UNIVERSITÀ DEGLI STUDI DI MILANO
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Dottorato in Scienze Farmacologiche Sperimentali e Cliniche
XXIX CICLO

Inflammation and vulnerability for major depression: in search of common molecular pathways

Settore Scientifico-Disciplinare BIO/14

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Anno Accademico 2015/2016

Abstract

The comprehension of the molecular mechanisms underpinning Major Depression (MD) is becoming a crucial issue in public health, considering that this psychiatric disorder has been estimated to become the leading cause of disability within 2020. To sustain the critical relevance of the investigation of the molecular bases of this pathology, it is important to underline that a high percentage of patients do not respond to the current pharmacological treatments, despite the number of antidepressant drugs available in the market.

MD is a very complex and invalidating pathology, characterized by neuro-vegetative and cognitive symptoms. Among them, the most relevant are alterations in mood and anhedonia, the latter defined as the incapability of feeling pleasure in pleasant circumstances. Although the causes of MD are not fully understood, it is known that the insurgence of this pathology is ascribable to the interaction between a genetic background of susceptibility and environmental factors. Among these factors, stress exposure play a pivotal role in the development of the psychopathology. However, it is important to mention that not all the subjects exposed to stressful situations develop a mental illness, indeed only a small percentage become affected by MD after stress exposure. In this context, people capable to cope with the consequences of stress are defined as resilient and the term "Stress-Resilience" refers to the ability of the subject to actively respond against adverse stimuli. The investigation and the identification of the molecular mechanisms underpinning stress vulnerability and stress resilience appear, thus, of critical importance to identify new therapeutic targets.

Among the molecular mechanisms involved in depression pathophysiology, compelling clinical and preclinical evidence support a role for alteration of the inflammatory system, which is also affected by stressful experiences.

With these premises, the general aim of my study was to investigate the relationship between major depression and neuroinflammation, in order to provide new information about the molecular background of this pathology. In particular, by the use of different experimental approaches, we evaluated the impact of stress on neuroinflammation and the potential anti-inflammatory properties of pharmacological treatment with the antidepressants agomelatine and imipramine or the antipsychotic lurasidone.

Our results demonstrated that neuroinflammation is strictly associated with the insurgence of stress-induced behavioral alterations in adult male rats tested for sucrose consumption.

Indeed anhedonic-like animals showed increased levels of pro-inflammatory cytokines and markers of microglia activation, especially in the dorsal hippocampus. Moreover, we found that chronic pharmacological treatment with agomelatine, imipramine and lurasidone was not only able to normalize the alterations in sucrose intake, but also to modulate the pro-inflammatory effects of chronic stress exposure.

In this context, we found that agomelatine was able to modulate the feedback inhibition pathway of interleukin-6 signaling. Indeed, we observed that chronic administration of the antidepressant potentiated the activity of the suppressor of cytokine signaling (SOCS)3 in the prefrontal cortex of stressed animals, thus promoting the shutdown of IL-6 pathway.

Subsequently, we used an unbiased genome-wide approach to characterize with a broader point of view the potential protective properties of agomelatine on a strong immune challenge such as the acute injection of lipopolysaccharide (LPS) in the rat ventral hippocampus. In particular, we enlightened molecules and pathways potentially important for its therapeutic effects in the context of neuroinflammation.

Pursuing the idea that stress-Resilient animals actively cope with stress-induced alteration/priming of inflammation within the brain, we exposed adult male rats to two weeks of Chronic Mild Stress (CMS), followed by an immune challenge with LPS. Specifically, we found that stress-Resilient rats could better respond to LPS-induced behavioral alterations in sucrose intake. Moreover, our molecular analyses pointed out that dysregulated activation of microglia may play a pivotal role in the insurgence of altered behaviors in anhedonic-like animals, thus indicating these cells as main actors in the mechanisms of stress-Resilience.

Lastly, we found that the altered expression of brain-derived neurotrophic factor (BDNF), a key molecule involved in the etiology of MD and in the therapeutic activity of antidepressants, influenced the inflammatory response within the brain. Specifically, we found that male and female mice heterozygous for this neurotrophic factor, differentially respond to an immune challenge with LPS when compared to wild-type animals, with a genotype*LPS interaction dependent on the brain area examined.

Summarizing, the data obtained during my PhD strongly support the direct involvement of neuroinflammation in the insurgence of depressive-like phenotype, in the mechanism of stress resilience and in the molecular activity of diverse psychotropic drugs.

La comprensione dei meccanismi molecolari alla base della Depressione Maggiore (MD) sta diventando un fattore importante nella salute pubblica, basti considerare che è stato stimato che questa patologia psichiatrica diventerà la seconda causa di disabilità al mondo entro in 2020. A sostegno della criticità dello studio delle basi molecolari della malattia, è importante sottolineare che una alta percentuale di pazienti non risponde correttamente ai trattamenti farmacologici oggi disponibili sul mercato.

La MD è una patologia invalidante e altamente complessa, la cui sintomatologia è caratterizzata da sintomi neuro-vegetativi e cognitivi. Tra questi, i più importanti sono l'alterazione del tono dell'umore e l'anedonia, quest'ultima definita come l'incapacità di provare piacere in situazioni che dovrebbero suscitarlo. Nonostante le cause della MD non siano ancora del tutto note, è risaputo che l'insorgenza della patologia è imputabile all'interazione tra un background di suscettibilità genetica e fattori ambientali. Tra questi, l'esposizione a stress gioca un ruolo fondamentale nello sviluppo della psicopatologia. Tuttavia, è importante sottolineare che non tutti i soggetti esposti a situazioni stressanti sviluppano un disturbo mentale, infatti solo una piccola percentuale risulta affetta da MD in seguito all'esposizione a stress. In questo contesto, persone in grado di affrontare positivamente le conseguenze dello stress sono definite resilienti e il termine "Resilienza allo Stress" si riferisce all'abilità di un soggetto di rispondere attivamente e positivamente contro uno stimolo esterno. Lo studio e l'identificazione dei meccanismi molecolari alla base della vulnerabilità allo stress appaiono, dunque, di importanza critica per la scoperta di nuovi target farmacologici.

Tra i meccanismi molecolari coinvolti nella patofisiologia della depressione, diversi dati clinici e preclinici supportano il ruolo delle alterazioni a carico del sistema infiammatorio, il quale risulta essere influenzato anche dall'esposizione a situazioni stressanti.

Con queste premesse, lo scopo generale del mio studio è stato quello di indagare la relazione tra depressione maggiore e neuroinfiammazione, con il fine di fornire nuove informazioni sul substrato molecolare della patologia. In particolare, attraverso diversi approcci sperimentali, abbiamo valutato l'impatto dello stress sulla neuroinfiammazione e il potenziale effetto anti-infiammatorio del trattamento farmacologico con gli antidepressivi agomelatina e imipramina o l'antipsicotico lurasidone.

I nostri risultati hanno dimostrato che la neuroinfiammazione è strettamente associata con l'insorgenza di alterazioni comportamentali indotte dallo stress in ratti maschi adulti, testati con il test del consumo del saccarosio. Infatti, animali con un comportamento simil-anedonico hanno mostrato aumentati livelli di citochine pro-infiammatorie e marcatori di attivazione microgliale, con una specificità particolare per l'ippocampo dorsale. Inoltre, abbiamo mostrato come il trattamento cronico con agomelatina, imipramina e lurasidone non sia stato solo in grado di normalizzare le alterazioni a livello del consumo di saccarosio, ma anche di modulare gli effetti pro-infiammatori dell'esposizione a stress cronico.

In questo contesto, è emerso come agomelatina sia in grado di modulare i meccanismi di feedback negativo del pathway dell'interleuchina 6. Infatti, abbiamo osservato che la somministrazione cronica dell'antidepressivo è stata in grado di potenziare l'attività del soppressore del signaling delle citochine (SOCS)3 nella corteccia prefrontale degli animali stressati, promuovendo, quindi, lo spegnimento del pathway di IL-6.

Successivamente, abbiamo adottato un approccio genome-wide per caratterizzare -da un punto di vista più ampio- le potenziali proprietà protettive di agomelatina su di un challenge immunologico con lipopolisaccaride (LPS). Nell'ippocampo ventrale di ratto, abbiamo evidenziato diverse molecole e pathway potenzialmente importanti per gli effetti terapeutici dell'antidepressivo in un contesto di neuroinfiammazione.

Perseguendo l'idea che gli animali resilienti allo stress siano in grado di contrastare attivamente le alterazioni/il priming infiammatorio nel cervello, abbiamo sottoposto ratti maschi adulti a due settimane di CMS, seguito da un challenge immunologico con LPS. Nel dettaglio, abbiamo dimostrato che i ratti resilienti allo stress sono in grado di rispondere meglio alle alterazioni nel consumo di saccarosio in seguito a stress. Inoltre, le nostre analisi molecolari hanno evidenziato come l'alterata attivazione della microglia possa giocare un ruolo chiave nell'insorgenza delle alterazioni comportamentali degli animali simil-anedonici, indicando che questa popolazione cellulare partecipa attivamente ai meccanismi di resilienza allo stress.

*Infine, abbiamo mostrato come l'alterata espressione di BDNF, una molecola chiave coinvolta nell'eziologia della depressione e nell'attività terapeutica degli antidepressivi, possa influenzare la risposta infiammatoria nel sistema nervoso centrale. In particolare, topi maschi e femmina, eterozigoti per il fattore neurotrofico, hanno risposto diversamente al challenge con LPS, quando paragonati agli animali wild-type, con un'interazione genotipo*LPS dipendente dalla regione cerebrale analizzata.*

In conclusione, i dati ottenuti durante la mia tedi di dottorato supportano fortemente il diretto coinvolgimento della neuroinfiammazione nella comparsa di un fenotipo comportamentale simil-depressivo, nei meccanismi di resilienza allo stress e nell'attività molecolare di diversi farmaci psicoattivi.

Index

1.Introduction

Major depression disorder (MDD), the mostly diffused psychiatric illness among mood disorders. This pathology is a common, costly and recurrent disorder, associated with considerable morbidity and excess mortality and has been projected to become the second leading cause of disability worldwide by 2020 (second to ischemic heart disease) (Sullivan et al., 2000).

MDD is a complex pathology, almost twice as common in females than males. Indeed, almost 20-25% of women are affected by the pathology, while only 7-12% of the men suffer from this disease (Kessler et al., 2009). Moreover, the probability to have a depressive episode before 70 years old is higher in women (45%) with respect to men (17%). This psychiatric disease is mainly characterized by affective, vegetative and cognitive symptoms that show a relapsing-remitting course. Depression has been described by mankind for several millennia. The term melancholia (which means black bile in Greek) was first used by Hippocrates around 400 a.C. (Nestler et al., 2002). Most of the major symptoms of depression observed today were recognized in ancient times, as were the contributions of innate predispositions and external factors in causing the illness. Even though similarities between ancient descriptions of depression and those of the modern era are outstanding, only in the middle part of the 19th century the brain become the focus of efforts to understand the pathophysiology of this disorder.

Since the 1960s, depression has been diagnosed as “major depression” based on symptomatic criteria set forth in the Diagnostic and Statistical Manual (DSM IV, 2000). Specifically, depressed patients may show:

- *Depressed mood for the larger part of the day;*
- *Lack of interest and pleasure for all, or almost all, the activities done for the larger part of the day, every day;*
- *Insomnia or hypersomnia almost every day;*
- *Psychomotor alterations;*
- *Reduced energy almost every day (asthenia);*
- *Feelings of guilt (exaggerated or unappropriated);*
- *Reduced capability to concentrate, clearly think almost every day;*
- *Recurrent suicidal thoughts.*

It is obvious from these criteria that the diagnosis of depression, as opposed to most diseases of other organ systems, is not based on objective diagnostic test but rather on a highly variable set of symptoms. Accordingly, depression should not be viewed as a single disease but as a heterogeneous syndrome comprised of numerous diseases of distinct causes and pathophysiology.

1.1 Etiology of major depressive disorder

Epidemiologic studies show that approximately 40%-50% of the risk for depression is genetic (Fava and Kendler, 2000). This makes depression a highly heritable disorder, at least as heritable as several common complex medical conditions (e.g. type II diabetes, asthma and certain cancers), which are often thought of as genetic. Yet, the search for specific genes that confer this risk has been daunting, with no genetic abnormality being identified to date with certainty. The difficulty in finding depression vulnerability genes parallels the difficulty in finding genes for other psychiatric disorders and, in fact, for most common complex diseases. Thus, any single gene might produce a relatively small effect and would therefore be difficult to detect experimentally. It is also possible that variants in different genes may contribute to depression in each family, which further complicates the search for depression genes (Nestler et al., 2002).

Most of the published genetic association studies of mood disorders have focused on functional polymorphisms (DNA sequence variations that alter the expression and/or functioning of the gene product) in the loci encoding the serotonin transporter (SLC6A4), serotonin 2A receptor ($5HTR_{2A}$); tyrosine hydroxylase (TH, the limiting enzyme for dopamine synthesis); tryptophan hydroxylase 1 (TPH1) involved in serotonin synthesis; and catechol-o-methyltransferase (COMT) that is an enzyme related to dopamine catabolism. All these molecules result implicated in the monoamine neurotransmitter system that is known to be involved in mood control (Levinson, 2006).

As research advances, detailed studies have led to formulate different molecular theories of depression and, among others, some of the most important are the “monoamine hypothesis” and the “neuroplasticity hypothesis”.

The “monoamine hypothesis” of depression, which asserts that depression is caused by decreased monoamine function in the brain, originated from early clinical observations (Pittenger and 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. Moreover Reserpine, an old antihypertensive agent that depletes monoamine stores, produced depressive symptoms in a subset of patients. Despite these evidences, treatment with currents antidepressant, designed to increase monoamine transmission acutely, reveals a relevant percentage of patients which not show an adequate response to the therapy and which have persistent symptomatology. Therefore, although these monoamine-based agents are potent antidepressants, alterations in central monoamine function might contribute marginally to genetic vulnerability (López-León et al., 2008; Ruhé et al., 2007) and 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 an impaired 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 include 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. In this scenario, neurotrophic factors (NTFs) -a family of proteins that are responsible for the growth and survival of developing neurons and also for network construction (Poo, 2001)- play a key role as mediators of neuroplasticity. It is now well established that NTFs are important mediators of neuronal plasticity also in adulthood, where they modulate axonal and dendritic growth and remodeling, membrane receptor trafficking, neurotransmitter release, synapse formation and function (Lu et al., 2005).

In addition to all these evidences, also non-genetic factors such as stress and emotional trauma, viral infections and even stochastic processes during brain development have been implicated in the etiology of depression (Fava and Kendler, 2000).

The role of stress warrants particular comment. Depression is often described as a stress-related disorder, and there is good evidence that episodes of depression often occur in the context of some form of stress. However, stress per se is not sufficient to cause depression. Most people do not become depressed after serious stressful experiences, whereas others develop the pathology under stress situations that might be considered mild for the majority of the population. Conversely, severe stress such as that experienced during combat, rape, or

physical abuse, does not typically induce depression, but instead causes post-traumatic stress disorder (PTSD) that is distinct from depression based on symptomatology, treatment and longitudinal course of illness. This underscores the view that depression in most people is caused by the interaction between a genetic predisposition and some environmental factors, which makes the mechanism of such interactions an important focus of investigation.

A further non-genetic factor implicated in the etiology of depression is inflammation and one promising development in regard to identify novel pathophysiologic targets is the emergence of a number of experimental evidences that support its in depression. Particularly, it is known that depression is accompanied by alterations of inflammatory system and, in fact, 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). It is moreover 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 involved in the etiopathogenesis of depression.

1.2 Inflammation

As discussed above stress exposure lead to an activation of the immune response, thus suggesting a potential role of the inflammatory response in the development of MDD. It is commonly known that following a viral or bacterial infection, there are subjective symptoms like general malaise, fatigue loss of appetite etc. The psychological and behavioral symptoms of the pathology represent, together with the febrile response and the associated neuroendocrine alterations, a well-organized strategy to cope infections (Dantzer et al., 2008; Howren et al., 2009; Raison et al., 2006). This strategy, called “Sickness behavior”, its activated by mediators of the inflammatory response, such as pro-inflammatory cytokines or pathogen associated molecular patters (PAMPs). These molecules coordinate the local and systemic response to pathogens; however, they can also act at central level, causing the psychologic symptomatology associated to infections.

In the last years, the dysregulation of these molecules has been proposed as a key feature of the pathophysiology of MDD (Dantzer et al., 2008; Howren et al., 2009; Raison et al., 2006).

1.2.1 Association between depression and inflammation

Several evidences suggest that neuroinflammation is associated with the insurgence of depressive phenotype at both pre-clinical and clinical level.

- ***Increased expression of pro-inflammatory mediators in depressed patients***

  As abovementioned depressed patients present increased levels of proinflammatory cytokines and their receptors, altered peripheral levels of chemokines and other adhesion molecules in the blood and in the cerebrospinal fluid (Maes, 1994; Miller et al., 2009). In addition, post-mortem studies, have shown increased levels of several genes and proteins related to the inflammatory response in the brain of suicidal depressed patients. Among these markers, we find the main pro-inflammatory cytokines interleukin (IL)-1β, TNF-α, IL-6 and toll-like receptors (Brambilla et al., 2014; Drago et al., 2015; Maes, 1995). Moreover, other studies suggested that increased levels of C reactive protein (CRP) and IL-1β are predictive of the development of depressive pathology, thus proposing inflammation as a potential cause of the pathology (van den Biggelaar et al., 2007).

  Lastly, inflammation has been also related to the lack of therapeutic response to antidepressant treatment (Cattaneo et al., 2013; Miller et al., 2013).

- ***The administration of pro-inflammatory cytokines*** **induces depression**

  The administration of pro-inflammatory cytokines (such as interferons) or their inductors in non-depressed subjects, induce the development of a depressive phenotype (Bonaccorso et al., 2002; Capuron et al., 2002; Reichenberg et al., 2001). As demonstration of this effect, the 30% of patients affected by hepatitis C and treated with interferons develop depression (Asnis and De La Garza, 2006). A similar effect has been observed also in cancer patients treated with immunotherapy (Capuron et al., 2002).

- ***Comorbidity with pathologies characterized by an altered inflammatory state.***

  Of note, that depression shows high comorbidity with pathologies like cancer, rheumatoid arthritis, multiple sclerosis, cardiovascular and metabolic diseases and neurodegenerative diseases (Benton et al., 2007).

