances towards an immune suppression or activation have detrimental consequences for the healthy brain (Hewett et al., 2012; Santello et al., 2012; Yirmiya and Goshen, 2011). A relevant theoretical consequence of the present findings is that the ultimate effect of anti- and

pro-inflammatory challenges is determined by the baseline inflammatory condition of the individual. Indeed, the beneficial or detrimental impact should be evaluated according to the capability to normalize or exacerbate the inflammatory balance. This view is in line with a number of preclinical and clinical data markers (Iyengar et al., 2013; Kohler et al., 2014; Tyring et al., 2006). For instance, a study examining the effects of the anti-inflammatory compound infliximab, a TNF-α inhibitor, as adjunctive treatment option to improve the effectiveness of antidepressants, showed that depressed patients with high baseline levels of peripheral inflammation profit from the adjunctive anti-inflammatory treatment, while those with low baseline inflammation show even a worse outcome, compared to patients receiving placebo (Raison et al., 2013). This confirms that lowering inflammation beyond optimal levels reduces the potential beneficial effects of the treatment.

Finally, previous studies showed that an increase in neural plasticity, induced through selective serotonin reuptake inhibitor administration, counterbalances both the activation and the suppression of the immune response (Alboni et al., 2016), keeping inflammation in a strict range. Integrating these results with the present ones, a clear interplay between inflammation and neural plasticity emerges indicating that these are mutually regulating processes. Therefore, therapeutic interventions aimed at targeting specifically neural plasticity may result in a more effective outcome if designed to simultaneously act on both the processes.

References

Alboni, S., Benatti, C., Montanari, C., Tascedda, F., Brunello, N., 2013. Chronic antidepressant treatments resulted in altered expression of genes involved in inflammation in the rat hypothalamus. European journal of pharmacology 721, 158-167.

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., 2016. Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment. Brain, behavior, and immunity 58, 261-271.

Alboni, S., van Dijk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I., Cirulli, F., Maggi, L., Branchi, I., 2017. Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Molecular psychiatry 22, 635.

Arend, W.P., Malyak, M., Guthridge, C.J., Gabay, C., 1998. Interleukin-1 receptor antagonist: role in biology. Annual review of immunology 16, 27-55.

Bellinger, F.P., Madamba, S., Siggins, G.R., 1993. Interleukin 1 beta inhibits synaptic strength and long-term potentiation in the rat CA1 hippocampus. Brain research 628, 227-234.

Benatti, C., Alboni, S., Montanari, C., Caggia, F., Tascedda, F., Brunello, N., Blom, J.M., 2011. Central effects of a local inflammation in three commonly used mouse strains with a different anxious phenotype. Behavioural brain research 224, 23-34.

Bliss, T.V., Collingridge, G.L., 2013. Expression of NMDA receptor-dependent LTP in the hippocampus: bridging the divide. Molecular brain 6, 5.

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

Chang, L.H., Huang, H.S., Wu, P.T., Jou, I.M., Pan, M.H., Chang, W.C., Wang, D.D., Wang, J.M., 2012. Role of macrophage CCAAT/enhancer binding protein delta in the pathogenesis of rheumatoid arthritis in collagen-induced arthritic mice. PloS one 7, e45378.

Chen, C., Magee, J.C., Bazan, N.G., 2002. Cyclooxygenase-2 regulates prostaglandin E2 signaling in hippocampal long-term synaptic plasticity. Journal of neurophysiology 87, 2851-2857.

Chen, W.W., Zhang, X., Huang, W.J., 2016. Role of neuroinflammation in neurodegenerative diseases (Review). Molecular medicine reports 13, 3391-3396.

Chertov, O., Yang, D., Howard, O.M., Oppenheim, J.J., 2000. Leukocyte granule proteins mobilize innate host defenses and adaptive immune responses. Immunological reviews 177, 68-78.

Dantzer, R., 2004. Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. European journal of pharmacology 500, 399-411.

Deng, H., Maitra, U., Morris, M., Li, L., 2013. Molecular mechanism responsible for the priming of macrophage activation. The Journal of biological chemistry 288, 3897-3906.

Dudele, A., Fischer, C.W., Elfving, B., Wegener, G., Wang, T., Lund, S., 2015. Chronic exposure to low doses of lipopolysaccharide and high-fat feeding increases body mass without affecting glucose tolerance in female rats. Physiological reports 3.

Ferrero-Miliani, L., Nielsen, O.H., Andersen, P.S., Girardin, S.E., 2007. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clinical and experimental immunology 147, 227-235.

Hart, P.H., Whitty, G.A., Piccoli, D.S., Hamilton, J.A., 1988. Synergistic activation of human monocytes by granulocyte-macrophage colony-stimulating factor and IFN-gamma. Increased TNF-alpha but not IL-1 activity. Journal of immunology 141, 1516-1521.

Hawkey, C.J., 1999. COX-2 inhibitors. Lancet 353, 307-314.

Hein, A.M., O'Banion, M.K., 2009. Neuroinflammation and memory: the role of prostaglandins. Molecular neurobiology 40, 15-32.

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.

Hotamisligil, G.S., Erbay, E., 2008. Nutrient sensing and inflammation in metabolic diseases. Nature reviews. Immunology 8, 923-934.

Hurley, L.L., Tizabi, Y., 2013. Neuroinflammation, neurodegeneration, and depression. Neurotoxicity research 23, 131-144.

Ikegaya, Y., Delcroix, I., Iwakura, Y., Matsuki, N., Nishiyama, N., 2003. Interleukin-1beta abrogates long-term depression of hippocampal CA1 synaptic transmission. Synapse 47, 54-57.

Iyengar, R.L., Gandhi, S., Aneja, A., Thorpe, K., Razzouk, L., Greenberg, J., Mosovich, S., Farkouh, M.E., 2013. NSAIDs are associated with lower depression scores in patients with osteoarthritis. The American journal of medicine 126, 1017 e1011-1018.

Jenkins, J.K., Arend, W.P., 1993. Interleukin 1 receptor antagonist production in human monocytes is induced by IL-1 alpha, IL-3, IL-4 and GM-CSF. Cytokine 5, 407-415.

Kaufmann, W.E., Worley, P.F., Pegg, J., Bremer, M., Isakson, P., 1996. COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. Proceedings of the National Academy of Sciences of the United States of America 93, 2317-2321.

Kent, S., Bluthe, R.M., Kelley, K.W., Dantzer, R., 1992. Sickness behavior as a new target for drug development. Trends in pharmacological sciences 13, 24-28.

Kohler, O., Benros, M.E., Nordentoft, M., Farkouh, M.E., Iyengar, R.L., Mors, O., Krogh, J., 2014. Effect of anti-inflammatory treatment on depression, depressive symptoms, and adverse effects: a systematic review and meta-analysis of randomized clinical trials. JAMA psychiatry 71, 1381-1391.

Kubera, M., Curzytek, K., Duda, W., Leskiewicz, M., Basta-Kaim, A., Budziszewska, B., Roman, A., Zajicova, A., Holan, V., Szczesny, E., Lason, W., Maes, M., 2013. A new animal model of (chronic) depression induced by repeated and intermittent lipopolysaccharide administration for 4 months. Brain, behavior, and immunity 31, 96-104.

Libby, P., 2006. Inflammation and cardiovascular disease mechanisms. The American journal of clinical nutrition 83, 456S-460S.

Lopez-Castejon, G., Brough, D., 2011. Understanding the mechanism of IL-1beta secretion. Cytokine & growth factor reviews 22, 189-195.

Lu, B., Nagappan, G., Lu, Y., 2014. BDNF and synaptic plasticity, cognitive function, and dysfunction. Handbook of experimental pharmacology 220, 223-250.

Martin, S.A., Pence, B.D., Greene, R.M., Johnson, S.J., Dantzer, R., Kelley, K.W., Woods, J.A., 2013. Effects of voluntary wheel running on LPS-induced sickness behavior in aged mice. Brain, behavior, and immunity 29, 113-123.

McEachern, J.C., Shaw, C.A., 1999. The plasticity-pathology continuum: defining a role for the LTP phenomenon. Journal of neuroscience research 58, 42-61.

Medzhitov, R., 2010. Inflammation 2010: new adventures of an old flame. Cell 140, 771-776.

Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1993. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proceedings of the National Academy of Sciences of the United States of America 90, 11693-11697.

Morris, M.C., Gilliam, E.A., Button, J., Li, L., 2014. Dynamic modulation of innate immune response by varying dosages of lipopolysaccharide (LPS) in human monocytic cells. The Journal of biological chemistry 289, 21584-21590.

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L., Ragozzino, D., Gross, C.T., 2011. Synaptic pruning by microglia is necessary for normal brain development. Science 333, 1456-1458.

Pecchi, E., Dallaporta, M., Jean, A., Thirion, S., Troadec, J.D., 2009. Prostaglandins and sickness behavior: old story, new insights. Physiology & behavior 97, 279-292.

Rainsford, K.D., 2013. Ibuprofen: from invention to an OTC therapeutic mainstay. International journal of clinical practice. Supplement, 9-20.

Raison, C.L., Capuron, L., Miller, A.H., 2006. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends in immunology 27, 24-31.

Raison, C.L., Rutherford, R.E., Woolwine, B.J., Shuo, C., Schettler, P., Drake, D.F., Haroon, E., Miller, A.H., 2013. A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. JAMA psychiatry 70, 31-41.

Santello, M., Cali, C., Bezzi, P., 2012. Gliotransmission and the tripartite synapse. Advances in experimental medicine and biology 970, 307-331.

Shaw, K.N., Commins, S., O'Mara, S.M., 2003. Deficits in spatial learning and synaptic plasticity induced by the rapid and competitive broad-spectrum cyclooxygenase inhibitor ibuprofen are reversed by increasing endogenous brain-derived neurotrophic factor. The European journal of neuroscience 17, 2438-2446.

Shaw, K.N., Commins, S., O'Mara, S.M., 2005. Cyclooxygenase inhibition attenuates endotoxin-induced spatial learning deficits, but not an endotoxin-induced blockade of long-term potentiation. Brain research 1038, 231-237.

Tay, T.L., Savage, J.C., Hui, C.W., Bisht, K., Tremblay, M.E., 2017. Microglia across the lifespan: from origin to function in brain development, plasticity and cognition. The Journal of physiology 595, 1929-1945.

Tong, L., Balazs, R., Soiampornkul, R., Thangnipon, W., Cotman, C.W., 2008. Interleukin-1 beta impairs brain derived neurotrophic factor-induced signal transduction. Neurobiology of aging 29, 1380-1393.

Tremblay, M.E., Stevens, B., Sierra, A., Wake, H., Bessis, A., Nimmerjahn, A., 2011. The role of microglia in the healthy brain. The Journal of neuroscience : the official journal of the Society for Neuroscience 31, 16064-16069.

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.

Tyring, S., Gottlieb, A., Papp, K., Gordon, K., Leonardi, C., Wang, A., Lalla, D., Woolley, M., Jahreis, A., Zitnik, R., Cella, D., Krishnan, R., 2006. Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. Lancet 367, 29-35.

Ulich, T.R., Yin, S., Guo, K., Yi, E.S., Remick, D., del Castillo, J., 1991. Intratracheal injection of endotoxin and cytokines. II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. The American journal of pathology 138, 1097-1101.

Wieseler-Frank, J., Maier, S.F., Watkins, L.R., 2005. Immune-to-brain communication dynamically modulates pain: physiological and pathological consequences. Brain, behavior, and immunity 19, 104-111.

Wyss-Coray, T., Mucke, L., 2002. Inflammation in neurodegenerative disease--a double-edged sword. Neuron 35, 419-432.

Yamagata, K., Andreasson, K.I., Kaufmann, W.E., Barnes, C.A., Worley, P.F., 1993. Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. Neuron 11, 371-386.

Yirmiya, R., 1996. Endotoxin produces a depressive-like episode in rats. Brain research 711, 163-174.

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

Yirmiya, R., Pollak, Y., Barak, O., Avitsur, R., Ovadia, H., Bette, M., Weihe, E., Weidenfeld, J., 2001. Effects of antidepressant drugs on the behavioral and physiological responses to lipopolysaccharide (LPS) in rodents. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 24, 531-544.

