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INFLAMMATORY MECHANISMS AND VULNERABILITY FOR MAJOR DEPRESSION: FROM ANIMAL MODELS TO DRUG TREATMENT

Doctorate thesis

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Abstract

Major depressive disorder (MDD) is a common disorder that represents a leading cause of disability in the world. It is thought to originate from the interaction between susceptibility genes and environmental events, such as stress, to which an individual can be exposed in different moments of life. One of the major problems of depression is the relevant percentage of patients who do not show an adequate response to antidepressant therapy, as well as the high rate of relapse. Growing evidence suggest that the activation of the inflammatory/immune system contributes to the pathogenesis of depression. Indeed, depressed patients exhibit increased levels of inflammatory markers in both the periphery and the brain, and high comorbidity exists between major depression and diseases associated with inflammatory alterations.

In order to characterize the link between depression and inflammation, the aim of the present study was on one hand, to evaluate the effect of antidepressant treatment on systems that might be relevant for the relationship and mutual modulation of depression and inflammation. Indeed, since depression can be considered a functional disease characterized by an impairment in coping mechanisms, LPS administration was used as an "inflammatory challenge" to investigate how the pharmacological treatment might affect brain responsiveness under a challenging condition. Specifically, male rats were treated for 21 days with agomelatine and they were then challenged with LPS. Animals were sacrificed after 2, 6, or 24 h from the challenge, in order to study the temporal progression of the inflammatory response mediated by LPS injection.

We found that agomelatine significantly reduced the LPS-induced up-regulation of interleukin-1β and interleukin-6 in different brain regions as well as at peripheral level. Moreover, pre-treatment with the antidepressant was also able to interfere with mechanisms responsible

for microglia activation following LPS injection and to modulate the expression of kynurenine pathway enzymes, which may also represent important mediators for inflammation-related depression.

We also investigated if genetic susceptibility to depression was associated with changes of the immune/inflammatory system. Indeed, since mood disorders originate from the interaction between environmental factors and a vulnerable genetic background, we aim to establish if the deletion of serotonin transporter (SERT) gene, a susceptibility gene for depression, was associated with altered expression of inflammatory markers under basal conditions. Secondly, we investigated the functional consequences of exposing these animals to an inflammatory challenge with LPS, which may precipitate the depressive phenotype. We found that animals with deletion of the SERT gene have, under basal conditions, enhanced levels of circulating immune proteins and increased expression of IL-1β in the hippocampus. Moreover, we found that genetic deletion of SERT gene in rats was associated with alterations of pro-inflammatory cytokines and markers of microglia activation following the immune challenge.

1. INTRODUCTION

MAJOR DEPRESSION

Major depression is a severe psychiatric disorder that is the fourth leading cause of disability in the world accounting for a relevant percentage of morbidity (Moussavi *et al.*, 2007). It is a complex disorder characterized by the interaction of genetic, biological, social and environmental factors that play in concert to determine the development of the disease. Core symptoms include depressed mood, anhedonia (reduced ability to experience pleasure from natural rewards), irritability, difficulties in concentrating, abnormalities in appetite and sleep ('neurovegetative symptoms') and suicidal thoughts (Nestler *et al.*, 2002). In addition to mortality associated with suicide, depressed patients are more likely to develop coronary artery disease and type 2 diabetes (Knol *et al.*, 2006). The chronic, festering nature of depression contributes substantially to the global burden of disease and disability.

Despite the prevalence of depression and its considerable impact, the knowledge about its pathophysiology is poor compared with knowledge of other common chronic and potentially fatal multifactorial conditions. Available techniques to document the aberrant function of brain circuits depend on either post-mortem studies, which have numerous limitations, or neuroimaging techniques, which rely on detecting changes in neuronal activity by using indirect markers of activation (Phelps & LeDoux, 2005). Despite these difficulties, several brain regions and circuits regulating emotion, reward, executive function and dysfunctional changes within these highly interconnected 'limbic' regions have been implicated in depression and antidepressant action (Berton & Nestler, 2006). A large body of post-mortem (Sheline, 2003) and neuroimaging (Harrison, 2002) studies on depressed patients have reported reductions in grey-matter volume and glial density in the prefrontal cortex and in the hippocampus, regions thought to mediate the cognitive aspects of depression, such

as feelings of worthlessness and guilt. Although these approaches have provided important insights into candidate brain regions, simple increases or decreases in regional brain activity are probably insufficient to explain the complex array of symptoms caused by depression.

Therefore a clear pathogenetic mechanism is yet to be determined, but detailed studies have led to formulate different molecular theories of depression such as the "monoamine hypothesis" and the "neuroplasticity hypothesis".

The "monoamine hypothesis" of depression, which states that depression is caused by decreased monoamine function in the brain, originated from early clinical observations (Berton & Nestler, 2006; Pittenger & Duman, 2008). Two structurally unrelated compounds developed for non-psychiatric conditions, namely iproniazid and imipramine, had potent antidepressant effects in humans and were later shown to enhance central serotonin or noradrenaline transmission. Reserpine, an old antihypertensive agent that depletes monoamine stores, produced depressive symptoms in a subset of patients. Today's antidepressant agents offer a better therapeutic index and lower rates of side effects for most patients, but they are still designed to increase monoamine transmission acutely (Berton & Nestler, 2006), either by inhibiting neuronal reuptake (for example, selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine) or by inhibiting degradation (for example, monoamine oxidase inhibitors such as tranylcypromine). However, while monoamine oxidase inhibitors and SSRIs produce immediate increases in monoamine transmission, their mood-enhancing properties require weeks of treatment. Moreover, a relevant percentage of patients does not show an adequate response to antidepressant therapy, or relapses into the pathology, possibly due to persistent symptomatology. Although these monoamine-based agents are potent antidepressants, and alterations in central monoamine function might contribute marginally to genetic vulnerability (Ruhe *et al.*, 2007; Lopez-Leon *et al.*, 2008), the cause

of depression is far from being a simple deficiency of central monoamines.

The "neuroplasticity hypothesis" of depression, suggests that mood disorders are caused by problems in information processing within particular neuronal circuits in the brain due to altered neuroplasticity, and that treatment with antidepressant drugs, may improve this deficit. Neuroplasticity is the ability of the brain to respond and adapt to environmental challenges and encompasses a series of functional and structural mechanisms that may lead to neuronal remodeling, formation of novel synapses and birth of new neurons. Failure of such mechanisms might enhance the susceptibility to environmental challenges, such as stress, and ultimately lead to psychopathology. Neurotrophic factors play a key role as mediators of neuroplasticity.

However, in recent years it has become more evident that many factors can contribute to the development of this disease, such as the association between a vulnerable genetic background and different environmental factors, such as stress.

Therefore, given the poor knowledge of the molecular mechanisms contributing to depression etiopathology and the unsuitability of the pharmacological treatment, several animal model of depression have been used, which could further our understanding of the pathophysiology of the disease, and also yield novel molecular mechanisms that may prove to be therapeutic targets for different antidepressants drugs.

In the context of mood disorder the serotonin transporter gene is particularly relevant. The human serotonin transporter is encoded by a single gene, SLC6A4 (solute carrier family 6 member 4), whose transcriptional activity is modulated by genetic variants, including a functional polymorphism in the promoter region (5-HTTLPR), characterized by a long (L) and a short (S) variant. Specifically, functional studies confirmed that the L allele of the functional polymorphism 5-HTTLPR is associated with higher levels of transcriptional activity and higher rate of serotonin uptake with

respect to the S variant (Murphy & Lesch, 2008). Moreover, the L allele could be a protective factor for major depression, whereas the low-functioning allele is associated with increased disease susceptibility upon exposure to adverse life events (Caspi *et al.*, 2003; Uher & McGuffin, 2008; Karg *et al.*, 2011).

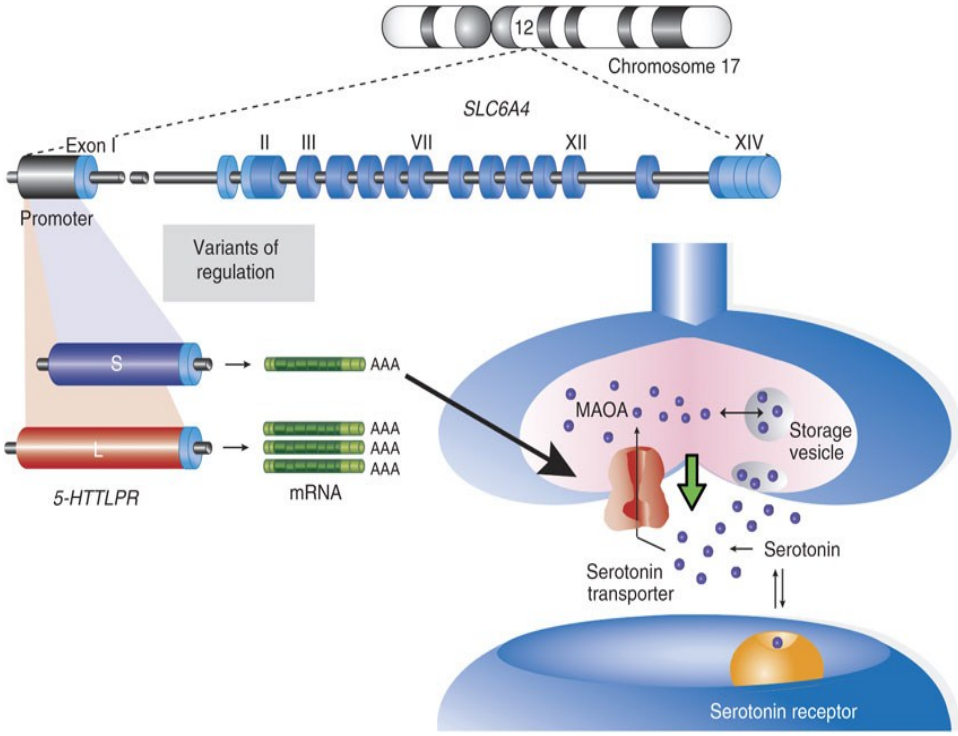

Figure 1
Polimorphism of Serotonin transporter gene

Given that this polymorphism is present in humans and non-human primates, but not in rodents, rats and mice with a partial or total deletion of the SERT gene have been generated. Animal with a total deletion of the serotonin transporter displayed increased levels of anxiety- and depression-like behaviors (Holmes *et al.*, 2003; Lira *et al.*, 2003; Olivier *et al.*, 2008). Particularly, SERT knock-out rat was subjected to a series of specific tests in order to assess the state of anxiety and the depression like behavior. The altered response in those tests promote the use of this model for the study of the role of the serotonin transporter in vulnerability to mood disorders. Indeed, the results of behavioral studies in both male and female rats homozygous for the serotonin transporter gene have confirmed a heightened state of anxiety in the Open Field Test, in the Elevated Plus Maze Test, in Novelty Suppressed Feeding Test and the Home Cage Emergence Task Test and a depression-like behavior in the Forced Swim Test and in the Sucrose preference test.

However, the increased knowledge of depression biology has not led to an improvement in the overall impact of pharmacotherapy, possibly because a number of systems that are affected in mood disorders may not be adequately modulated by pharmacological treatments.
On these bases, in the attempt to clarify the etiopathological bases of depression, it is crucial to consider different components and also their possible interaction. In line with this point of view, incomplete response to pharmacological treatment might be due to the limited impact that available drugs may exert on the above-mentioned players, the result being the failure to reach remission.
Moreover, as mentioned above, growing body of evidence described and partially characterized dysfunction of multiple systems, including hormones, inflammatory markers, signaling pathways, neurotrophic and neuroplastic molecules.

With regard to inflammation, a number of experimental evidences support its role in depression. Particularly, it is known that depression is accompanied by immunosuppression as well as alteration of inflammatory systems. Indeed, patients with depression exhibit increased levels of inflammatory markers including interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF), in both the periphery and the brain (Dowlati *et al.*, 2010). Furthermore, the high co-morbidity that depression presents with various diseases associated with inflammatory conditions such as cardiovascular disease, neurodegenerative diseases, tumors (Yirmiya, 1996) supports the importance that immune/inflammatory component may have in mood disorders. In agreement with this hypothesis, the use of interferon in patients with multiple sclerosis causes neurotransmitter abnormalities that may contribute to the development of depression (Asnis & De La Garza, 2006; Lotrich, 2009; Raison *et al.*, 2009).

It is therefore important not only to characterize the changes in immune/inflammatory responses in people with depression or in animal models of depression, but also to identify and investigate the possible link between the components of the immune/inflammatory response and systems that play a key role in the etiopathogenesis of depression.

INFLAMMATION

Inflammation is the biological response of the tissue to harmful stimuli. Indeed, when tissue receives a detrimental stimulus that causes the death or the suffering of some of its cells, it generates a series of vascular reactions aimed to the elimination of the harmful and the removal of cellular debris from the injured tissue. The classical signs of acute inflammation are rubor (redness), calor (heat), dolor (pain), tumor (swelling) and loss of function. The purpose of an

inflammatory process is defensive and seeks to circumscribe the lesion, eliminate the cause of the damage and repair the tissue to restore function. In the early stages of the inflammatory process, the injure tissue is reconstructed by the proliferation of parenchymal cells or replaced by fibrous tissue. However, inflammation can become harmful when the stimulation persists for long time, causing an excess of fibrosis which can lead to severe organ dysfunctions. The main factors involved in inflammation are both exogenous, such as viral, bacterial, fungal, trauma, thermal, environmental toxins and endogenous, such as ischemia, cancer, autoimmune diseases. Inflammation can be classified as either acute or chronic. Acute inflammation is relatively short (hours or days) and is characterized by the movement of fluid and cells from the vascular bed to the extravascular tissue in order to border the lesion, eliminate the causes of the damage and repair tissues. Prolongued inflammation (weeks or months), known as chronic inflammation, characterized by simultaneous inflammation, destruction and healing of tissues. Both the acute and chronic inflammation are regulated and amplified by a wide range of soluble mediators, which derived from plasma or cell and that participate with positive or negative signals to the development of the inflammatory response.

SOLUBLE MEDIATORS

A lot of soluble factors regulate and amplify the inflammatory process in the early stage and participate to the resolution of the damage. These factors have a specific receptor and can stimulate the release of other mediators by second messengers with an amplification effect, modulation or regulation. The mediators may act on one or more cell types and may exert different effects depending on the type of tissue or cell. Based on the anatomical distribution and the origin of tissue we can classified these soluble mediators into three different classes: plasma mediators, that circulate in plasma, neo-synthesis mediators,

proteins that are synthesized by different tissues and preformed mediators.

Plasma mediators

There are three different protein system of plasma mediator:

• The complement system: consists of about 20 different plasma proteins founded in the blood, in general synthesized by the liver, and normally circulating as inactive precursors (pro-protein). The complement system play a crucial role in the innate immune system. However, it can be recruited and brought into action by the adaptive immune system. It can be activated in two ways: a classical one, activated by the presence of antigen-antibody complexes, and an alternative one that depends on the presence of bacterial products, polysaccharides or aggregates of antibodies. Once activated the complement leads to cell death.

• The kinin-kallikrein system: increased capillary permeability causing vasodilation and pain. Its components have short life and are quickly inactivated by enzymes.

• The coagulation/fibrinolysis system. This system leads to the production of thrombin and derivatives of fibrinogen with pro inflammatory activities, such the increasing of the vascular permeability, adhesion of leukocyte and proliferation of fibroblasts.