1.2.2 Cytokines and neurotransmitters

As commonly known, monoamines have a crucial role in the regulation of mood and in the pathophysiology of depression. Pro-inflammatory cytokines have an important impact on the monoamine system and on the glutamatergic system. Specifically, the can lead to the reduced availability of synaptic monoamines. As an example, IL-1β and TNF-α stimulate serotonin reuptake, an effect mediated by MAP kinases (Zhu et al., 2010).

The alteration of neurotransmitter homeostasis induced by cytokines involves also the activation of the enzyme indoleamine 2,3 dioxygenase (IDO), that is responsible of the generations of kynurenines from tryptophan (a key precursor for serotonin synthesis). Moreover, kynurenines can be metabolized by microglial cells into quinolinic acid, a neurotoxic molecule that acts as NMDA agonist (Maes et al., 2011).

Cytokines can also cause the decrease of the neurotrophin Brain-derived neurotrophic factor (BDNF) a key regulator of synaptic plasticity and brain homeostasis (Calabrese et al., 2014).

1.2.3 Microglia

Microglia cells are the macrophagic resident cells of the brain, responsible of the first line defense against immune alterations within the brain.

In the so called “resting state” microglia surveil the microenvironment searching for pathogens or signs of disuse damage. At this stage microglia have a dendritic morphology characterized by long and ramified processes. In the “activated state” microglia have an amoeboid form with high mobility toward the site of damage. Moreover, active microglia trigger different pathways related to the production of pro-inflammatory mediators, modelling the function and activity of neurons.

In physiological conditions microglia is regulated by soluble factors released by neurons. Among them we find CX3CL1 (or fractalkine), colony stimulating factor1 (CSF1), transforming growth factor beta (TGF-β), IL-34, CD47 and CD200 (Kiedorf 2013; Butovsky 2014). These molecules bind to their cognate receptors laying on microglia. One of the most important is CX3CR1, the receptor of fractalkine a mediator constitutively expressed by neurons to control microglia state (Biber et al., 2007).

Alterations in the response of microglia can contribute to the development of MDD, in particular it has been reported that microglia mediate stress-induced production of pro-inflammatory mediators (Frank et al., 2007). The mechanisms responsible of microglia activation after stress are not completely resolved, however a pivotal role seems to be exerted

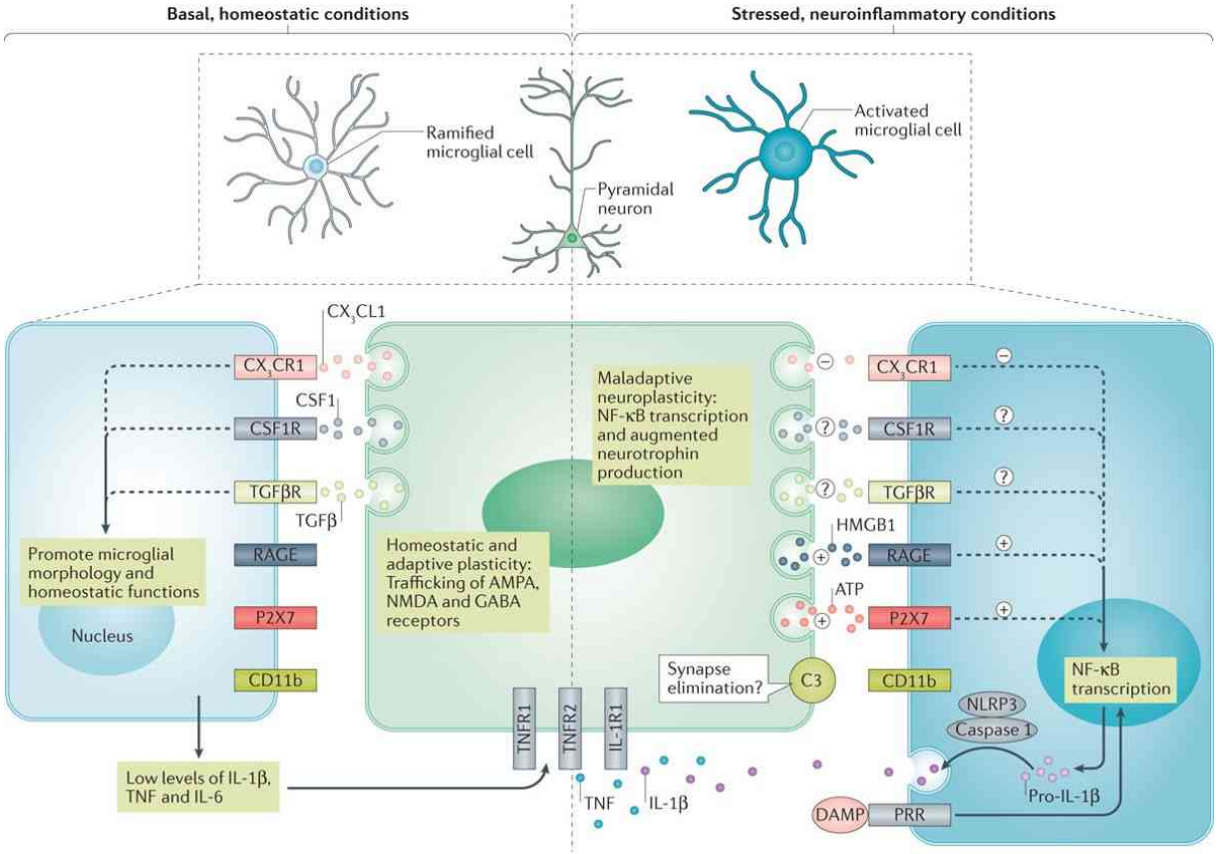
by pattern recognition receptors (PPRs). In this context, PAMPs and danger associated molecular patterns (DAMPs) may contribute to the dysregulation of microglia activation, thus leading to the exacerbation of the depressed phenotype. One of the most relevant PPR is the toll-like receptor-4. This receptor, present on microglia surface, is upregulated after stress exposure in animal models (Frank et al., 2012), while the blockage of its activation prevents microglia activation after stress. Another agonist of PPRs is high mobility group box 1 (HMGB1), another key mediator upregulated after stress able to activate microglia. Lastly ATP represent another important signal at central level in the context of neuroinflammation. More in detail ATP binds both purinergic metabotropic (P2Y) and ionotropic (P2X) receptors. The activation of microglial P2Ys receptors induces a decrease of the activation of proinflammatory pathways, through the release of anti-inflammatory mediators; whereas the activation of the ionotropic receptors triggers the inflammatory response led by microglia (Harry, 2013). Among the latter receptors, P2X7 seem to play a crucial role in the context of psychiatric disorders, indeed mice with impaired expression of P2X7 show increased resilience to stress-induced behavioral alterations (Basso et al., 2009).

1.3 Preclinical models

1.3.1 Chronic mild stress (CMS) exposure

One of the mostly used experimental approach to generate animal models of depression, is the exposure to paradigms of chronic stress. Among them the chronic mild stress (CMS) paradigm developed by Willner and collaborators in 1997, lead to the development of a depressed like phenotype in rodents that resemble the hallmark symptoms of depression: anhedonia and behavioral despair, respectively evaluated with the so-called sucrose consumption test and with the forced swim test (Willner, 1997). It is important to note that in this experimental paradigm not all the animals exposed to CMS develop an altered phenotype. This is in line with the fact that not all the human subjects routinely exposed to environmental stressors develop psychiatric disorders, on the contrary they are able to cope with an adverse situation. Similarly, it is possible to distinguish in rodents exposed to chronic stress, two populations that differently respond to stress: one susceptible that present alterations at behavioral level, the other resilient to the detrimental impact of stress. The nature of this different response is multifactorial and depends on the interaction between several systems both genetic and environmental. At molecular level, many systems are altered by the

exposure to chronic stress. As example, beyond the mediators of neuroplasticity and the glucocorticoid system, stressful events are associated with alterations of mediators of inflammation at both peripheral and central level.



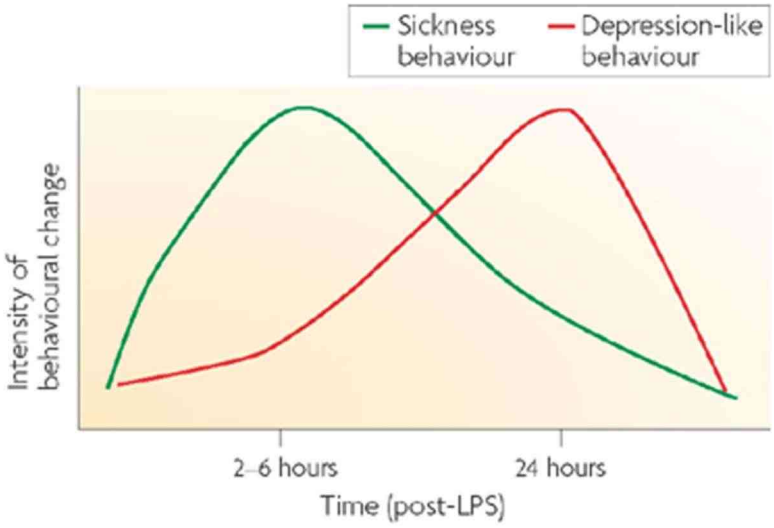
Microglia-neuron interactions in physiological conditions and after stress response (adapted from Wohleb et al. 2016).

1.3.2 Immune challenge with lipopolysaccharide

Another demonstration of the involvement of the immune system in the development of MDD is the fact that the administration of the bacterial toxin lipopolysaccharide (LPS) in rodents, induce the development of depressive-like phenotypes (Frenois et al., 2007; Zhu et al., 2010). LPS administration mimic a bacterial infection, by massively inducing the production of pro-inflammatory cytokines that -from the periphery- reach the CNS. Animals treated with LPS show the so-called sickness behavior, as a consequence of the systemic effects of the activation of the immune response. In general, these animals present impaired locomotor activity, decreased interest for the environment, reduced social interactions, reduced water and food consumption and cognitive alterations (Dantzer et al., 2008).

Usually sickness behavior resolves within 24hours from LPS administration and -subsequently- depressive-like behavior emerges. At molecular level, within the first 6 hours from the immune challenge, there is a massive induction of pro-inflammatory cytokines, a peak is usually decreased in 24 hours.

Lipopolysaccharide exerts its action binding to its specific receptor, the TLR-4. As previously described, this PPR receptor may mediate the activation of the immune signaling within the brain thus promoting the production of pro-inflammatory mediators, the activation of microglia and the activation of the kynurenine pathway.



Temporal profile of the behavioral effect of LPS administration. Peripheral administration of lipopolysaccharide (LPS) induces sickness behavior that peaks 2 to 6 hours later and gradually wanes. The development of sickness behavior is paralleled by the activation of pro-inflammatory cytokine signaling in the brain in response to peripheral LPS. Depression-like behavior, as measured by increased immobility in the forced-swim test or the tail-suspension test and decreased preference for a sweet solution, emerges on this background 24 h later (Dantzer et al., 2008).

1.4 Pharmacological treatment

In contrast to our limited understanding of depression, there are many effective treatments. The large majority (80%) of people with depression show some improvement with any of several antidepressant medications or electroconvulsive seizures (ECS). In addition, several forms of psychotherapy (in particular, cognitive and behavioral therapies) can be effective for patients with mild to moderate cases, and the combination of medication and psychotherapy can exert a synergistic effect.

The treatment of depression was revolutionized about 50 years ago, when two classes of agents were discovered -entirely by serendipity- to be effective antidepressant: the tricyclic antidepressants and the monoamine oxidase inhibitors, the original tricyclic agents (imipramine) arose from anti-histamine research, whereas the early monoamine oxidase inhibitors (iproniazid) were derived from work on antitubercular drugs. The discovery that depression could be treated with these medications provided one of the first clues into the types of chemical changes in the brain that regulate depressive symptoms. Indeed, much depression research over the last half-century was based on the notion that understanding how these treatments work would reveal new insight into the causes of depression.

The acute mechanisms of action of antidepressant medications were identified: inhibition of serotonin or norepinephrine reuptake transporters by the tricyclic antidepressants, and inhibition of monoamine oxidase (a major catabolic enzyme for monoamine neurotransmitters) by monoamine oxidase inhibitors. These discoveries led to the development of numerous second-generation medications (e.g. serotonin-selective reuptake inhibitors [SSRIs] and norepinephrine-selective reuptake inhibitors) which are widely used today. The availability of clinically active antidepressants also made it possible to develop and validate a wide range of behavioral tests to study depression-like phenotypes in animal models. Moreover, these medications and behavioral tests represent important tools for the study of brain function under normal conditions and for identify a range of proteins in the brain that might serve as targets for novel antidepressant treatments.

Furthermore, the mechanism of action of antidepressant medications is far more complex that their acute mode of action might suggest. Inhibition of serotonin or norepinephrine reuptake or catabolism would be expected to result in enhanced actions of these transmitters. However, all available antidepressants exert their mood-elevating effects only after prolonged administration (several weeks to months), which means that enhanced serotonergic or

noradrenergic neurotransmission per se is not responsible for the clinical actions of these drugs. Rather, some gradually developing adaptations to this enhanced neurotransmission would appear to mediate drug action. Important progress has made in the search for such drug-induced plasticity but definitive answers are still out of reach. Moreover, several generations of research have failed to provide convincing evidence that depression is caused by abnormalities in the brain's serotonin or norepinephrine systems. This is consistent with the ability of antidepressant medications to treat a wide range of syndrome far beyond depression, including anxiety disorders, PTSD, obsessive-compulsive disorder, eating disorders, and chronic pain syndrome.

As mentioned previously, an important shift in emphasis has occurred in the past 2 decades with the discovery that physical or psychological stress, and the resulting activation of the inflammatory cascade, plays an increasingly important role in MDD. It can be therefore hypothesized that the anti-inflammatory drug would exhibit antidepressant activity and there is experimental (Manji et al., 2003) and clinical evidence (Müller et al., 2006) that cyclo-oxygenase (COX) and nitric oxide synthase (NOS) inhibitors have antidepressant-like activity.

Likewise, several lines of evidence indicate that antidepressants produce various immunomodulatory effects. In depressed patients, the effects of antidepressants are variable and seem to be related to the immune status of the subjects at the initiation of the treatment. Antidepressants reduced immune function and cytokine secretion and, for example, the increased plasma levels of IL-6 during acute depression were normalized by antidepressant treatment (Lanquillon et al., 2000). Indeed, treatment with antidepressant appears also to have an effect in lowering levels of IL-1β, a cytokine for which evidence of an elevation in depression is controversial (Dowlati et al., 2010). On the other hand, when immune functions were found to be normal, antidepressants had no immunological effects; for example, chronic moclobemide treatment had no effect on monocytes functions, TNF-α production or IFN-γ levels (Landmann et al., 1997). In experimental animals, TCAs as well as SSRIs produce mainly immune suppression and anti-inflammatory effects. For example, administration of the tricyclic antidepressant desipramine in rats has been shown to result in a virtual ablation of neuro-derived TNF-α (Reynolds et al., 2005). Antidepressants are also able to decrease peripheral inflammation and recently, preventive treatment with bupropion-amfebutamone, a noradrenaline-dopamine reuptake inhibitor, was shown to reduce TNF-α release and mortality in a murine model of severe sepsis (Brustolim et al., 2006).

In addition to their effects on immune functions, antidepressants were also found to attenuate the behavioral effects of immune activation. Specifically, chronic but not acute administration of imipramine attenuated LPS-induced decrease in the consumption of and preference for saccharine solution, which is considered as a good animal model of anhedonia, as well as others sickness behavior symptoms including anorexia, weight loss, and reduced social, locomotor, and exploratory behavior (Yirmiya et al., 2001).

Among others, one antidepressant that has shown modulatory properties on the inflammatory response is Agomelatine. Specifically, previous data from our laboratory demonstrated that chronic pretreatment with this antidepressant mitigated the inflammatory response induced in the rat by acute injection of lipopolysaccharide. Indeed, it has been found that agomelatine is able to act on the early phase of the inflammatory response (2-6 h after LPS), as well as in the late phase (24h after LPS) by acting on specific mediators. For example, the antidepressant 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 (Molteni et al., 2013).

2. Aim of the project

Major depression is a severe psychiatric disorder characterized by a complex etiology and a heterogeneous symptomatology. The hallmark symptoms in depressed patients are alterations in mood and anhedonia, defined as the incapability of feeling pleasure in hedonic circumstances. Despite the numerous drugs available in the market, there are several unmet needs in the pharmacological treatment of major depression. Indeed, their therapeutic effect appears only after several weeks of treatment, often preceded by adverse effects, and it is not always related to a complete remission of the pathology. For these reasons a high percentage of patients do not respond to the pharmacological treatment. With these premises, the comprehension of the molecular mechanism altered in major depression appears fundamental to find new potential targets and develop new pharmacological entities.

Despite the huge complexity of depression pathophysiology, it is well established that the insurgence of the disease is based on the interaction between a genetic background of susceptibility and environmental factors; among them, stressful events during life seem to play a pivotal role. It is important to note that not all the people exposed to stressful situations develop a mental illness, indeed only a small percentage of subjects become affected by major depression after stress exposure. In this sense the term “Resilience” refers to the ability of the subject to actively cope against adverse stimuli. The investigation and the identification of mechanisms underpinning stress vulnerability and stress resilience are, thus, of critical importance to identify new therapeutic targets.

In addition, compelling evidence support the idea that neuroinflammation is involved in the etiology of psychiatric disorders and -in this context- it has been demonstrated that alterations in the inflammatory system may lead to the insurgence of depressive phenotype in animal models and in humans.

With these premises, the aim of this work was to characterize the response of animal models of the pathology to chronic stress and/or to immune challenges to identify from one side the molecular systems mainly involved in stress resilience and, on the other, to better understand how different classes of antidepressant drugs may intervene on altered expression of mediators of inflammation. To pursue this end, we used different experimental approaches.