Study 3. Combined fluoxetine and metformin treatment potentiates antidepressant efficacy increasing IGF2 expression in the hippocampus dorsal hippocampus

Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed drugs for the treatment of major depressive disorder (MDD), which constitutes an enormous medical, individual, societal and economical challenge and afflicts up to 10-15% of the population worldwide [1]. 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 [2]. To explain such incomplete efficacy, a novel hypothesis – named undirected susceptibility to change -- posits that the increase in serotonin levels induced by SSRIs does not affect mood per se, but enhances brain plasticity and thus amplifies the influence of the environment on the individual [3-6]. Therefore, SSRI treatment has not a univocal effect but, in a favorable environment, it would lead to a reduction of symptoms while in a stressful environment, has limited efficacy and may even lead to a worse prognosis [7].

A number of evidence supports a role for serotonin in increasing brain plasticity and enhancing susceptibility to environment [3, 8, 9]. In addition, the undirected susceptibility to change hypothesis has been recently demonstrated at preclinical and clinical levels. In preclinical studies, it has been shown that fluoxetine (FLX), one of the most commonly prescribed SSRIs, affects the molecular and behavioral depression-like phenotype according to the quality of the living environment: when administered in an enriched environment, it led to an improvement while, when administered in a stressful environment, FLX treatment led to a worsening of depression-like endpoints such as an enhanced anhedonic behavior and a reduced neurogenesis [5, 10]. At clinical level, it has been demonstrated that the commonly used SSRI citalopram amplifies the influence of the living conditions on mood, confirming that SSRI effects are affected by the environment [11].

The SSRI action on brain plasticity and susceptibility to environment opens new perspectives on how to improve the efficacy of these antidepressants by improving the quality of the patients' living environment. However, often it is not possible to act on the environment because of constraints due to patient's personal history and unchangeable life circumstances. In these cases, the pharmacological modulation of the factors underlying the link between living environment and SSRI action represents a novel and desirable strategy to improve treatment outcome in patients living in adverse conditions, very common in depressed patients.

Metabolism is markedly affected by the quality of the living environment. For instance, having an active physical and social life profoundly modulates metabolic markers [12, 13]. In turn, metabolic profile affects vulnerability to MDD and antidepressant efficacy. As an example, normalization of metabolic markers has been associated to remission following antidepressant treatment, while no change occurs in non-remitters [14]. Therefore, modification of metabolism represents a potential approach to modulate the interplay between environment and SSRIs in order to improve treatment outcome.

The aim of the present study was to assess whether the pharmacological modulation of metabolism may improve the limited efficacy of FLX when administered in a stressful environment. To this purpose, we used metformin (MET), a widely used drug to treat type II diabetes and other metabolic syndromes [15]. It affects metabolic profile both at peripheral and central levels since it crosses the blood-brain barrier [16, 17]. Though the underlying molecular mechanisms of MET action are yet to be fully determined, it has been reported in preclinical models that MET affects brain plasticity, increasing Long Term Potentiation (LTP) in the hippocampal CA1 region [18] and modulating neurotrophic factor levels, such as Brain Derived Neurotrophic factor (BDNF) [19].

Our prediction was that the combined FLX and MET treatment is more effective than FLX alone in counteracting depression-like phenotype in a stressful environment. According to our hypothesis, such enhanced efficacy is ascribable to the combined action of the two drugs: FLX increases brain plasticity, favoring a

change in mood, while MET, which affects the metabolic profile, drives the change toward an improvement. To this aim, we measured BDNF expression and LTP as molecular and cellular markers of neural plasticity, in addition to liking- and wanting-type anhedonia as endpoints of depression-like response.

Material and methods

Animals

C57BL/6 male mice 12–15 weeksold were used and kept under 12-hour light-dark cycle at 22–25°C. Mice were purchased at Envigo Italia (Udine, Italy). All procedures were carried out in accordance with the European law (EEC Council Directive 2010/63/UE86/609 1987), Italian legislation on animal experimentation (Decreto Legislativo 26/2014). In addition, animals were routinely examined for signs of discomfort as indicated by the animal care and use National Academy of Sciences of USA guidelines (National Research Council 2003).

Housing condition

For the entire duration of the experiment, animals were housed in the Intellicage system (TSE-system, NewBehavior AG, Zürich, Switzerland), which is an apparatus for automatic monitoring of mouse behavior. It consists of a large acrylic cage (20.5 cm high, 58 cm × 40 cm at the top and 55 cm × 37.5 cm at the base, Model 2000 Tecniplast, Buguggiate, VA, Italy) with 4 walls separating each corner from the center so that they form 4 identical triangular conditioning chambers (15 × 15 × 21 cm). Animals have access to the chamber by entering a front hole (chamber entrance). Only one mouse at a time can enter the chamber. Once entered, it is identified through a transponder-antenna system. The system is able to collect data about number and duration of visits and number, duration and side (right or left) of nosepokes and licks. The floor of the cage was covered with bedding and contains four sleeping shelters in the center while on the top a food rack is present filled with standard mouse chow (food ad libitum). An additional cage (SocialBoxes) was used to expand existing Intellicage to a multiarea system, thus we increased the capacity of the system to test

simultaneously more mice. One week before being moved to the Intellicage, each animal was injected with a subcutaneous transponder (T-IS 8010 FDX-B; Datamars SA, Switzerland). Mice have been gradually habituated to the Intellicage environment during a 14-days period (habituation period).

Treatment

After the first two weeks of stressful condition, aimed at inducing a depression-like phenotype, mice continued to be exposed to the unpredictable chronic mild stress (see below) for 4 weeks receiving one of the following treatments: VEH, FLX, MET or FLX and MET together.

FLX (Fluoxetine HCl, SantaCruz, USA) and MET (Metformin, SigmaAldrich, St Louis, MO, USA) were dissolved in water and in saccharin solution and delivered ad libitum in the drinking bottles for 4 weeks. Compared to injection, this administration method allows avoiding the stress due to the manipulation. The solutions were prepared according to the mouse average weight and daily water consumption in order to provide respectively an average daily intake of 30 mg/kg of FLX [20] and 200 mg/kg of MET [21, 22]. Bottles with FLX and with FLX and MET were wrapped in tin foil to protect the substance from light. Metformin, fluoxetine and their combination were dissolved in both water and saccharin solution to avoid that saccharin preference could affect the amount of drug received. The average amount of fluoxetine or metformin administered did not differ among the experimental groups receiving the same compound. Though we did not perform a pharmacokinetic analysis, to our knowledge, no interaction between fluoxetine and metformin has been reported.

Environmental conditions

All mice were exposed to the stressful condition for two weeks to induce the depression-like behavior. For the following 4 weeks, the subjects went on being exposed to the stressful condition or were exposed to the standard condition.

Stressful condition: mice were exposed to unpredictable chronic mild stress procedure to induce depression-like behavior (Figure S1). To prevent habituation to stress, mice were exposed each day to a different stressful procedure, randomly chosen among the procedures provided by the Intellicage. The procedures were Short open door: door to access water or saccharin solution remains open for only 1.5 seconds; Delay: door opens with a delay of 1, 1.5, 2, 2.5 seconds after the first nosepoke; Open door 25%: door opens only following 25% of nosepokes; Air puff: when the mouse performs a visit, it has a 20% chance to receive an air puff (2 bar) which lasts 1-sec or until the animal leaves the corner. In the latter case, the doors remain closed. Once each one of these procedures ended, in order to re-open the doors and drink again, the animals had to leave the corner and start a new visit. The duration of each stressful procedure was randomly chosen: 12, 18 or 24 hrs. In addition, during the stressful condition, no shelter or tissue paper was provided.

Standard condition: mice were socially housed in the Intellicages 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 (Figure S2a).

Behavioral tests

Behavioral endpoints investigated are liking- and wanting-type anhedonia. These were automatically assessed by the Intellicage avoiding any bias or stress due to the experimenter.

Liking-type anhedonia - Saccharin preference

To assess liking-type anhedonia we measured saccharin preference. Two bottles were present in each corner of the Intellicage , one containing tap water and

the other containing the saccharin solution 0.1%; both were freely available 24/24 h. Water and saccharin solution were substituted everyday. The position of water and saccharin in each corner was counterbalanced across the four corners. Saccharin preference was determined as follows: (saccharin solution consumed/ saccharin solution consumed + water consumed) × 100. We measured the baseline saccharin preference across a two days period (i) at the end of the habituation period, (ii) at the end of the first two weeks of exposure to the stressful condition (aimed at inducing the depression-like phenotype), (iii) 1 week after the beginning of the treatment period, (iv) at the end of the treatment period. Mice were exposed to the saccharin solution only (all bottles filled with saccharin solution) during the first two days of the Intellicage habituation period in order to make them used to the saccharin flavor. In the remaining 12 days, mice could choose between water and saccharin solution.

Wanting-type anhedonia - Progressive Ratio schedule

To assess wanting-type anhedonia, i.e. the drive for obtaining a reward, we used the Progressive Ratio reinforcement schedule that utilizes a multiplicative increase in the number of responses (nosepokes) required to dispense a unit of reinforce (i.e. access to saccharin). In particular, water was always accessible after one nosepoke while saccharin solution was accessible only after a specific number of nosepokes that increases progressively. After each series of 8 visits, the number of nosepokes required to access saccharin increases according to the following schedule: 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20, 24. After reaching the 24 nosepoke level, mice had freely access to saccharin following one nosepoke. The time for performing the nosepokes increased gradually according to the number of nosepokes requested from one to 24 sec. Mice were exposed to this test at the end of the habituation period, immediately before the treatment period and after both 1 and 4 weeks of treatment. To make the mice aware of the progressive ratio testing, the three LEDs on the top of each door were kept turned on throughout the test. Each test session lasted two days.

RNA extraction and RT-RTqPCR

Following 4 weeks of treatment in the stressful condition animals were sacrificed by decapitation, the brains removed and the dorsal and ventral parts of the hippocampus dissected, rapidly frozen and then stored at -80 °C for further molecular analyses. The same animals tested for behavior were analyzed for mRNA expression. Total RNA, from ventral and dorsal hippocampi, was prepared combining extraction whit TRI Reagent® and GenElute™ Mammalian Total RNA Miniprep Kit and (Sigma Aldrich ®, Milan, Italy) as previously described [4]. Two µg of total RNA were reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA USA) in a final reaction volume of 20 µL [23]. The cDNA was stored at -20 °C until Real Time PCR that was performed in Roche LightCycler® 480 (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) using Power SYBR Green mix (Life Technologies Corporation, Carlsbad, CA, USA). The following forward and reverse sequences were used at the final concentration of 150 nM: for IGF1 F5'-TGCTCTTCAGTTCGTGTG-3' and R5'-ACATCTCCAGTCTCCTCAG-3'; for IGF2 F5'-CGCTTCAGTTTGTCTGTTCG-3' and R5'-GGAAGTACGGCCTGAGAGGTA-3'; for BDNF F5'-CCATAAGGACGCGGACTTGTA C-3' and R5'-AGACATGTTTGCGGCATCCAGG-3'; for p11 (S100a10) F5'-CTTCAA AATGCCATC CCAAA-3' and R5'-TATTTTGTCCACAGCCAGAGG-3', for leptin F5'-AAGAAGATCCCAGGGAGGA and R5'-TGATGAGGGTTTTGGTGTCA, and for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) F5'-TTCGCAAAACAAGTTCACCA-3' and 5'-TCGTTGTGGTTGTAAATGGAA-3' as a house-keeping gene. Melt curve analyse and agarose gel separations were performed at the end of every RTqPCR to confirm formation of a single PCR product. Ct (cycle threshold) value was determined by the LightCycler® 480 Software (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) and mRNA expression was calculated with the ΔΔCt method with GAPDH as endogenous control as previously described [24].

Relative expression of the genes of interest was performed by using as calibrator (RQ value=1) expression levels in the ventral hippocampus of vehicle treated animals. All qPCR reactions based upon the same primer-set were run in the same amplification plate to compare the levels of mRNAs expression between the two parts of the hippocampus.

Electrophysiology

Hippocampal slice preparation

In order to perform electrophysiological experiments, acute hippocampal slices were collected. At the end of treatment period in the stressful condition, animals were anesthetized by inhalation of halothane (Sigma-Aldrich S.r.l., Milan, Italy) and decapitated. The brain was rapidly removed from the skull and immersed in ice-cold artificial cerebrospinal fluid (ACSF) solution composed of (in mM): NaCl 125, KCl 4, CaCl2 2.5, MgSO4 1.5, NaHPO4 1, NaHCO3 26 and glucose 10). ACSF was continuously bubbled with 95% O2 + 5% CO2 to maintain a pH close to 7.4.