NEO-SYSTESIS MEDIATORS

The mediators of neosynthesis include products that derived from lipid, prostaglandins, leukotrienes, and cytokines.

• Lipidic mediators: prostaglandins and leukotrienes derived from the peroxidation of the arachidonic acid through distinct enzymatic pathways, the cyclooxygenase and lipoxygenase way. The way of cyclo-oxygenase produces a series of prostanoids involved in the early

stages of the inflammatory process. Among these, we can found the thromboxanes with vessel constrictive activity and aggregating, and the prostaglandins that act as vasodilators, aggregating and cause an increase in vascular permeability. The lipoxygenase pathway produces leukotrienes with different activity. They are bronchial constrictor, vasodilator and they increase capillary permeability. They are also strong chemotactic and increase the adhesion of leukocytes to the endothelium vessel.

• Protein mediators: the inflammatory cytokines represent the soluble mediators involved in the activation or the inhibition of the different cell types involved in the phenomena of protection and repair. Among the most studied cytokines there are the inflammatory interleukins involved in the acute processes.

- Interleukin-1 (IL-1): induces fever through the production of prostaglandins and it is considered an endogenous pyrogen. IL-1 stimulates macrophages to produce IL-6 and additional IL-1; it induces the expression of molecules on vascular endothelium that produce the adhesion of leukocytes. IL-1 is synthesized by many cell types, especially monocytes/macrophages, neutrophils and endothelial cells, under stimulation of immunocomplexes, physical damage and other cytokines (tumor necrosis factor).

- Interleukin-6 (IL-6): proinflammatory cytokine that stimulates liver cells to produce acute phase proteins. It acts as an endogenous pyrogen causing fever and inhibits the production of IL-1 and interferon gamma (INF-γ) by macrophages.

- Tumor Necrosis Factor (TNF): is the main mediator of the acute inflammatory response in the presence of gram-negative bacteria. It is mainly produced by monocytes/macrophages

activated, but also by T lymphocytes, natural-killer cells (NK cells) and mast cells. TNF acts on endothelial cells by increasing the expression of adhesion molecules and by the synthesis of chemotactic factors. Moreover, it amplifies the inflammatory response inducing the synthesis of IL-1 and IL-6.

- Interleukin-10 (IL -10): on the contrary of the previously cytokines described is an anti-inflammatory cytokine that acts by inhibiting the production of IL-1, TNF and interferon. It suppresses the expression and activation of the receptors for cytokines.

PREFORMED MEDIATORS

The main mediator preformed is represented by histamine. It causes dilation of arterioles, increases the permeability of venules, but coerses the large arteries. Histamine is also the main cause of the increase of vascular permeability in the first hours after the damage.

ROLE OF MICROGLIA IN THE INFLAMMATORY RESPONSE

The microglial cells, astrocytes, oligodendrocytes and Schwann cells, constitute glial cells that, together with the neurons, forming the nervous system. Microglia has nutritive and supporting activity against neuronal, provide to the isolation of nerve tissue and to the protection in case of injury. The microglial cells act as the first and main form of active immune defense in the CNS, then, will be the recruitment of immune cells from the bloodstream, such as neutrophils and macrophages. Microglia constitute 10-15% of the total glial cell population within the brain, and are called "residents" to distinguish them from other immune cells recruited externally to the CNS during the inflammatory process. The microglial cells in a healthy brain tissue

are in a state of "resting", a state of quiescence in which microglia is constantly moving in the CNS in search of damaged neurons, plaques and infectious agents (Gehrmann *et al.*, 1995). Since the brain and spinal cord are separated from the rest of the body by a series of endothelial cells known as the blood-brain barrier (BBB) that prevents the majority of infections to reach the nerve tissue, they are considered organs "immune-privileged". When infectious agents are directly introduced into the brain or cross the BBB, microglial cells must react quickly to decrease inflammation and destroy the infectious agents before they damage the sensitive neural tissue. Due to the unavailability of antibodies from the rest of the body (few antibodies are small enough to cross the BBB), microglia must be able to recognize foreign bodies, swallow them, and act as antigen-presenting cells (APCs) activating T-cells. Given that this process must be done quickly to prevent potentially fatal damage, microglia are extremely sensitive to even small pathological changes in the CNS (Dissing-Olesen *et al.*, 2007). In the state of "resting" microglia presents a dendritic morphology that is characterized by a small cell body with many processes branched in all directions, these appendages are mobile and supervise the surrounding microenvironment in search of signals of pathogens or tissue damage. In the activeted state, microglia cells turn in an amoeboid form and acquire a high motility that allow them to reach areas of the injured. The transition from the resting state to the activated state is not reversible and after cessation of their activities in the inflammatory process such cells will undergo apoptosis. Recently it has been demonstrated that neurons may control the microglia through signals "on-off". Indeed, microglia is controlled by signals that keep it in a resting state or that induce its activation (Biber *et al.*, 2007). One of the mechanisms involved in this regulation is the glia-neuron cross-talk. Among this system, the fractalkin (CX3CL1) and its receptor (CX3R1) play a crucial role. This chemokine is produced by neurons and recent studies in vitro have demonstrated that it is able to inhibit

microglial activation once bound to its receptor on microglia (Corona *et al.*, 2010). Furthermore, KO mice for CX3CR1 receptor show a prolonged microglial activation after administration of lipopolysaccharide (LPS) and an increased production of IL-1β by microglia (Corona *et al.*, 2010). Once activated microglia can lead to an exacerbation of the damage that causes a state of chronic inflammation or can trigger remodeling and repair of tissue that contribute to the functional recovery. This different activity is defined neurotoxic and neuroprotective respectively. Moreover, studies of hippocampal slices have shown that these mechanisms are triggered by different factors. Indeed, the response of the neurotoxic microglia may be caused by the injection of LPS, while administration of IL-4 induces the neuroprotective phenotype (Butovsky *et al.*, 2005). In general, microglia could produced neurotoxicity after an excessive and uncontrolled microglial stimulation, or when its function is impaired. Therefore, depending on the pathologic stimulus, microglia may undergo proliferation, migration through the damaged area or can induce the release of several factors, such as cytokines and reactive oxygen species (ROS).

DEPRESSION AND INFLAMMATION

Recently, has been paid particular attention to the relationship between the activation of the immune system and the development of depressive illness. Indeed, a large body of evidence, both clinical and pre-clinical, suggest a link between this two pathology (Connor & Leonard, 1998; Maes, 1999; Miller, 2010).

The first aspect to take into account is the similarity between typical symptoms of depression and the events that develop in patients with an altered inflammatory state. Indeed, these subjects show typical sign of sicknes behavior, like fever, nausea, irritability, loss of appetite

and changes in sleep-wake cycle (Dantzer & Kelley, 2007). The second evidence concerns the increased activation of the inflammatory system observed in depressed patients, in which it has been observed an increase of pro-inflammatory cytokines and chemokines (Raison *et al.*, 2006). Indeed, recent meta-analysis conducted by Howren and co-workers show an increase in the periphery of IL-6 and C-reactive protein (CRP) in depressed patients (Howren *et al.*, 2009). Further studies carried out by van den Biggelaar show as high concentrations of CRP and IL-1β are predictive of a late onset of depressive illness, which suggests that inflammation precedes and is potentially able to lead to depression (van den Biggelaar *et al.*, 2007). One other aspect to take into account is the increased vulnerability to develop depression in subjects treated with drugs with pro-inflammatory activity (Gimeno *et al.*, 2009). Indeed, patients vaccinated for Salmonella show low mood, fatigue, mental confusion, and psychomotor slowness (Brydon *et al.*, 2009). These depressive symptoms are associated with increased concentrations of inflammatory mediators in the periphery. Other studies have shown that chronic administration of inflammatory cytokines such as IFN-α, induces depressive symptoms (Miyaoka *et al.*, 1999; Asnis & De La Garza, 2006; Lotrich, 2009). Moreover, it was shown for the first time that antidepressant treatment may prevent the development of depression during chronic immune stimulation (Musselman *et al.*, 2001,Raison, 2005 #399). In clinical trials, chronic treatment of viral diseases or cancer carried out with recombinant cytokines has led patients to manifest episodes of acute psychosis, characterized by depression (Capuron & Dantzer, 2003). Finally, a relationship between depression and inflammation was demonstrate also by the comorbidity between this two pathology (Benton *et al.*, 2007; Anisman *et al.*, 2008a). Indeed, the onset of depressive disorders is more prevalent in patients suffering from conditions that alter the inflammatory system, such as cardiovascular disease, cancer,

diabetes type 2, rheumatoid arthritis. For example, the probability to develop depression in patients with coronary heart disease is three times higher compared to healthy individuals (Frasure-Smith & Lesperance, 2006). Moreover, genetic vulnerability to developing depression is also associated with alterations in cytokines expression. Indeed, Fredericks and colleagues show that healthy subjects carrying the *short* variant of the serotonin stransporter gene have a high ratio of IL-6/IL-10 compared to individuals with the *long* one (Fredericks *et al.*, 2010), suggesting that an unbalanced between pro and anti-inflammatory components could contribute to increased susceptability to the development of depression.

Moreover, polymorphisms of genes coding for interleukins seem to influence the response to drug treatment. Indeed, recent studies show that functional variations of IL-1β and TNF-α genes, two cytokines involved in the pathology of depression (Thomas *et al.*, 2005), may underlie the increased risk of developing depression and reduced responsiveness to antidepressant therapy (Jun *et al.*, 2003; Yu *et al.*, 2003). In particuar, patients with depressive disorders that have specific polymorphisms of IL-1β gene are less responsive to treatment with antidepressants (Baune *et al.*, 2010). In brain imaging studies this polymorphism is associated with a diminished response to emotional stimuli in the amygdala and in the anterior cingulate cortex, indicating a possible lack of activity of antidepressant in this specific brain regions. In addition to clinical studies, there are a number of pre-clinical evidence supporting the relationship between inflammation and depression. Indeed, it has been demonstrated that the induction of inflammation in rodents is associated to the development of a depressive phenotype. One of the most widely experimental approaches that is been used is the administration of lipopolysaccharide (LPS), a component of gram negative bacteria's wall that activates the immune system and induces an inflammatory response (Raetz & Whitfield, 2002).

In particular, a study performed in mice has shown that, together with the inflammatory response, the injection of LPS induces the onset of the depressive-like behavior (Dantzer *et al.*, 2008). Interestingly, these effects follow a specific temporal profile. Indeed, it is possible to distinguish two phases: the first, which is observed after 2-6 hours after the inflammatory challenge, characterized by the production of pro-inflammatory cytokines and the appearance of the so-called sickness behavior, and a second one, after 24 hours, where the levels of cytokines tend to normalize and became manifest the depressive phenotype.

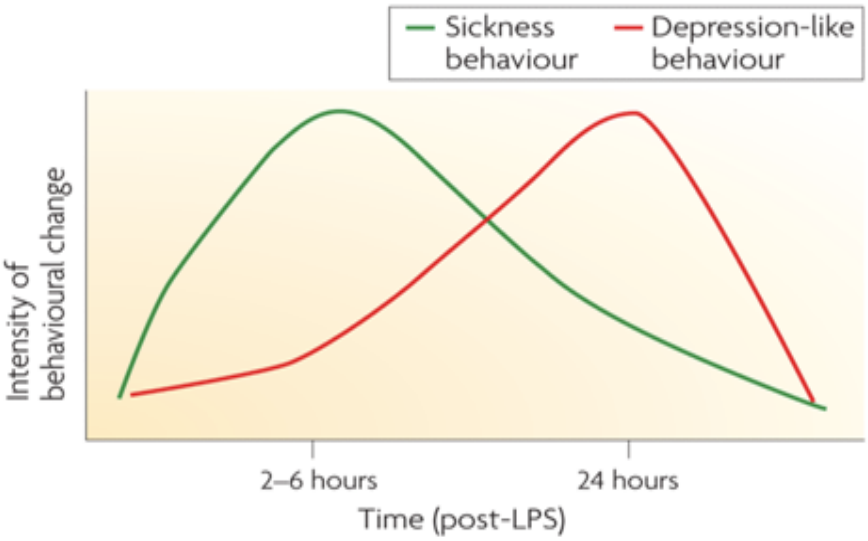

Figure 2

Temporal profile of LPS effect.

2. AIM OF THE STUDY

Major depression is a psychiatric disorder characterized by the interaction of genetic, biological, social and environmental factors, which play in concert to determine the development of the disease. One of the major problems associated with this disease is the high percentage of patients who do not respond adequately to drug therapy. Indeed, despite the large number of drugs available on the market, about 30% of depressed patients do not adequately respond to treatment. It is therefore evident the need to identify novel pharmacological 'targets' in order to improve the clinical outcome. Recently, particular attention has been paid to the relationship between the central nervous system and the peripheral mediators, identifying in inflammation a possible component that could contribute to the development of many psychiatric disorders, including depression (Raison *et al.*, 2006; Dantzer *et al.*, 2008; Haroon *et al.*, 2012). In fact, pre-clinical and clinical evidence have shown a close relationship between depression and disorders characterized by an altered inflammatory state such as cancer, chronic degenerative diseases and cardiovascular disorders (Benton *et al.*, 2007; Anisman *et al.*, 2008b). In addition, depressed patients show increased levels of inflammatory mediators in both peripheral and central level. Finally, experiments carried out in animals have shown that immune and inflammatory challenge can lead to the development of a depressive-like phenotype (Frenois *et al.*, 2007).

On these bases, the aim of the present study was to investigate the relationship between depression, antidepressant treatment and inflammation using two different experimental approaches.

In the first study, we evaluated the ability of the new antidepressant agomelatine to modulate specific components of the immune response under basal conditions as well as following an acute inflammatory challenge, achieved through acute administration of the bacterial toxin lipopolysaccharide (LPS) in adult rats. Specifically, we examined the possibility that a chronic treatment with agomelatine could influence the inflammatory response induced by LPS. To this purpose, we

investigated the expression of pro-inflammatory cytokines, markers of microglial activation and enzymes involeved in the metabolic pathway of tryptophan (Molteni *et al.*, 2013)

In the second study, since it is well known that a genetic background of vulnerability contribute to the onset of depression, we analyzed the inflammatory state and responsiveness in a genetic animal model of depression susceptability. In particular, since one of the genes that has been consistently associated with depression vulnerability is the serotonin transporter (SERT) gene, we conducted our experiments in rats with partial or total deletion of this gene in basal condition and after the immune challenge (Macchi *et al.*, 2013).

3. RESULTS

CHAPTER I

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MODULATION OF THE INFLAMMATORY RESPONSE IN RATS CHRONICALLY TRATED WITH THE ANTIDEPRESSANT AGOMELATINE

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Abstract

Growing evidence suggests that the activation of the inflammatory/immune system contributes to depression pathogenesis, a hypothesis that might hold strong clinical implication. Indeed more than 30% of depressed patients fail to achieve remission, which poses the necessity to identify systems that may represent novel targets for medications. Accordingly, goal of this study was to evaluate the ability of the antidepressant agomelatine to modulate specific components of the immune response in the rat brain following an inflammatory challenge with lipopolysaccharide (LPS). To this aim, adult male rats were chronically treated with agomelatine before being acutely challenged with LPS 16 h after the last drug administration. Rats were sacrificed 2, 6, or 24 h after the challenge and several components of the inflammatory response have been investigated by using real time PCR or ELISA.