Firstly, we characterized the behavioral response of rats chronically exposed to the chronic mild stress (CMS) procedure -a well-established model of depression- by the mean of sucrose consumption test. The identification of a stress-Resilient population of animals gave us the possibility to investigate the alterations of mediators of inflammation in the different context of susceptibility, with a focus on brain areas involved in the pathophysiology of depression, such as the dorsal and ventral hippocampus and the prefrontal cortex. Then we evaluated how the antidepressant drugs agomelatine and imipramine and the antipsychotic lurasidone were able to modulate the alterations at behavioral and molecular levels in stress-Responsive rats. We then decided to focus our attention to a specific molecular signaling pathway, to identify a peculiar mechanism of action of antidepressant in the context of neuroinflammation. We chose the IL-6 pathway, because it is characterized by a feedback inhibition mechanism led by the suppressor of cytokine signaling (SOCS)3. We deepened the characterization of the activity of agomelatine on this system, considering the pronounced activity of this drug on IL-6 expression in the rat prefrontal cortex.

Subsequently, we used an unbiased genome-wide approach to characterize the potential protective properties of agomelatine on a strong immune challenge such as the acute injection of lipopolysaccharide in the rat ventral hippocampus. The aim of this new experimental methodology was to look at the molecular effect of an antidepressant from a broader point of view, to enlighten molecules and pathway potentially important for its therapeutic effects. We, then, pursued the idea that stress-resilient animals were more able to cope with stress-induced alteration/priming of inflammation within the brain. Animals were exposed to two weeks of CMS, followed by an immune challenge with LPS, to test -at behavioral and molecular levels- the capability of the different stressed populations to respond to the massive induction of the inflammatory system after stress. This study was focused on the investigation on the molecular systems -related to neuroinflammation- underlying the response to CMS-induced susceptibility, with a specific interest on the protective mechanisms that are involved in resilience.

Lastly, considering the key role of BDNF in the etiopathology of depression, we investigated its contribution in the response to inflammatory stimuli, exposing heterozygous male and female mice for the neurotrophin to a single injection of lipopolysaccharide.

3. Materials and Methods

3.1 Animals

3.1.1 Chronic mild stress study: evaluation of CMS-induced neuroinflammation (Exp.1), effects of pharmacological treatment on CMS-induced neuroinflammation (Exp.2) and the impact of a challenge with Lipopolysaccharide (LPS) in animals exposed to CMS (Exp. 3)

Adult male Wistar rats (Charles River, Germany) were brought into the laboratory one month before the start of the experiments. The animals were singly housed with food and water freely available, and were maintained on a 12-h light/dark cycle in a constant temperature (22 ± 2° C) and humidity (50 ± 5%) conditions. All procedures used in this study were conformed to the rules and principles of the 2010/63/EU Directive and were approved by the Local Bioethical Committee at the Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.1.2 Genome wide analysis of agomelatine anti-inflammatory activity (Microarray study - Exp. 4)

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 (12h 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 (D.Leg. 2014/26), the EC (EEC Council Directive 2010/63/UE), 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.

3.1.3 Immune challenge in animals heterozygous for the neurotrophin BDNF ($BDNF^{+/-}$ study – Exp. 5)

Wild type (C57BL/6 males and females) and $BDNF^{+/-}$ male and female mice on a mixed C57BL/6 SV129 background were taken from animal house of the Central Institute of Mental Health (Mannheim, Germany). All the animals were housed individually at the age of 13-19 weeks in standard macrolon cages (type II - 26 cm x 20 cm x 14cm) with bedding and nesting material (paper tissue). They were acclimatized at least for 2 weeks to a reserved 12 hour

dark-light cycle (lights off 8 am – 8 pm) at 22 ± 1°C room temperature and the humidity 35 %. Animals received a standard pellet diet and water *ad libitum*. Bodyweight was assessed once a week when the cages were changed. All animal experiments were approved by the Animal Welfare Office of the Regierungspräsidium Karlsruhe, Germany.

3.2 Experimental procedures

3.2.1 Chronic mild stress study

Each week of the stress regimen consisted of two periods of food or water deprivation, two periods of 45-degree cage tilt, two periods of intermittent illumination (lights on and off every 2 hours), two periods of soiled cage (250 ml water in sawdust bedding), one period of paired housing, two periods of low intensity stroboscopic illumination (150 flashes/min), and three periods of no stress. All stressors were 10–14 hours of duration and were applied individually and continuously, day and night. Control animals were housed in separate rooms and had no contact with the stressed animals. They were deprived of food and water for 14 hours preceding each sucrose test, but otherwise food and water were freely available in the home cage

***Experiment 1*.** Animals were subjected to the stress procedure for two weeks, tested for the sucrose consumption. Based on the results of this test, the stressed animals were divided into 2 groups: “stress-Responsive” (i.e. showing at least 50% decrease of sucrose consumption) and “stress-Resilient” (i.e. showing small or no decrease of sucrose consumption) to be compared versus un-stressed rats. This experimental design implied three experimental groups: unstressed animals used as control group (n=10 animals); stressed animals that showed a decrease in sucrose consumption (“stress-Responsive” animals, n=10); stressed animals that were resilient to the CMS (“stress-Resilient”, n=10). 24 hours after the final sucrose test the rats were killed by decapitation, the brains were removed and hippocampus (dorsal and ventral) and prefrontal cortex were dissected as fresh tissues. Specifically, the dorsal hippocampus corresponds to the plates 25-33 according to the atlas of Paxinos and Watson (Paxinos and Watson, 2006), whereas the ventral hippocampus corresponds to the plates 34-43. The prefrontal cortex (defined as Cg1, Cg3, and IL sub regions corresponding to the plates 6-10 according to the atlas of Paxinos and Watson) was dissected from 2-mm-thick slices, whereas the hippocampus was dissected from the whole brain. The brain specimens were then rapidly frozen in dry ice/isopentane and stored at -80° C for the molecular analyses.

Experiment 2. Animals were subjected to the stress procedure for 7 weeks. Based on the results of the final sucrose test carried out following first 2 weeks of stress, both control and stress-Responsive groups were further divided into matched subgroups and for the subsequent five weeks they received intraperitoneal injections (i.p.) of vehicle (hydroxy-ethyl-cellulose, HEC 1%), imipramine (10 mg/kg daily) or agomelatine (40 mg/kg daily) with a dosage chosen according with previous data (Papp et al., 2003). Another group of animals received oral administration (by gavage) of vehicle (HEC 1%) or lurasidone (3 mg/kg daily); this dose and route of delivery were chosen based on previous studies (Ishiyama et al., 2007; Tarazi and Riva, 2013). The stress was continued throughout the entire period of drug administration. According with this experimental design the animals were divided into matched subgroups: rats that were left undisturbed and received the appropriate vehicle (i.p. or per os according with the respective drug) and used as control group (CTRL, n=10); CMS-exposed animals that received the appropriate vehicle for five weeks (STRESS; n=10); un-stressed rats that received only the chronic pharmacological treatment (IMI or AGO or LUR, n=10/group); rats that were subjected to the CMS procedure in parallel with pharmacological treatment (STRESS/IMI; STRESS/AGO; STRESS/LUR n=10/group). After five weeks, the treatments were terminated and control and stressed animals were killed by decapitation 24h after the last drug administration, their brains removed and dissected for dorsal hippocampus as fresh tissue. All samples were then rapidly frozen in dry ice/isopentane and stored at -80° C for the further molecular analyses.

Experiment 3 Animals exposed to two weeks of chronic mild stress received Lipopolysaccharide (LPS from E. coli, serotype 026:B6; 250 μg/kg, i.p.) or saline administration 24 hours after the sucrose consumption test. The dose of LPS was based on previously published studies of the laboratory (Macchi et al., 2013; Molteni et al., 2013) and was chosen as a sub-septic dose. The experimental design generated six experimental groups: animals that were not exposed to CMS that received saline (No Stress) or LPS (LPS); animals that showed a decreased sucrose intake after stress, that were administered with saline (Responsive) or LPS (Responsive/LPS); animals that did not present alterations at behavioral level that received saline (Resilient) or LPS (Resilient/LPS).

In order to evaluate the effects of the immune challenge at both early and later time points, the animals were sacrificed 24 hours or six days after the LPS injection. During these six days,

the stress procedure was not interrupted. Dorsal hippocampi were dissected, frozen on dry ice and stored at -80°C for the molecular analyses.

3.2.2 Microarray study - Exp. 4

Rats were chronically (21 days) treated by oral gavage with vehicle (VEH; hydroxy-ethyl-cellulose 1%, 1 ml/kg) or agomelatine (AGO; 40 mg/kg) at 5 pm (2h before the dark phase) to mimic the evening administration of agomelatine in clinics. Animals were challenged with lipopolysaccharide (LPS from E. coli, serotype 026:B6; 250 μg/kg, i.p.) or saline (SAL) 16h after the last drug administration. The choice of agomelatine dose was based on previous work demonstrating its activity in different animal models of depression (Papp et al., 2003) and for its anti-inflammatory properties in a previous study (Molteni et al., 2013). This experimental design implied 4 experimental groups: animals that received saline and vehicle (VEH/SAL), animals challenged with LPS without pharmacological pre-treatment (VEH/LPS), animals treated with agomelatine without the inflammatory challenge (AGO/SAL) and animals treated with agomelatine and injected with LPS (AGO/LPS).

The animals were sacrificed by decapitation 2h (11 am) post LPS injection, ventral hippocampus was rapidly dissected, frozen on dry ice and stored at -80°C for the molecular analyses.

3.2.3 BDNF$^{+/-}$ study - Exp.5

After the acclimatization phase, wild-type and heterozygous mice were randomly divided to received saline or LPS. The bacterial toxin (from E. coli; serotype 026:B6) was dissolved in sterile, endotoxin-free isotonic saline and injected i.p. at the dose of 400 μg/kg. Intraperitoneal injections were prepared from 1 mg/ml stock solution and the dose of LPS was chosen after a pilot study (unpublished data). With this experimental design, we obtained eight groups of animals: male and female wild type mice treated with saline or LPS; male and female BDNF$^{+/-}$ mice that received saline or the bacterial toxin. After 6 hours from the injection, animals were tested with the Open Field (OF) test to evaluate alterations in the locomotor activity; whereas, after 18 hours from the immune challenge, we assessed the insurgence of depressive-like behavior with the Forced Swim test (FST). At the end of the FST the mice were dried and sacrificed 5 minutes later, the brains were harvested and the different brain regions (total hippocampus and frontal lobe) were dissected form both hemispheres. The tissues were frozen on dry ice and stored at -80°C until the molecular analyses.

3.3 Behavioral tests

3.3.1 Sucrose consumption test (Chronic mild stress study)

After a period of adaptation to laboratory and housing conditions, the rats (220 ± 7g) were trained to consume a 1% sucrose solution. Training consisted of nine 1h-baseline tests, in which sucrose was presented in the home cage, following 14h of food and water deprivation. The sucrose intake was measured at the end of the test by weighing pre-weighed bottles (300 ml Polythene bottles equipped with Stainless steel ball sippers, North Kent Plastics, UK) containing the sucrose solution. Based on their sucrose intake in the final baseline test, animals were divided into two matched groups to be subjected or not to a chronic mild stress procedure (Papp, 2012) for a period of two (**Experiment 1**) or seven (**Experiment 2**) weeks.

Sucrose consumption was used to discriminate between stress-Responsive and stress Resilient rats after two weeks of CMS. Subsequently, sucrose consumption was monitored, under similar conditions, at weekly intervals throughout the whole experiments.

A similar procedure was used in **Experiment 3** to assess the insurgence of anhedonic-like phenotype (after two weeks of CMS) and to evaluate the behavioral impact of LPS administration (6 days after LPS injection).

3.3.2 Open Field test ($BDNF^{+/-}$ study - Exp.5)

Locomotor activity monitoring was conducted in a square shaped, white openfield, measuring 50 x 50 cm^2 and illuminated from above by 25 lx. Mice were placed individually into the arena and monitored for 10 minutes by a Video camera (Sony CCD IRIS). The resulting data were analyzed using the image processing system Etho Vision 3.0 (Noldus Information Technology, Wageningen, the Netherlands). For each sample, the system recorded position and the status defined events. Parameters assessed were total distance moved, velocity, distance to the walls and time in the center, which was defined as the area 10 cm distant from the walls.

3.3.3 Forced Swim test ($BDNF^{+/-}$ study - Exp. 5)

Briefly, mice were placed individually into a glass cylinder (23 cm height, 13 cm diameter), which was filled with water (21°C) up to height of 12 cm. The water was changed between testing sessions. A testing period of 6 minutes was used to determinate the onset and the percentage of time spent immobile (´floating´). Mice were monitored by a video camera (Sony CCD IRIS) from sideward. The resulting data were analyzed using the image processing system EthoVision 3.0 (Noldus Information Technology, Wageningen, the Netherlands). For each

sample, the system recorded position, object area and the status of defined events. Parameters assessed were latency to start floating, total immobility time, mobility, where mobility was defined as percentage change between 11.5% and 17% in the object area between samples.

3.4 Molecular analyses

3.4.1 *RNA preparation and real time RT-PCR*

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) accordingly to the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were then processed for real-time polymerase chain reaction (PCR) to assess mRNA levels of different markers of inflammation. 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. 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 Life Technologies Italia and Eurofins MWG-Operon (the complete list is presented in Table 1).

Gene	Forward Primer	Reverse Primer	Probe
IL-1b	Purchased from Applied Biosystem (Italy) cod. Rn99999009_m1		
IL-6	Purchased from Applied Biosystem (Italy) cod. Rn99999011_m1		
TGFb	CAACAATTCCTGGCGTTACC	ACTGAAGCGAAAGCCCTGTA	TGAGTGGCTGTCTTTTGACG
CD11b	TTCCGGACTCACTTCACCTT	TTCAGCTGCCTTATGGGTCT	TTCAAGAGAAACCCTGACCC
CX3CL1	ACAAGATGACCTCGCCAATC	TGGACCCATTTCTCCTTTGG	CCTTGCTCATCCACTATCAACTGAACCA
CX3CR1	TTCCCTAGTTGTGGCATGAAG	ACCTTCTGAACTTTTCCCCAG	TTAGTGTGACGGAGACAGTGGCG
Socs3	AGAGCGGATTCTACTGGAGT	TCGACGCTCAGTGTGAAGAA	TTTCTTATCCGCGACAGCTC
Caspase1	TGCCCTCATTATCTGCAGCA	CACAGTATACCCCAGATCCTGC	AGAGTCGGAGCTGATGTTGA
Caspase3	GCTGGACTGCGGTATTGAGA	AGGAATAGTAACCGGGTGCG	AGAAGATACCAGTGGAGGCC
Bcl-xl	GAACTCTTTCGGGATGGGGTAA	ACTTGCAATCCGACTCACCA	AGCGTAGACAAGGAGATGCA
TNFa	Purchased from Applied Biosystem (Italy) cod. Rn99999017_m1		
TLR4	AGTTGGCTCTGCCAAGTCTCAGAT	TGGCACTCATCAGGATGACACCAT	GAAATGCCATGAGCTTTAGAGGTT
Arg1	TGTACATCGGCTTGCGAGAT	TTGCCAATTCCCAGCTTGTC	GGACCCTGGGGAACACTATATA
CD68	TTACGGACAGCTTACCTTTGG	CTTGAAGAGATGAATTCTGCGC	CAAACAGGACCGACATCAGAGCCA
Grm2	TGACATTGCGCTGTAACCAC	TTGCGGGTCTTGAAGGCATA	TACAATGTGCTCCTCATCGC
β-actin	CACTTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG	TCTGGGTCATCTTTTCACGGTTGGC
Gapdh	Purchased from Applied Biosystem (Italy) cod. Rn99999916_s1		

Table 1 List of primers and probes used in the different studies presented.

3.4.2 Microarray procedures

Gene expression microarray assays were performed using Rat Gene 2.1ST Array Strips on Gene Atlas™ platform (Affymetrix), following the WT Expression Kit protocol described in the "Affymetrix Gene Chip Expression Analysis Technical Manual" and in the GeneAtlas™ WT Expression Kit User Manual. Briefly, starting from 250 ng of total RNA, cDNA was synthetized with the Gene Atlas WT Expression Kit (Affymetrix, Santa Clara, CA, USA). The concentration and quality of cRNA and cDNA samples were determined by measuring its absorbance at 260 nm using NanoDrop Spectrophotometer. After fragmentation and labeling procedures, 5.5 µg of cDNA were hybridized using Rat Gene 2.1ST Array Strip. The hybridization, the fluidics and the imaging were performed on the Affymetrix Gene Atlas instrument following the manufacturer's instructions.

3.4.3 Protein extraction and preparation of subcellular fractions

Frozen brain regions from the different studies were manually homogenized in a glass-glass potter in ice-cold 0.32M sucrose buffer (pH 7.4) containing 1mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) 0.1mM ethylene glycol tetra-acetic acid (EGTA) and 0.1mM phenylmethylsulphonyl fluoride in the presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors. An aliquot of the homogenate (OMO) was sonicated and stored at -20°C, then the remaining part was

clarified at 1000g for 10 minutes. The pellet (P1) was kept as nuclear fraction and re-suspended in a proper buffer (20mM HEPES, 0.1mM dithiothreitol, 0.1mM EGTA) supplemented with protease and phosphatase inhibitors, while the supernatant (S1) was centrifuged at 13000g for 15 minutes. The resulting supernatant (S2) was recovered as the cytosolic fraction, while the pellet (P2), corresponding to the crude membrane fraction, was re-suspended in the re-suspension buffer described above. Total protein content was measured accordingly to the Bradford Protein Assay procedure (Bio-Rad Laboratories) using bovine serum albumin as the calibration standard.

3.4.4 Western blot analyses

Equal amounts of protein (12-15 μg, depending on the target of the analysis) were run under reducing conditions on 8% or 10% SDS-PAGE (PolyAcrylamide Gel Electrophoresis) and then electrophoretically transferred onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes. Unspecific binding sites were blocked for 1 hour in 10% nonfat dry milk in Tris-buffered saline, and membranes were then incubated overnight with the proper primary antibody (the complete list is presented in Table 2) at 4°C in blocking solution and then with the corresponding secondary antibody for 1 hour at room temperature. Immunocomplexes were visualized by chemiluminescence using ECL (Perkin Elmer) and the Chemidoc MP imaging system (BioRad Laboratories). Results were normalized using β-actin (mouse polyclonal antibody, Sigma-Aldrich, 1:10000 in 3% nonfat dry milk in Tris-buffered saline) as internal standard.