Following removal, the brain was hemisected along the longitudinal fissure to separate the two hemispheres. Brain dissection was carried out according to the slicing plane chosen and the structure to be investigated. Specifically, for experiments on the ventral hippocampus, slices were cut perpendicular to the longitudinal axis from the temporal pole of the brain. For experiments on the dorsal hippocampus, coronal slices were cut from the frontal pole. Dorsal and ventral slices have been identified as the distance, in μm, from the frontal and temporal pole respectively (approximately from 400 to 1750 μm). The brain tissues were blocked on the stage of a vibrating microtome (Thermo Scientific, USA) and 350 μm thick slices were cut in ice-cold ACSF. The slices were then transferred to an incubation chamber containing oxygenated ACSF, where they were allowed to recover for 1 h at 30 °C prior to electrophysiological recording. After this period, the slices were transferred to the interface slice-recording chamber (BSC1, Scientific System Design Inc) to perform experiments within 1–6 h after slice preparation. Dorsal and ventral slices were prepared from separate hemispheres of the same brain and were obtained alternately from right or left hemisphere.

Extracellular field recordings

For field recordings, individual slices were maintained at 30–32 °C and superfused with ACSF at 2 ml/min by a peristaltic pump. A concentric bipolar stimulating electrode (SNE-100 × 50 mm long, Elektronik–Harvard Apparatus GmbH) was placed in the stratum radiatum to stimulate Schaffer collateral fibers. Stimuli consisted of 100 μs constant current pulses of variable intensity, applied at 0.05 Hz. A glass micropipette (0.5–1 MΩ) filled with ACSF was placed in the CA1 hippocampal region, at 200–600 μm from the stimulating electrode, in order to measure orthodromically-evoked field extracellular postsynaptic potentials (fEPSP). Stimulus intensity was adjusted to evoke fEPSPs of amplitude about 50% of maximal amplitude with minimal contamination by a population spike. Evoked responses were monitored online and stable baseline responses were recorded for at least 10 min. Only the slices that showed stable fEPSP amplitudes were included in the experiments. LTP was induced by high-frequency stimulation (HFS, 1 train of stimuli at 100 Hz of 1 s duration), repeated after 30 min. To analyze the time course of fEPSP slope, the recorded fEPSP was routinely averaged over 1 min (n = 3). fEPSP slope changes following the LTP induction protocol at 31 and 61 min post tetanus were calculated with respect to the baseline (1 minute before induction). N/n refers to the number of slices on total number of mice analyzed.

The paired-pulse ratio (PPR) was measured from responses to two synaptic stimuli at 50 ms inter-stimulus interval. PPR was calculated as the ratio between the fEPSP amplitude evoked by the second stimulus (A2) over the first (A1; A2/A1).

fEPSPs were recorded and filtered (low pass at 1 kHz) with an Axopatch 200 A amplifier (Axon Instruments, CA) and digitized at 10 kHz with an A/D converter (Digidata 1322 A, Axon Instruments). Data acquisition was stored on a computer using pClamp 9 software (Axon Instruments) and analyzed off-line with Clampfit 10 program (Axon Instruments).

Data and Statistical analysis

All data were analyzed with one-way ANOVA with the Statistical software Statview II (Abacus Concepts, CA, USA), comparing VEH versus FLX, MET and FLX plus MET treated mice. When a significant main effect was found, selected pairwise comparisons were made using Tukey post-hoc analysis.

Results

FLX and MET combination is effective in alleviating depression-like behavior

As behavioral phenotypes of depression, we assessed the liking- and wanting-type anhedonia, which have been previously shown to be susceptible to stress and SSRI treatment [5, 10, 25].

The two weeks of chronic stress before treatment were effective in inducing a depression-like profile. In particular, saccharin preference (liking-type anhedonia) dropped from around 90 to 55 percent [$F(1,37)=87.870$, $p<0.0001$, Figure 1a] and the breakpoint level (wanting-type anhedonia) was significantly reduced [$F(1,39)=17.874$, $p<0.0001$, Figure 1b]. No difference in weight between treated and control groups was found (data not shown), indicating that the stress procedure did not differentially affect the experimental groups. Following the induction of a depression-like profile, mice receiving the combination of FLX and MET showed an improvement of their behavioral phenotype when compared to the other experimental groups. Specifically, following 1 week of treatment, liking-type anhedonia was significantly affected by treatment [$F(3,34)=6.126$, $p=0.0019$], post-hoc analysis revealing that FLX-MET mice displayed an increased saccharin preference compared both to VEH, FLX and MET mice (respectively, $p<0.05$, $p<0.05$ and $p<0.001$; Figure 1a). Wanting-type anhedonia was significantly affected by treatment as well [$F(3,35)=3.047$, $p=0.0414$]. FLX-MET mice showed a significant increase of the breakpoint level compared both to FLX and MET mice (respectively, $p<0.05$ and $p<0.05$, Figure 1b]). At the end of the treatment, the prolonged exposure to stress (6 weeks) led to a marked anhedonic profile in all groups, flattening the potential differences in liking-type anhedonia. As for wanting-type anhedonia, a significant main effect of treatment was found [$F(3,35)=4.329$, $p=0.0107$]. In particular, FLX-MET mice reached a higher breakpoint level compare to MET mice ($p<0.001$, Figure 1b).

All mice to be treated in the standard condition showed a significant increase of the depression-like phenotype following the two weeks of exposure to the stressful condition (Figure S2b and c). In particular, both liking-type anhedonia [$F(1,41)=43.721$, $p<0.0001$] and wanting-type anhedonia [$F(1,40)=10.681$, $p=0.0022$] were significantly reduced. Afterwards, when receiving VEH, FLX, MET or FLX-MET in a standard condition, they showed no difference in depression-like behavior. In particular, all experimental groups showed a full recovery, displaying no anhedonic response, both at 1 and 4 weeks of treatment.

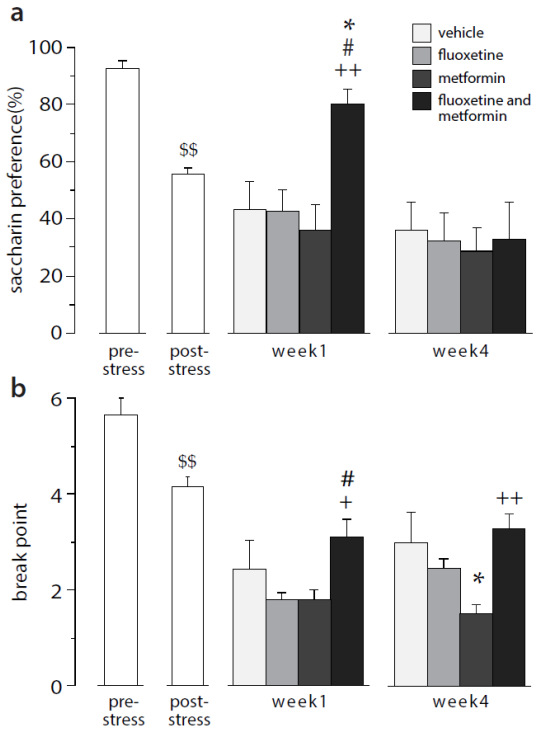

Figure 1. Effects of treatment with fluoxetine, metformin or their combination on depression-like behavior. **(a)** liking-type anhedonia. Saccharin preference significantly decreased following exposure to stressful procedure. After the first week of treatment FLX-MET mice displayed an increased saccharin preference compared both to VEH, FLX and MET mice; **(b)** wanting-type anhedonia. The breakpoint level was significantly reduced after the unpredictable chronic mild stress. Following the first week of treatment, FLX-MET mice showed a significant increase of the breakpoint level compared both to FLX and MET mice. Treatments as indicated in the legend, n=9-10 mice per group. $$p<0.0001 pre- vs post-stress, *p<0.05 and **p<0.001 vs VEH, #p< 0.05 and ##p<0.0001 vs FLX, +p>0.05 and ++p<0.001 vs MET. Data are presented as mean + S.E.M.

IGF2 mRNA levels are increased in the dorsal hippocampus of mice receiving the combined treatment

To explore the molecular bases of treatment effect, we analyzed gene expression of selected targets reported to be involved in MDD and metabolism. In particular, we focused on IGF2 and IGF1, p11, BDNF and leptin mRNAs expression in the dorsal and ventral hippocampus. These hippocampal areas have been reported to be differently involved in MDD and antidepressant efficacy [26-30].

IGF2 analysis revealed a significant main effect of treatment [$F(3,50)=3.370$, $p=0.0256$] and a significant interaction treatment x hippocampal region [$F(3,50)=5.912$, $p=0.0015$; Figure 2a]. Post-hoc analysis revealed that, overall, mice receiving the combined treatment showed higher IGF2 expression compared to VEH and FLX groups. With regard to the dorsal hippocampus, they displayed higher IGF2 levels compared to VEH ($p < 0.05$), MET ($p < 0.05$) and FLX ($p < 0.01$). FLX-MET mice showed also significantly higher IGF2 levels in the dorsal compared to the ventral region ($p < 0.01$). As for the other genetic markers investigated, IGF1, BDNF, p11 and leptin, we found no effect of treatment but a significant main effect of hippocampal region [$Fs(1,50)= 29.161, 96.221, 114.972, 126.865$, $ps <0.001$, Figure 2b-e)]. In particular, IGF1 and BDNF levels were higher in dorsal, while p11 and leptin were higher in ventral hippocampus.

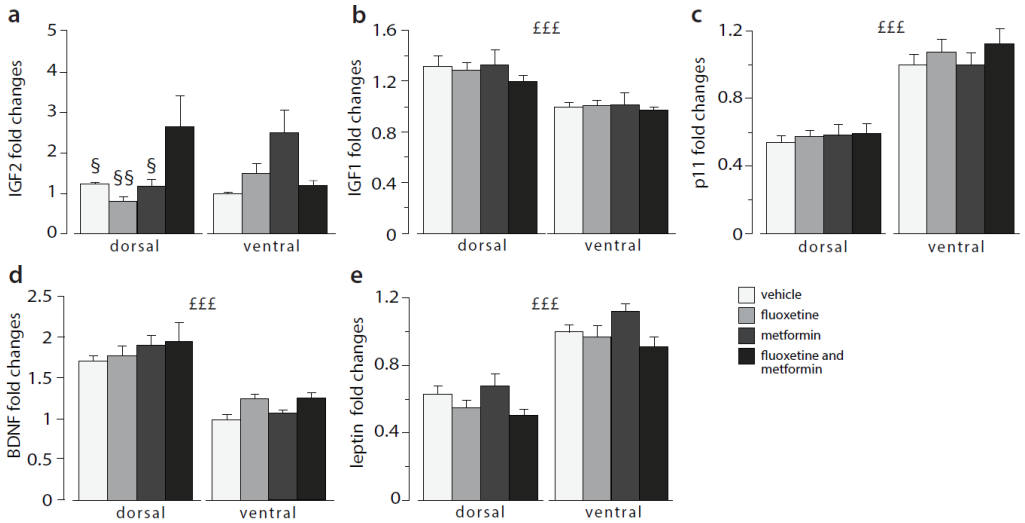

Figure 2. Effects of fluoxetine, metformin or their combination on expression of genes involved in brain plasticity in the dorsal and ventral hippocampus. (a) IGF2 is significantly increased by the combined treatment compared to VEH and FLX alone, suggesting that this growth factor is involved in the antidepressant action of the FLX-MET treatment. Such effect concerned mainly the dorsal hippocampus, where FLX-MET treatment increased IGF2 expression compared to all the other groups. (b) IGF1, (c) p11, (d) BDNF and (e) leptin expression was not affected by treatment but IGF1 and BDNF levels were overall higher in dorsal, while p11 and leptin were higher in ventral hippocampus. Treatments as indicated in the legend, n = 6-8 mice per group. £££ $p<0.001$, main effect of hippocampal region, § $p<0.05$ and §§ $p<0.01$ vs FLX-MET in the dorsal region. Data are presented as mean + S.E.M.