We found that agomelatine significantly reduced the LPS-induced up-regulation of the pro-inflammatory cytokines interleukin-1β and interleukin-6 in the rat brain as well as at peripheral level. At central level, these effects are associated to the inhibition of NF-κB translocation as well as to alterations of mechanisms responsible for microglia activation. In addition, we found that agomelatine was also able to alter the expression of enzymes related to the kynurenine pathway that are thought to represent important mediators to inflammation-related depression.

These data disclose novel properties that may contribute to the therapeutic effect of agomelatine providing evidence for a crucial role of specific components of the immune/inflammatory system in the antidepressant response and thereby in depression etiopathology.

Key words: depression, lipopolysaccharide, hippocampus, microglia, tryptophan catabolism, gene expression

1. Introduction

Major depression is a severe psychiatric disorder that has lifetime prevalence in excess of 15% and is the fourth leading cause of disability worldwide (Moussavi et al., 2007). One of the major problems associated with depression is the relevant percentage of patients who do not adequately respond to antidepressant therapy, as well as the high rate of relapse. For this reason there has always been a great deal of interest in understanding the molecular mechanisms contributing to depression etiopathology as well as in identifying systems and pathways that may play a critical role in antidepressant response. It is known that this complex disorder is characterized by the interaction of genetic and environmental factors that play in concert. Moreover, a growing body of evidence described and partially characterized dysfunction of multiple systems, including neurotransmitters, hormones, signaling pathways, neurotrophic and neuroplastic molecules. Nevertheless, the increased knowledge of depression biology has not led to an improvement in the overall impact of pharmacotherapy, possibly because a number of systems that are affected in mood disorders may not be adequately modulated by pharmacological treatments.

Currently, there is strong evidence that depression is associated with alterations of immune/inflammatory system (Dantzer et al., 2008; Haroon et al., 2012; Raison et al., 2006). Particularly, it is known that depression is accompanied by both immune-suppression (decreases in natural killer cell activity and lymphocyte proliferation) and immune-activation (immune cell proliferation and increased production of inflammatory markers) (Irwin and Miller, 2007). Also, increased concentration of pro-inflammatory cytokines including interleukin (IL)-6 and tumor necrosis factor (TNF)-a as well as their soluble receptors have been found in the blood and in the cerebrospinal fluid of depressed patients (Dowlati et al., 2010). Moreover, increases of

acute phase proteins, such as C-reactive protein, chemokines, and adhesion molecules have been described in subjects suffering from depression (Howren et al., 2009). In addition, patients carrying a genetic variant of the pro-inflammatory cytokine IL-1β fail to achieve remission (Baune et al., 2010).

A role for inflammation in depression is also supported by the findings that cytokine administration induces depressive symptoms, as occurs in the 30% of hepatitis C patients who are treated with the immune activator interferon-alpha (Asnis and De La Garza, 2006; Miyaoka et al., 1999). Finally, depression shows elevated comorbidity with diseases such as cancer, arthritis rheumatoid, cardiovascular and neurodegenerative diseases, which are associated with an altered inflammatory state (Benton et al., 2007).

In line with these observations, preclinical studies have demonstrated that administration of the cytokine inducer lipopolysaccharide (LPS) elicits several depressive–like behaviors (Frenois et al., 2007; O'Connor et al., 2009; Zhu et al., 2010). It has to be noted that this effect follows a precise temporal profile with an earlier "sickness behaviour", which peaks in the first 2-6 h and a "depressive-like behaviour", which became manifest 24 h later when sickness behaviour is diminished (Dantzer et al., 2008).

Based on these observations, the aim of the present study was to evaluate the ability of chronic treatment with agomelatine, a new antidepressant with a unique receptor profile as a MT1/MT2 melatonergic agonist and 5-HT2C receptor antagonist (de Bodinat et al., 2010), to modulate the inflammatory response in rodents. The antidepressant properties of this drug have been validated in different animal models (Bertaina-Anglade et al., 2006; Morley-Fletcher et al., 2011; Papp et al., 2003; Rainer et al., 2011) and may also rely on the ability to regulate neurogenesis and neurotrophic mechanism, through a synergistic activity between the two receptor moieties (Calabrese et al., 2011; Molteni et al., 2010; Racagni et al., 2011). In addition,

agomelatine is able to resynchronize disturbed circadian rhythms often reported by depressed patients, an effect observed both at clinical and preclinical level (De Berardis et al., 2011; Srinivasan et al., 2012).

To evaluate the impact of agomelatine on the inflammatory system, rats chronically treated with agomelatine were injected with LPS and sacrificed at different time points in order to establish the ability of the antidepressant to interfere with the temporal progression of the inflammatory response.

2. Experimental procedures

General reagents were purchased from Sigma–Aldrich (Milan, Italy) whereas molecular biology reagents were obtained from Applied Biosystem Italia (Monza, Italy), Eurofins MWG-Operon, (Ebersberg, Germany) and Bio-Rad Laboratories S.r.l. (Segrate, Italy). Agomelatine was provided by IRIS (Institut de Recherches Internationales Servier, France) whereas lipopolysaccharide was purchased by Sigma–Aldrich.

2.1. Animals

Adult male Sprague-Dawley rats (Charles River, Calco, Italy) weighing 300-350 g were used throughout the experiments. Rats were housed in groups of 4 per cage under standard conditions (12-h light/dark cycle with food and water *ad libitum*) and were exposed to daily handling for 1 week before any treatment. All animal handling and experimental procedures were approved by the University of Milan Institutional Animal Care and Use Committee and adhered to the Italian legislation on animal experimentation (Decreto Legislativo 116/92), the EC (EEC Council Directive 86/609 1987), and the National Institutes of Health Guide for the Care and Use of Laboratory

Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Experimental design and pharmacological treatments

Rats were chronically (21 days) treated by oral gavage with vehicle (VEH; hydroxyethylcellulose, 1%, 1 ml/kg) or agomelatine (AGO; 40 mg/kg) at 5 pm (2 h before the dark phase to mimic the evening administration suggested for agomelatine therapy) before being challenged with lipopolysaccharide (LPS; 250 □g/kg, i.p.) or saline (SAL) 16 h later (VEH or AGO at 5 pm and LPS or SAL at 9 am of the next day). The choice of agomelatine dose was based on previous work demonstrating its activity in different animal models of depression and anxiety (Papp et al., 2003; Papp et al., 2006). In order to establish the ability of agomelatine to interfere with the initial (2-6 h) or the later phase (24 h) of the inflammatory response, rats were sacrificed by decapitation at three different time points post-LPS treatment: 2 h (11 am), 6 h (3 pm), and 24 h (9 am next day). This experimental design implies twelve experimental groups (n=10 rats per group). Blood samples were taken for the analysis of circulating levels of cytokines, whereas brain regions of interest (ventral hippocampus: VH, dorsal hippocampus: DH and prefrontal cortex: PFC) were rapidly dissected, frozen on dry ice and stored at -80 °C for molecular analyses. These regions were chosen based on their involvement in depression etiophatology. Particularly, ventral hippocampus relates to emotions and stress whereas dorsal hippocampus is primarily engaged in cognitive functions (Fanselow and Dong, 2010).

2.3. Cytokines plasma measurements

Samples of blood from each rat were collected in heparinized tubes. Plasma was separated by centrifugation (5000 rpm for 10 min at 4°C) and IL-1β and IL-6 protein levels were simultaneously quantified by

Luminex Technology using a Rat Multianalyte Profiling kit (R&D Systems, Inc.) according to the manufacturer's instructions. Briefly, analyte-specific antibodies were pre-coated onto color-coded microparticles. Microparticles, standards and samples were added into wells for the binding of the analytes of interest with the immobilized antibodies. After washing to remove unbound substances, a biotinylated antibody cocktail specific to the analytes of interest was added to each well. Following a second wash, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the captured biotinylated detection antibodies, was added to each well. A final wash removed unbound Streptavidin-PE, the microparticles were re-suspended in buffer and read using the Luminex analyzer. One laser is microparticle-specific and determines which analyte is being detected whereas the other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound.

2.4. RNA preparation and gene expression analyses

For gene expression analyses, total RNA was isolated from the different brain regions by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l.; Segrate, Italy) according with the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were then processed for real-time polymerase chain reaction (PCR) as previously reported (Calabrese et al., 2012) to assess mRNA levels of: interleukin-1□ (IL-1□); interleukin-6 (IL-6); integrin alpha M or cluster of differentiation CD11b; cluster of differentiation CD68; chemokine (C-X3-C Motif) ligand 1 (CX3CL1; fractalkine) and its receptor CX3R1; kynurenine-3-monooxygenase (KMO); kynurenine aminotransferase (KAT II).

Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination and subsequently analyzed by TaqMan qRT–PCR instrument (CFX384 real-time system, Bio-Rad Laboratories S.r.l.)

using the iScript one-step RT–PCR kit for probes (Bio-Rad Laboratories S.r.l.). Samples were run in 384-well format in triplicates as multiplexed reactions with a normalizing internal control (□-actin). Thermal cycling was initiated with incubation at 50 °C for 10 min (RNA retrotranscription), and then at 95 °C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95 °C for 10 s to enable the melting process, and then for 30 s at 60 °C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression. Probe and primer sequences used were purchased from Applied Biosystem Italia and Eurofins MWG-Operon.

2.5. Preparation of nuclear fraction and evaluation of NF-κB dependent transcription

Ventral hippocampus was manually homogenized using a glass-glass potter in a pH 7.4 cold buffer containing 0.32 M sucrose, 0.1 mM EGTA, 1 mM HEPES solution in presence of a complete set of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors. The total homogenate was centrifuged at 2500 g for 10 min at 4 °C, thus obtaining a pellet (P1) corresponding to the nuclear fraction, which was re-suspended in a buffer (20 mM HEPES, 0.1 mM dithiothreitol DTT, 0.1 mM EGTA) with protease and phosphatase inhibitors. Total protein content was measured according to the Bradford protein assay procedure (Bio-Rad Laboratories S.r.l.), using bovine serum albumin as calibration standard. The same quantity of nuclear proteins was used to assess nuclear factor (NF)-κB dependent transcription using the NF-κB (p65) transcription factor assay kit (Cayman Chemical Company, Michigan, USA). Briefly, 10 □g of nuclear extract of each sample were loaded in duplicate in a 96 well plate to specifically bind to the NF-κB response element containing in double stranded DNA sequence immobilized to the wells. Following incubation with specific primary and secondary antibodies, NF-κB

(p65) was detected by spectroscopy (signal read 450 nm, 0.1 s). Parthenolide at 10 mM was used as a reference inhibitor of NF-κB driven transcription.

2.6. Statistical analyses

All the analyses were carried out in individual animals (independent determinations) by using different statistical test according to the effect examined. Specifically, the effect of LPS on vehicle- or agomelatine-treated animals was analyzed by one-way analysis of variance (ANOVA). Conversely, the influence of the antidepressant on LPS-induced inflammatory response was evaluated by two-way ANOVA performed at each specific time point, with treatment (vehicle vs. agomelatine) and challenge (LPS vs. saline) as independent factors. When appropriate, further differences were analyzed by Fisher's Protected Least Significant Difference (PLSD) or Single Contrast post-hoc test (SCPHT). Significance for all tests was assumed for $p < 0.05$. For graphic clarity, data are presented as means percent ± standard error (SEM) of control group, namely vehicle-pretreated rats received saline (VEH/SAL).

3. Results

3.1. Effect of agomelatine on IL-1□ and IL-6 basal and LPS-induced levels

As a first step, we evaluated the ability of chronic agomelatine to modulate the inflammatory response to LPS injection by measuring the mRNA levels of the pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6) in the hippocampus (ventral VH and dorsal DH subregions) and prefrontal cortex (PFC) of rats pre-treated with vehicle or agomelatine. Although basal cytokine gene expression was slightly increased by chronic antidepressant treatment (See

supplemental table 1), this effect was not significant in a global statistical analysis and is probably negligible with respect to the robust induction of IL-1□ and IL-6 observed after LPS in all the brain regions considered. Indeed, as shown in figure 1, both cytokines were strongly up-regulated by the inflammatory challenge with specific temporal and anatomical profiles. Specifically, in the ventral hippocampus (Fig. 1A) IL-1□□mRNA levels were markedly increased [$F(1,32) = 190.545$, $P < 0.001$] 2 and 6 h after LPS injection remaining significantly elevated also 24 h after the challenge. A quite similar profile was observed in the dorsal hippocampus (Fig. 1B) and in prefrontal cortex (Fig. 1C), where IL-1□ gene expression was increased by LPS [DH: $F(1,35) = 47.471$, $P < 0.001$; PFC: $F(1,34) = 32.072$, $P < 0.001$, respectively] at 2 and 6 h but not at 24 h. Interestingly, chronic pre-treatment with agomelatine significantly reduced the LPS-induced up-regulation of IL-1□. Indeed, as shown in figures 1A, 1B, and 1C, chronic agomelatine attenuated the increase of IL-1□ mRNA levels observed 2 and 6 h after the challenge in all the brain regions examined (VH: AGO/LPS 2 h -439% $P < 0.01$ and 6 h -1422% $P < 0.05$ vs. VEH/LPS; DH: AGO/LPS 2 h -1347% and 6 h -1379% $P < 0.001$ vs. VEH/LPS; PFC: AGO/LPS 2 h -1064% $P < 0.001$ and 6 h -1174% $P < 0.001$ vs. VEH/LPS).

The expression profile of IL-6 mRNA levels (Fig. 1D, 1E, 1F) was modulated by the inflammatory challenge similarly to what observed for IL-1□ [VH: $F(1,39) = 80.062$, $P < 0.001$; DH: $F(1,35) = 149.834$, $P < 0.001$; PFC: $F(1,35) = 41.501$, $P < 0.001$] as the cytokine was mainly up-regulated at the same time points. The maximal increase was observed at 2 h in all the brain regions examined, an effect that was back towards controls in the next 24 h. Once again, agomelatine was able to limit the LPS-induced cytokine up-regulation (Fig. 1D, VH: AGO/LPS 2 h -191% and 6 h -122% $P < 0.001$ vs. VEH/LPS; Fig. 1E, DH: AGO/LPS 2 h -478% and 6 h -515% $P < 0.001$ vs. VEH/LPS; Fig. 1F, PFC: AGO/LPS 6 h -211% $P < 0.01$ vs. VEH/LPS).

All the percentage of changes for the pro-inflammatory cytokines IL-1□ and IL-6 in different brain regions and the corresponding *P* values for statistical significant are listed in supplementary Table 1.

We next examined peripheral changes in IL-1β and IL-6 by measuring their plasma levels 6 h after the inflammatory challenge. Chronic agomelatine treatment completely prevented the increase of IL-1β (Fig. 2A) and IL-6 (Fig. 2B) protein levels produced by LPS injection in vehicle-treated animals.

3.2. Effect of agomelatine on NF-κB dependent transcription

It is known that LPS-induced cytokine production is dependent on the activation of the transcription factor NF-κB (Li and Verma, 2002). Therefore, we investigated the effect of agomelatine on the nuclear translocation of the transcription factor at the earlier time point (2 h), focusing the analysis on the ventral hippocampus that, as stated before, is more relevant for depression (Fanselow and Dong, 2010). We found that chronic agomelatine treatment did not alter nuclear levels of NF-κB under basal condition but was able to affect its stimulation by LPS, as indicated by the significant Treatment x Challenge interaction [$F(1,48)=4.148$, $P < 0.05$]. Indeed, as shown in figure 3, nuclear translocation of NF-κB was increased following LPS injection [$F(1,48)=226.203$, $P < 0.001$] in vehicle (VEH/LPS +56%, $P < 0.001$ vs. VEH/SAL) as well as in agomelatine-treated rats (AGO/LPS +43%, $P < 0.001$ vs. AGO/SAL), although such effect was significantly attenuated in antidepressant pre-treated animals (AGO/LPS -22%, $P < 0.001$ vs. VEH/LPS).