Target protein	Primary antibody	Secondary antibody
IL-6 (21 kDa)	1:500 BSA 5% in TBS-t Santa Cruz Biotech.	HRP conjugated anti-rabbit IgG 1:500
pSTAT3 Tyr705 (86 kDa)	1:500 BSA 5% in TBS-t Cell Signaling	HRP conjugated anti-rabbit IgG 1:1000
pSTAT3 Ser727 (86 kDa)	1:500 BSA 5% in TBS-t Cell Signaling	HRP conjugated anti-rabbit IgG 1:1000
STAT3 (86 kDa)	1:2000 BSA 5% in TBS-t Cell Signaling	HRP conjugated anti-rabbit IgG 1:4000
SOCS3 (23 kDa)	1:1000 BSA 5% in TBS-t Cell Signaling	HRP conjugated anti-rabbit IgG 1:1000
pJAK Ser1022/1023 (120 kDa)	1:500 BSA 5% in TBS-t Cell Signaling	HRP conjugated anti-rabbit IgG 1:1000
JAK (120 kDa)	1:500 BSA 5% in TBS-t Cell Signaling	HRP conjugated anti-rabbit IgG 1:500
pp38 Thr180/Tyr182 (43kDa)	1:500 BSA 5% in TBS-t Cell Signaling	HRP conjugated anti-rabbit IgG 1:500
p38 (43kDa)	1:1000 BSA 5% in TBS-t Cell Signaling	HRP conjugated anti-rabbit IgG 1:2000
pERK1/2 Thr202/Tyr204 (42/44 kDa)	1:1000 3% nonfat dry milk in TBS-t Cell Signaling	HRP conjugated anti-rabbit IgG 1:2000
ERK1/2 (42/44 kDa)	1:5000 1% nonfat dry milk in TBS-t Santa Cruz Biotech	HRP conjugated anti-rabbit IgG 1:5000
Caspase3 (32 kDa)	1:1000 3% nonfat dry milk in TBS-t Genetex	HRP conjugated anti-rabbit IgG 1:2000
CD11b (160 kDa)	1:500 BSA 5% in TBS-t Serotec	HRP conjugated anti-mouse IgG 1:500
TLR4 (95 kDa)	1:1000 3% nonfat dry milk in TBS-t Santa Cruz Biotech	HRP conjugated anti-rabbit IgG 1:750
IBA1 (17 kDa)	1:1000 3% nonfat dry milk in TBS-t Sigma-Aldrich	HRP conjugated anti-rabbit IgG 1:1500
β-actin (43 kDa)	1:10000 3% nonfat dry milk in TBS-t Sigma-Aldrich	HRP conjugated anti-mouse IgG 1:20000

Table 2 List of primary and secondary antibodies used throughout the different studies presented.

3.5 Statistics

All behavioral and molecular analyses were carried out in individual animals (independent determinations) by using different statistical tests according to the effect examined.

In **Experiment 1** and **Experiment 2** the behavioral and molecular impacts of two weeks of stress were analyzed by one-way analysis of variance (ANOVA). Conversely, the effect of the pharmacological treatment was evaluated by two-way ANOVA, with treatment (vehicle vs. imipramine/agomelatine/lurasidone) and stress (stress vs. no stress) as independent factors. When appropriate, further differences were analyzed by Fisher's Protected Least Significant Difference (PLSD). In addition, to evaluate the association between the development of the anhedonic phenotype and the alteration of gene expression, Pearson product-moment correlation coefficients (r) were calculated between sucrose consumption levels of single animals and the corresponding mRNA levels of IL-1β, IL-6 and CD11b. Significance for all tests was assumed for $P<0.05$. Gene expression and protein data are expressed as mean ± standard error (SEM) and presented for graphic clarity as mean percent of the control group.

In **Experiment 3** analyses of sucrose consumption were performed with ANOVA with repeated measures, whereas the molecular impact of stress and LPS was evaluated with Two-way ANOVA, followed -when appropriate- by a PLSD test Significance for all was assumed for $P<0.05$. For graphical clarity graphs are presented as % differences between saline and LPS treated rats.

For the data processing in the **Microarray study (Exp.4)**, Affymetrix CEL files were imported into Partek Genomics Suite version 6.6 for data visualization and statistical testing. Quality control assessment was performed using Partek Genomic Suite 6.6. All samples passed the criteria for hybridization controls, labeling controls and 3'/5' Metrics. Background correction was conducted using Robust Multi-strip Average (RMA) (Irizarry et al., 2003) to remove noise from auto fluorescence. After background correction, normalization was conducted using Quantiles normalization (Bolstad et al., 2003) to normalize the distribution of probe intensities among different microarray chips. Subsequently, a summarization step was conducted using a linear median polish algorithm to integrate probe intensities to compute the expression levels for each gene transcript. Pre-processing of CEL data for the complete data set was performed using ANOVA to assess the effects of the different treatments. Subsequently, to investigate the effects of LPS challenge, agomelatine treatment and their combinations, a four linear contrast was performed (VEH/LPS vs. VEH/SAL; AGO/SAL vs. VEH/SAL; AGO/LPS vs.

VEH/SAL; AGO/LPS vs. VEH/LPS). In this comparison, a maximum filter of $P<0.05$ and a minimum absolute fold-change cut-off of ±1.2 was applied. Genes that passed these criteria were used to run further analyses. Ingenuity Pathway Analyses (IPA) Software was then used to identify regulation of molecular signaling pathways, network and GO terms in each condition, using a significance threshold of -Log p value equal to 1.3 (P value = 0.05).

For Real time-PCR, we used two-way ANOVA with treatment (Vehicle vs. Agomelatine) and challenge (LPS vs. Saline) as independent factors. When appropriate, further differences were analyzed by PLSD test or Single Contrast post-hoc test (SCPHT). Significance was assumed for $P<0.05$. For graphic clarity, data are presented as means percent ± standard error (SEM) of control group, namely vehicle-pre-treated rats received saline (VEH/SAL).

In the **BDNF$^{+/-}$ study (Exp.5)** behavioral data were analyzed using Repeated Measurement ANOVA (time × treatment × genotype); One-Way ANOVA (treatment) Two-Way ANOVA (treatment × genotype). When appropriate, Bonferroni Post-Hoc-Tests was used to evaluate further differences between groups.

Two-way ANOVA with treatment (Saline vs. LPS) or genotype (wild type vs. BDNF$^{+/-}$) as independent factors was used for the molecular analyses. When appropriate, direct contrasts were analyzed with PLSD test. For graphic clarity, data are presented as means percent ± standard error (SEM) of control group, with significance threshold set at $P<0.05$.

4 Results

4.1 Stress induced anhedonia is associated with the activation of the inflammatory system in the rat brain: restorative effect of pharmacological intervention

Rossetti A.C., Papp M., Gruca P., Paladini M.S., Racagni G., Riva M.A., Molteni R. *Pharmacological Research 2016 Jan; 103:1-12. doi: 10.1016/j.phrs.2015.10.022. Epub 2015 Nov 1.*

4.1.1 Introduction

Major depression is a severe psychiatric disorder estimated to become the second leading cause of disability in the world by 2020 (Kessler et al., 2009). Although its etiology has not yet been fully elucidated, it is known that the exposure to stressful events may significantly contribute to the development of the disease (Juruena, 2014; Klengel and Binder, 2013; Shapero et al., 2014). However, even if depression occurs in a significant percentage of stress-exposed sub-jects, most of them are able to successfully cope with the adverse situation and avoid such psychopathology (Feder et al., 2009; Franklin et al., 2012). The nature of this differential vulnerability is probably multi-factorial and involves a complex interplay between stress and the genetic and biological personal background. Over the past decade, there has been increasing attention to the involvement of the inflammatory system in the etiology of depression (Dantzer et al., 2008; Leonard and Maes, 2012; Miller et al., 2009). In particular, it has been reported that depressed subjects exhibit increased levels of inflammatory markers both in the periphery and in brain (Dowlati et al., 2010) and several pathologies associated with a moderate grade of inflammation present high co-morbidity with depression (Benton et al., 2007). Furthermore, a high percentage of patients with cancer or hepatitis C receiving immunotherapy with interferon-alpha develop major depression (Udina et al., 2012), suggesting that the activation of the immune system may effectively contribute to the onset of the disease. In addition, it has been described that stress may activate pro-inflammatory mediators at both peripheral and central level. For example, an increased inflammatory response has been observed in depressed subjects who experienced early life adversities (Danese et al., 2008; Danese et al., 2007; Pace et al., 2006) and similar effects were reported in laboratory animals exposed to different stress paradigms (Couch et al., 2013; Gibb et al., 2011; Girotti et al., 2011; You et al., 2011). However, whether the neuroinflammation plays a pathogenic role in the insurgence of depression or it represents a merely epiphenomena is still elusive. In order to clarify this issue, in the present study we evaluated to what extent the development of a stress-induced anhedonic-like phenotype is associated with brain inflammation. To this purpose, we exposed adult male rats to a chronic mild stress (CMS) paradigm, an experimental procedure that considers the naturally occurring variation in the stress response. Indeed, CMS leads to two distinct behavioral responses in the rat: a “susceptible” response characterized by anhedonic-like symptoms as well as a “resilient“

response where the animals appear able to avoid the pathological consequences of the stress exposure (Bergström et al., 2008). Given that, it is thought to be a well-established model of depression and has been widely used to evaluate stress-related molecular mechanisms (Hill et al., 2012; Pochwat et al., 2014; Zurawek et al., 2013). On these bases, we first exposed the animals to 2 weeks of CMS, a period sufficient to identify rats that were "susceptible" or "resilient" to the development of a decrease in the sucrose intake, a test used as measure of anhedonia in the CMS (Willner, 2005) as well as in other animal models of depression (Vollmayr et al., 2004). We then assessed the contribution of specific mediators of the immune/inflammatory system during this initial phase of stress by a detailed analysis of the expression of pro- and anti-inflammatory cytokines and markers of microglia activation and regulation in the hippocampus and prefrontal cortex, two brain regions that play a critical role in the pathophysiology of depression (Duman and Aghajanian, 2012; Krishnan and Nestler, 2010). Next, we established if these molecular changes persisted following exposure to an additional 5 weeks of CMS. Last, we used two antidepressant drugs characterized by different primary mechanism of action, namely the classic tricyclic imipramine and agomelatine. Imipramine was chosen as a gold standard inhibitor of monoamine uptake, whereas agomelatine was selected based on its novel mechanism as melatonergic (MT1/MT2) agonist and serotonergic ($5HT_{2C}$) antagonist. Moreover, a separate cohort of animals received the antipsychotic lurasidone, to evaluate to what extent pharmacological intervention with different class of drugs could normalize the behavioral and molecular consequences set in motion by CMS.

4.1.2 Experiment 1: expression profiling of inflammatory mediators in stress-responsive and stress-resilient rats

4.1.2.1 Sucrose consumption test

Approximately 70% of the animals exposed to the CMS paradigm for 2weeks showed a reduction in sucrose consumption (−4.6 g vs. No Stress, P<0.001). In particular, in the final baseline test, i.e. before the stress protocol had been initiated, we found that all animals drank approximately 12 g of sucrose solution and following two weeks of CMS the intake remained at similar level in control, non stressed animals but fell to approximately 6 g in stressed rats. We defined these animals as "Stress-Responsive" to distinguish them from stressed rats that did not show reduced sucrose intake, which were termed as "Stress-Resilient" (Fig. 1). The reduced sucrose intake was not associated with weight loss (data not shown).

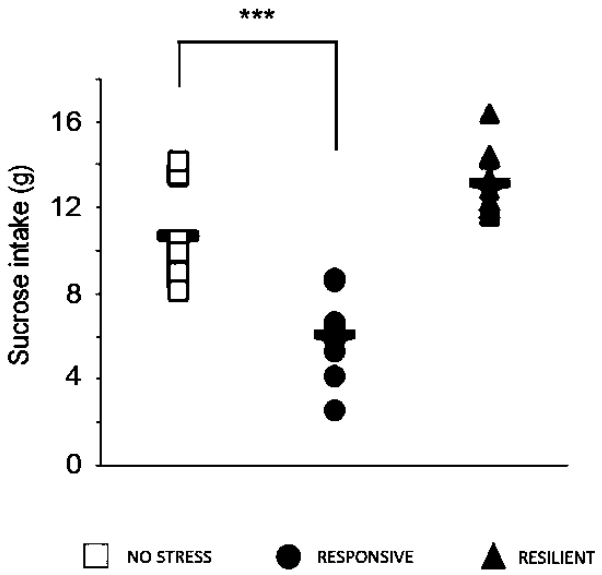


Figure 1. Effect of 2 weeks of chronic mild stress (CMS) on sucrose preference. Rats were divided into animals reactive (RESPONSIVE) and non-responsive (RESILIENT) to CMS depending on sucrose intake (*n*=10 each experimental group) and compared to control unstressed rats (NO STRESS). The data represent the sucrose intake expressed in grams (g) of each animal included in the study. ****P*<0.001 vs. No Stress (One-way ANOVA with PLSD).

4.1.2.2 Cytokine gene expression analysis.

To investigate a possible link between the CMS-induced anhedonic phenotype and inflammation, we investigated some critical mediators of the inflammatory response in Responsive and Resilient animals. Specifically, we analyzed the mRNA levels of the pro-inflammatory cytokines IL-1β and IL-6 and the anti-inflammatory cytokine TGF-β in the dorsal and ventral hippocampal sub regions and in the prefrontal cortex, three brain areas mainly involved in the pathophysiology of depression. As shown in Fig. 2, stress significantly affected the expression of both IL-1β and IL-6 in the dorsal hippocampus ($F_{2,26}$= 7.721 P=0.003; $F_{2,28}$= 7.469 P=0.003, respectively). Specifically, the mRNA levels of the two pro-inflammatory cytokines were increased by CMS only in Responsive animals (+52%, P<0.001 and +27%, P<0.05 vs. No Stress. respectively), whereas no changes were found in Resilient rats. Conversely, CMS did not alter TGF-β mRNA levels in any experimental group. In the ventral hippocampus, IL-1β was specifically up-regulated by stress in reactive animals ($F_{2,27}$= 4.003 P=0.032; +71% vs. No Stress, P<0.01), with no effect of CMS on IL-6 and TGF-β expression. Two weeks of CMS significantly modulated the expression of IL-1β also in the prefrontal cortex ($F_{2,24}$= 3.116 P=0.05), an effect selectively observed in reactive rats (+41% vs. No Stress, P<0.05). In this experimental group, stress up-regulated also the expression of IL-6 ($F_{2,28}$= 4.003 P=0.022), which was significantly different from non-reactive rats (+29% vs. Resilient, P<0.01). Conversely, TGF-β gene expression was not altered neither in reactive nor in non-reactive rats.

4.1.2.3 Gene expression analysis of microglial markers

Given the increased expression of pro-inflammatory cytokines in animals that were reactive to CMS, we next investigated microglial response that represents a key component for brain inflammation (Saijo et al 2011). Specifically, we assessed the expression of CD11b, a marker for the activated state of this cellular population (Perego et al. 2011) as well as the mRNA levels of fractalkine (CX3CL1) and its receptor (CX3CR1), which control microglia activation. In particular, the interaction between the neuronal protein fractalkine and its receptor expressed by microglia plays a crucial role to maintain these cells in a resting. As shown in Fig. 3A, we found that CD11b mRNA levels in the dorsal hippocampus were significantly up-regulated in Responsive animals when compared with control animals or Resilient animals ($F_{2,24}$= 6,633 P=0.006; +56% vs. No Stress, P<0.01 and +63% vs. Resilient, P<0.001). A similar

effect was observed for CX3CL1 ($F_{2,28}$= 7.812 P=0.002; +37%vs. No Stress, P<0.001) and its receptor ($F_{2,30}$= 5.026 P=0.014; +32% vs. No Stress, P<0.01; Fig. 4A and B). Conversely, CMS exposure did not alter the mRNA levels for CD11b in the ventral hippocampus (Fig. 3B), although the neuronal-glial cross talk was dysregulated. Indeed, a slight but significant decrease of CX3CL1 mRNA levels was observed in CMS-reactive animals (−12% vs. No Stress, P<0.05; Fig. 4C) whereas the expression of its receptor was up-regulated ($F_{2,28}$= 7.551 P=0.003; +32% vs. No Stress, P<0.001; Fig. 4D).