LTP in the dorsal and ventral hippocampal regions is differentially affected by treatment

We explored plasticity processes in CA1 hippocampal region by recording LTP evoked by two spaced (30 minutes apart) Schaffer collaterals stimulations in both dorsal and ventral hippocampus. Interestingly, during the second stimulation, the main effect of treatment emerged [$F(3,60) = 3.321$, $p=0.026$]. In addition, the main effect of hippocampal area and treatment x hippocampal region interaction were very close to reach statistical significance [$F(1,60)=3.473$, $p=0.067$ and $F(3,60)=2.523$, $p=0.066$, respectively]. Post-hoc analysis revealed that, in the dorsal hippocampus, FLX-MET treated mice show an increased LTP amplitude (1.495±0.065) compared to MET (1.226±0.061, $p < 0.05$) and VEH (1.260±0.058, $p < 0.05$, Figure 3a, left), but not to FLX (1.406 ± 0.069). By contrast, in the ventral region, the combined treatment showed a trend toward a reduction of LTP amplitude compared to FLX alone and VEH (1.387±0.058 *vs* 1.525±0.065 and 1.479±0.061, Figure 3a, right) but was similar to MET alone (1.323±0.0759). Finally, in the VEH group, the magnitude of LTP was higher in the ventral compared the dorsal hippocampus ($p=0.012$).

With regard to PPR, a main effect of hippocampal region was observed following treatment [$F(1,139)=157.357$, $p<0.001$], being its value higher in dorsal hippocampus for all treatments ($p<0.001$, Figure 3b). In the VEH group, PPR was 1.402 ± 0.034 and 1.081 ± 0.041, in the dorsal and the ventral hippocampus, respectively. No treatment effect was observed.

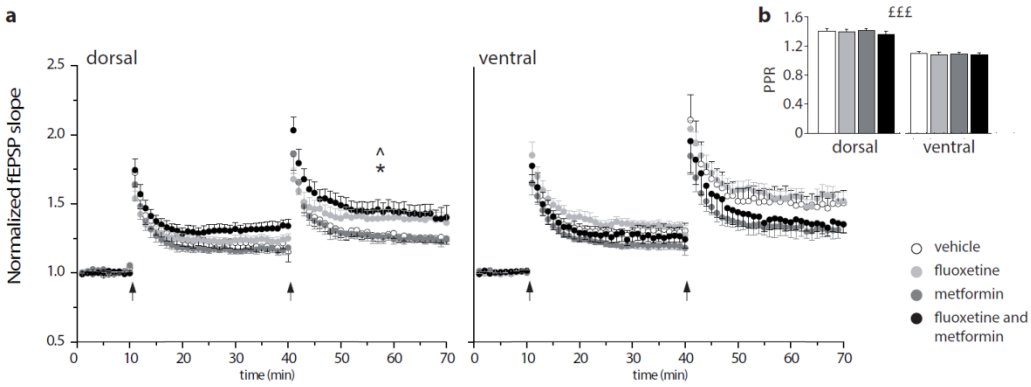

Figure 3. LTP in the dorsal and ventral hippocampus is differentially affected by fluoxetine, metformin or their combination. (a) LTP from extracellular records in dorsal and ventral hippocampus. Time course of fEPSP slope responses evoked at 0.05 Hz and normalized as detailed in the Methods. Arrows indicate repeated spaced HFS (2 train at 100 Hz of 1 s duration, 30 minutes apart). Treatments as indicated in the legend (in dorsal hippocampus, VEH: n=12/8; FLX: n=10/9, MET: n=12/9 and FLX-MET: n=10/7; in ventral hippocampus, VEH: n=10/10, FLX: n=8/8, MET: n=9/8 and FLX-MET: n=11/9). Note that in dorsal hippocampus, FLX-MET mice show an increased LTP compared to MET or VEH and that in VEH, LTP is higher in ventral compared to dorsal. Tukey t-test post hoc analysis, 20 minutes after the second HFS. * $p< 0.05$ FLX-MET vs VEH, ^ $p< 0.05$ FLX-MET vs MET. (b) PPR. Bar histogram indicate averaged PPR values for dorsal hippocampus (VEH: n=20/12, FLX: n=20/12, MET: n=19/11 and FLX-MET: n=21/12) and ventral hippocampus (VEH: n=14/11, FLX: n=16/11, MET: n=21/12 and FLX-MET: n=16/10). Note that PPR is not affected by treatments and it is higher in dorsal compared to ventral hippocampus. Treatments as indicated in the legend, £££ $p< 0.001$ main effect of hippocampal region. Data are presented as mean + S.E.M.

Discussion

The present results show that the combination of FLX and MET administered in the stressful condition ameliorates the depression-like phenotype compared to FLX alone and VEH after one but not after four weeks. The combination of FLX and MET led also to increased IGF2 expression and enhanced LTP, specifically in the dorsal hippocampus, at the end of treatment.

Previous findings by us [5, 10] indicate that FLX alone administered in a chronic stress condition has limited beneficial effects or leads to a worsening of depression-like behavior. This is in line with previous studies [31-34]. However other studies found that mice treated with SSRI in a stressful environment show an improvement of the depression-like profile [35, 36]. Here, we confirm that, compared to VEH, FLX has limited beneficial effects when administered in adverse conditions. However, the co-treatment of MET and FLX counteracts the detrimental effects induced by the exposure to stress following 1 week of treatment. In particular, FLX and MET combination increases saccharin preference to the level the mice had before chronic stress compared to both VEH and FLX alone. Similar results have been found for wanting type anhedonia, mice treated with the combined treatment showing a higher motivation to obtain the reward compared to those receiving FLX only. The results collected at four weeks show no difference among the experimental groups, indicating that the combined treatment has not long lasting beneficial effects on depression-like behavior and should be used for subacute interventions. It is worth noting that, when administered in standard condition, treatments did not produced different effects, all experimental groups showing a recovery of the anhedonic profile both at week 1 and 4. These results confirm that the combination MET-FLX is an effective therapeutic approach when administered to subjects living in stressful conditions and support our hypothesis that FLX treatment outcome depends on the quality of the environment [7].

To explore the molecular mechanisms associated to the therapeutic action of the combined treatment, we analyzed the expression of selected genes reportedly involved in MDD and modulated by FLX and MET [37, 38]. In particular, we focused

on IGF2 that is a key molecule in vulnerability to stress and a potential molecular target able to trigger antidepressant action [39-41]. A decrease in IGF2 hippocampal expression is significantly associated to depression-like behavior induced through chronic restraint stress [40-42]. Accordingly, IGF2 overexpression was found to rescue the neurobehavioral effects of stress exposure [40]. In addition, recent evidence indicates that IGF2 administration enhances adult neurogenesis in the hippocampal dentate gyrus [43], considered a marker of recovery from MDD [44, 45], indicating IGF2 as involved in switching from depressive-like to healthy phenotype. IGF2 has been also reported to be a key target of ketamine [39], a novel antidepressant drug, which has a rapid but not long-lasting action [46], similarly to the effect of the combined FLX-MET treatment reported here. This suggests that IGF2 might be involved mainly in first phase recovery from MDD. Here we found that IGF2 is significantly increased by the combined treatment compared to VEH and FLX alone, suggesting that this growth factor might be involved in the antidepressant action of the FLX-MET treatment. However, since IGF2 expression has been associated to enhanced learning and memory [47], the differences in wanting-type anhedonia, assessed through a progressive ratio learning paradigm, could be ascribed also to the differences in learning abilities associated to IGF2 levels. The differences in the IGF2 expression levels concern mainly the dorsal hippocampus. Despite the classic view on anatomical segregation of the hippocampal function considers the dorsal part to be involved in learning and memory while the ventral in emotional and stress response [48, 49], an increasing number of studies is challenging this dichotomy view [50, 51]. Indeed, novel evidence indicates that dorsal hippocampus is implicated in MDD [52-54] and is an important target for antidepressants [29, 30, 53-55]. For instance, though the ventral region shows the highest expression levels of most markers of antidepressant action, such as the 5-HT1a receptor in the dentate gyrus [56], the dorsal region expresses at high levels specific markers, including the 5-HT6 receptor, emerging as relevant regulators of depression-like behavior as well [57, 58].

We also analyzed the expression levels of other metabolic markers related to MDD such as IGF1, p11, BDNF and leptin, but these were not affected by treatments. Nevertheless, all of them showed a significantly different expression in the two hippocampal regions. The adipose-derived hormone leptin is well known for its function in controlling energy homeostasis and has been recently involved in regulating mood and emotion [59, 60]. Low levels of leptin are associated to depression in humans and preclinical models as well as pharmacological studies indicate leptin as a potential antidepressant drug [61]. Here, we observe an increased leptin expression in ventral compared to dorsal hippocampus. Such specificity is in line with previous data showing that leptin differently affects memory and food intake when administered in the dorsal or the ventral hippocampus [62]. Similar to leptin, the expression of p11 (also known as S100A10), involved in the regulation of depression-like behavior and response to antidepressants [63-65], was not modified by treatments, but its expression levels were higher in the ventral compared to the dorsal hippocampus.

BDNF is a neurotrophic factor particularly abundant in hippocampal neurons [66] that has been indicated as key player in the pathophysiology of MDD. Indeed, according to the "neurotrophic hypothesis of depression", the psychopathology is associated with the reduction of brain BDNF levels and antidepressant treatments alleviate depressive symptoms increasing its levels [6, 67]. BDNF is reported to be expressed at higher levels in dorsal compared to ventral hippocampus [68]. We here confirm this finding. In addition, we replicate data from our and other research groups showing that FLX treatment does not increase the levels of this neurotrophin in a stressful environment [4]. In line with previous data, BDNF levels were not affected also by MET [69]. Akin to IGF1, BDNF levels were higher in dorsal compared to ventral hippocampus.

Similar to gene expression, physiological properties differ along the longitudinal axis of the hippocampus. For instance, in the CA1 region [70-72], LTP magnitude is smaller in the ventral than in the dorsal hippocampus [73, 74, 75] and is differentially modulated by stress in the two regions [76-81], being reduced or not

affected in dorsal but increased in ventral hippocampus following both acute [82, 83] and chronic stress [27]. In line with these findings, we show here that the LTP magnitude was smaller in dorsal compared to ventral hippocampus. This might be due to the different distribution and effects exerted by the corticosteroid receptors, mineralcorticoids (MRs) and glucocorticoids (GRs), on LTP after exposure to stress. In particular, it has been reported that MRs, more expressed in the ventral part [84], facilitate LTP [85, 86], while GRs, more abundant in the dorsal part of the hippocampus [84], impair LTP [87].

Interestingly, the treatments differentially affected LTP in the two hippocampal regions. In particular, the co-treatment FLX-MET produced a significant increase in LTP amplitude in dorsal hippocampus, which parallels the significant IGF2 expression increase observed in this region. Given the role of IGF2 in modulating biological processes involved in neuronal plasticity [88], such as promoting dendritic spine formation[89] and enhancing pERK1/2 and GluR1 [47], the IGF2 increase might be involved in the plasticity enhancement we observed. By contrast, no significant difference in LTP was found in the ventral part, suggesting that FLX and MET, alone or in combination, do not regulate plasticity in this area. This evidence suggests that the combination FLX and MET affects the electrophysiological activity specifically in the dorsal hippocampus which has been reported as potential target for antidepressant treatments [29, 30, 53-55]. Such LTP amplitude enhancement, in addition to the increased IGF2 expression, in a brain region reportedly involved in learning processes further support that these changes might contribute to the differences in progressive ratio paradigm used to assess wanting-type anhedonia.

The major limitations of the present study include the lack of the analysis of the molecular and cellular endpoints after 1 week of treatment in order to better investigate the association between behavioral changes and modifications in neurophysiological substrates. In addition, a pharmacokinetic analysis of the possible interaction between metformin and fluoxetine would have better illustrated whether the co-administration affects their bioavailability. Finally, given that MDD affects

mostly female with a female:male ratio of approximately 2:1, the assessment of the effect of the treatments not only in males, but also in female individuals will be extremely relevant.