3.3. Effect of agomelatine on microglia activation

Beside cytokine production, one key aspect in the inflammatory response is microglia activation (Saijo and Glass, 2011). Accordingly, we evaluated the potential of agomelatine to interfere with LPS-induced microglia activation by measuring the expression of CD11b as

marker for this cellular phenotype (Perego et al., 2011). As shown in figure 4A, we found that LPS increased CD11b gene expression in the ventral hippocampus starting at 6 h post-injection with a maximal up-regulation at 24 h, while these changes were significantly attenuated by agomelatine pre-treatment (AGO/LPS 6 h: -124% $P < 0.001$ and 24 h: -156% $P < 0.01$ vs. VEH/LPS). We also measured the expression of CD68, as marker of active phagocytosis (de Beer et al., 2003; Ramprasad et al., 1996). We found that LPS injection increased the mRNA levels for CD68 in vehicle pre-treated rats (Fig. 4B) with a profile similar to microglia activation but the changes at 24 h were larger in animals received agomelatine (AGO/LPS +94% vs. VEH/LPS, $P < 0.001$).

All the percentage of changes for the pro-inflammatory cytokines CD11b and CD68 in ventral hippocampus and the corresponding *p* values for statistical significant are listed in supplementary Table 2.

3.3. Effect of agomelatine on neuron-glia cross talk

One potential mechanism by which agomelatine may interfere with microglia activation is the modulation of the cross-talk between neuron and glia. Indeed, recent findings indicate that neurons may control microglia through signals that keep these cells in a resting state or signals that favour their activation (Biber et al., 2007). The interaction of fractalkine (CX_3CL1) with its receptor (CX_3CR1) is one of the main neuronal signals that maintain microglia in a resting state (Harrison et al., 1998). On these bases, we measured CX_3CL1 and CX_3CR1 gene expression in the ventral hippocampus of rats pre-treated with agomelatine, challenged with LPS and sacrificed 24 h later, when the effect of the antidepressant on LPS-induced microglia activation was maximal. As indicated by two-way ANOVA analysis, both agomelatine and LPS regulated CX_3CL1 mRNA levels [$F(1,24) = 29.861$, $p < 0.001$ and $F(1,24) = 15.559$, $P < 0.001$ respectively]. Specifically, as shown in figure 5A, the antidepressant was able to increase fractalkine gene expression (AGO/SAL +73% vs. VEH/SAL, *P*

< 0.001), while preventing its reduction following LPS injection (AGO/LPS +46% vs. VEH/LPS, *P* < 0.01). A similar profile was observed for CX_3CR1 since agomelatine increased the expression of fractalkine receptor in both saline- (AGO/SAL +27% vs. VEH/SAL, *P* < 0.05) and LPS- (AGO/LPS +20% vs. VEH/LPS, *P* < 0.05) treated animals.

3.4. Effect of agomelatine on kynurenine pathway

One important consequence of LPS-induced cytokine increase, which may bear relevance for depression, is the activation of indolamine 2,3 dioxigenase (IDO), the rate-limiting enzyme for the synthesis of kynurenine within the tryptophan catabolism pathway (Schwarcz et al., 2012). Kynurenine is degraded along two catabolic branches that result in antagonistic products: kynurenine-3-monooxygenase (KMO), responsible for the conversion of kynurenine into quinolinic acid that my activate glutamate N-Methyl-D-aspartate (NMDA) receptors, and kynurenine aminotransferase II (KAT II) that metabolizes kynurenine into kynurenic acid, an antagonist of NMDA receptors. Accordingly, we investigated the ability of chronic agomelatine treatment to affect KMO and KAT II gene expression under basal condition or 24 h after the inflammatory challenge. As indicated by two-way ANOVA analysis, the expression of KMO was significantly regulated by agomelatine [$F(1,19) = 39.944$, $P < 0.001$] as well as LPS [$F(1,19) = 38.804$, $P < 0.001$]. Specifically, as shown in figure 6A, KMO mRNA levels were strongly up-regulated by LPS only in vehicle-treated rats (VEH/LPS +128% vs. VEH/SAL, *P* < 0.001), but not in animals that received agomelatine, as indicated by the significant Treatment x Challenge interaction [$F(1,19) = 6.960$, $P < 0.01$]. Indeed, agomelatine pre-treatment significantly reduced LPS-dependent KMO increase (AGO/LPS -129% vs. VEH/LPS, *P* < 0.001). Furthermore, we found that chronic antidepressant treatment *per se* significantly reduced KMO expression (AGO/SAL -53% vs. VEH/SAL, *P* < 0.05).

In addition, we found that KAT II mRNA levels were altered by the antidepressant and LPS [$F(1,26) = 13.947$, $P < 0.001$ and $F(1,26) = 6.267$, $P < 0.01$ respectively]. Particularly, the expression of this enzyme was significantly up-regulated only in rats that were pre-treated with agomelatine and challenged with LPS (AGO/LPS +34% vs. VEH/LPS, $P < 0.01$ and +26% vs. AGO/SAL, $P < 0.01$).

4. Discussion

Our results demonstrate that the novel antidepressant agomelatine attenuates the inflammatory response induced by LPS injection, showing for the first time *in vivo* that chronic antidepressant treatment may affect different components of the immune/inflammatory system. Moreover we show that agomelatine acts on the whole temporal progression of the inflammatory response mediated by LPS injection (Dantzer et al., 2008), which includes an early phase characterized by the release of pro-inflammatory cytokines and a later phase that may be characterized by changes in microglia as well as of systems that may lie downstream cytokine induction.

Previous studies reported that antidepressant drugs have immunoregulatory effects in normal volunteer as well as in animals. In particular, both *in vitro* and *in vivo* studies in animals and humans -including depressed patients- have analyzed the influence of antidepressants on cytokine levels finding that -despite some controversial results- these drugs are able to limit the production of pro-inflammatory cytokines (Janssen et al., 2010). Our results are in agreement with this notion, since chronic agomelatine pre-treatment reduced the pro-inflammatory cytokine up-regulation mediated by LPS. Specifically, the effect of agomelatine on IL-1□ and IL-6 was observed at both peripheral level -where the antidepressant

completely blocked the effect of LPS- as well as in different brain regions. Given the intraperitoneal LPS administration that leads to peripheral acute inflammation, different mechanisms may contribute to the action of agomelatine in the two compartments. However, a feasible hypothesis implies the involvement of the transcription factor NF-κB, which on one hand may be activated by a variety of stimuli including LPS and selected cytokines in peripheral immune cells and in the brain, and on the other it regulates the transcription of pro-inflammatory cytokine genes (Haddad, 2009; Li and Verma, 2002). In line with these considerations we found that agomelatine prevented the nuclear translocation of NF-κB induced by LPS in the ventral hippocampus.

We also found that chronic agomelatine treatment can alter microglia activation following LPS injection. These cerebral immune cells are normally present in the healthy brain were they actively survey their surrounds, ready to rapidly respond to any microenvironment alteration (Hanisch and Kettenmann, 2007). As expected, the inflammatory challenge induced microglia activation, as demonstrated by the progressive increase of CD11b gene expression that was maximal at the later phase of the response to LPS, whereas agomelatine was able to prevent -at least in part- CD11b up-regulation. In parallel, agomelatine amplified the LPS-induced phagocytic activity, as indicated by the up-regulation of the marker of phagocytic macrophages CD68 observed in animals pre-treated with the antidepressant and given LPS. Indeed, by measuring CD11b we do not distinguish between resident activated microglial cells and recruited macrophages and only through the analysis of CD68 we differentiated the effect of the antidepressant on resident microglia from that on invading macrophages (de Beer et al., 2003; Ramprasad et al., 1996). Given that microglial phagocytosis of dead or dying neurons -namely secondary phagocytosis- can be useful to prevent the release of damaging or pro-inflammatory intracellular components

(Neher et al., 2012), its increase by agomelatine suggest that antidepressant treatment may be beneficial.

One of the mechanisms that may underline the reduction of microglia activation by agomelatine is neuronal-glia cross-talk. Indeed, microglia is maintained under resting phenotype through neuron-derived signals including CX3-chemokine ligand 1 (CX3CL1, fractalkine), which acts on the corresponding receptor CX3CR1 expressed by microglia (Hughes et al., 2002; Nishiyori et al., 1998). We found that chronic agomelatine treatment promoted the interaction between fractalkine and its receptor, identifying for the first time the possibility that antidepressants may regulate CX3CL1 and therefore control mechanisms that may be responsible for microglia activation. Although many others "off and on signals" might contribute to the reduction of microglia activation by agomelatine (Saijo and Glass, 2011), a recent work highlights the role of fractalkine in inflammation-related depression. Indeed, it has been reported that CX3CR1 knock-out mice challenged with LPS display increased levels of IL-1□ and persistent microglia activation, and that such alterations were associated with protracted depressive-like behavior (Corona et al., 2010). Hence the modulatory effect of agomelatine on CX3CL1 does not only have implication for inflammation but may actually be relevant for its antidepressant properties.

A further mechanism that may contribute to cytokine-related depression is the modulation of the tryptophan catabolism pathway (Dantzer et al., 2011). Indeed, it is well established that several pro-inflammatory cytokines can stimulate the enzyme indoleamine 2,3-dioxygenase (IDO) which converts the essential aminoacid tryptophan into the intermediate metabolite kynurenine, which is itself inactive. IDO also oxidizes other indole-derived molecules such as melatonin (Mackay et al., 2009), since agomelatine is a potent mono-indole MT1/MT2 receptor agonist, it may compensate the reduction of melatonergic function. Here we show for the first time that chronic

antidepressant treatment *in vivo* modulates the expression of two of the major enzymes involved in the subsequent steps of tryptophan catabolism: kynurenine 3-monooxygenase (KMO) and kynurenine aminotransferase (KAT)-II that acting on kynurenine may switch the path toward neurotoxic or neuroprotective arms respectively (Muller and Schwarz, 2008; Myint and Kim, 2003). Our data indicate that agomelatine prevented the LPS-dependent increase of KMO and was also able to reduce *per se* the expression of this enzyme. Given that KMO converts kynurenine to quinolinic acid, an N-Methyl-D-aspartate (NMDA) agonist (Schwarcz et al., 2012), it is feasible to postulate that diminished KMO expression after the antidepressant reduces NMDA receptor activation, which may also relevant for antidepressant properties (Hashimoto, 2009). Indeed, it been reported that infusion of the NMDA receptor antagonist ketamine exerts rapid antidepressant effect even in resistant depressed patients (Zarate et al., 2006). In addition, we have recently demonstrated that chronic stress increases NMDA receptor expression and activation, an effect that may be normalized by chronic antidepressant treatment (Calabrese et al., 2012).

In line with the effect of agomelatine on cytokines and KMO expression, *in vitro* studies indicated that IL-1□ enhances NMDA receptor activation (Viviani et al., 2003).

Interestingly, although the expression of KAT II was not altered by agomelatine, we found that chronic antidepressant treatment up-regulated the enzyme only in LPS-injected animals, to suggest that under challenging conditions agomelatine may stimulate this enzyme, which will eventually shift the biosynthetic pathway toward the formation of kynurenic acid that acts as NMDA receptor antagonist (Schwarcz et al., 2012). It should be noted that, despite the functional importance of the steps regulated by KMO and KAT, the majority of the studies focused on IDO (O'Connor et al., 2009; Salazar et al., 2012) and only *in vitro* studies examined the effect of antidepressant drugs on the subsequent metabolic steps (Kocki et al.,

2012; Zunszain et al., 2012). Although further studies are required to understand if the observed effects are peculiar for agomelatine, the ability to interfere with the kynurenine pathway appear to be of clinical relevance considering the correlation between elevated cerebrospinal fluid levels of quinolinic acid and depressive symptoms observed in patients treated with interferon-□□(Raison et al., 2010). Moreover, it is important to consider that within the brain there may be a cellular distinction in the two pathways (KMO vs. KAT II): microglial cells contain KMO and therefore can produce quinolic acid whereas astrocytes contain KAT to generate kynurenic acid. According to our data, we may speculate that activated microglia cells might represent another important target for the modulatory activity of agomelatine beside its well-known effect in regulating circadian rhythms (de Bodinat et al., 2010).

We are aware that acute administration of LPS may result in an exaggerated inflammatory response that does not exactly reproduce the low grade of inflammation reported in depression (Evans et al., 2005). It will therefore be important to investigate in future studies the ability of agomelatine to interfere with inflammatory changes in models that may resemble more closely the clinical situation.

In conclusion, the present study provides novel evidence on the ability of the antidepressant agomelatine to interfere with molecular systems involved in inflammatory response. Both clinical (Muller et al., 2006) and preclinical studies (Brunello et al., 2006) suggest that combined use of anti-inflammatory and antidepressant drugs may have beneficial effects on major depression. Accordingly, in a translational perspective, since inflammation may contribute to the development of depression in a significant number of patients (Evans et al., 2005) and may be responsible for residual symptoms that impair or limit clinical remission, we suggest that the ability of agomelatine to modulate or interfere with immune/inflammatory system may represent an add-on value for its therapeutic activity.