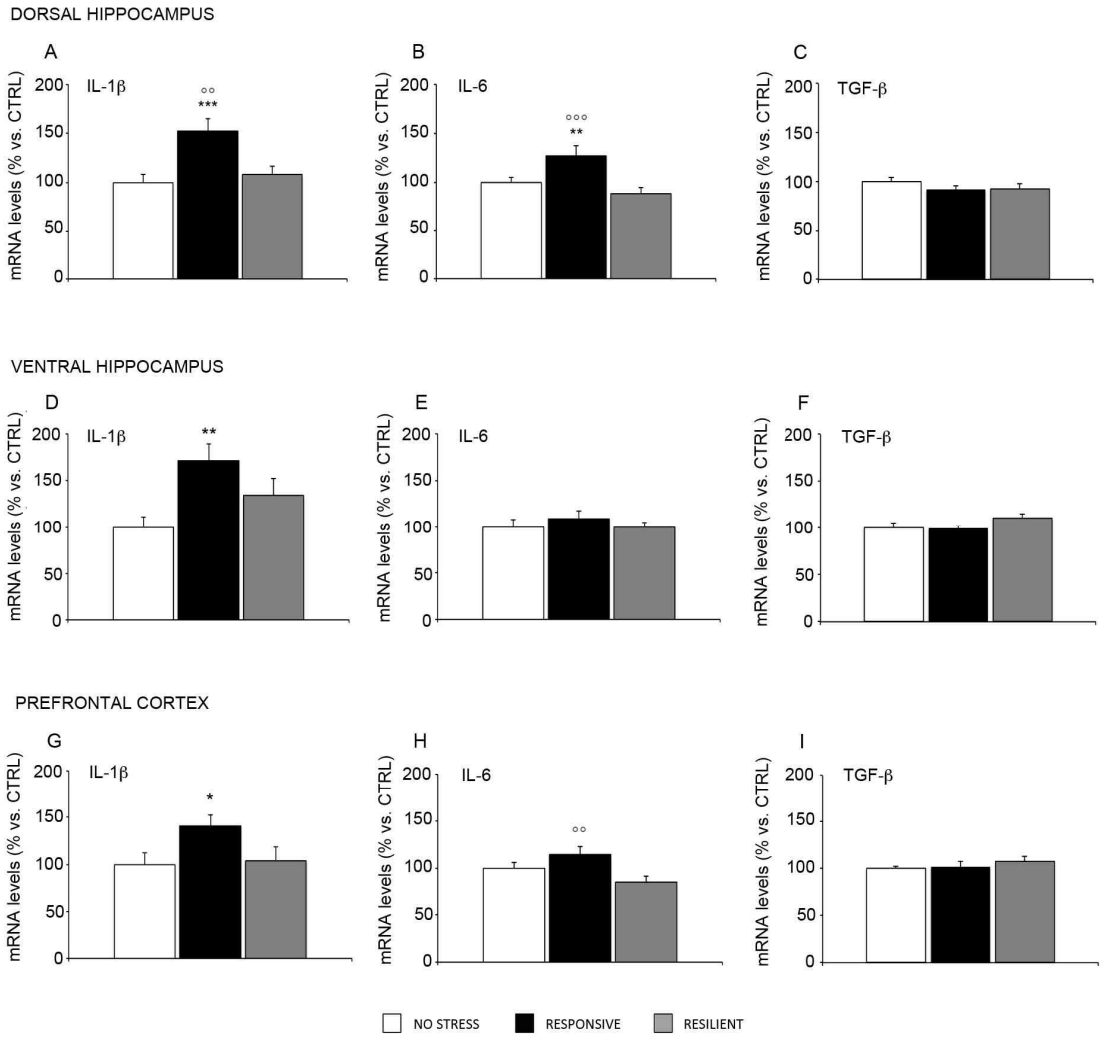


Figure 2. Effect of 2 weeks of chronic mild stress (CMS) on cytokine gene expression in the rat brain. The mRNA levels of the pro-inflammatory cytokines IL-1β and IL-6 and the anti-inflammatory cytokine TGF-β were measured in the dorsal hippocampus (A, B, C), in the ventral hippocampus (D, E, F) and in the prefrontal cortex (G, H, I) of stressed (Responsive or Resilient) rats in comparison with unstressed animals (No Stress). The data, expressed as a percentage of No Stress animals (set at 100%), are the mean ± SEM of at least eight independent determinations. **P*<0.05; ***P*<0.01; ****P*<0.001 vs. No Stress; °°*P*<0.01 °°°*P*<0.001 vs. Responsive (One-way ANOVA with PLSD).

In line with the findings in the ventral hippocampus, CD11b gene expression was not affected by CMS exposure in the prefrontal cortex (Fig. 3C), whereas a significant decrease of CX3CL1 mRNA levels was found in stressed-Responsive animals ($F_{2,30}$= 5.226 P=0.012; −15% vs. No Stress, P<0.01; −11% vs. Resilient, P<0.05; Fig. 4E) without concomitant changes of CX3CR1 expression (Fig. 4F).

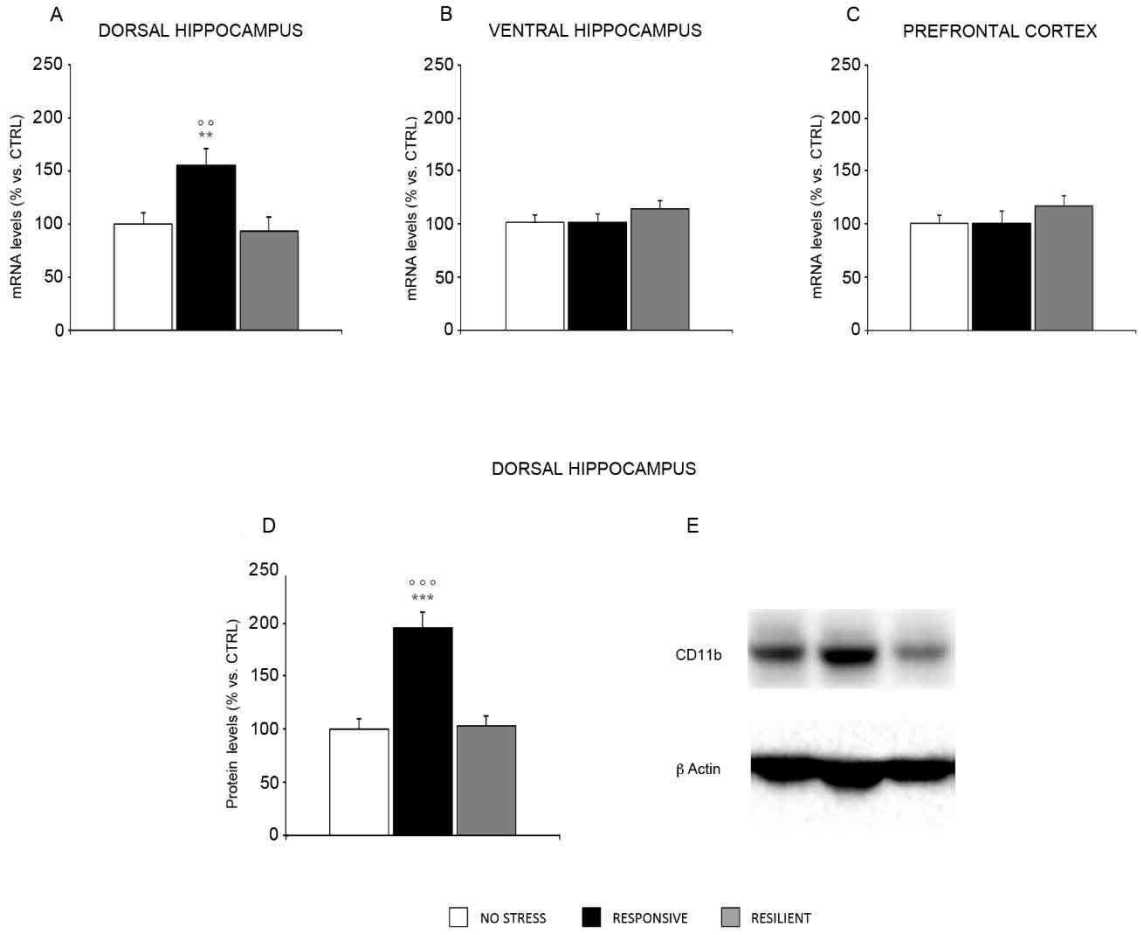


Figure 3. Effect of 2 weeks of chronic mild stress (CMS) on the microglia marker CD11b. The gene expression of CD11b was measured in the dorsal hippocampus (A), in the ventral hippocampus (C) and in the prefrontal cortex of stressed (Responsive or Resilient) rats in comparison with unstressed animals (No Stress). The protein levels of CD11b (panel D) were measured by western blot analysis in the in the dorsal hippocampus of stressed (Responsive or Resilient) rats in comparison with unstressed animals (No Stress). The data, expressed as a percentage of No Stress animals (set at 100%), are the mean ± SEM of at least eight independent determinations. ***P*<0.01, ****P*<0.001 vs. No Stress; °° P<0.01, °°°*P*<0.001 vs. Responsive (One-way ANOVA with PLSD). In panel E are shown representative bands from the western blot analysis.

4.1.2.4 Protein analysis of microglial activation (CD11b)

The changes of CD11b mRNA levels were paralleled by significant modifications of its protein levels in the crude membrane fraction. Indeed, as shown in Fig. 3, we found a main effect of stress ($F_{2,25}$= 15.121 P<0.001) with a significant up-regulation of CD11b only in Responsive animals when compared to both the control group (+95%, P<0.001) and the Resilient animals (+92%, P<0.001).

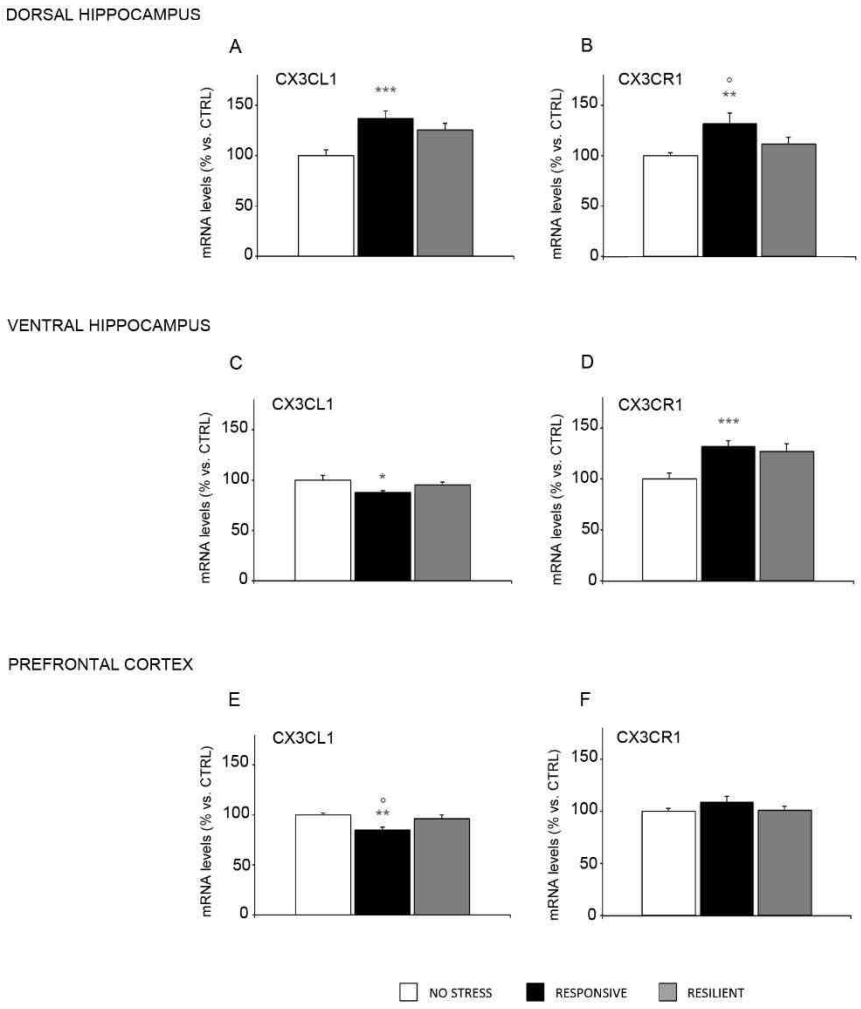


Figure 4. Effect of 2 weeks of chronic mild stress (CMS) on fractalkine (CX3CL1) and its receptor (CX3CR1) in the rat brain. The mRNA levels of CX3CL1 and CX3CR1 were measured in the dorsal hippocampus (A, B), in the ventral hippocampus (C, D) and in the prefrontal cortex (E, F) of stressed (reactive or non-reactive) rats in comparison with unstressed animals (No Stress). The data, expressed as a percentage of No Stress animals (set at 100%), are the mean ± SEM of at least eight independent determinations. **P*<0.05; ***P*<0.01; ****P*<0.001 vs. No Stress; °*P*<0.01; vs. Responsive (One-way ANOVA with PLSD).

4.1.2.5 Pearson correlation analysis between sucrose intake and IL-1β, IL-6 and CD11b gene expression levels

To evaluate if the molecular changes induced by CMS in the stress-Responsive rats were associated with changes in sucrose intake, we calculated the Pearson product-moment correlation coefficient between the mRNA levels of IL-1β, IL-6 and CD11b and sucrose consumption. As shown in Fig. 5, in the dorsal hippocampus all the molecular variables considered were associated with the intake of sucrose. Specifically, we found a significant inverse linear correlation between IL-1β gene expression and sucrose consumption ($r = -0.510$, $P<0.01$; Fig. 5A) and a similar result was also observed for IL-6 ($r = -0.532$, $P<0.01$; Fig. 5B) and CD11b ($r = -0.409$, P 0.05, Fig. 5C). For all these inflammatory mediators, the highest mRNA levels were measured in animals consuming less sucrose, suggesting that the development of anhedonia at an early stage of stress exposure correlates with the activation of the inflammatory response in the dorsal hippocampus. Conversely, there was no correlation between changes in sucrose consumption and the expression of these inflammatory markers in the ventral hippocampus (Fig. 5D–F), whereas in the prefrontal cortex (Fig. 5G–I) only the mRNA levels of IL-6 significantly correlated with the intake of sucrose ($r = -0.570$, $P<0.01$).

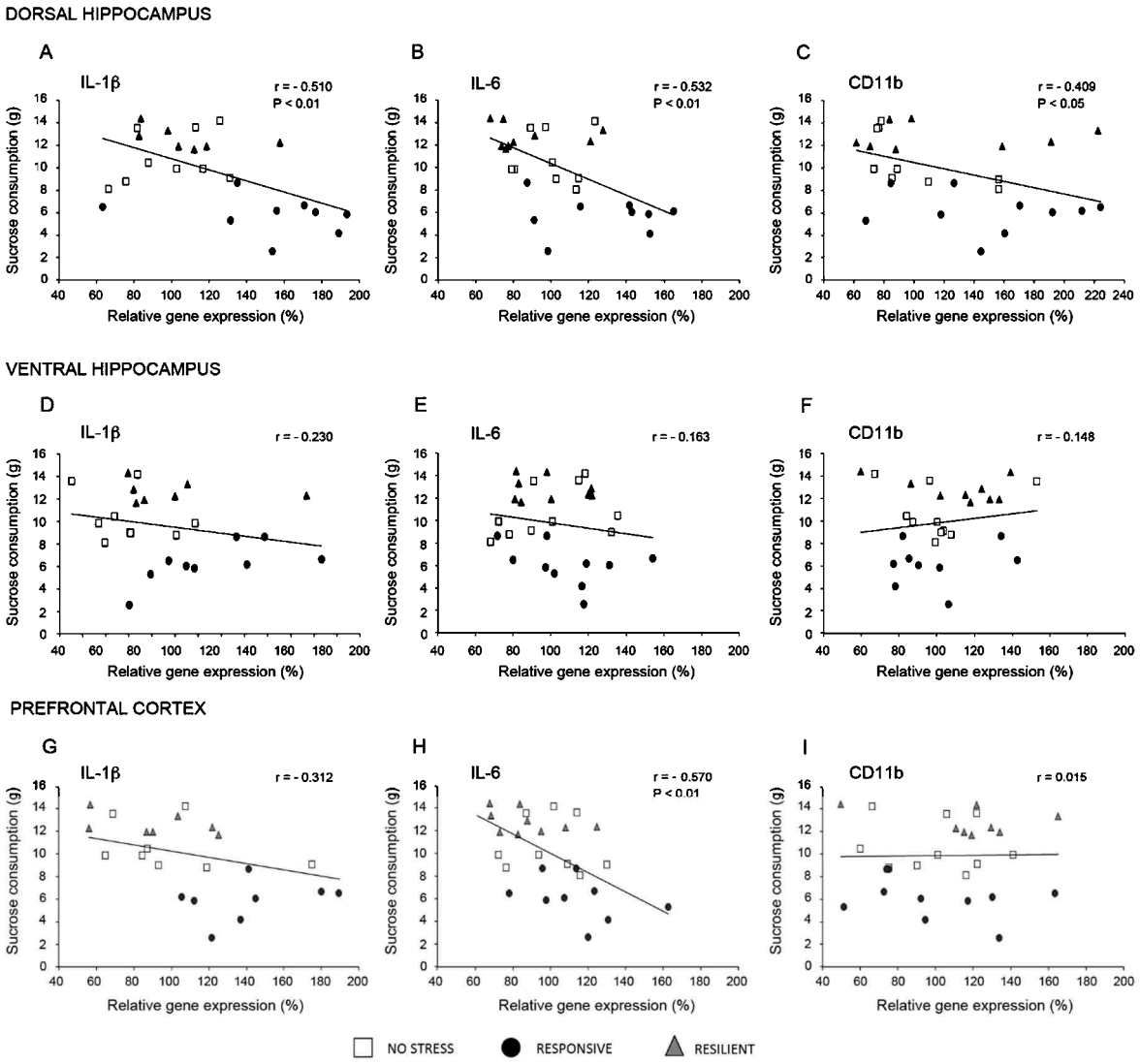


Figure 5. Pearson correlation analysis. The correlation analysis between sucrose consumption and relative gene expression (expressed as percentage) of IL-1β, IL-6 and CD11b in the dorsal hippocampus (A, B, C), in the ventral hippocampus (D, E, F) and in the prefrontal cortex (G, H, I) of unstressed (No Stress), stress-Responsive and stress-Resilient animals. The statistical significance was assumed with $P<0.05$.

4.1.3 Experiment 2: effect of long-term stress exposure on the inflammatory mediators: impact of pharmacological treatment

4.1.3.1 Sucrose consumption test

As in Experiment 1, two weeks of chronic stress reduced the consumption of 1% sucrose solution, an effect that persisted for the subsequent 5 weeks of CMS. As compared to vehicle administration, chronic treatment with imipramine, agomelatine and lurasidone did not affect sucrose intake in control animals (IMI: $F_{1,40}$= 0.067, P=0.797; AGO: $F_{1,40}$= 0.023, P=0.880; LUR: $F_{1,39}$= 0.259, P=0.614), however they all increased sucrose consumption in stressed animals (Fig. 6). Specifically, as compared to week 0 scores, the increases in sucrose intake of stressed animals that receive imipramine (Fig. 6A) and agomelatine (Fig. 6B) reached statistical significance after 1 week of treatment (IMI: $F_{1,40}$= 4.819, P=0.035; AGO: $F_{1,40}$= 6.705, P=0.014). These effects were maintained and further enhanced thereafter, and at week 5 the amount of sucrose solution drunk by these animals was comparable to that of vehicle-treated control rats and significantly higher than that of vehicle-treated stressed animals (IMI: $F_{1,40}$= 4.624, P=0.038; AGO: $F_{1,40}$= 5.753, P=0.022). Similarly, the overall effect of 5 weeks of lurasidone treatment (Fig. 6C) led to increased sucrose consumption in stressed-rats (LUR: $F_{1,40}$= 8.494, P=0.006). The recovery of sucrose preference in CMS rats treated with lurasidone was apparent during the first 2 weeks of treatment and reached first statistical significance after 3 weeks (LUR: $F_{1,39}$= 15.452, P<0.001). All the changes of the sucrose consumption at the different weeks of treatment and the corresponding P values for statistical significance are listed in supplementary Tables S1–S3.