Conclusions

In conclusion, previous works by us and others have found that FLX administration has beneficial effects in an enriched environment but has no effects or even leads to detrimental outcome when administered in a stressful environment [4, 5, 90]. Here we show that the combined treatment FLX and MET is more effective than FLX alone and VEH in the short-term when administered in individuals exposed to a stressful condition. Therefore, this polypharmacological strategy appears effective to counteract the potential limited efficacy of FLX in individuals living in adverse conditions. This might be highly relevant in the clinic because, with very few exceptions, people cannot rapidly and effectively change their life circumstance, and adverse conditions are very common in depressed patients.

Supplementary materials

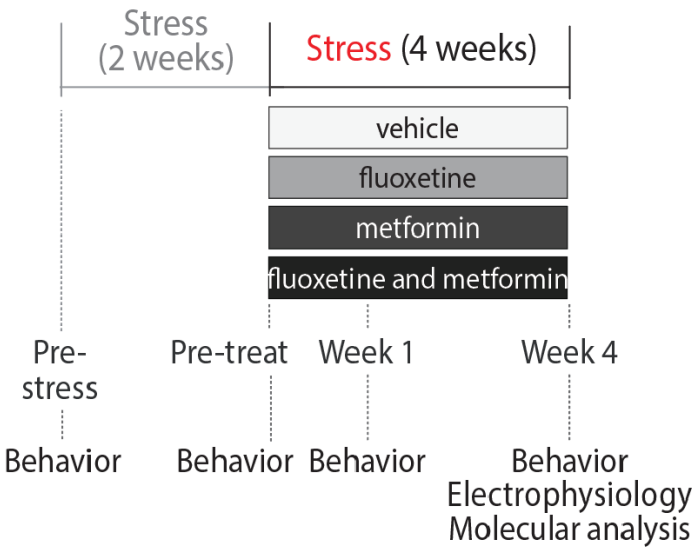

Figure S1 - Experimental design of the stressful condition. Mice were exposed to stress for six weeks. The first two induced the depression-like phenotype. During the following four weeks of stress, the subjects were treated for with fluoxetine, metformin or their combination.

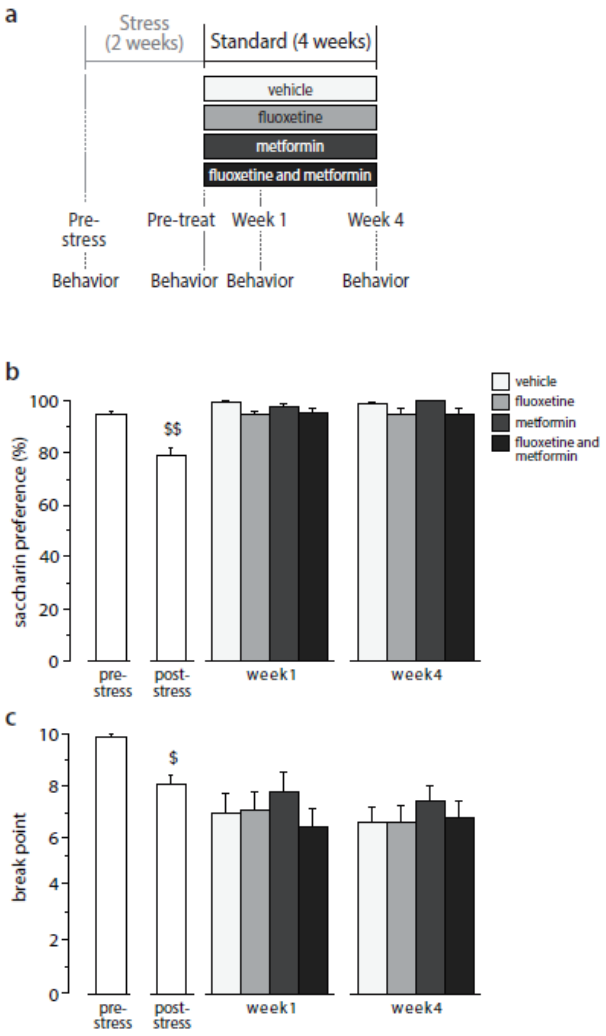

Figure S2 - Experimental design and effects of fluoxetine, metformin or their combination on depression-like behavior in standard condition: (a) First mice were exposed for two weeks to the stressful condition to induce a depression-like phenotype. Afterwards, they were treated for four weeks in the standard condition. (b) Liking-type anhedonia. Saccharin preference significantly decreased following exposure to stress. Treatments did not produce different effects. (c) Wanting-type anhedonia. The breakpoint level was significantly reduced after the unpredictable chronic mild stress. Treatments did not produce different effects.

References

1. WHO. Depression and Other Common Mental Disorders: Global Health Estimates. 2017.
2. Trivedi MH, Rush AJ, Wisniewski SR, Nierenberg AA, Warden D, Ritz L, et al. Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice. Am J Psychiatry. 2006;163(1):28-40.
3. Maya Vetencourt JF, Sale A, Viegi A, Baroncelli L, De Pasquale R, O'Leary OF, et al. The antidepressant fluoxetine restores plasticity in the adult visual cortex. Science. 2008;320(5874):385-8.
4. Alboni S, van Dijk RM, Poggini S, Milior G, Perrotta M, Drenth T, et al. Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Molecular psychiatry. 2017;22(4):552-61.
5. Branchi I, Santarelli S, Capoccia S, Poggini S, D'Andrea I, Cirulli F, et al. Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice. PLoS One. 2013;8(4):e62226.
6. Castren E, Kojima M. Brain-derived neurotrophic factor in mood disorders and antidepressant treatments. Neurobiol Dis. 2017;97(Pt B):119-26.
7. Branchi I. The double edged sword of neural plasticity: increasing serotonin levels leads to both greater vulnerability to depression and improved capacity to recover. Psychoneuroendocrinology. 2011;36(3):339-51.
8. Belsky J, Jonassaint C, Pluess M, Stanton M, Brummett B, Williams R. Vulnerability genes or plasticity genes? Molecular psychiatry. 2009;14(8):746-54.
9. Bakermans-Kranenburg MJ, van IMH. The hidden efficacy of interventions: genexenvironment experiments from a differential susceptibility perspective. Annual review of psychology. 2015;66:381-409.
10. Alboni S, van Dijk M, Poggini S, Milior G, Perrotta ML, Drenth T, et al. Hippocampus-related effects of fluoxetine treatment under stressful vs enriched conditions. Molecular psychiatry. 2017;22(4):483.
11. Chiarotti F, Viglione A, Giuliani A, Branchi I. Citalopram amplifies the influence of living conditions on mood in depressed patients enrolled in the STAR*D study. Transl Psychiatry. 2017;7(3):e1066.
12. Hafner S, Zierer A, Emeny RT, Thorand B, Herder C, Koenig W, et al. Social isolation and depressed mood are associated with elevated serum leptin levels in men but not in women. Psychoneuroendocrinology. 2011;36(2):200-9.

13. Corpeleijn E, Feskens EJ, Jansen EH, Mensink M, Saris WH, Blaak EE. Lifestyle intervention and adipokine levels in subjects at high risk for type 2 diabetes: the Study on Lifestyle intervention and Impaired glucose tolerance Maastricht (SLIM). Diabetes care. 2007;30(12):3125-7.
14. Weber-Hamann B, Kratzsch J, Kopf D, Lederbogen F, Gilles M, Heuser I, et al. Resistin and adiponectin in major depression: the association with free cortisol and effects of antidepressant treatment. Journal of psychiatric research. 2007;41(3-4):344-50.
15. Pernicova I, Korbonits M. Metformin--mode of action and clinical implications for diabetes and cancer. Nat Rev Endocrinol. 2014;10(3):143-56.
16. Wang Y, Li G, Goode J, Paz JC, Ouyang K, Screaton R, et al. Inositol-1,4,5-trisphosphate receptor regulates hepatic gluconeogenesis in fasting and diabetes. Nature. 2012;485(7396):128-32.
17. Grommes C, Karlo JC, Caprariello A, Blankenship D, Dechant A, Landreth GE. The PPARgamma agonist pioglitazone crosses the blood-brain barrier and reduces tumor growth in a human xenograft model. Cancer chemotherapy and pharmacology. 2013;71(4):929-36.
18. Lennox R, Porter DW, Flatt PR, Holscher C, Irwin N, Gault VA. Comparison of the independent and combined effects of sub-chronic therapy with metformin and a stable GLP-1 receptor agonist on cognitive function, hippocampal synaptic plasticity and metabolic control in high-fat fed mice. Neuropharmacology. 2014;86:22-30.
19. Ghadernezhad N, Khalaj L, Pazoki-Toroudi H, Mirmasoumi M, Ashabi G. Metformin pretreatment enhanced learning and memory in cerebral forebrain ischaemia: the role of the AMPK/BDNF/P70SK signalling pathway. Pharm Biol. 2016;54(10):2211-9.
20. Dulawa SC, Holick KA, Gundersen B, Hen R. Effects of chronic fluoxetine in animal models of anxiety and depression. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2004;29(7):1321-30.
21. Cheng DM, Pogrebnyak N, Kuhn P, Poulev A, Waterman C, Rojas-Silva P, et al. Polyphenol-rich Rutgers Scarlet Lettuce improves glucose metabolism and liver lipid accumulation in diet-induced obese C57BL/6 mice. Nutrition. 2014;30(7-8 Suppl):S52-8.
22. Green BD, Irwin N, Duffy NA, Gault VA, O'Harte F P, Flatt PR. Inhibition of dipeptidyl peptidase-IV activity by metformin enhances the antidiabetic effects of glucagon-like peptide-1. European journal of pharmacology. 2006;547(1-3):192-9.

23. Alboni S, Benatti C, Montanari C, Tascedda F, Brunello N. Chronic antidepressant treatments resulted in altered expression of genes involved in inflammation in the rat hypothalamus. European journal of pharmacology. 2013;721(1-3):158-67.
24. Alboni S, Benatti C, Capone G, Corsini D, Caggia F, Tascedda F, et al. Time-dependent effects of escitalopram on brain derived neurotrophic factor (BDNF) and neuroplasticity related targets in the central nervous system of rats. European journal of pharmacology. 2010;643(2-3):180-7.
25. Alboni S, Poggini S, Garofalo S, Milior G, El Hajj H, Lecours C, et al. Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment. Brain Behav Immun. 2016;58:261-71.
26. Tanti A, Belzung C. Neurogenesis along the septo-temporal axis of the hippocampus: are depression and the action of antidepressants region-specific? Neuroscience. 2013;252:234-52.
27. Pinto V, Costa JC, Morgado P, Mota C, Miranda A, Bravo FV, et al. Differential impact of chronic stress along the hippocampal dorsal-ventral axis. Brain Struct Funct. 2015;220(2):1205-12.
28. Hawley DF, Leasure JL. Region-specific response of the hippocampus to chronic unpredictable stress. Hippocampus. 2012;22(6):1338-49.
29. McLaughlin RJ, Hill MN, Morrish AC, Gorzalka BB. Local enhancement of cannabinoid CB1 receptor signalling in the dorsal hippocampus elicits an antidepressant-like effect. Behavioural pharmacology. 2007;18(5-6):431-8.
30. Padovan CM, Guimaraes FS. Antidepressant-like effects of NMDA-receptor antagonist injected into the dorsal hippocampus of rats. Pharmacol Biochem Behav. 2004;77(1):15-9.
31. Brenes JC, Fornaguera J. The effect of chronic fluoxetine on social isolation-induced changes on sucrose consumption, immobility behavior, and on serotonin and dopamine function in hippocampus and ventral striatum. Behavioural brain research. 2009;198(1):199-205.
32. Prendergast MA, Yells DP, Balogh SE, Paige SR, Hendricks SE. Fluoxetine differentially suppresses sucrose solution consumption in free-fed and food-deprived rats--reversal by amantadine. Medical science monitor : international medical journal of experimental and clinical research. 2002;8(10):BR385-90.
33. Sammut S, Bethus I, Goodall G, Muscat R. Antidepressant reversal of interferon-alpha-induced anhedonia. Physiology & behavior. 2002;75(5):765-72.