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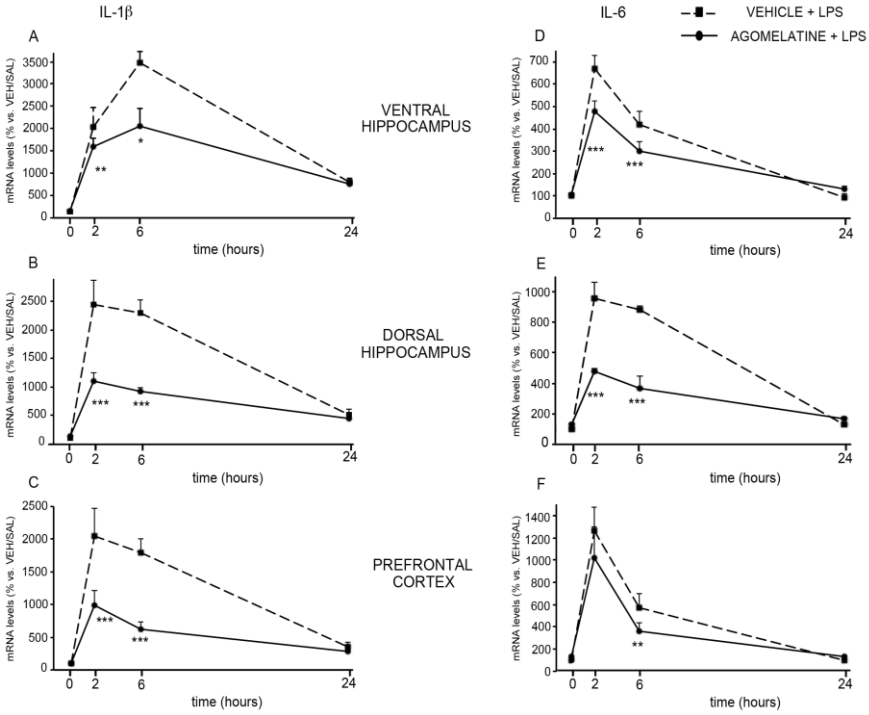
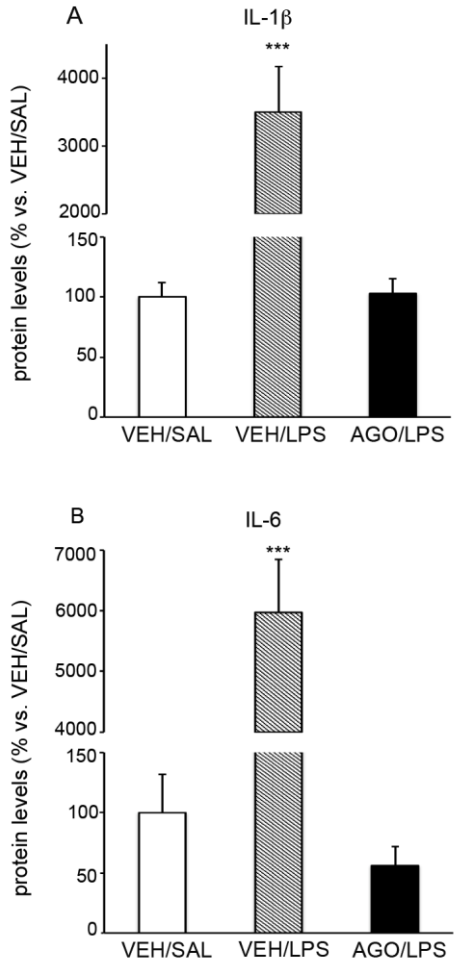
Figure 1



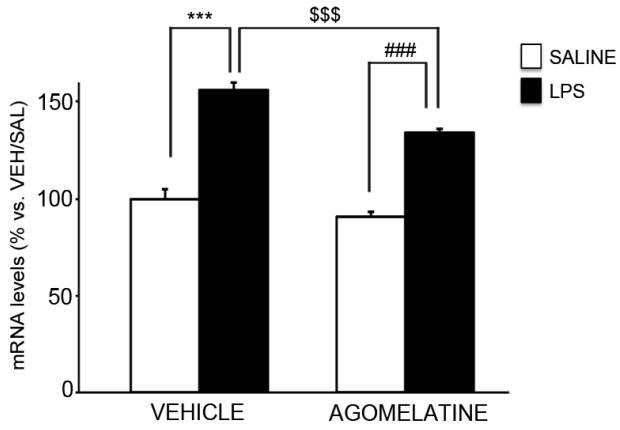
Effect of agomelatine on LPS-induced changes of cytokine gene expression. Chronic pre-treatment with agomelatine significantly reduced the LPS-induced up-regulation of Interleukin (IL)-1□ and Interleukin (IL)-6 mRNA levels in ventral hippocampus (A, D), dorsal hippocampus (B, E) and prefrontal cortex (C, F). Rats were chronically treated with vehicle (dashed line) or agomelatine (solid line) for 3 weeks before being challenged with LPS and sacrificed 2, 6 or 24 h later. "0" indicates "NO LPS injection". The data, expressed as a percentage of VEH/SAL treated animals (set at 100%), are the mean ± SEM of at least five independent determinations. The statistical effects illustrated in the figures refer to the comparison between AGO/LPS vs. VEH/LPS. Please refer to the text and table in supplemental information for all other comparisons. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001

Figure 2



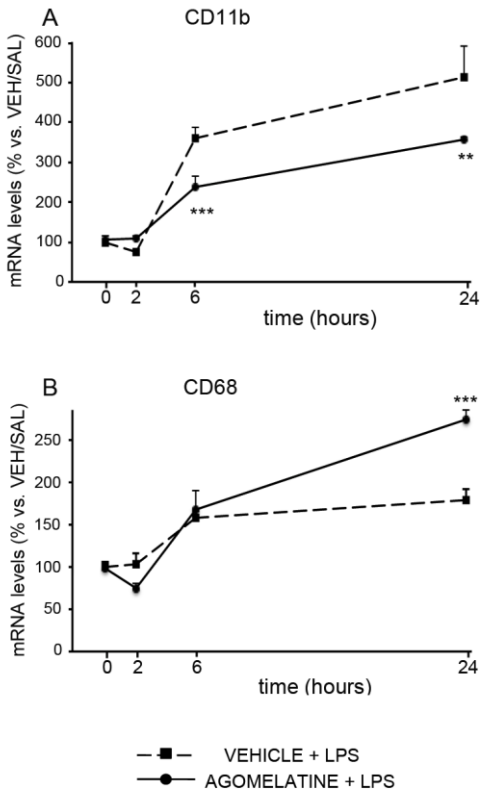
Effect of agomelatine on LPS-induced changes of cytokine plasma levels. Chronic pretreatment with agomelatine prevented the increase of IL-1β (A) and IL-6 (B) protein plasma observed 6 h after LPS injection. Rats were chronically treated with agomelatine for 3 weeks before being challenged with LPS and sacrificed 6 h later. The data, expressed as a percentage of VEH/SAL treated animals (set at 100%), are the mean ± SEM of at least five independent determination. ***P < 0.001 vs. VEH/SAL (Two-way ANOVA with SCPHT).

Figure 3



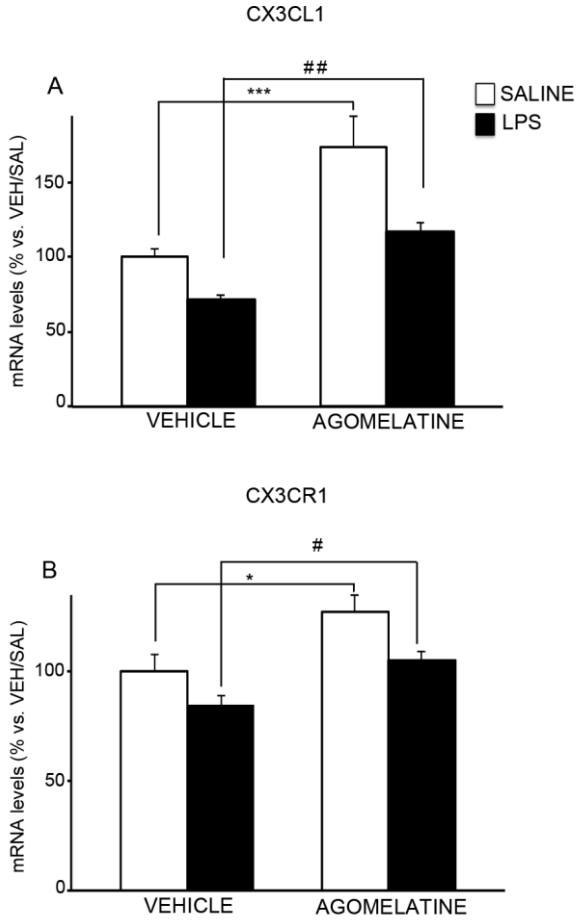
Effect of agomelatine on LPS-induced regulation of NF-κB translocation. Pre-treatment with agomelatine reduced the increase of LPS-dependent NF-κB translocation assessed in the nuclear fraction of ventral hippocampus of rats treated with the antidepressant for 3 weeks, challenged with LPS and sacrificed 2 h later. The data, expressed as a percentage of VEH/SAL treated animals (set at 100%), are the mean ± SEM of at least six independent determination. ***$P < 0.001$ vs. VEH/SAL, $^{\$\$\$}P < 0.001$ vs. VEH/LPS, $^{\#\#\#}P < 0.001$ vs. AGO/SAL (Two-way ANOVA with SCPHT).

Figure 4



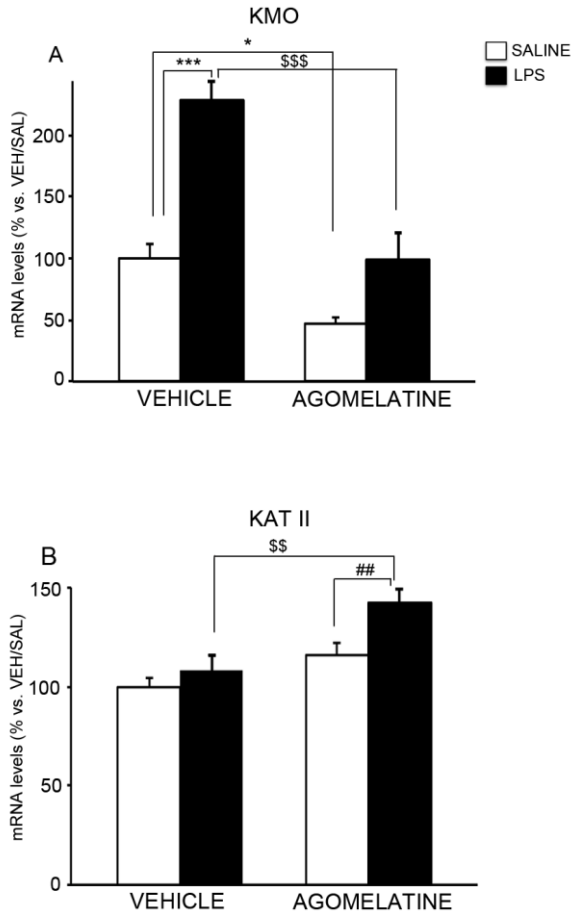
Effect of agomelatine on LPS-induced regulation of microglia activation. Chronic pre-treatment with agomelatine attenuated LPS-induced microglia activation through a reduction of CD11b gene expression (A) and an increase of CD68 gene expression (B). mRNA levels of CD11b and CD68 were measured in the ventral hippocampus of rats chronically treated with vehicle (dashed line) or agomelatine (solid line) for 3 weeks before being challenged with LPS and sacrificed 2, 6 or 24 h later. "0" indicates "NO LPS injection". The data, expressed as a percentage of VEH/SAL treated animals (set at 100%), are the mean ± SEM of at least six independent determinations. The statistical effects illustrated in the figures refer to the comparison between AGO/LPS vs. VEH/LPS. Please refer to the text and table 2 in supplemental information for the other comparisons. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. VEH/LPS (Two-way ANOVA with SCPHT).

Figure 5



Effect of agomelatine on neuron-glia cross-talk. CX3CL1 (A) and CX3CR1 (B) gene expression was measured in the ventral hippocampus of rats chronically treated with agomelatine for 3 weeks before being challenged with LPS and sacrificed 24 h later. Chronic agomelatine modulated mRNA levels of fractalkine and its receptor under basal condition as well as after the inflammatory challenge. The data, expressed as a percentage of VEH/SAL treated animals (set at 100%), are the mean ± SEM of at least five independent determination. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. VEH/SAL, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. VEH/LPS (Two-way ANOVA with SCPHT).

Figure 6



Effect of agomelatine on kynurenine pathway. KMO (A) and KAT II (B) gene expression was measured in the ventral hippocampus of rats chronically treated with agomelatine for 3 weeks before being challenged with LPS and sacrificed 24 h later. The data, expressed as a percentage of VEH/SAL treated animals (set at 100%), are the mean ± SEM of at least five independent determination. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. VEH/SAL; $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ vs. VEH/LPS; $^{\#\#}P < 0.01$ vs. AGO/SAL (Two-way ANOVA with SCPHT).

Supplementary Table 1

Brain region	Gene (mRNA)	Experimental Group	% change (vs. VEH no LPS)	P-Value vs. no LPS	P-Value vs. LPS 2h	P-Value vs. LPS 6h	Figure
Ventral Hippocampus	IL-1β	VEH- no LPS	0	-	-	-	Fig 1A
		VEH- LPS 2h	+1910	0,15E-12	-	-	
		VEH- LPS 6h	+3358	0,99E-15	0,51E-08	-	
		VEH- LPS 24h	+668	0,000098	0,10E-06	0,24E-14	
		AGO- no LPS	+33	-	-	-	Fig 1A
		AGO- LPS 2h	+1471	0,88E-09	-	-	
		AGO- LPS 6h	+1936	0,58E-11	0,038772	-	
		AGO- LPS 24h	+639	0,000577	0, 000203	0,58E-06	
	IL-6	VEH- no LPS	0	-	-	-	Fig 1D
		VEH- LPS 2h	+570	0,99E-15	-	-	
		VEH- LPS 6h	+321	0,704208	0,99E-15	-	
		VEH- LPS 24h	-8	0,805524	0,99E-15	0,613021	
		AGO- no LPS	+8	-	-	-	Fig 1D
		AGO- LPS 2h	+379	0,15E-13	-	-	
		AGO- LPS 6h	+199	0,26E-06	0,000033	-	
		AGO- LPS 24h	+31	0,431862	0,19E-10	0,000052	

Brain region	Gene (mRNA)	Experimental Group	% change (vs. VEH no LPS)	P-Value vs. no LPS	P-Value vs. LPS 2h	P-Value vs. LPS 6h	Figure
Dorsal Hippocampus	IL-1β	VEH- no LPS	0	-	-	-	Fig 1B
		VEH- LPS 2h	+2341	0,17E-09	-	-	
		VEH- LPS 6h	+2194	0,76E-10	0,609935	-	
		VEH- LPS 24h	+409	0,100084	0,20E-06	0,22E-06	
		AGO- no LPS	+27	-	-	-	Fig 1B
		AGO- LPS 2h	+994	0,39E-12	-	-	
		AGO- LPS 6h	+815	0,17E-08	0,122754	-	
		AGO- LPS 24h	+341	0,001397	0,71E-06	0,000288	
	IL-6	VEH- no LPS	0	-	-	-	Fig 1E
		VEH- LPS 2h	+854	0,99E-15	-	-	
		VEH- LPS 6h	+780	0,34E-14	0,268175	-	
		VEH- LPS 24h	+31	0,544509	0,28E-13	0,11E-11	
		AGO- no LPS	+28	-	-	-	Fig 1E
		AGO- LPS 2h	+376	0,50E-09	-	-	
		AGO- LPS 6h	+265	0,38E-06	0,030866	-	
		AGO- LPS 24h	+70	0,279384	0,46E-06	0,000211	

Brain region	Gene (mRNA)	Experimental Group	% change (vs. VEH no LPS)	P-Value vs. no LPS	P-Value vs. LPS 2h	P-Value vs. LPS 6h	Figure
Prefrontal Cortex	IL-1β	VEH- no LPS	0	-	-	-	Fig 1C
		VEH- LPS 2h	+1946	0,11E-07	-	-	
		VEH- LPS 6h	+1696	0,13E-07	0,493718	-	
		VEH- LPS 24h	+257	0,302778	0,000004	0,000011	
		AGO- no LPS	+3	-	-	-	Fig 1C
		AGO- LPS 2h	+882	0,16E-08	-	-	
		AGO- LPS 6h	+522	0,000010	0,015374	-	
		AGO- LPS 24h	+185	0,082512	0,000009	0,010351	
	IL-6	VEH- no LPS	0	-	-	-	Fig 1F
		VEH- LPS 2h	+1165	0,88E-11	-	-	
		VEH- LPS 6h	+474	0,000038	0,000007	-	
		VEH- LPS 24h	-4	0,968696	0,24E-09	0,000305	
		AGO- no LPS	+34	-	-	-	Fig 1F
		AGO- LPS 2h	+922	0,68E-07	-	-	
		AGO- LPS 6h	+263	0,079016	0,000155	-	
		AGO- LPS 24h	+31	0,994773	0,000003	0,154348	

Summary of the effect of lipolysaccharide (LPS; 250 μg/kg, o.p.) on the pro-inflammatory cytokines IL-1β and IL-6 in different brain regions of rats chronically pre-treated with vehicle (VEH; hydroxyethilcellulose, 1%, 1 ml/kg) or agomelatine (AGO; 40 mg/kg) before receiving the inflammatory challenge.