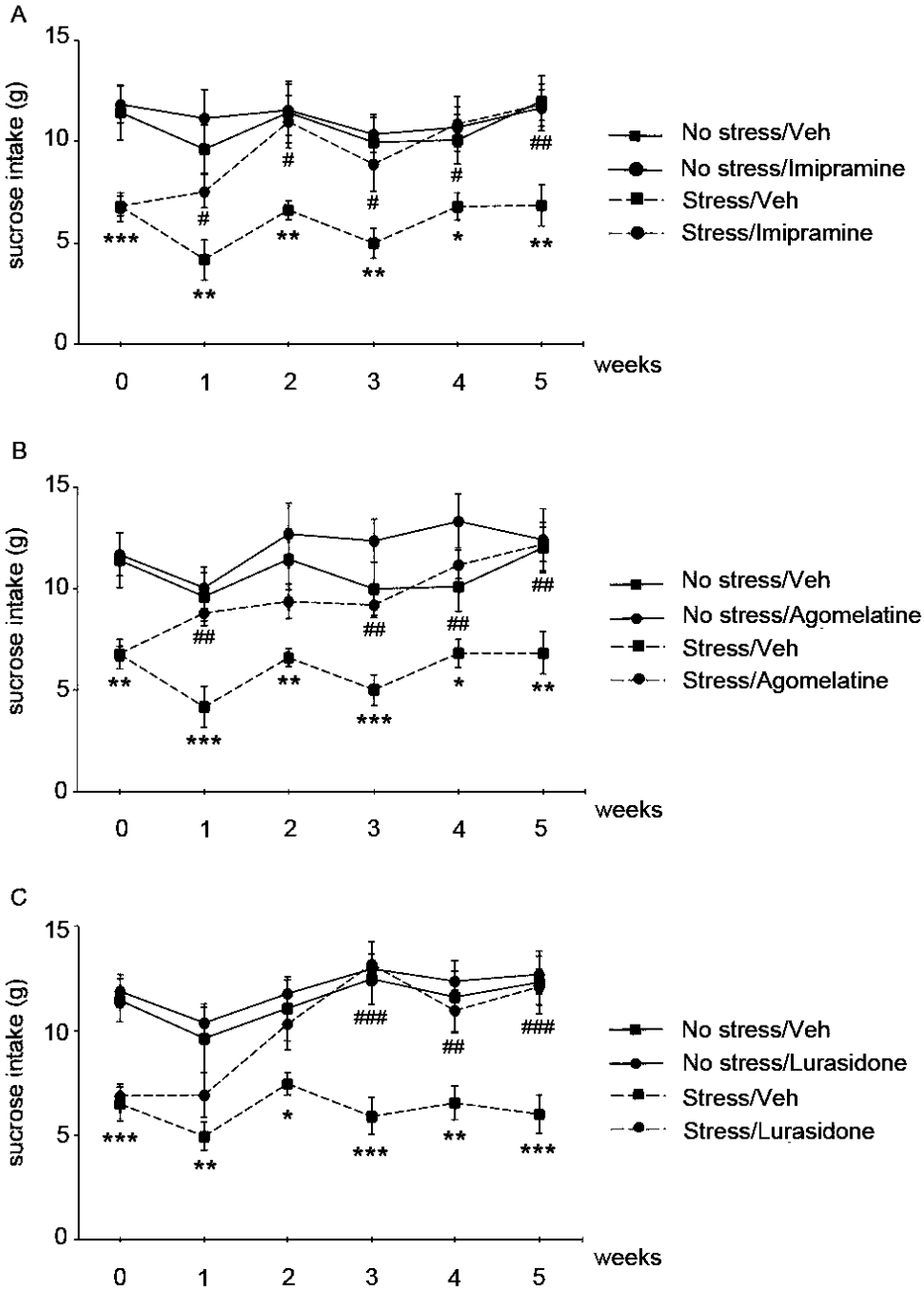


Figure 6. Effect of pharmacological intervention on sucrose intake following exposure to prolonged chronic mild stress (CMS). The sucrose intake was measured weekly during the whole experiment in rats (*n*=10 each experimental group) exposed to CMS combined with chronic treatment with imipramine (A), agomelatine (B) and lurasidone (C) for further 5 weeks starting after 2 weeks of only CMS. The data, expressed as gram (g) of sucrose intake, are the mean ± SEM of at least nine independent determinations. **P*<0.05; ***P*<0.01; ****P*<0.001 vs. No stress/Veh; $^{\#}$*P*<0.05; $^{\#\#}$*P*<0.01; $^{\#\#\#}$*P*<0.001 vs. Stress/Veh (Two-way ANOVA with PLSD).

4.1.3.2 Cytokine gene expression analysis

We next investigated if the ability of pharmacological treatment to normalize the depressive-like phenotype of stressed animals was associated with an effect on the inflammatory changes produced by chronic stress exposure. These analyses were performed in the dorsal hippocampus, the area in which we previously observed the major differences between reactive and non-reactive animals and where we found a significant correlation between sucrose consumption and the gene expression of IL-1β, IL-6 and CD11b. As shown in Fig. 8, the expression of IL-1β was still significantly up-regulated after 7 weeks of CMS; these changes were normalized by the chronic treatment with imipramine, agomelatine as well as lurasidone. Of note, agomelatine per se was able to reduce basal levels of IL-1β mRNA (−36% vs. no Stress/Veh $P<0.01$, Fig. 7B), whereas imipramine (Fig. 7A) or lurasidone (Fig. 7C) did not produce any significant change on the inflammatory cytokine when administered to control (non-stressed) animals. The expression of IL-6 was significantly increased in stressed animals, but the pharmacological treatment did not interfere with this effect (Fig. 7C–E). Finally, the expression of TGF-β was slightly but significantly decreased by chronic stress, whereas pharmacological treatment did not produce any change (IMI: $F_{1,35}= 2.973$, $P=0.095$, Fig. 7G; AGO: $F_{1,36}= 2.523$, $P=0.122$, Fig. 7H; LUR: $F_{1,38}= 0.015$, $P=0.905$, Fig. 7I) except for imipramine that -per se- caused a modest reduction of TGF-β expression. All the percentage of changes of the cytokine expression and the corresponding P values for statistical significance are listed in supplementary Table S4.

4.1.3.3 Gene expression analysis of microglial markers

We then investigated the modulation of microglia activation through the analysis of CD11b expression in the dorsal hippocampus. As shown in Fig. 8, CD11b mRNA levels were still up-regulated after 7 weeks of CMS. These changes were completely normalized by chronic treatment with imipramine ($F_{1,32}= 13.355$, $P=0.001$, Fig. 8A) and partially restored by agomelatine (Fig. 8B) and lurasidone treatment (Fig. 8C). We next examined CX3CL1 expression and, at variance from what we observed after 2 weeks of CMS, we found that prolonged exposure to the stress paradigm caused a modest but significant decrease of fractalkine mRNA levels. This reduction was normalized by chronic lurasidone treatment ($F_{1,36}= 7.031$, $P=0.012$, Fig. 8C) while imipramine and agomelatine (Fig. 8A and B) did not show

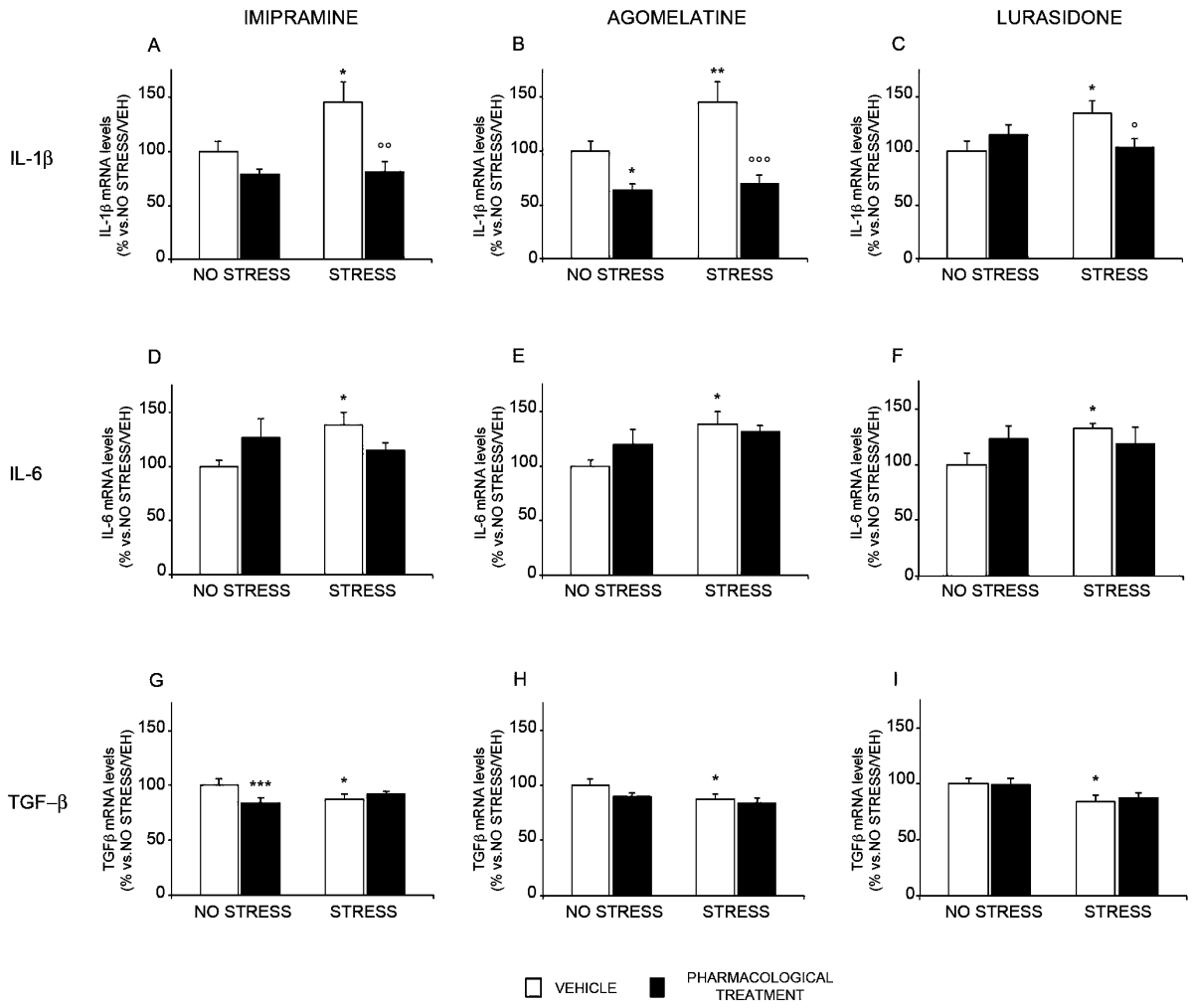


Figure 7. Modulation of cytokine gene expression following CMS and pharmacological treatment in the dorsal hippocampus. The mRNA levels of IL-1β (A, B, C), IL-6 (D, E, F) and TGF-β (G, H, I) were analyzed in rats exposed to CMS and to the treatment with imipramine (A, D, G), agomelatine (B, E, H) or lurasidone (C, F, I) for 5 weeks. The data, expressed as a percentage of unstressed rats treated with vehicle (No stress/Veh animals, set at 100%), are the mean ± SEM of at least seven independent determinations. **P*<0.05; ***P*<0.01; ****P*<0.001 vs. No stress/Veh. °*P*<0.01; °°*P*<0.01; °°°*P*<0.001 vs. Stress/treated animals (Two-way ANOVA with PLSD).

any effect. The expression of the fractalkine receptor CX3CR1 was not significantly affected by 7 weeks of CMS, although imipramine and lurasidone per se produced a modest, though significant, reduction of its mRNA levels (IMI: $F_{1,38}$= 9.726, P=0.004, Fig. 8G; LUR: $F_{1,37}$= 21.455, P<0.001, Fig. 8I). All the percentage of changes of microglia markers and the corresponding P values for statistical significance are listed in Supplementary Table S5.

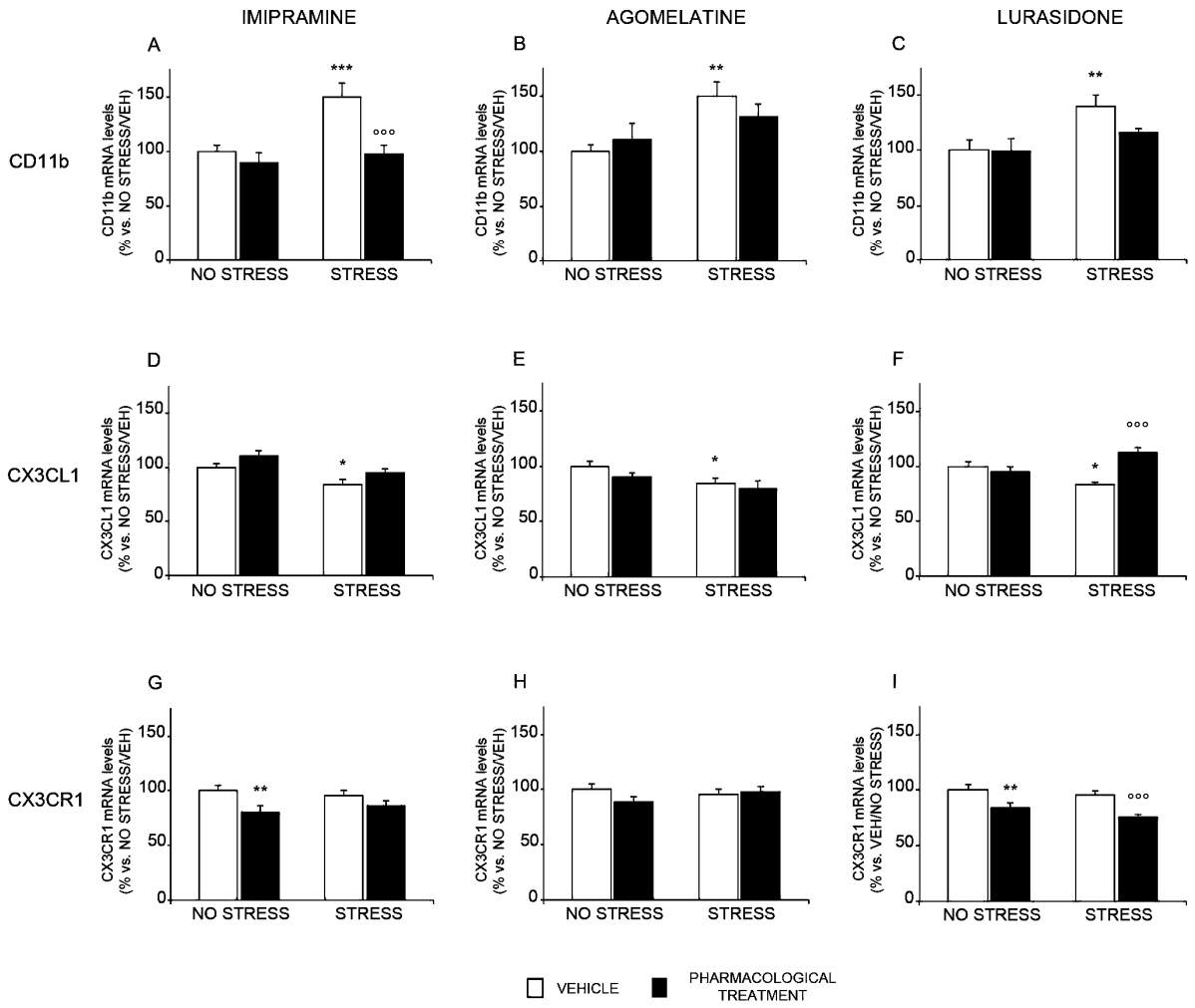


Figure 8. Modulation of microglia markers following CMS and pharmacological treatment in the dorsal hippocampus. The mRNA levels of CD11b (A, B, C), CX3CL1 (D, E, F) and CX3CR1 (G, H, I) were analyzed in rats exposed to CMS and to the treatment with imipramine (A, D, G), agomelatine (B, E, H) or lurasidone (C, F, I) for 5 weeks. The data, expressed as a percentage of unstressed rats treated with vehicle (No stress/Veh animals, set at 100%), are the mean ± SEM of at least eight independent determinations. **P*<0.05; ***P*<0.01; ****P*<0.001 vs. No stress/Veh; °°°*P*<0.001vs. Stress/treated animals (Two-way ANOVA with PLSD).