34. Tonissaar M, Mallo T, Eller M, Haidkind R, Koiv K, Harro J. Rat behavior after chronic variable stress and partial lesioning of 5-HT-ergic neurotransmission: effects of citalopram. Progress in neuro-psychopharmacology & biological psychiatry. 2008;32(1):164-77.
35. Bessa JM, Mesquita AR, Oliveira M, Pego JM, Cerqueira JJ, Palha JA, et al. A trans-dimensional approach to the behavioral aspects of depression. Frontiers in behavioral neuroscience. 2009;3:1.
36. Rygula R, Abumaria N, Flugge G, Hiemke C, Fuchs E, Ruther E, et al. Citalopram counteracts depressive-like symptoms evoked by chronic social stress in rats. Behavioural pharmacology. 2006;17(1):19-29.
37. Tunc-Ozcan E, Wert SL, Lim PH, Ferreira A, Redei EE. Hippocampus-dependent memory and allele-specific gene expression in adult offspring of alcohol-consuming dams after neonatal treatment with thyroxin or metformin. Molecular psychiatry. 2017.
38. Smieszek A, Czyrek A, Basinska K, Trynda J, Skaradzinska A, Siudzinska A, et al. Effect of Metformin on Viability, Morphology, and Ultrastructure of Mouse Bone Marrow-Derived Multipotent Mesenchymal Stromal Cells and Balb/3T3 Embryonic Fibroblast Cell Line. BioMed research international. 2015;2015:769402.
39. Grieco SF, Cheng Y, Eldar-Finkelman H, Jope RS, Beurel E. Up-regulation of insulin-like growth factor 2 by ketamine requires glycogen synthase kinase-3 inhibition. Progress in neuro-psychopharmacology & biological psychiatry. 2017;72:49-54.
40. Luo YW, Xu Y, Cao WY, Zhong XL, Duan J, Wang XQ, et al. Insulin-like growth factor 2 mitigates depressive behavior in a rat model of chronic stress. Neuropharmacology. 2015;89:318-24.
41. Andrus BM, Blizinsky K, Vedell PT, Dennis K, Shukla PK, Schaffer DJ, et al. Gene expression patterns in the hippocampus and amygdala of endogenous depression and chronic stress models. Molecular psychiatry. 2012;17(1):49-61.
42. Jung S, Lee Y, Kim G, Son H, Lee DH, Roh GS, et al. Decreased expression of extracellular matrix proteins and trophic factors in the amygdala complex of depressed mice after chronic immobilization stress. BMC Neurosci. 2012;13:58.
43. Bracko O, Singer T, Aigner S, Knobloch M, Winner B, Ray J, et al. Gene expression profiling of neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult hippocampal neurogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2012;32(10):3376-87.

44. Ferron SR, Radford EJ, Domingo-Muelas A, Kleine I, Ramme A, Gray D, et al. Differential genomic imprinting regulates paracrine and autocrine roles of IGF2 in mouse adult neurogenesis. Nature communications. 2015;6:8265.
45. Kita Y, Ago Y, Higashino K, Asada K, Takano E, Takuma K, et al. Galantamine promotes adult hippocampal neurogenesis via M(1) muscarinic and alpha7 nicotinic receptors in mice. Int J Neuropsychopharmacol. 2014;17(12):1957-68.
46. Mathews DC, Zarate CA, Jr. Current status of ketamine and related compounds for depression. J Clin Psychiatry. 2013;74(5):516-7.
47. Chen DY, Stern SA, Garcia-Osta A, Saunier-Rebori B, Pollonini G, Bambah-Mukku D, et al. A critical role for IGF-II in memory consolidation and enhancement. Nature. 2011;469(7331):491-7.
48. Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, et al. Regional dissociations within the hippocampus--memory and anxiety. Neurosci Biobehav Rev. 2004;28(3):273-83.
49. O'Leary OF, Cryan JF. A ventral view on antidepressant action: roles for adult hippocampal neurogenesis along the dorsoventral axis. Trends Pharmacol Sci. 2014;35(12):675-87.
50. Strange BA, Witter MP, Lein ES, Moser EI. Functional organization of the hippocampal longitudinal axis. Nat Rev Neurosci. 2014;15(10):655-69.
51. Fanselow MS, Dong HW. Are the dorsal and ventral hippocampus functionally distinct structures? Neuron. 2010;65(1):7-19.
52. Rodriguez-Landa JF, Contreras CM, Garcia-Rios RI. Allopregnanolone microinjected into the lateral septum or dorsal hippocampus reduces immobility in the forced swim test: participation of the GABAA receptor. Behavioural pharmacology. 2009;20(7):614-22.
53. Ho YC, Wang S. Adult neurogenesis is reduced in the dorsal hippocampus of rats displaying learned helplessness behavior. Neuroscience. 2010;171(1):153-61.
54. Goeldner C, Reiss D, Kieffer BL, Ouagazzal AM. Endogenous nociceptin/orphanin-FQ in the dorsal hippocampus facilitates despair-related behavior. Hippocampus. 2010;20(8):911-6.
55. Liu R, Dang W, Jianting M, Su C, Wang H, Chen Y, et al. Citalopram alleviates chronic stress induced depression-like behaviors in rats by activating GSK3beta signaling in dorsal hippocampus. Brain Res. 2012;1467:10-7.
56. Tanaka KF, Samuels BA, Hen R. Serotonin receptor expression along the dorsal-ventral axis of mouse hippocampus. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2012;367(1601):2395-401.

57. Carr GV, Schechter LE, Lucki I. Antidepressant and anxiolytic effects of selective 5-HT6 receptor agonists in rats. Psychopharmacology. 2011;213(2-3):499-507.
58. Liu KC, Li JY, Tan HH, Du CX, Xie W, Zhang YM, et al. Serotonin(6) receptors in the dorsal hippocampus regulate depressive-like behaviors in unilateral 6-hydroxydopamine-lesioned Parkinson's rats. Neuropharmacology. 2015;95:290-8.
59. Milaneschi Y, Simmons WK, van Rossum EFC, Penninx BW. Depression and obesity: evidence of shared biological mechanisms. Molecular psychiatry. 2018.
60. Bond DJ, Andreazza AC, Hughes J, Dhanoa T, Torres IJ, Kozicky JM, et al. A Longitudinal Study of the Relationships Between Mood Symptoms, Body Mass Index, and Serum Adipokines in Bipolar Disorder. J Clin Psychiatry. 2017;78(4):441-8.
61. Lu XY, Kim CS, Frazer A, Zhang W. Leptin: a potential novel antidepressant. Proc Natl Acad Sci U S A. 2006;103(5):1593-8.
62. Kanoski SE, Hayes MR, Greenwald HS, Fortin SM, Gianessi CA, Gilbert JR, et al. Hippocampal leptin signaling reduces food intake and modulates food-related memory processing. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2011;36(9):1859-70.
63. Svenningsson P, Kim Y, Warner-Schmidt J, Oh YS, Greengard P. p11 and its role in depression and therapeutic responses to antidepressants. Nat Rev Neurosci. 2013;14(10):673-80.
64. Warner-Schmidt JL, Chen EY, Zhang X, Marshall JJ, Morozov A, Svenningsson P, et al. A role for p11 in the antidepressant action of brain-derived neurotrophic factor. Biol Psychiatry. 2010;68(6):528-35.
65. Lu B, Nagappan G, Lu Y. BDNF and synaptic plasticity, cognitive function, and dysfunction. Handb Exp Pharmacol. 2014;220:223-50.
66. Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S. Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1997;17(7):2295-313.
67. Duman RS. Pathophysiology of depression: the concept of synaptic plasticity. Eur Psychiatry. 2002;17 Suppl 3:306-10.
68. Faure J, Uys JD, Marais L, Stein DJ, Daniels WM. Early maternal separation alters the response to traumatization: resulting in increased levels of hippocampal neurotrophic factors. Metab Brain Dis. 2007;22(2):183-95.

69. Allard JS, Perez EJ, Fukui K, Carpenter P, Ingram DK, de Cabo R. Prolonged metformin treatment leads to reduced transcription of Nrf2 and neurotrophic factors without cognitive impairment in older C57BL/6J mice. Behavioural brain research. 2016;301:1-9.
70. Papatheodoropoulos C, Kostopoulos G. Dorsal-ventral differentiation of short-term synaptic plasticity in rat CA1 hippocampal region. Neurosci Lett. 2000;286(1):57-60.
71. Maruki K, Izaki Y, Nomura M, Yamauchi T. Differences in paired-pulse facilitation and long-term potentiation between dorsal and ventral CA1 regions in anesthetized rats. Hippocampus. 2001;11(6):655-61.
72. Colgin LL, Kubota D, Jia Y, Rex CS, Lynch G. Long-term potentiation is impaired in rat hippocampal slices that produce spontaneous sharp waves. J Physiol. 2004;558(Pt 3):953-61.
73. Maggio N, Segal M. Unique regulation of long term potentiation in the rat ventral hippocampus. Hippocampus. 2007;17(1):10-25.
74. Papatheodoropoulos C, Kostopoulos G. Decreased ability of rat temporal hippocampal CA1 region to produce long-term potentiation. Neurosci Lett. 2000;279(3):177-80.
75. Milior G, Di Castro MA, Sciarria LP, Garofalo S, Branchi I, Ragozzino D, et al. Electrophysiological Properties of CA1 Pyramidal Neurons along the Longitudinal Axis of the Mouse Hippocampus. Sci Rep. 2016;6:38242.
76. Alfarez DN, Joels M, Krugers HJ. Chronic unpredictable stress impairs long-term potentiation in rat hippocampal CA1 area and dentate gyrus in vitro. The European journal of neuroscience. 2003;17(9):1928-34.
77. Maggio N, Segal M. Striking variations in corticosteroid modulation of long-term potentiation along the septotemporal axis of the hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2007;27(21):5757-65.
78. Garcia R. Stress, metaplasticity, and antidepressants. Curr Mol Med. 2002;2(7):629-38.
79. Kim JJ, Yoon KS. Stress: metaplastic effects in the hippocampus. Trends Neurosci. 1998;21(12):505-9.
80. Kim JJ, Song EY, Kosten TA. Stress effects in the hippocampus: synaptic plasticity and memory. Stress. 2006;9(1):1-11.
81. Maras PM, Molet J, Chen Y, Rice C, Ji SG, Solodkin A, et al. Preferential loss of dorsal-hippocampus synapses underlies memory impairments provoked by short, multimodal stress. Molecular psychiatry. 2014;19(7):811-22.

82. Vlachos A, Maggio N, Segal M. Lack of correlation between synaptopodin expression and the ability to induce LTP in the rat dorsal and ventral hippocampus. Hippocampus. 2008;18(1):1-4.
83. Maggio N, Segal M. Differential modulation of long-term depression by acute stress in the rat dorsal and ventral hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009;29(27):8633-8.
84. Robertson DA, Beattie JE, Reid IC, Balfour DJ. Regulation of corticosteroid receptors in the rat brain: the role of serotonin and stress. The European journal of neuroscience. 2005;21(6):1511-20.
85. Avital A, Segal M, Richter-Levin G. Contrasting roles of corticosteroid receptors in hippocampal plasticity. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006;26(36):9130-4.
86. Korz V, Frey JU. Stress-related modulation of hippocampal long-term potentiation in rats: Involvement of adrenal steroid receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2003;23(19):7281-7.
87. Wiegert O, Pu Z, Shor S, Joels M, Krugers H. Glucocorticoid receptor activation selectively hampers N-methyl-D-aspartate receptor dependent hippocampal synaptic plasticity in vitro. Neuroscience. 2005;135(2):403-11.
88. Schmeisser MJ, Baumann B, Johannsen S, Vindedal GF, Jensen V, Hvalby OC, et al. IkappaB kinase/nuclear factor kappaB-dependent insulin-like growth factor 2 (Igf2) expression regulates synapse formation and spine maturation via Igf2 receptor signaling. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2012;32(16):5688-703.
89. Pascual-Lucas M, Viana da Silva S, Di Scala M, Garcia-Barroso C, Gonzalez-Aseguinolaza G, Mulle C, et al. Insulin-like growth factor 2 reverses memory and synaptic deficits in APP transgenic mice. EMBO Mol Med. 2014;6(10):1246-62.
90. Cohen A, Houck PR, Szanto K, Dew MA, Gilman SE, Reynolds CF, 3rd. Social inequalities in response to antidepressant treatment in older adults. Archives of general psychiatry. 2006;63(1):50-6.