The table shows the percentage of change for each inflammatory protein and the corresponding significant P obtained analyzing the LPS effect at the different time points by One-way ANOVA.

Supplementary Table 2

Brain region	Gene (mRNA)	Experimental Group	% change (of VEH no LPS)	P-Value Vs. no LPS	P-Value Vs. LPS 2h	P-Value Vs. LPS 6h	Figure
Ventral Hippocampus	CD11b	VEH- no LPS	0	-	-	-	Fig 4A
		VEH- LPS 2h	-15	0,566613	-	-	
		VEH- LPS 6h	+261	0,000002	0,000006	-	
		VEH- LPS 24h	+412	0,47E-10	0,74E-09	0,007887	
		AGO- no LPS	+6	-	-	-	Fig 4A
		AGO- LPS 2h	+9	0,700497	-	-	
		AGO- LPS 6h	+137	0,67E-08	0,14E-06	-	
		AGO- LPS 24h	+256	0,99E-15	0, 24E-13	0,000001	
	CD68	VEH- no LPS	0	-	-	-	Fig 4B
		VEH- LPS 2h	+4	0,746399	-	-	
		VEH- LPS 6h	+58	0,000182	0,004012	-	
		VEH- LPS 24h	+80	0,12E-06	0,000015	0,049978	
		AGO- no LPS	-1	-	-	-	Fig 4B
		AGO- LPS 2h	-15	0,166990	-	-	
		AGO- LPS 6h	+68	0,000135	0,000044	-	
		AGO- LPS 24h	+174	0,49E-12	0,46E-11	0,000006	

Summary of the effect of lipolysaccharide (LPS; 250 μg/kg, o.p.) on the markers of microglia activation/macrophage phagocytosis CD11b and CD68 in ventral hippocampus of rats chronically pre-treated with vehicle (VEH; hydroxyethilcellulose, 1%, 1 ml/kg) or agomelatine (AGO; 40 mg/kg) before receiving the inflammatory challenge.

The table shows the percentage of change for each inflammatory protein and the corresponding significant P obtained analyzing the LPS effect at the different time points by One-way ANOVA.

CHAPTER II

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ALTERED INFLAMMATORY RESPONSIVENESS IN SEROTONIN TRANSPORTER MUTANT RATS

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Abstract

Background

Growing evidence suggests that alterations of the inflammatory/immune system contribute to the pathogenesis of depression. Indeed, depressed patients exhibit increased levels of inflammatory markers in both the periphery and the brain, and high comorbidity exists between major depression and diseases associated with inflammatory alterations. In order to characterize the link between depression and inflammation, we aimed to investigate whether an altered inflammatory system is present in a genetic model of vulnerability for depression, namely rats with partial or total deletion of the serotonin transporter (SERT) gene.

Methods

Wild-type, heterozygous and homozygous SERT rats were analyzed under basal condition or following a challenge with an acute injection of lipopolysaccharide (LPS) and killed 24 h or 5 days later.

Results

We found that SERT mutant rats show altered cytokine expression in the dorsal and ventral hippocampus at basal conditions, and they also display an exacerbated cytokine response to the LPS challenge. Moreover, mutant rats exhibit differences in the expression of markers for microglia activation.

Conclusion

Based on these data, we suggest that basal or functional alterations of immune/inflammatory systems might contribute to the phenotype of SERT rats and to their heightened susceptibility to depressive-like behavior.

Keywords

Inflammation, Lipopolysaccharide (LPS), Cytokines, Microglia, Animal model

Background

Major depressive disorder (MDD) is a leading cause of disability in the world supposedly originating from the interaction between a background of genetic vulnerability and adverse environmental factors. Compelling evidence also suggests that inflammation may contribute to specific dysfunctions associated with depression [1,2]. Accordingly, depression shows elevated comorbidity with immune-related diseases such as cancer, cardiovascular and neurodegenerative diseases that are characterized by the presence of inflammatory alterations [3,4]. In addition, depressed patients exhibit all the cardinal features of inflammation [5]. Indeed, elevated blood levels of the proinflammatory cytokines, including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, are commonly found in depressive subjects [6-9]. It has also been shown that depressed suicidal attempters display elevated levels of IL-6 in the cerebrospinal fluid [10]. Moreover, it has been demonstrated that IL-6 is related to the stress-induced development of depression-like behaviors in mice [11].

Furthermore, the endotoxin lipopolysaccharide (LPS), a proinflammatory agent, can trigger the development of depressive symptoms in humans as well as depressive-related behavior in rodents [1,12-14]. In line with this, patients treated with interferons and interleukins often develop depression [15]. This evidence suggests that immune-inflammatory signals may represent an 'environmental' condition relevant for the etiology of mood disorders, which may also unmask a latent genetic vulnerability.

In this respect, one of the most extensively investigated susceptibility genes in depression encodes for the serotonin transporter (5-HTT or SERT), which is responsible for the re-uptake of serotonin into the presynaptic terminal [16]. The 5-HTT gene exists in two major allelic variants, a long (L) form and a short (S) form. It has been demonstrated that the S variant leads to reduced expression of the

transporter [17] and might enhance the sensitivity to the pro-depressive effects of stressful life events in rhesus monkeys [18] as well as in humans [19,20]. Furthermore, SERT knockout mice [21-23], as well as SERT mutant rats [24], show depression and anxiety-related behaviors, as well as impaired neuronal plasticity [25-27], supporting the possibility that altered function of SERT may be associated with increased risk of mood disorders [28]. Interestingly, it has been recently shown that the short variant of SERT is also associated with higher risk of developing depression in patients following INF-α treatment [29].

Based on these observations, the purpose of the present study was twofold. First, we established whether partial or total deletion of the SERT gene is associated with altered expression of inflammatory markers in basal conditions. Next, in order to establish whether genetic susceptibility to depression is associated with significant changes in the inflammatory/immune system, we investigated whether SERT heterozygous rats show altered responsiveness to an inflammatory challenge with LPS. We chose $SERT^{+/-}$ animals since the partial SERT deletion better mimics the condition of humans carrying the short variant of the 5-HTTLPR, which has been associated with enhanced susceptibility to environmental adversities [30]. The higher validity of SERT heterozygous models has already been discussed [31,32], and the behavioral vulnerability of SERT heterozygous animals has been previously demonstrated for mice [21,33].

Methods

General reagents were purchased from Sigma-Aldrich (Milan, Italy), and molecular biology reagents were obtained from Applied Biosystem Italia (Monza, Italy), Eurofins MWG-Operon (Ebersberg, Germany) and Bio-Rad Laboratories S.r.l. (Segrate, Italy). Lipopolysaccharide

(from *Escherichia coli* 026:B6 ≥10,000 eu/mg) was purchased from Sigma-Aldrich (code L8274).

Animals and experimental paradigm

Serotonin transporter knockout rats (Slc6a4^{1Hubr}) were generated in a Wistar background by N-ethyl-N-nitrosurea (ENU)-induced mutagenesis [34]. Experimental animals were derived from crossing heterozygous SERT knockout rats that were out crossed for at least ten generations with wild-type Wistar rats obtained from Harlan Laboratories. All subjects were bred and reared in the Central Animal Laboratory of the University of Nijmegen. After weaning at the age of 21 days, ear cuts were taken for genotyping. In all experiments, adult male SERT$^{+/+}$(WT), SERT$^{+/-}$ and SERT$^{-/-}$ rats were used. Animals were housed in groups of four per cage under standard conditions (12-h light/dark cycle with food and water *ad libitum*) and were exposed to daily handling for 1 week before any treatment.

Rats were randomly divided into two experimental groups: control (39 rats) and treated animals (40 rats); the first group received saline, whereas the second one was treated with a single injection of lipopolysaccharide (250 μg/Kg, i.p.). They were killed 1 or 5 days later. Our analyses were carried out in the ventral and dorsal hippocampus, which were rapidly dissected, frozen on dry ice and stored at −80°C for molecular analyses.

All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used in accordance with the Guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Protein analysis of plasma inflammatory mediators

Samples of blood from each rat were collected in heparinized tubes. Plasma was separated by centrifugation (5,000 rpm for 10 min at 4°C), and interleukin (IL)-6, tumor necrosis factor (TNF)-α, cytokine-induced neutrophil chemoattractant (CINC)-1, CINC-3 and macrophage inflammatory protein (MIP)-1α protein levels were quantified using a Rat Cytokine Array kit (R&D Systems, Inc.) according to the manufacturer's instructions. Briefly, plasma was diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture was then incubated with the rat cytokine array membrane where capture and control antibodies have been spotted. Any cytokine/detection antibody complex present was bound by its cognate immobilized capture antibody on the membrane. Following a wash to remove unbound material, streptavidin-HRP and chemiluminescent detection reagents were applied and a signal was produced at each spot corresponding to the amount of cytokine bound. Protein levels were calculated by measuring the optical density of the autoradiographic bands using Quantity One software (Bio-Rad Laboratories). To ensure that autoradiographic bands were in the linear range of intensity, different exposure times were used.

RNA preparation and gene expression analyses

For gene expression analysis, total RNA was isolated from the different brain regions by single-step guanidinium isothiocyanate/phenol extraction using the PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l.; Segrate, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were then processed for real-time polymerase chain reaction (PCR) as previously reported [35] to assess levels of interleukin 1β (IL-1β) interleukin-6 (IL-6), integrin alpha M (cluster of

differentiation molecule CD11b), chemokine (C-X3-C motif) ligand 1 (CX3CL1; fractalkine) and integrin-associated protein (CD47).
Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination and subsequently analyzed by the TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories S.r.l.) using the iScript one-step RT-PCR kit for probes (Bio-Rad Laboratories S.r.l.). Samples were run in 384-well format in triplicate as multiplexed reactions with a normalizing internal control (β-actin). Thermal cycling was initiated with incubation at 50°C for 10 min (RNA retrotranscription) and then at 95 °C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95 °C for 10 s to enable the melting process, and then for 30 s at 60 °C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression. Probe and primer sequences used were purchased from Applied Biosystem Italia and Eurofins MWG-Operon.

Statistical analyses

The effect of the genotype on gene expression was analyzed with a one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference (Fisher PLSD), whereas the effect of the LPS was analyzed with a two-way ANOVA with genotype (WT vs. $SERT^{+/-}$ rats) and treatment (Sal vs. LPS) as independent factors and mRNA levels as dependent variable. When appropriate, further differences were analyzed by single contrast post hoc test (SCPHT). Significance for all tests was assumed at $P < 0.05$. Data are presented as means ± standard error (SEM). For graphic clarity, results are presented as mean percent of WT rats (basal effect analysis) and WT or $SERT^{+/-}$ rats treated with saline (LPS effect analysis).

Results

We first analyzed different markers of the immune system at the peripheral level of $SERT^{+/-}$ and $SERT^{-/-}$ animals. Specifically, we measured the protein level of two cytokines, IL-6 and TNF-α and CINC-1, CINC-3 and MIP-1α, which belong to the family of chemokines. As shown in Table 1, all these markers were strongly upregulated in the plasma of $SERT^{-/-}$ rats, whereas we did not find any change in SERT heterozygous animals.

On these bases, we decided to analyze the expression levels of inflammatory markers in the hippocampus of mutant rats under resting conditions or following an acute challenge with LPS. We chose to investigate this brain area because it is highly relevant for depression and it is also vulnerable to environmental challenges [36-38]. Moreover, we decided to compare the ventral and dorsal part of the hippocampus since the two subregions subserve different functions. In particular, the dorsal part (DH) has been linked to cognition, whereas the ventral portion (VH) has been associated with emotion and stress responses [39].

IL-1β expression

We first measured IL-1β mRNA and, as shown in Figure 1, we found that under basal conditions the expression of IL-1β was significantly increased in the dorsal hippocampus (A) of $SERT^{+/-}$ and $SERT^{-/-}$ animals (+55%, $P < 0.001$; +37%, $P < 0.05$ respectively), whereas, in the ventral hippocampus, (B) the cytokine mRNA levels were increased only in rats with partial deletion of the gene (+68%, $P < 0.01$). Next, we investigated the responsiveness of $SERT^{+/-}$ rats to a challenge with the proinflammatory agent LPS. The analyses were carried out only in $SERT^{+/-}$ rats, which more closely mimic the genetic vulnerability associated with patients carrying the S variant of the 5-HTT gene. In the dorsal hippocampus (Figure 1C), we found that LPS

produced similar changes in both genotypes. Indeed, the mRNA levels for IL-1β were significantly increased 1 day after LPS injection in WT (+634%, $P < 0.01$) as well as in SERT$^{+/-}$ rats (+513%, $P < 0.05$), but not after 5 days when the cytokine expression levels had returned to control levels. Conversely, in the ventral hippocampus (Figure 1D), LPS injection produced larger changes in SERT$^{+/-}$ rats since IL-1β upregulation at 24 h was more pronounced in mutant animals (+964% vs. +370 % in WT rats) and also persisted up to 5 days after the proinflammatory challenge (+156%, $P < 0.05$) when the cytokine expression had returned to control levels in wild-type rats.

IL-6 expression

Unlike what we observed for IL-1β, the mRNA levels of IL-6 (Figure 2) were not significantly altered under basal conditions in the dorsal (A) or ventral hippocampus (B) of SERT mutant rats. However, we found that the SERT genotype had a significant effect on the modulation of IL-6 gene expression after LPS challenge. In the dorsal hippocampus (Figure 2C), 24 h after LPS injection, IL-6 mRNA levels were significantly increased in SERT$^{+/-}$ (+136%, $P < 0.001$) but not wild-type animals (+58%, $P = 0.067$), whereas the cytokine expression was back to control levels in both genotypes 5 days post-LPS. A similar profile was found in the ventral hippocampus (Figure 2D), since IL-6 expression was upregulated 24 h post LPS administration in SERT$^{+/-}$ (+58%, $P < 0.01$) but not in WT rats (−11%, $P = 0.565$). Also in this brain region, no significant changes of IL-6 mRNA levels were found 5 days after LPS challenge in both genotypes.

CD11b expression

Besides cytokine production, another important aspect of the inflammatory response is microglia activation [40]. In order to evaluate if the basal activity of microglia was altered as a

consequence of SERT gene deletion, we assessed the expression of CD11b, a marker for this cellular phenotype [41]. As shown in Figure 3A, we found that the basal expression of CD11b was significantly higher in dorsal hippocampus of $SERT^{+/-}$ (+56%, $P < 0.05$) but not in $SERT^{-/-}$ rats. A similar change was also observed in the ventral hippocampus (Figure 3B) where the expression of the microglial marker was significantly upregulated under basal conditions in $SERT^{+/-}$ (+120%, $P < 0.01$) as well as in $SERT^{-/-}$ rats (+173%, $P < 0.001$).