4.1.4 Discussion

In the current study, we demonstrate that the development of the anhedonic-like phenotype in response to chronic stress is associated with neuroinflammation, sustained by the increased expression of pro-inflammatory cytokines IL-1β and IL-6 and the marker of microglial activation CD11b. These changes were selectively observed in stressed animals showing a reduction of sucrose intake, but not in resilient rats. The expression of IL-1 β was increased in stress-reactive rats in all the brain regions examined. Moreover, the evidence that pharmacological inhibition (Koo et al., 2008) or genetic deletion of IL-1β receptor (van Heesch et al., 2013) blocks the anhedonic behavior induced by chronic stress clearly supports the involvement of this cytokine in pathological impact of stress. Similarly, the increased expression of IL-6 observed in stressed rats with the anhedonic-like phenotype is in line with the reduced behavioral despair, enhanced hedonic behavior and resistance to stress-induced helplessness shown by IL-6 knockout mice (Goshen et al., 2008). Moreover, administration of IL-6 in the rat hippocampus increased immobility time in the forced swim test, whereas its inhibition has an opposite effect (Koo and Duman, 2008). It has to be noted that the association between increased pro-inflammatory cytokines and the pathological consequence of stress exposure has been also reported by a recent study showing a main involvement of TNF-α (Couch et al. 2013), a discrepancy that may be due to differences in the experimental paradigm. Beside the up-regulation of pro-inflammatory cytokines, the neuroinflammatory response observed in our study included microglia activation, adding an important information about the role of these cells on the effect of stress exposure (for review see Thase, 2006). Among the maladaptive mechanisms set in motion by stress that may result in microglia activation, our data point to the involvement of neuron-microglia cross-talk that regulates the state of these cells (Uher et al., 2012). Indeed, the expression of fractalkine and its receptor were increased after 2 weeks of stress. We hypothesize that the initial fractalkine up-regulation may represent an attempt to counteract the elevated neuroinflammatory response induced by the early phase of the CMS exposure, in agreement with data reporting that a short exposure to stress can lead to microglial activation (Cattaneo et al., 2013). Interestingly, a recent study by Milior et al. showed that CX3CR1 KO mice do not present an anhedonic-like phenotype after two weeks of stress (Walker et al., 2013). Moreover, the increased expression of CX3CL1 and CX3CR1 observed in our study may contribute to the enhanced IL-1β release

by microglia, as recently reported (Biber et al., 2007). All in all, these data suggest a potential role of CX3CR1 and its ligand in the behavioral response to chronic stress, as sustained also by a significant linear correlation between the increased gene expression of CX3CR1 and the decrease in sucrose intake of reactive animals (data not shown). We found a similar significant negative correlation between the expression of IL-1β, IL-6 and CD11b observed in the dorsal hippocampus of reactive animals and their intake of sucrose, providing support for the relationship of these molecular alterations with the development of the anhedonic-like phenotype in this brain region. These effects are in line with data demonstrating the association between anhedonia and neuroinflammation following the administration of the cytokine inducer lipopolysaccharide (Kreisel et al., 2014) suggesting that neuroinflammation is closely associated with the development of the depressive-like behavior, rather than being a consequence of stress exposure. However, further studies are demanded to establish whether the decreased sucrose consumption is a consequence of the inflammatory state or if the latter develops in close association with the behavioral deficit. The increased expression of inflammatory markers, as well as the dysregulation of the fractalkine system, persists after 7 weeks of CMS suggesting that such changes may be intimately associated with the persistence of the anhedonic phenotype in stressed rats in line with previous reports (Hinwood et al., 2013; Raison et al., 2006). Of note, the increase of pro-inflammatory cytokines was paralleled by a reduction of TGF-β supporting its potential role in the psychoimmunology of depression (Dhabhar et al., 2009) and suggesting that the pathological phenotype observed after a long exposure to stress may be due to an unbalance between pro- and anti-inflammatory cytokines, as observed in clinical studies (de Bodinat et al., 2010). Interestingly, we found that drugs characterized by different mechanisms of action were able to normalize the decrease of sucrose intake and ameliorate the neuroinflammatory sig-nature observed in CMS rats. Indeed, an overall dampening of stress-induced neuroinflammation was observed following chronic treatment with the tricyclic antidepressant imipramine, with the novel antidepressant agomelatine that acts as MT1/MT2 melatonergic agonist and $5HT_{2C}$ antagonist (Janssen et al., 2010), as well as with the multireceptor antipsychotic drug lurasidone, which has high affinity for dopamine D2 receptors as well as for 5-HT_{1A}, 5-HT_{2A} and 5-HT_7 serotonin receptors (Tarazi and Riva, 2013). These results suggest that the ability of these drugs to modulate CMS-induced inflammatory changes appears to be independent from their primary effect at synaptic level, but may be due to shared long-term adaptive mechanisms induced by

their repeated administration. The role of inflammatory mediators as target of psychotropic drugs has been reported in in vitro and in in vivo studies (Brunello et al., 2006) and beneficial effects have been demonstrated with the combined use of anti-inflammatory and antidepressant drugs in animal models of depression (Mutlu et al., 2012). Moreover, in line with our results, Mutlu and colleagues demonstrated that chronic administration of agomelatine normalized the enhanced levels of IL-6 observed in the plasma of chronically stressed rats (Mutlu et al., 2013).The anti-depressant activity of lurasidone in the CMS paradigm is in agreement with data obtained using the forced swim test, an effect that appears to rely on its ability to block 5-HT7receptors (Cates et al., 2013). Moreover, we have recently reported that the ability of lurasidone to normalize the anhedonic-like phenotype induced by CMS may be also due to the modulation of synaptic and neuroplastic mechanisms (Luoni et al., 2014). It has to be noted that the main target of our pharmacological treatment appears to be IL-1β. In fact, stress-induced IL-1 up-regulation was completely normalized by all the drugs examined, differently from what observed for IL-6, whose changes were ameliorated only in part by imipramine and agomelatine. Given the apparent 'resistance' of IL-6 to the pharmacological treatment, it may be inferred that the elevation of its levels contributes to residual symptoms that may impair or limit clinical remission of depression. Several mechanisms may underline the overall anti-inflammatory properties of the drugs used. Among these, one intriguing possibility is a role for the kynurenine pathway (Chourbaji et al., 2006), which represents an important link between inflammation and depression (Wu and Lin, 2008). We have previously demonstrated that chronic agomelatine treatment is able to modulate the expression of two of the major enzymes involved in this pathway, namelykynurenine-3-monooxygenase (KMO) and kynurenine amino-transferase (KAT)-II, (Schwarcz et al., 2012) that, by acting on kynurenine, may switch the pathway toward neurotoxic or neuroprotective arms respectively (Chourbaji et al., 2006). In line with these data, preliminary results point to an unbalance between these two enzymes in response to stress, which can be regulated by chronic treatment with antidepressant drugs (Molteni et al., unpublished).

4.2 Effect of the antidepressant agomelatine on the IL-6 pathway in rats exposed to chronic mild stress: role of suppressor of cytokine signaling 3 (SOCS3)

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Manuscript in preparation

4.2.1 Introduction

In the context of affective disorders, the role of neuroinflammation is gaining increasing importance (Haroon et al., 2012; Wohleb et al., 2016), as a matter of fact, different meta-analyses have shown that pro-inflammatory cytokines are strictly associated with the insurgence of psychopathologies such as major depressive disorder (MDD) (Dowlati et al., 2010; Howren et al., 2009) In addition, it appears that an altered inflammatory response in the patients may play a pivotal role not only in the severity of the pathology, but also in the positive outcome of pharmacological therapy. Currently, standard therapies fail to reach the complete remission of the pathology in a large number of patients, thus suggesting an urgent need of new therapeutic targets and a better understanding of the molecular basis of MDD (Sukoff Rizzo et al., 2012)

Interleukin (IL)-6 is a pleiotropic cytokine which, depending on the cellular context, may have pro or anti-inflammatory properties after rapid induction or following homeostatic regulation (Hunter and Jones, 2015). In the context of psychiatric disorders, this cytokine, together with tumor necrosis factor (TNF)-α, resulted to have the most robust association with MDD, with a peculiar contribution to treatment-resistant depression (Maes, 1994; Maes et al., 2014). Among the signaling pathways activated by IL-6, one of the most important is mediated by the JAK/STAT proteins. Janus Kinase (JAK) 1 is a kinase non-covalently associated with the cytokine receptor responsible of the first phosphorylation processes of the cascade, due to the lack of intrinsic kinase activity of the receptor (Garbers et al., 2015). The signal transducer and activator of transcription (STAT) 3 is the downstream target of JAK1 that, upon activation, translocates into the nucleus to promote the transcription of several genes involved in both positive and detrimental effects of IL-6. The peculiarity of this system is the intrinsic feedback inhibition mechanism led by the suppressor of cytokine signaling (SOCS) 3. This protein is a member of the SOCS family, which is constituted by eight members; among them SOCS1 and SOCS3 are the unique proteins to possess a kinase inhibitory region (KIR) domain, which is able to inhibit the activity of the target receptor (Baker et al., 2009; Qin et al., 2008). In particular, it has been demonstrated that SOCS3 is able to block the pathway of the IL-6 family cytokines, showing a particular affinity for IL-6 signaling (Babon et al., 2014). The inhibitory action of SOCS3 is exerted through diverse mechanisms: firstly, with the inhibition of STAT3 activation in the cytosol and secondly, through the blockage of JAK1, a protein that is

fundamental for the initiation of the downstream signaling (Babon et al., 2014; White and Nicola, 2013).

In our study, we used the chronic mild stress procedure (CMS), a well-known model of depression, extensively used to investigate stress-related molecular alterations (Hill et al., 2012; Pochwat et al., 2014). More in details, we exposed adult male rats to seven weeks of CMS paralleled with the pharmacological treatment with agomelatine for the last five weeks. Considering the ability of this antidepressant to modulate the expression of several mediators of inflammation (Molteni et al., 2013; Rossetti et al., 2016), our aim was to investigate the potential mechanisms underlying this activity on the IL-6 pathway in the rat prefrontal cortex, a brain region particularly involved in stress response and in the etiopathology of MDD, with a particular attention to SOCS3 and the potentiality of the IL-6 feedback inhibition.

4.2.2 Results

4.2.2.1 Agomelatine modulates IL-6 increase in the prefrontal cortex of rats exposed to CMS.

In order to investigate the impact of seven weeks of CMS on the expression of IL-6 in the rat brain, we performed the analyses of gene and protein expression in the dorsal, ventral hippocampus and in the prefrontal cortex.

Despite the similar effects exerted by chronic stress exposure on the gene expression of IL-6 in the ventral (+43% vs. No Stress/Vehicle, P<0.01; Table3) and dorsal (+38% vs. No Stress/Vehicle, P<0.05; Tabl3) hippocampus, we found a significant effect of the drug administration ($F_{1,32}$= 7.892, P<0.01) and an interaction between stress and the pharmacological treatment ($F_{1,32}$= 9.868, P<0.01) only in the prefrontal cortex. More in detail, while stress led to an increase of 51% when compared to control animals (P<0.01; Fig.9A), agomelatine had a normalizing effect only in this brain area. Indeed, in stressed animals the increase of IL-6 mRNA was normalized by the drug (-67% vs. Stress/Vehicle, P<0.001; Fig.9A).

To deepen our analyses, we investigated the modulation of IL-6 protein levels in the prefrontal cortex. As shown in figure 1B -although not significant- we observed a trend toward increase in stress animals (+38% vs. No Stress/Vehicle) and an expression profile comparable to control rats in stress animals treated with agomelatine, with a potential normalizing effect of the drug (-47% vs. Stress/Vehicle).

In addition, we analyzed the activation of JAK1, an accessory protein that mediates the cascade if IL6 interacting with its membrane receptor. The analysis of JAK1 phosphorylation (at tyrosine 1022 and 1023) showed that the main effect was due to a significant interaction stress*agomelatine ($F_{1,32}$ =15.135, P<0.001; Fig.9C), while the pharmacological treatment did not reach the statistical significance ($F_{1,32}$= 4.094, P=0.053 Fig.9C). More in detail, we observed an increase of JAK1 activation in the stress group when compared to control animals (+34% P<0.001 vs. Non Stress; Fig.9C). On the other hand, the pharmacological treatment was able to normalize the alterations due to stress (-35% P<0.001 vs. Stress; Fig.9C), with a profile similar to IL-6 expression.

	CTRL	Agomelatine	Stress	Agomelatine/Stress
Ventral hippocampus	100±9	128±7*	143±15**	122±9
Dorsal hippocampus	100±6	119±14	138±11*	131±6

Table3. Gene expression analysis of IL-6 in the ventral and dorsal hippocampus. The mRNA levels of *Il-6* were analyzed in rats exposed to CMS and/or to the treatment with agomelatine for 5 weeks. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle, set at 100%), are the mean ± SEM of independent determinations. *P<0.05; **P<0.01 vs. No Stress/Vehicle (two-way ANOVA with PLSD).

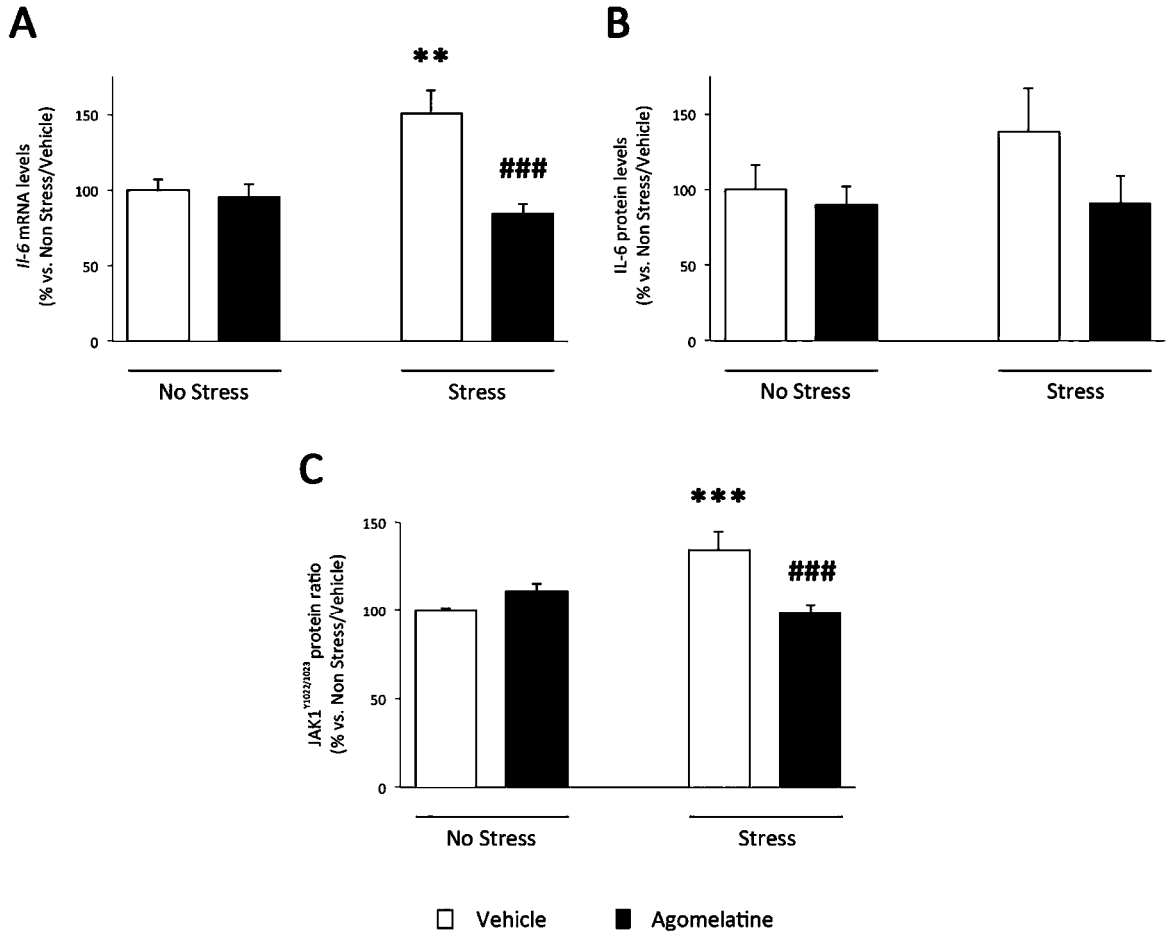

Figure 9. Gene and protein expression analyses of IL-6 and activation of JAK1. The gene (A), protein (B) expression of IL-6 and the analysis of JAK1 phosphorylation on tyrosine 1022/1023 (C) were conducted in the prefrontal cortex of rats exposed to CMS and/or to the treatment with agomelatine for 5 weeks. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle, set at 100%), are the mean ± SEM independent determinations. **P<0.01, ***P<0.001 vs. No Stress/Vehicle; ###P<0.001 vs. Stress/Vehicle (two-way ANOVA with PLSD).

4.2.2.2 Impact of CMS and drug treatment on the IL-6 signaling pathway in the prefrontal cortex.

To better characterize the modulation of IL-6 pathway, we analyzed the expression of different molecules involved in its signaling. More specifically, we investigated in the cytosolic compartment the modulation of STAT3-activating phosphorylation at tyrosine 705 ($pSTAT3^{Y705}$) and the protein expression of SOCS3. Moreover, to evaluate the activity of the transcription factor, we analyzed $pSTAT3^{Y705}$ levels in the nucleus and the mRNA levels of *Socs3*.

The up-regulation of IL-6 after stress exposure was paralleled by the activation of STAT3 in the cytosol as indicated by the significant up-regulation of $pSTAT3^{Y705}$ in stressed animals (+37% $P<0.05$ vs. No Stress/Vehicle; Fig.10A). Interestingly, this increase was normalized by the pharmacological treatment (-41% $P<0.01$ vs. Stress/Vehicle; Fig.10A) that had no effect on control rats, as demonstrated by the significant stress*agomelatine interaction ($F1,36=6.764$, $P<0.05$). On the contrary, no changes were found on the total form of STAT3 protein (Fig.10B, 10E).

In line with the increase of STAT3 activation in the cytosolic compartment, we found a similar expression profile in the nucleus. Animals exposed to CMS showed an increase of $pSTAT3^{T705}$ (+75% $P<0.01$ vs. No Stress/Vehicle; Fig.10D), an alteration normalized by the pharmacological treatment (-59% $P<0.05$ vs. Stress/Vehicle; Fig.10D). However, agomelatine *per se*, was able to induce $pSTAT3^{Y705}$ levels in the nucleus, with a marked increase of the activated protein (+124% $P<0.001$ vs. No Stress/Vehicle; Fig.10D). Similarly to what previously observed,in this cellular compartment we did not found any change in the total form of STAT3.

The expression level of *Socs3* mRNA, whose transcription is promoted by the active form of STAT3, was modulated by stress ($F_{1,33}= 6.216$, $P<0.05$) and also by pharmacological treatment ($F_{1,33}= 11.077$, $P<0.01$). Indeed, as shown in figure 10C, stressed animals showed a 30% increase of *Socs3* ($P<0.05$, vs. No Stress/Vehicle) and agomelatine up-regulated *Socs3* in both control (+49% $P<0.01$ vs. Non Stress; Fig.10C) and in stressed animals (+69% $P<0.001$ vs. No Stress/Vehicle; Fig.10C). In line with these results,SOCS3 protein levels were increased in the cytosol in all the experimental groups (Fig. 10F). Indeed, SOCS3 was up-regulated by stress exposure (+35% $P<0.05$ vs. No Stress/Vehicle; Fig. 10F), and by agomelatine in both Non Stress and Stress conditions (+61% $P<0.001$ and +48% $P<0.01$ vs. No Stress/Vehicle respectively).