GENERAL DISCUSSION AND CONCLUSIONS

General discussion

The purpose of the present work was to unravel the factors determining the limited efficacy of antidepressant drugs, with the final aim of developing novel and more effective therapeutic strategies for MDD. To this aim, we leveraged our previous results pointing out the relevance of neural plasticity in SSRI outcome (Alboni et al., 2017; Branchi et al., 2013). In order to identify the biological processes affecting treatment efficacy, we focused on the immune system, which is considered to be involved both in neural plasticity and SSRI action (Carvalho et al., 2013; Eller et al., 2008; Lanquillon et al., 2000; Tuglu et al., 2003). In particular, **Study 1** and **2** were aimed at exploring the interaction between neural plasticity, inflammation and serotoninergic antidepressant treatment outcome. We demonstrated that enhanced neural plasticity induced by the administration of SSRIs is able to keep inflammation within a strict physiological range and that any deviation from such range may result in plasticity impairment. In addition, in **Study 3**, we explored a polypharmacological strategy aimed at increasing SSRI efficacy through the modulation of metabolism (Corpeleijn et al., 2007; Hafner et al., 2011). In particular we assessed the effects of the combination of fluoxetine and metformin on mood. The results reported suggest that such combination has an improved efficacy compared to SSRI alone in adverse conditions.

Overall, the present work shows that the increase in neural plasticity, a key effector of serotoninergic antidepressants action, is determined by inflammation. Therefore, the regulation of the immune system activation is of critical importance to improve SSRI efficacy. Finally, our results show that the neural plasticity induced by serotoninergic antidepressant - and the consequent increased capability to improve mood - can be favored by the co-administration of compounds affecting the metabolic profile.

Main findings

Neural plasticity induced by SSRI treatment regulates the inflammatory response

Previous studies reported contradictory results about the effects of SSRI treatment on inflammation, showing that these drugs can act either as pro- or anti-inflammatory agents (Basterzi et al., 2005; Chen et al., 2010; Haastrup et al., 2012; Hannestad et al., 2011; Jazayeri et al., 2010; Kagaya et al., 2001; Kim et al., 2013; Lanquillon et al., 2000; Leo et al., 2006; Song et al., 2009; Tuglu et al., 2003; Yoshimura et al., 2009). Here, we obtained results that might reconcile such divergent views, demonstrating that the effects of fluoxetine administration on inflammation depend on the baseline inflammatory levels (Chabry et al., 2015; Goshen et al., 2008; Singhal et al., 2014; Wohleb et al., 2011). In particular, when the inflammatory levels are high, as following the exposure to a chronic stressful environment, SSRIs decrease inflammation. By contrast, when the inflammatory levels are low, as following the exposure to an enriched environment, SSRIs have a pro-inflammatory effect. The literature supports our hypothesis since the anti-inflammatory action of SSRIs has been described mainly by studies in which these compounds were administered to individuals exposed to a stressful environment. Whereas, studies reporting a pro-inflammatory action, investigated the effects of SSRIs in an enriched environment (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). These findings may contribute to elucidate the biological bases of the heterogeneity in the antidepressant response among patients, which may depend on the baseline inflammatory conditions of each patient. Consequently, for a personalized and more effective pharmacological approach, the inflammatory levels of the patients before receiving and during the treatment period should be monitored and, in case, modified, in order to favor neural plasticity and thus recovery from MDD. A further concept supported by our results is that increased neural plasticity induced by SSRIs (Branchi, 2011; Ruiz-Perera et al., 2015; Wang et al., 2008) keeps inflammatory levels in a strict range, which

consequently appears permissive for functional and structural remodeling of brain networks. This is in line with previous studies demonstrating a strictly regulated interaction between neurons and immune system, including microglia and cytokines, which is crucial for plasticity processes, such as synaptic shaping and neuronal excitability (Yirmiya and Goshen, 2011; Figure 8).

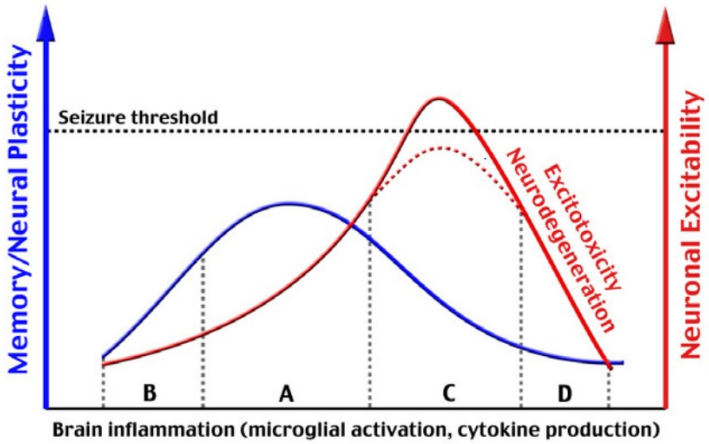

Figure 8. Inverted-U curve describes the interaction between inflammatory levels and neural plasticity. Memory, neural plasticity and neuronal excitability as a function of brain inflammation. Immune processes in the brain, including microglial activation and inflammatory cytokine production play a complex dual role in learning, memory, and neural plasticity (blue graph), as well as neuronal excitability (red graph) (Yirmiya and Goshen, 2011).

Deviations from the physiological range of inflammation determine neural plasticity

With the aim of demonstrating that inflammatory levels within a specific physiological range favor neural plasticity, we treated mice with ibuprofen and LPS that, respectively, decreases and increases inflammatory levels (Martin et al., 2013; Rainsford, 2013; Shaw et al., 2003), and assessed the effects of these treatments on molecular and cellular neural plasticity. The results showed that, as expected, the two compounds have opposite effects on sickness behavior and inflammatory response. Nevertheless, they produced overlapping effects on neural plasticity, both determining plasticity impairment. Such effect appears to be dose-dependent since only high, but not low, doses of both compounds produced such effect.

The present findings suggest that neural plasticity and inflammation are mutually regulating processes and inflammatory levels should be kept within a strict physiological range to allow for a structural remodeling of brain networks. It is worth noting that the present results indicate that not only an iper- but also an ipo-activation of the immune system could have detrimental effect. Accordingly, Miller and Raison have shown that low levels of inflammatory activation might increase the vulnerability to psychopathologies, such as depression (Raison and Miller, 2011). Indeed, inflammatory processes, including the phagocytic activity of microglia or the production of cytokines, underlie numerous key physiological processes and too low inflammatory levels could impair them affecting brain functioning. The strict interplay between neural plasticity and inflammation is not unexpected when considering the inflammatory pathways as fully integrate and key elements within the brain. Such interplay is described by an inverted-U curve where intermediate levels of inflammation are associated with higher neural plasticity levels and any deviation from this range, caused by either a pro- or anti-inflammatory action, leads to reduced plasticity (Figure 9).

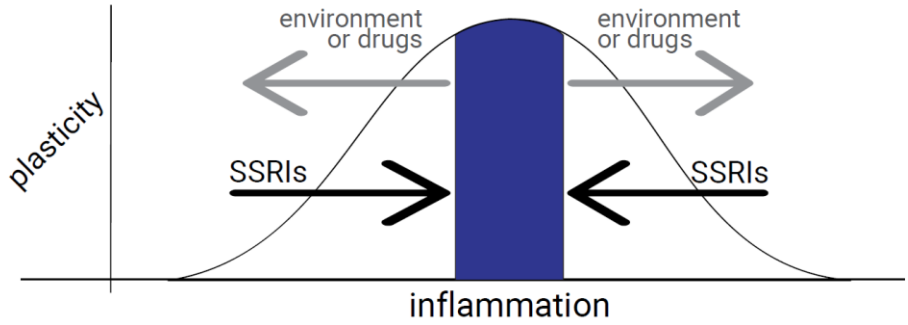

Figure 9. The curve describes the interaction between neural plasticity and inflammatory levels. When the inflammatory levels are kept in the physiological range the neural plasticity reaches its maximal level. By contrast, both immune activation and suppression lead to reduced neural plasticity.

SSRI efficacy in adverse condition could be increased by the co-treatment with metformin

As further step, we explored novel strategies aimed at exploiting the effect of SSRI treatment on neural plasticity. In particular, according to the *undirected susceptibility to change* hypothesis, the increase in serotonin levels induced by SSRIs enhances brain plasticity and thus amplifies the influence of the environment on the individual (Branchi, 2011). Consequently, SSRI treatment has not a univocal effect but, in a favorable environment, it would lead to a reduction of symptoms while in a stressful environment might lead to a worse prognosis. Such SSRI action on the susceptibility to environment opens new perspectives on how to increase their efficacy by improving the quality of the patients' living environment. However, since often it is not possible to act on the environment because of constraints due to patients' personal history, a pharmacological modulation of the factors underlying the link between living environment and SSRI action represents a novel strategy to improve treatment outcome. Given that metabolism is affected by the quality of the living environment and, in turn, metabolic profile affects antidepressant efficacy (Corpeleijn et al., 2007; Hafner et al., 2011), the pharmacological regulation of metabolism represents a potential approach to improve treatment outcome. To this aim, we exploited a polypharmacological strategy consisting in the co-treatment with the SSRI fluoxetine and metformin, a commonly used drug to improve metabolic

dysfunction. Our results demonstrated that the exploitation of a polypharmacological approach, considering (i) metabolic profile as a targetable proxy for the beneficial effect of a favorable living environment on mood, and (ii) SSRIs as enhancers of such beneficial effect, allows for a more effective antidepressant treatment (Figure 10).

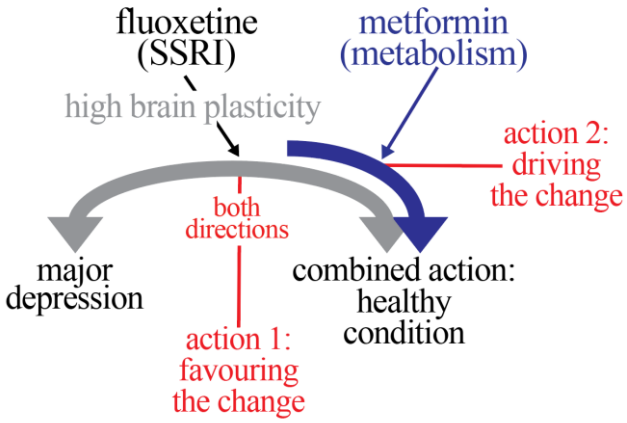

Figure 10. Two-targets combined action strategy to enhance SSRI efficacy. The approach exploited is to develop a concerted action to produce two different effects – i.e. favouring the change (i.e. SSRI) and driving the change (i.e. regulation of the metabolism) – in order to develop highly effective therapeutic strategies for MDD.

Limits

Although reporting novel findings, the present work presents some limitations. First, all the studies have been conducted on male subjects. Future studies should employ both male and female individuals. This appears even more relevant considering that MDD affects mostly female with a female:male ratio of approximately 2:1 (Albert, 2015).

A further limitation is the lack of a detailed and comprehensive investigation of molecular mechanisms underlying the action of the psychoactive compounds tested here. However, given the well-described effects of these compounds in both preclinical and clinical studies, including the description of their potential side effects (Altamura et al., 1994; Davies, 1998; Maruthur et al., 2016; Sirtori et al., 1978), such characterization was beyond the aims of the present work. In addition, the molecular data presented here comprise mainly mRNA transcript levels, thus further analyses devoted to measure protein levels may strengthen the relevance of the present results.

Finally, with regard to the analysis of immune activation, it should be taken into account that our data focused on the central levels of inflammation, while, in clinical settings, peripheral levels are routinely measured. Therefore, future studies should assess both central and peripheral markers of inflammation to have a higher translational value and to better illustrate the link between central and peripheral inflammation

General conclusions

MDD represents a relevant global public health burden at clinical, social and economic level. One of the most relevant causes of such burden is the incomplete efficacy of antidepressant treatments. In particular, the absence of a substantial progress in improving SSRI effectiveness over the last decades could be ascribed to the limited understanding of the action of these drugs. In the present study, we strived to identify the factors involved in determining SSRI outcome with the final aim of developing a more effective antidepressant treatment. Our starting point was that, though inflammation and neural plasticity have been widely reported as key factors in SSRI action, their interplay in determining antidepressant outcome has been limitedly explored.