Next, we examined CD11b mRNA levels following the acute LPS challenge. In the dorsal hippocampus (Figure 3C), the mRNA levels of CD11b were significantly increased 1 day after LPS treatment in WT (+312%, $P < 0.001$) as well as in $SERT^{+/-}$ rats (+274%, $P < 0.001$), while its expression returned to basal levels in both genotypes 5 days post-LPS. In the ventral hippocampus (Figure 3D), we found a significant increase of CD11b mRNA levels 24 h after LPS injection in WT (+436%, $P < 0.001$) and $SERT^{+/-}$ rats (+332%, $P < 0.001$). However, 5 days after LPS challenge the mRNA levels of CD11b were upregulated in WT rats (140%, $P < 0.001$), but they had returned to baseline in $SERT^{+/-}$ rats.

CX3CL1 and CD47 expression

In order to gain further insight into the changes of microglial function, we investigated the glia-neuron cross-talk. Indeed, microglial function may be controlled through signals that keep it in a resting state or that may favor its activation [42]. In this respect, the interactions between fractalkine (CX3CL1) and CD47 with their receptors play a crucial role in maintaining microglia in a resting state [43]. Therefore, we analyzed the expression of CX3CL1 and CD47 in the dorsal and ventral hippocampus of $SERT^{+/-}$ and wild-type animals.

As shown in Figure 4A, the expression of CX3CL1 was not altered in the dorsal hippocampus of $SERT^{+/-}$ rats under basal conditions, while

its mRNA levels were increased in the ventral subregion of mutant rats (+36%, $P < 0.001$).

When considering the inflammatory challenge, CX3CL1 mRNA levels were not altered in the dorsal hippocampus of WT rats 24 h after LPS treatment (Figure 4C), while they were significantly increased in $SERT^{+/-}$ animals (+42%, $P < 0.001$). Conversely, 5 days after the LPS challenge, the expression of CX3CL1 was significantly reduced in both genotypes (WT: -19%, $P < 0.05$; SERT+/−: -20%, $P < 0.05$). In the ventral hippocampus (Figure 4D), the mRNA levels of CX3CL1 were upregulated 1 day after LPS injection in WT rats (+144%, $P < 0.001$), but not in $SERT^{+/-}$ animals, whereas no significant changes were observed 5 days post-LPS challenge.

Conversely, as shown in Figure 5, basal expression of CD47 was reduced in the dorsal hippocampus of $SERT^{+/-}$ rats (−34%, $P < 0.01$), while it was increased in the ventral hippocampus (+144%, $P < 0.001$). Following LPS challenge, the expression of CD47 was not altered in the dorsal hippocampus of WT rats 24 h post-injection, and it was reduced 5 days after (−23%, $P < 0.05$), (Figure 5C). Conversely, the expression of CD47 was decreased at 24 h post-LPS in $SERT^{+/-}$ animals (−30%, $P < 0.05$), but was significantly upregulated 5 days post-LPS injection (+30%, $P < 0.05$). In the ventral hippocampus (Figure 5D), the mRNA levels of CD47 were upregulated 1 day after LPS administration in WT rats (+99%, $P < 0.001$) but not in $SERT^{+/-}$ animals. On the contrary, 5 days after the inflammatory challenge the expression of the microglial marker had returned to control values in WT rats, while being significantly increased in SERT mutant rats (+29%, $P < 0.05$).

Discussion

Our results suggest that genetic deletion of the serotonin transporter in rats is associated with alterations of immune/inflammatory players, such as proinflammatory cytokines and markers of microglia

activation, under basal conditions or following an immune challenge. These results support the idea of a close and reciprocal modulation between a gene strongly associated with depression and systems involved in the immune response, in line with the idea that inflammation represents an important environmental factor for depression susceptibility [1,44,45]. Indeed, we demonstrate that animals with partial or total deletion of the SERT gene have, under basal conditions, enhanced levels of circulating immune proteins as well as increased expression of IL-1β in dorsal and ventral hippocampus. In this respect, it is interesting to notice that major changes occurred in heterozygous animals, which mimic more closely the human situation of individuals carrying the short (S) variant of the SERT gene that is associated with enhanced vulnerability to depressive disorders [19,20]. Besides this, Su and colleagues have demonstrated that in S-carrier subjects depressive symptoms are associated with elevated plasma levels of IL-6 [46]. Moreover, healthy SS carriers of the serotonin transporter show, when compared to LL carriers, a proinflammatory phenotype, measured as the ratio between IL-6 and IL-10, under resting conditions as well as following an acute stress [47]. These data are in good agreement with the results of our study, also in respect to the responsiveness to an immune challenge. In particular, while cytokine mRNA levels are similarly increased in the dorsal hippocampus of all genotypes, the response to LPS was exacerbated in the ventral hippocampus of $SERT^{+/-}$ rats, where the cytokine upregulation was not only quantitatively larger but also lasted up to 5 days after the inflammatory challenge. The anatomical specificity is in line with the potential role of these changes for depression considering that the ventral subregion of the hippocampus is primarily involved in emotional responses and stress regulation [48].

One key element of the inflammatory response is the activation and modulation of microglia [40]. These cerebral immune cells are normally present in the healthy brain where they actively survey the

system and may rapidly respond to any microenvironment alteration [49]. To substantiate the phenotype of SERT mutant rats, we found increased mRNA levels of CD11b in the ventral and dorsal hippocampus. In particular, $SERT^{+/-}$ rats displayed more obvious alterations, suggesting once again that heterozygous rats are more sensitive to an LPS challenge. Indeed, we observed a genotype-dependent increase in CD11b: after 24 h from LPS administration there was no difference, whereas after 5 days microglia was still activated in wild-type but not in $SERT^{+/-}$ animals. The interpretation of these results is not straightforward, since the mechanisms underlying microglia activation and its functional consequences are not completely understood. Indeed, microglia is considered to function as a double-edged sword since its response is not necessarily neurotoxic, but may be useful to control and clear damage resulting from challenging conditions [42]. In line with these considerations, our results suggest that SERT mutant rats show activated microglia under basal conditions, which may eventually be associated with a chronic inflammatory state, also suggested by increased expression of proinflammatory cytokines. On the other hand, the microglial response to the inflammatory challenge is largely similar in SERT wild-type and mutant rats, suggesting that the intensity of the challenge may not be adequate for discriminating between the two cohorts.

Microglia is maintained in a resting state through neuron-derived signals including CX3CL1 and CD47, which act on their respective receptors CX3CR1 and CD172A expressed by microglia [42,50,51]. However, according to our data, the activation of microglia observed in mutant rats under basal condition cannot be explained by changes in the expression of CX3CL1 and CD47, as their mRNA levels are even higher in the ventral hippocampus of SERT+/− rats. We may speculate that the basal upregulation of these genes may represent a compensatory mechanism aimed at limiting the microglial activation observed in SERT mutant rats under basal conditions. In line with this interpretation, 24 h after the LPS challenge CX3CL1 and CD47 mRNA

levels were markedly increased in wild-type animals but not in mutant rats, further suggesting a dysregulation of the mechanisms responsible for the control of microglial function and activation.

Multiple molecular mechanisms may be involved in the differential inflammatory response observed after LPS challenge in SERT mutant rats. For example, it is known that LPS can activate and recruit Toll-like receptors (TLRs), especially TLR4, which appear important in mood-related disorders [52]. However, since hippocampal TLR4 gene expression is not altered in SERT mutant rats (data not shown), other mechanism may be responsible for heightened responsiveness to LPS challenge of $SERT^{+/-}$ rats.

In conclusion, our results demonstrate that the inflammatory/immune system is altered in rats with genetic alterations of the serotonin transporter. Moreover, these animals show a different response to an inflammatory challenge, suggesting that some of the mechanisms that regulate these systems can be compromised, thus rendering the hippocampus more susceptible to the adverse influence of inflammatory mediators. Since depression vulnerability can be associated with increased inflammation, we suggest that the alterations of the immune system observed in animals with a deletion of the SERT gene may contribute to their pathologic phenotype. Furthermore, considering that depressed patients with higher levels of cytokines, as well as individuals carrying the S variant of the 5-HTTLPR polymorphism, are less responsive to antidepressant treatment [53,54], we may speculate that alterations of the immune/inflammatory system in depressed individuals may not only contribute to the pathologic phenotype, but also represent an important component for the response to pharmacological intervention.

Abbreviations

CX3CL1, Chemokine (C-X3-C Motif) ligand 1 or fractalkine; CX3R1, Chemokine (C-X3-C Motif) receptor 1; CINC, Cytokine-induced neutrophil chemoattractant; DH, Dorsal hippocampus; CD11b, Integrin alpha M or cluster of differentiation; CD47, Integrin-associated protein; IL, Interleukin; L, Long; LPS, Lipopolysaccharide; MIP, Macrophage inflammatory protein; PCR, Polymerase chain reaction; SERT/5-HTT, Serotonin transporter; S, Short; TNF, Tumor necrosis factor; VH, Ventral hippocampus; WT, Wild-type

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The authors FM, GR, JH, MAR and RM conceived and designed the experiments; the authors FM, FC and CZ performed the experiments and analyzed the data; the authors FM, MAR and RM wrote or contributed to the writing of the manuscript. All the authors have approved the final manuscript.

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Table 1

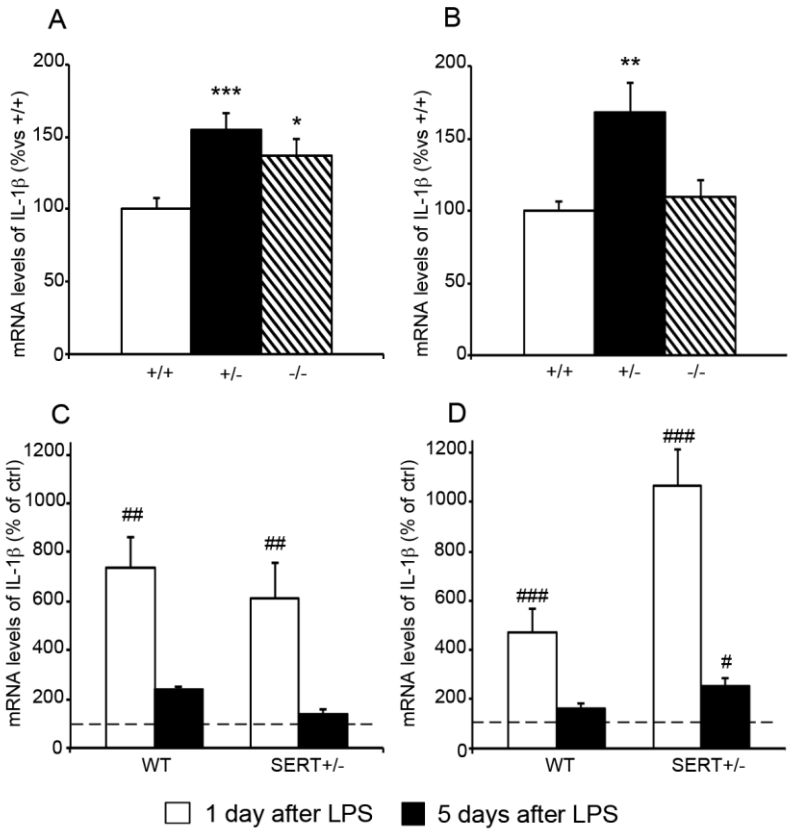
Protein analysis of inflammatory mediators in the plasma of SERT mutant rats.

Gene	**SERT +/-**	**SERT -/-**
CINC-1	=	+
CINC-3	=	+++
MIP-1□	=	+++
IL-6	=	++
TNF-□	=	+

The table shows the changes of interleukin 6 (IL-6), tumor necrosis factors (TNF-α), cytokine-induced neutrophil chemoattractant 1 (CINC-1), cytokine-induced neutrophil chemoattractant 3 (CINC-3), and macrophage inflammatory protein (MIP-1α) found in SERT mutant as compared to wild type animals.

=, no change; +, 5 to 10 fold increase; ++ 20 to 30 fold increase; +++ more that 30 fold increase.

Figure 1

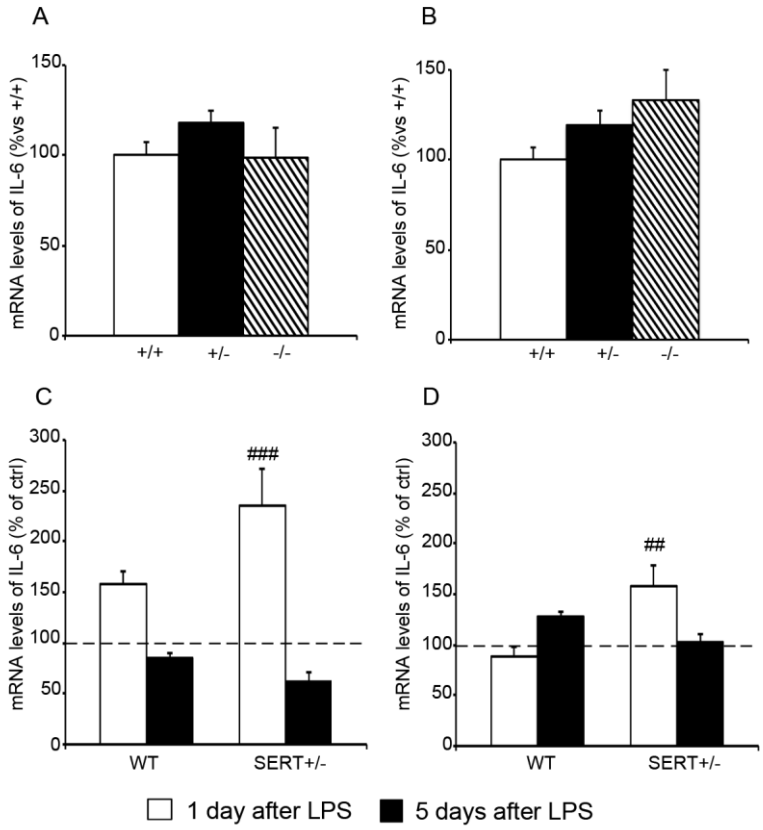


Analysis of Interleukin (IL)-1β gene expression in the hippocampus of SERT mutant rats.

Basal expression of IL-1β mRNA levels were measured in dorsal (A) and ventral (B) hippocampus of mutant rats. The data, expressed as percentage of $SERT^{+/+}$ (wild-type) animals (set at 100%), are the mean ± SEM of at least five independent determinations. * P<0.05, ** P<0.01 and *** P<0.001 vs. $SERT^{+/+}$ (One-way ANOVA with SCPHT).

The expression of IL-1β was also investigated in dorsal (C) and ventral (D) hippocampus following an acute LPS challenge, the animals being sacrificed 1 or 5 days after the immune challenge.. The data are expressed as percentage of the respective saline-injected counterpart (set at 100%, dashed line) and represent the mean ± SEM of at least five independent determinations. # P<0.05, ## P<0.01, ### P<0.001 vs. ctrl (One-way ANOVA with SCPHT).