The results obtained reveal that neural plasticity and inflammation are deeply intertwined processes. In particular, we first showed that an increase in neural plasticity, induced through SSRI administration, counterbalances both the activation and the suppression of the immune response. As second step, we demonstrated that neural plasticity is high when inflammatory markers are kept within a strict physiological range and any deviation towards an extreme activation or suppression of the immune response leads to a plasticity impairment. Together these findings indicate that neural plasticity and inflammation act as mutually regulating processes and that, to instate the neural plasticity needed for the SSRI beneficial action on mood, inflammation has to be tightly regulated. In a translational perspective, such concept entails that, to improve the efficacy of the antidepressant treatment in clinical settings, patients' inflammatory levels should be controlled before and during the pharmacological therapy. As a consequence, combining the antidepressant treatment with either a pro- or anti-inflammatory compounds aimed at counterbalancing any deviation in inflammatory levels, could result in a more effective antidepressant strategy. Although the beneficial effects of a co-treatment involving an antidepressant SSRI and an anti-inflammatory compound, as NSAIDs, has been already reported in both pre-clinical and clinical studies (Kohler et al., 2015; Kopschina Feltes et al.,

2017), new studies should be devoted to assess whether, as suggested by the theoretical framework proposed here, the add-on of pro-inflammatory agents could improve treatment outcome in patients showing extremely low inflammatory levels.

Finally, with the purpose to develop a novel and highly effective antidepressant treatment, we performed a further experiment exploiting a polypharmacological approach combining the SSRI fluoxetine, used to enhance neural plasticity, with metformin, a drug able produce metabolic effects overlapping with those induced by an healthy lifestyle. The exploitation of such approach is based on the view that a multi-target intervention, which act on different pathways and systems, has a greater clinical efficacy compared to the old "single-target" compound paradigm (Hopkins, 2008). The results obtained confirmed that, in adverse conditions, the combined treatment is more effective in improving mood than SSRI treatment alone.

Overall, the studies presented in this work indicate that, in order to improve the efficacy of antidepressant drugs, information concerning not only the patients' mood but also their living environment and selected physiological endpoints (e.g. inflammatory levels) should be considered for an effective antidepressant therapeutic strategy. This calls for the implementation of the precision medicine paradigm in the psychiatric field, which consists in personalized therapeutic interventions based on a comprehensive assessment of the patients' biological and psychosocial features. Such paradigm is relevant for a large number of psychopathologies and in particular for MDD, a disorder whose onset and progression are, according to an increasing number of studies, multifactorial rather than triggered by a single chemical imbalance (Lacasse JK, 2005).

References

Albert, P.R., 2015. Why is depression more prevalent in women? Journal of psychiatry & neuroscience : JPN 40, 219-221.

Alboni, S., van Dijk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I., Cirulli, F., Maggi, L., Branchi, I., 2017. Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Molecular psychiatry 22, 552-561.

Altamura, A.C., Moro, A.R., Percudani, M., 1994. Clinical pharmacokinetics of fluoxetine. Clinical pharmacokinetics 26, 201-214.

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.

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., 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.

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.

Corpeleijn, E., Feskens, E.J., Jansen, E.H., Mensink, M., Saris, W.H., Blaak, E.E., 2007. Lifestyle intervention and adipokine levels in subjects at high risk for type 2 diabetes: the Study on Lifestyle intervention and Impaired glucose tolerance Maastricht (SLIM). Diabetes care 30, 3125-3127.

Davies, N.M., 1998. Clinical pharmacokinetics of ibuprofen. The first 30 years. Clinical pharmacokinetics 34, 101-154.

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.

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. Molecular psychiatry 13, 717-728.

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.

Hafner, S., Zierer, A., Emeny, R.T., Thorand, B., Herder, C., Koenig, W., Rupprecht, R., Ladwig, K.H., Investigators, K.S., 2011. Social isolation and depressed mood are associated with elevated serum leptin levels in men but not in women. Psychoneuroendocrinology 36, 200-209.

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.

Hopkins, A.L., 2008. Network pharmacology: the next paradigm in drug discovery. Nature chemical biology 4, 682-690.

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.

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.

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.

Kohler, O., Petersen, L., Mors, O., Gasse, C., 2015. Inflammation and depression: combined use of selective serotonin reuptake inhibitors and NSAIDs or paracetamol and psychiatric outcomes. Brain and behavior 5, e00338.

Kopschina Feltes, P., Doorduin, J., Klein, H.C., Juarez-Orozco, L.E., Dierckx, R.A., Moriguchi-Jeckel, C.M., de Vries, E.F., 2017. Anti-inflammatory treatment for major depressive disorder: implications for patients with an elevated immune profile and non-responders to standard antidepressant therapy. Journal of psychopharmacology 31, 1149-1165.

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.

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.

Lacasse J.R, .Leo, j., 2007. The Media and the Chemical Imbalance Theory. of Depression. PLoS Med. 2005 Dec;2(12):e392.

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 17, 1501-1510.

Martin, S.A., Pence, B.D., Greene, R.M., Johnson, S.J., Dantzer, R., Kelley, K.W., Woods, J.A., 2013. Effects of voluntary wheel running on LPS-induced sickness behavior in aged mice. Brain, behavior, and immunity 29, 113-123.

Maruthur, N.M., Tseng, E., Hutfless, S., Wilson, L.M., Suarez-Cuervo, C., Berger, Z., Chu, Y., Iyoha, E., Segal, J.B., Bolen, S., 2016. Diabetes Medications as Monotherapy or Metformin-Based Combination Therapy for Type 2 Diabetes: A Systematic Review and Meta-analysis. Annals of internal medicine 164, 740-751.

Rainsford, K.D., 2013. Ibuprofen: from invention to an OTC therapeutic mainstay. International journal of clinical practice. Supplement, 9-20.

Raison, C.L., Miller, A.H., 2011. Is depression an inflammatory disorder? Current psychiatry reports 13, 467-475.

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.

Shaw, K.N., Commins, S., O'Mara, S.M., 2003. Deficits in spatial learning and synaptic plasticity induced by the rapid and competitive broad-spectrum cyclooxygenase inhibitor ibuprofen are reversed by increasing endogenous brain-derived neurotrophic factor. The European journal of neuroscience 17, 2438-2446.

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.

Sirtori, C.R., Franceschini, G., Galli-Kienle, M., Cighetti, G., Galli, G., Bondioli, A., Conti, F., 1978. Disposition of metformin (N,N-dimethylbiguanide) in man. Clinical pharmacology and therapeutics 24, 683-693.

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.

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., Team, S.D.S., 2006. Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice. The American journal of psychiatry 163, 28-40.

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.

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.

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.

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.

LIST OF PUBLICATIONS

- Viglione A, Chiarotti F, **Poggini S**, Giuliani A, Branchi I. "'Predicting antidepressant treatment outcome based on socioeconomic status and citalopram dose", Pharmacogenomics J, 2018, in press.
- **Poggini S**, Golia MT, Alboni S, Milior G, Pepè Sciarria L, Viglione A, Matte Bon G, Brunello N, Puglisi-Allegra S, Limatola C, Maggi L, Branchi I. 2018 "Combined fluoxetine and metformin treatment potentiates antidepressant efficacy increasing IGF2 expression in the dorsal hippocampus", Neural Plast, 2018, in press.
- Alboni S, **Poggini S,** Garofalo S, Milior G, El Hajj H, Lecours C, Girard I, Gagnon S, Boisjoly-Villeneuve S, Brunello N, Wolfer DP, Limatola C, Tremblay MÈ, Maggi L, Branchi I.(2016) "Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment". Brain Behav Immun. 2016 Nov;58:261-271. Epub 2016 Jul 27.
- Alboni S, van Dijk RM, **Poggini S**, Milior G, Perrotta M, Drenth T, Brunello N, Wolfer DP, Limatola C, Amrein I, Cirulli F, Maggi L, Branchi I. (2015) "Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment". Mol Psychiatry. 2015 Sep 15.
- Milior G, Lecours C, Samson L, Bisht K, **Poggini S**, Pagani F, Deflorio C, Lauro C, Alboni S, Limatola C, Branchi I, Tremblay ME, Maggi L (2015) "Fractalkine receptor deficiency impairs microglial and neuronal responsiveness to chronic stress". Brain Behav Immun. 2016 (55:114-25).
- Pendolino V, Ghiglieri V, Sgobio C, Morelli E, **Poggini S**, Branchi I, Latagliata E.C, Pascucci T, Puglisi-Allegra S, Calabresi P (2014) "L-DOPA reverses the impairment of Dentate Gyrus LTD in experimental parkinsonism via betanoradrenergic receptors". Experimental Neurology 261 (377-285).
- 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(4).

Acknowledgements

I would like to thank the people who contributed in some way to the work described in this thesis.

Foremost, I want to express my sincere gratitude to my advisor Igor Branchi for the continuous support, for his patience, enthusiasm, and knowledge. I am very grateful for having the possibility to carry out this and other interesting projects. I feel lucky to have him as advisor: he trusted me even when I did not trust myself and He always motivated and encouraged me. He became a guide not only for my professional career.

I reserve a special gratitude to Prof. Stefano Puglisi Allegra who has been an excellent tutor for my PhD at Sapienza, University of Rome.

I also thank all the committee members of this PhD School in Behavioral Neuroscience and the referees Dr. Marie-Eve Tremblay, professor at Université Laval and Dr. Francesco Benedetti, professor at Vita-Salute San Raffaele University, for their precious time and kind availability to review this work.

My gratitude is deserved for Prof. Enrico Alleva for giving me the opportunity to conduct my research in his laboratory. He is a constant source of scientific and cultural debate, and I had very interesting conversations with him.

I would like to thank all the students who earned their degree or are performing their thesis under the supervision of Dr. Branchi. The work reported in this thesis has been also realized thanks to their collaboration. In particular, I would like express a special thanks to Aurelia Viglione who conducted with me most of the experiments here described. She is a valid PhD student and I wish her a professional life full of successes.

I would like to thank Francesca Cirulli, who had contributed to my professional growth and whose successes are always a source of inspiration for me. I would like to express my gratitude for having the opportunity to work with her team, in particular with Alessandra Berry and Barbara Collacchi, who have enlarged my scientific background and given me precious advices.

I would like to thank all the members of the Center for Behavioral Sciences and Mental Health at the Istituto Superiore di Sanità. Nadia Francia and Daniela Santucci have been day by day a sort of second tutor for me. Their precious advices helped me in a long series of situations and their lively presence created an enjoyable environment in the lab. Thanks to Flavia Chiarotti for her useful advices on data analysis and for making statistic more clear. Thanks to Gemma Calamandrei, Aldina Pesciolini-Venerosi and Laura Ricceri for the many pleasant conversations, which have contributed to my professional and personal growth. Thanks to Simone Macrì

and Augusto Vitale for the interesting discussions concerning not only science and research. They have broadened my scientific and cultural background.

Special thanks go to all the present and former students at the Center for Behavioral Science and Mental Health because: with them I spent long and funny days in the lab. Foremost, I would like to thank the two persons who mostly helped and supported me before starting the PhD program, during these three intense years and while I am writing these pages: Marta Borgi and Angela Caruso. Thanks to Marta for understanding me as no one else, for her wise advices and for finding always a solution when I have professional and personal problems. Thanks to Angela for being the perfect listener. With her unlimited patience, she gave me the possibility to discover a point of view far from mine and to broaden my professional and personal horizons. With them, I could overcome the challenges encountered during these years and I feel lucky to be their colleague and friend. Thanks to Daniele Vigli who shared with me the ups and downs of the PhD journey. Our numerous conversations helped me to open my mind and to develop new perspectives. I would like to thank Francesca Zoratto: even if she is a very hard worker, she always had found time and patience to listen to and help me. Finally, thanks to Anna Maria Tartaglione because she showed me how passion and determination are important for this career.

Furthermore, I am very grateful to Prof. David Wolfer for given me the opportunity to carry out part of my research in his laboratory at the University of Zurich-Irchel. My deep gratitude goes to Dr. Irmgard Amrein for being my tutor during my stay in Zurich. I really appreciated her kind availability and I admired her strength and dedication at work. Spending part of my PhD in Zurich has been an unforgettable experience mostly thanks to her. I am very grateful to all the Swiss researchers and students, including Sonia, Marteen, Franzisca, Giovanni, and Ann Kristina, for having provided me with an excellent atmosphere and for have been considering me part of the team from the very first day.