Figure 2

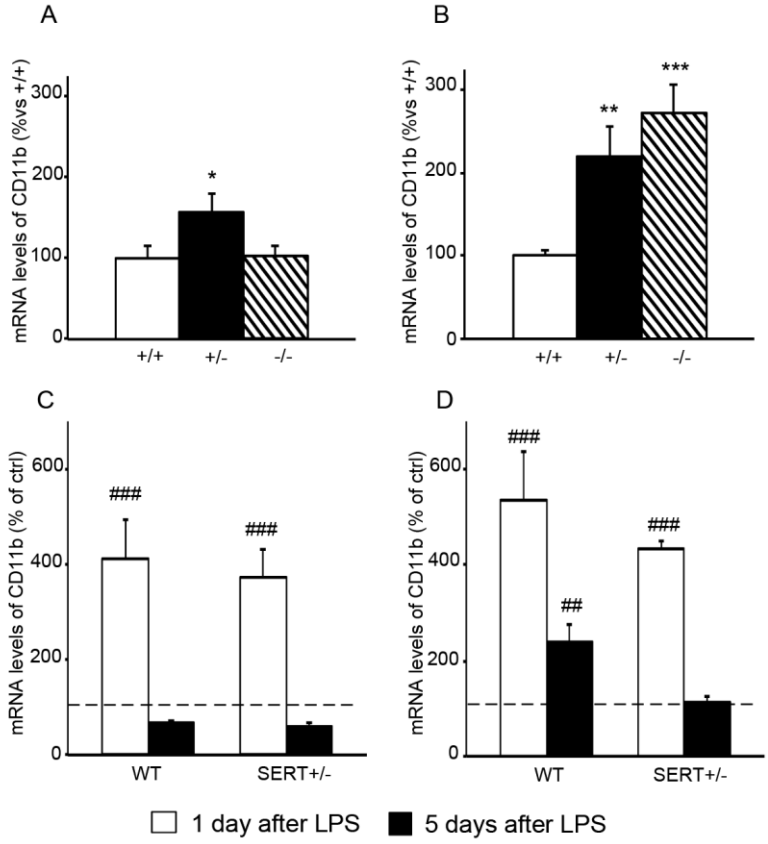


Analysis of Interleukin (IL)-6 gene expression in the hippocampus of SERT mutant rats.

Basal Interleukin (IL)-6 gene expression is not altered in dorsal (A) or ventral (B) hippocampus of mutant rats. The data, expressed as percentage of $SERT^{+/+}$ (wild-type) animals (set at 100%), are the mean ± SEM of at least five independent determinations.

The expression of IL-6 is up-regulated in both dorsal (C) and ventral (D) hippocampus only in $SERT^{+/-}$ animals 5 days after an lipopolysaccharide (LPS) challenge. The data are expressed as percentage of the respective saline-injected counterpart (set at 100%, dashed line) and represent the mean ± SEM of at least five independent determinations. ## $P<0.01$, ### $P<0.001$ vs. ctrl (One-way ANOVA with SCPHT).

Figure 3

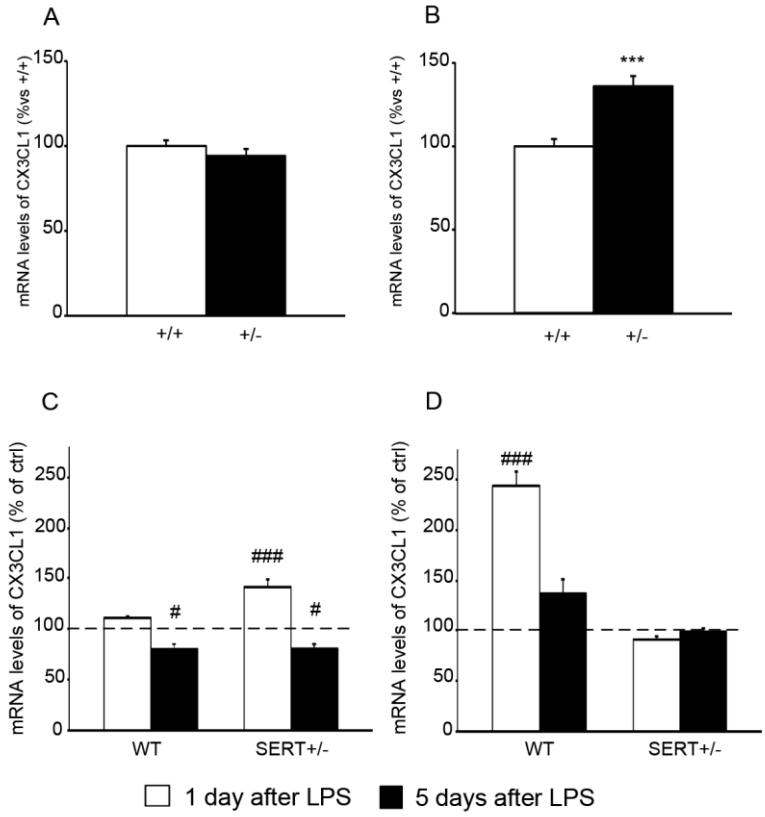


Analysis of integrin alpha M (CD11b) gene expression in the hippocampus of SERT mutant rats.

Basal CD11b gene expression is altered in the in dorsal (A) and ventral (B) hippocampus of mutant rats. The data, expressed as percentage of $SERT^{+/+}$ animals (set at 100%), are the mean ± SEM of at least five independent determinations. * P<0.05, ** P<0.01, and *** P<0.001 vs. $SERT^{+/+}$ (One-way ANOVA with SCPHT).

The expression of CD11b is up-regulated in dorsal (C) and ventral (D) hippocampus of WT or $SERT^{+/-}$ animals 1 or 5 days after an LPS challenge. The data are expressed as percentage of the respective saline-injected counterpart (set at 100%, dashed line) and represent the mean ± SEM of at least five independent determinations. ## P<0.01, ### P<0.001 vs. ctrl (One-way ANOVA with SCPHT).

Figure 4

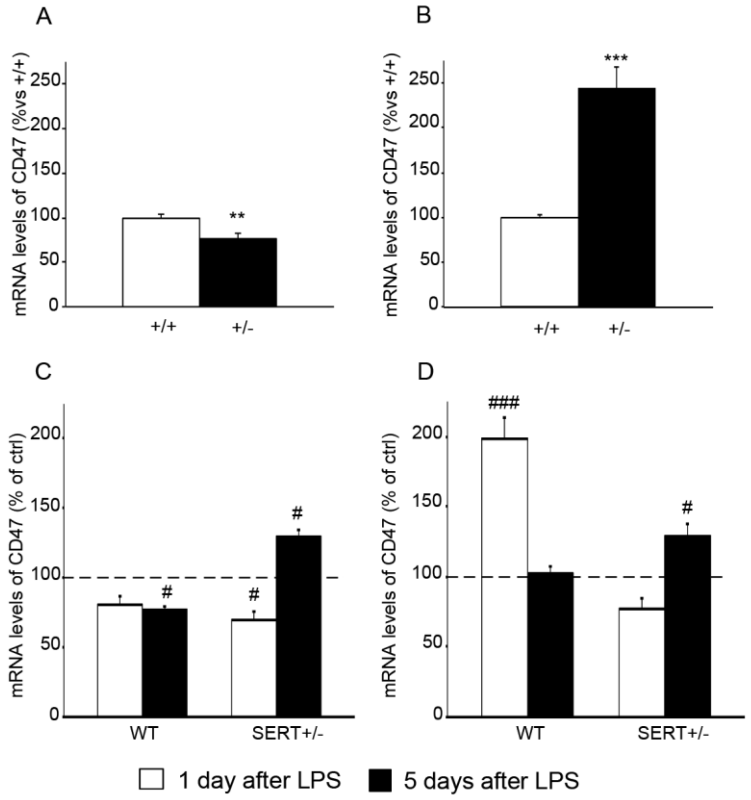


Analysis of chemokine ligand 1 (CX3CL1) gene expression in the hippocampus of SERT mutant rats.

Basal CX3CL1 gene expression is altered in the ventral hippocampus of mutant rats (B) but not in the dorsal hippocampus (A). The data, expressed as percentage of $SERT^{+/+}$ animals (set at 100%), are the mean ± SEM of at least five independent determinations. *** P< 0.001 vs. $SERT^{+/+}$ (One-way ANOVA with SCPHT).

The expression of CX3CL1 is modulated in dorsal (C) and ventral (D) hippocampus of WT or $SERT^{+/-}$ animals 1 or 5 days after an LPS challenge. The data are expressed as percentage of the respective saline-injected counterpart (set at 100%, dashed line) and represent the mean ± SEM of at least five independent determinations. ## P<0.01, ### P<0.001 vs. ctrl (One-way ANOVA with SCPHT).

Figure 5



Analysis of integrin-associated protein (CD47) gene expression in the hippocampus of SERT mutant rats.

Basal CD47 mRNA levels are decreased in the dorsal hippocampus (A) of $SERT^{+/-}$ rats, while they are increased in the ventral hippocampus (B). The data, expressed as percentage of $SERT^{+/+}$ animals (set at 100%), are the mean ± SEM of at least five independent determinations. ** $P<0.01$ and *** $P<0.001$ vs. $SERT^{+/+}$ (One-way ANOVA with SCPHT).

The expression of CD47 is modulated in dorsal (C) and ventral (D) hippocampus of WT or $SERT^{+/-}$ animals 1 or 5 days after an LPS challenge. The data are expressed as percentage of the respective saline-injected counterpart (set at 100%, dashed line) and represent the mean ± SEM of at least five independent determinations. # $P<0.05$, ### $P<0.001$ vs. ctrl (One-way ANOVA with SCPHT).

4. DISCUSSION and CONCLUSIONS

Major depressive disorders (MDD) is widespread disorder and represents the fourth leading cause of disability in the world. Supposedly it originates from the interaction between a background of genetic vulnerability and adverse environmental factors. Moreover, a growing body of evidence described, and partially characterized, dysfunction of multiple systems, including neurotransmitters, hormones, signaling pathways, neurotrophic and neuroplastic molecules.

In recent years, increasing evidence has suggested that inflammation may contribute to specific dysfunctions associated with depression (Dantzer *et al.*, 2008; Zunszain *et al.*, 2013). Accordingly, depression shows elevated comorbidity with diseases such as cancer, arthritis rheumatoid, cardiovascular and neurodegenerative diseases, which are associated with an altered inflammatory state (Benton *et al.*, 2007). In addition, depressed patients exhibit all the cardinal features of inflammation (Miller, 2010). Indeed, elevated blood levels of the pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, are commonly found in depressive subjects (Connor & Leonard, 1998; Maes, 1999; Hestad *et al.*, 2003; Thomas *et al.*, 2005).

Furthermore, the endotoxin lipopolysaccharide (LPS), a pro-inflammatory agent, can trigger the development of depressive symptoms in humans as well as depressive-related behavior in rodents (Yirmiya, 1996; Pucak & Kaplin, 2005; Frenois *et al.*, 2007; Dantzer *et al.*, 2008). In line with this, patients treated with interferons and interleukins often develop depression (Capuron *et al.*, 2000). All these evidence suggests that immune-inflammatory signals may represent an 'environmental' condition relevant for the etiology of mood disorders, which may also unmask a latent genetic vulnerability.

On these bases, it may be inferred that pharmacological treatments should also be effective in modulating or normalizing inflammatory changes, which may represent an important component of depression

symptomatology. We addressed this issue in the first paper and we demonstrate that chronic treatment with the antidepressant agomelatine is able to mitigate the inflammatory response associated with LPS injection, a strong stimuli of immune/inflammatory systems. We show that the effects of agomelatine rely on the modulation of microglial activation and may also affect the kynurenic pathway. These results suggest that anti-inflammatory properties may contribute to the long-term effects of the antidepressant on brain.
In the second study, we showed that a background of genetic vulnerability for depression is associated with altered immune/inflammatory state and responsiveness. Indeed, we demonstrate that the deletion of the serotonin transporter gene in rats is associated with alterations of immune/inflammatory system, including pro-inflammatory cytokines and markers of microglia activation, under basal conditions and following an immune challenge. These results suggest that the possibility to develop depression in comorbidity or following inflammatory conditions may depend on the genetic background.

In conclusion, these results provide further support to the idea of a close relationship between depression and inflammation. A better understanding of this relationship will be crucial at diagnostic level as well as to improve the effectiveness of pharmacological intervention.

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LIST OF PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

PUBLICATIONS

- **Macchi F**, Homberg JR, Calabrese F, Zecchillo C, Racagni G, Riva MA, Molteni R. Altered inflammatory responsiveness in serotonin transporter mutant rats. J Neuroinflammation. 2013 Sep 19;10(1):116. doi: 10.1186/1742-2094-10-116.
- Molteni R, **Macchi F**, Riva MA. Gene expression profiling as functional readout of rodent models for psychiatric disorders. Cell Tissue Res. 2013 Oct;354(1):51-60. doi: 10.1007/s00441-013-1648-0. Epub 2013 May 25.
- Molteni R, **Macchi F**, Zecchillo C, Dell'agli M, Colombo E, Calabrese F, Guidotti G, Racagni G, Riva MA. Modulation of the inflammatory response in rats chronically treated with the antidepressant agomelatine. Eur Neuropsychopharmacol. 2013 Nov;23(11):1645-55. doi: 10.1016/j.euroneuro.2013.03.008. Epub 2013 Apr 24.

Conference procedeedings

F. Macchi, R. Molteni, F. Calabrese, A. Cattaneo, G.A. Racagni, B.A. Ellenbroek and M.A. Riva. Modulation of the neurotrophin brain-derived neurotrophic factor in serotonin transporter mutant rats. ECNP Workshop on Neuropsychopharmacology for Young Scientists in Europe, Nice, France; 3-5 March 2011. In: European Neuropsychopharmacology 21, Suppl. 1: S23-S24 (2011).

G. Guidotti, F. Calabrese, R. Molteni, **F. Macchi**, G.A. Racagni and M.A. Riva. Chronic mild stress modulates the transcription of BDNF isoforms with brain region specificity: influence of antidepressant treatment. ECNP Workshop on Neuropsychopharmacology for Young Scientists in Europe, Nice, France; 3-5 march 2011. In: European Neuropsychopharmacology 21, Suppl. 1: S2-S3 (2011).

R. Molteni, F. Calabrese, **F. Macchi**, G. Guidotti, G. Racagni and M.A. Riva. Chronic stress modulates activity-dependent transcription of BDNF in rat hippocampal slices. 41st Annual Meeting of the Society for Neuroscience. Washington (DC) USA, November, 12-16, 2011.

F. Macchi, R. Molteni, C. Zecchillo, G. Racagni, J. Homberg and M.A. Riva. Altered inflammatory response of serotonin transporter mutant

rats, a genetic model of depression. 8th FENS Forum, 14-18 July 2012, Barcellona, Spain.

R. Molteni, **F. Macchi**, C. Zecchillo, M. Dell'Agli, M.A. Riva and G. Racagni. Modulation of the inflammatory response after the antidepressant agomelatine in rats. 50th ACNP Annual Meeting, 2-6 December 2012, Hollywood, Florida (USA).

R. Molteni, **F. Macchi**, C. Zecchillo, C. Gabriel, E. Mocaer, G. Racagni and M.A. Riva. Modulation of the inflammatory response in rats chronically treated with the antidepressant agomelatine. 25th ECNP Congress, 13-17 October 2012, Vienna, Austria.

F. Macchi, C. Zecchillo, R. Molteni, G. Racagni, M. Papp and M.A. Riva. Inflammatory response in the brain of rats exposed to chronic mild stress. 7th Meeting Steroids and Nervous System. 16-20 February 2013, Torino, Italy.

C. Zecchillo, **F. Macchi**, R. Molteni, G. Racagni, M. Papp and M.A. Riva. Inflammatory response in the brain of rats exposed to chronic mild stress. ECNP Workshop on Neuropsychopharmacology for Young Scientists in Europe, Nice, France; 7-10 March 2013.

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