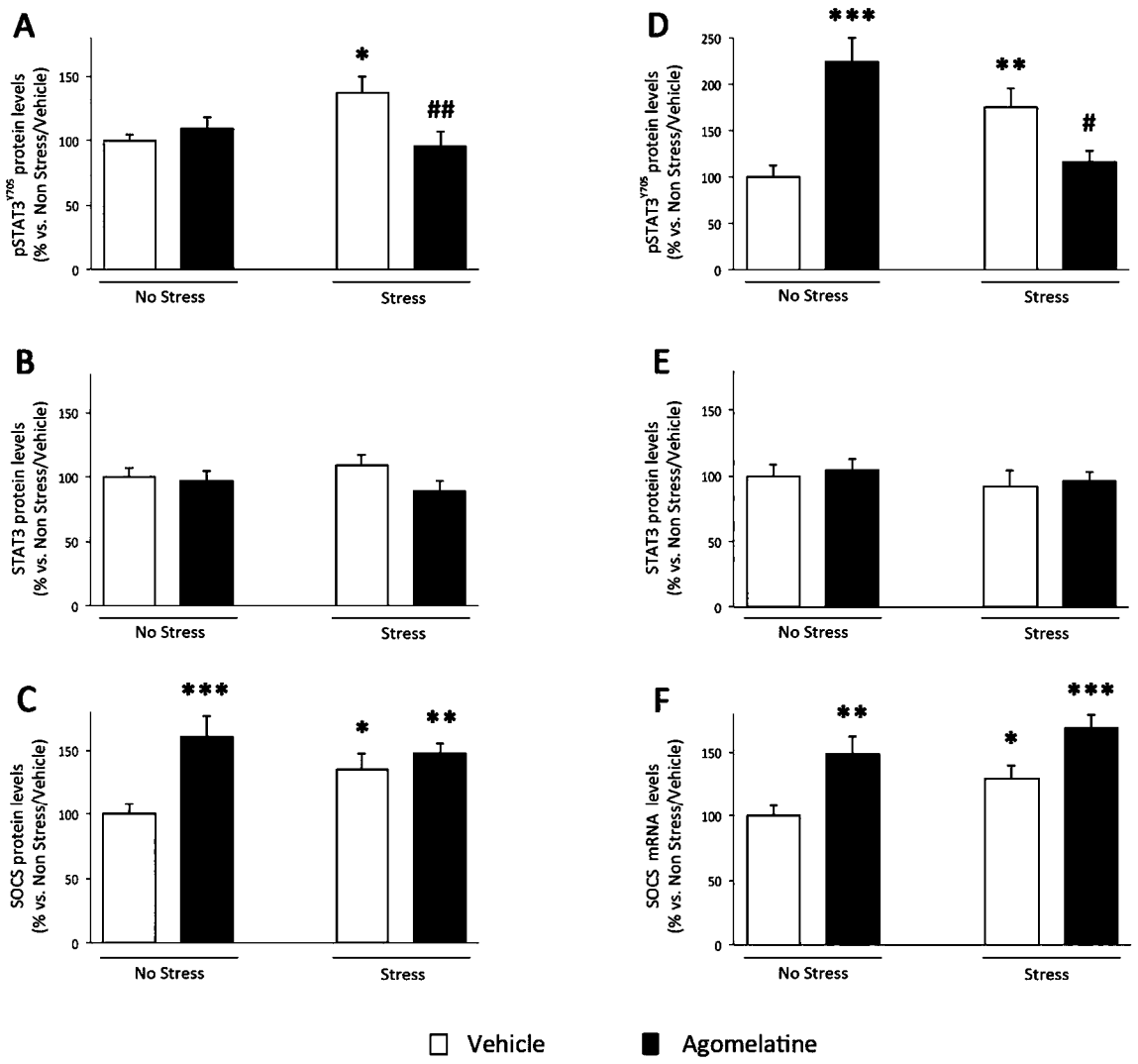

Figure 10. Analysis of the activation of IL-6 intracellular signaling. The protein expression of activated (phospho tyrosine 705) and total STAT3 were performed in the cytosolic (A, B) and in the nuclear compartments (D, E); SOCS3 levels were analyzed as protein (C) and as transcript (F). All the analyses were conducted in the prefrontal cortex of rats exposed to CMS and/or to the treatment with agomelatine for 5 weeks. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle, set at 100%), are the mean ± SEM of independent determinations. *P<0.05; **P<0.01; ***P<0.001 vs. No Stress/Vehicle; #P<0.05, ##P<0.01 vs. Stress/Vehicle (two-way ANOVA with PLSD).

4.2.2.3 Effect of CMS and agomelatine on the protein expression of pSTAT^{S727} and MAP kinases in the nuclear compartment.

Considering the diverse modulation of pSTAT3^{Y705} exerted by the pharmacological treatment *per se* in the cytosol and in the nucleus, we investigated if the regulation of the transcription factor at serine 727 (pSTAT^{S727}) or the activation of pp38$^{T180/Y182}$, pERK1$^{T202/Y204}$ and pERK2$^{T185/Y187}$ may be involved.

At first -as shown in figure 11- we analyzed the protein expression of pSTAT^{S727}. The phosphorylation at this site, however, was not altered by either the CMS, or the administration of agomelatine (Fig. 11A). Conversely, our experimental paradigm affected MAP kinases. Specifically, the levels of pp38$^{T180/Y182}$ (Fig. 11B) were significantly increased by stress exposure (+39% $P<0.05$ vs. No Stress/Vehicle) and by agomelatine, which up-regulated the activation of the enzyme in both non stressed (+46% $P<0.01$ vs. No Stress/Vehicle; Fig. 11B) and stressed (+39% $P<0.01$ vs. No Stress/Vehicle) animals. A different profile was observed for ERK1 (Fig. 11C), whose activation was increased only in stress animals (+58% $P<0.05$ vs. No Stress/Vehicle; Fig.11C), an effect normalized by the pharmacological treatment (-91% $P<0.01$ vs. Stress/Vehicle). Similarly, stress induced pERK2$^{T185/Y187}$ protein levels (+77% $P<0.001$ vs. No Stress/Vehicle; Fig. 11D), an effect normalized by agomelatine (-47% $P<0.01$ vs. Stress/Vehicle), which was also able to significantly increased the activation of the enzyme when administered to control rats (+48% $P<0.01$ vs. No Stress/Vehicle).

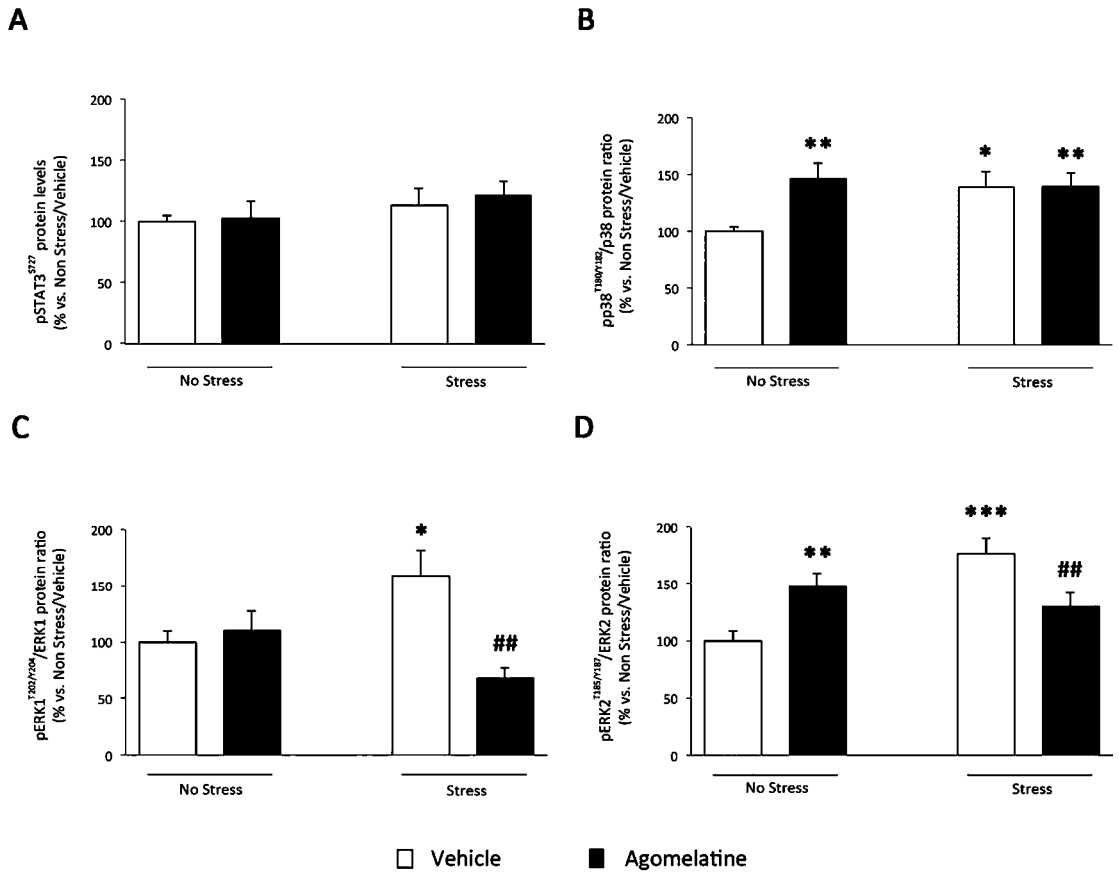

Figure 11. Protein levels of pSTAT at serine 727 and MAP kinases in the nuclear fraction. Panel A shows the protein levels of pSTAT3 (phospho serine 727) whereas the activation of nuclear MAP kinases p38, ERK1 and ERK2 are presented respectively in panels B, C and D. All the analyses were conducted in the prefrontal cortex of rats exposed to CMS and/or to the treatment with agomelatine for 5 weeks. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle, set at 100%), are the mean ± SEM of independent determinations and are presented as a ratio between phosphorylated and total form of the protein. *P<0.05; **P<0.01; ***P<0.001 vs. No Stress/Vehicle; ##P<0.01 vs. Stress/Vehicle (two-way ANOVA with PLSD).

4.2.2.4 Analysis of STAT3 transcriptional activity: gene expression of *Casp1*, *Casp3* and *Bcl-xl*

Lastly, we investigated the mRNA levels of three genes whose transcription is induced by STAT3 activity within the nucleus, namely caspase 1 (*Casp1*), caspase 3 (*Casp3*) and B-cell lymphoma-extra large (*Bcl-xl*).

As shown in figure 12, we did not observe statistically significant alterations of *Casp1* gene expression (Fig. 12A). On the contrary, we found a significant effect of stress exposure ($F_{1,35}$ = 5.061; P<0.05) on *Casp3* (Fig. 12B): its mRNA levels were slightly but significantly reduced in stressed rats (-16% P<0.01 vs. No Stress/vehicle). Similarly, agomelatine administration led to

a reduction of *Casp3* levels in both non stressed (-12% P<0.05 vs. No Stress/Vehicle; Fig.12B) and stressed (-10% P<0.05 vs. No Stress/Vehicle; Fig.12B) animals.

Regarding *Bcl-x* (Fig. 12C), we found a main effect of the pharmacological treatment ($F_{1,37}$= 16.929, P<0.001) that increased its gene expression in non-stressed animals (+17% P<0.05 vs. No Stress/Vehicle) and in animals subjected to CMS (+45% P<0.01 vs. Stress/Vehicle).

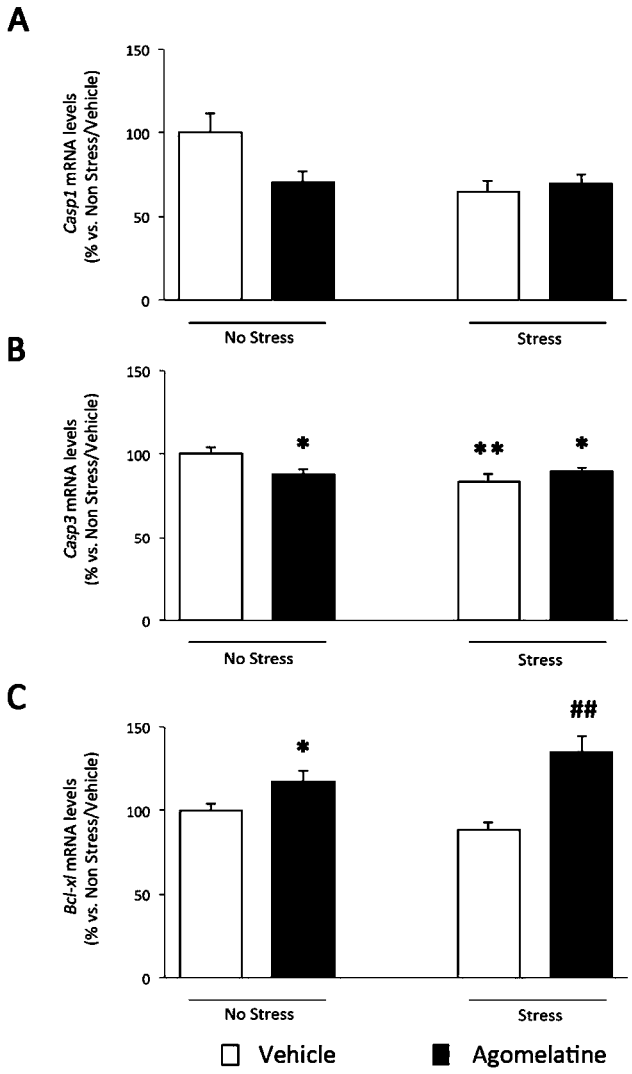

Figure 12. Gene expression analysis of STAT3 transcriptional activity. The mRNA levels of *Casp1* (A), *Casp3* (B), *and Bcl-xl* (C), were analyzed in rats exposed to CMS and/or to the treatment with agomelatine for 5 weeks. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle, set at 100%), are the mean ± SEM of independent determinations. *P< 005; **P<0.01vs. No Stress/Vehicle; ##P<0.01 vs. Stress/Vehicle (two-way ANOVA with PLSD).

4.2.3 Discussion

Our data showed that chronic exposure to stress increases the expression of IL-6 in the prefrontal cortex and in the hippocampus, two brain areas strictly interconnected and fundamental for the control of stress response and involved in the pathophysiology of depression (Radley et al., 2015). These results are in line with the reported association between IL-6 and the development of depressive-like behaviors in animal models (Sukoff Rizzo 2012) and with alterations observed in major depressive disorder (Money et al., 2016). Moreover, a meta-analysis showed that -among others pro-inflammatory cytokines- IL-6 and TNF-α have a strong association with the pathologic phenotype (Dowlati et al., 2010).

Interestingly, our results demonstrated that agomelatine, a peculiar antidepressant with melatonergic and serotonergic activity (Guardiola-Lemaitre et al., 2014), exerted a specific effect on IL-6 expression only in the prefrontal cortex. In this context, we already demonstrated that agomelatine possesses anti-inflammatory properties when administered to rats exposed to lipopolysaccharide (Molteni et al., 2013) or to chronic stress (Molteni et al., 2013; Rossetti et al., 2016), however, the underpinning molecular mechanisms are still elusive. Thus, we deepened our analysis on agomelatine activity by measuring the protein expression of key molecules involved in IL-6 pathway in the prefrontal cortex of rats. IL-6 is a pleiotropic cytokine with pro- or anti-inflammatory properties whose action is context-dependent. In the brain, this cytokine may have neurotrophic effects (Molteni et al., 2013; Rossetti et al., 2016), but also sustain chronic inflammation (Hunter and Jones, 2015). Interestingly IL-6 has an intrinsic feedback inhibitory mechanism, led by the protein SOCS3 (Babon et al., 2014).

Firstly, here we demonstrated that stress was able to activate IL-6 pathway in all the cellular compartments analyzed: starting from the receptor-bound protein JAK1, through the cytoplasmic and nuclear activation of STAT3, to the gene and protein expression of SOCS3. The increase of the inhibitory protein in the stress condition, however, did not seem to limit either the activity of JAK1 (via the inhibition of its phosphorylation), or the activation of STAT3 at tyr705, the latter fundamental for STAT3 nuclear translocation (Qi and Yang, 2014). The lack of the SOCS3-mediated feedback inhibition may be due to a sensitization of the system caused by the over-activation of the pathway that -in the stressed animals- is not dampened by the physiological activity of SOCS3. In this context, another contribution to SOCS3 induction may come from the activity of MAP kinases such as ERK1/2 and p38 that have been reported to be

involved in Socs3 expression also in the absence of STAT3 (Qin et al., 2007). In this regard, our data showed that chronic stress seems to over-activate nuclear p38, ERK1 and ERK2, a modulation that may be related to SOCS3 overexpression. These enzymes may be responsible for the activation of other transcription factors whose consensus regions lay on the promoter of *Socs3:* in this sense, it has been demonstrated -although in human non neuronal cell lines- that the intracellular increase of cAMP is capable of induce *Socs3* transcription independently from STAT3 binding (Wiejak et al., 2012).

Interestingly, the chronic treatment with agomelatine was able to normalize the alterations observed at intracellular level, without affecting the induction of SOCS3. Our hypothesis is that the effective inhibition of the pathway might occur through the inhibition of JAK1 and $pSTAT3^{Y705}$ due to the increased SOCS3 expression. The modulation of the antidepressant on these molecules seemed to start from the nucleus, through the induction of *Socs3* gene expression.

To the best of our knowledge there are only few studies on the effects of antidepressants on SOCS3 activity. In a work of 2016 Al-Samhari and colleagues showed that pharmacological treatment with fluoxetine or N-acetylcysteine after forced swim was able to normalize the activation of STAT3 and to increase the levels of Socs3 gene expression, with no changes in SOCS3 protein levels (Al-Samhari et al., 2016). Similarly, fluoxetine has been demonstrated to reduce SOCS3 protein levels in the hypothalamus of animals subjected to chronic stress (Pan et al., 2013). Lastly, the administration of minocycline -a well-studied microglia inhibitor- in bulbectomized rats led to an increase of *Socs3* levels, while the treatment per se caused a decrease in its gene expression (Burke et al., 2014). In our study we provide new insight in the action of antidepressants on this system, in particular, adding information on the activity of the drug itself. Nevertheless, the mechanism underlying *Socs3* expression has to be fully elucidated, especially considering that nuclear $pSTAT^{Y705}$ levels are normalized by the drug treatment. It is interesting to note that agomelatine normalizes the induction of ERK1/2 in the nucleus, without affect p38 activation, thus suggesting a potential contribution of this kinase in fostering the effect of the drug on SOCS3 inhibitory effect. Other analyses are needed to fully understand the role of MAP kinases in the stress-induced modulation of IL-6 signaling, especially keeping in mind that the control of SOCS3 expression by these enzymes has been only partially resolved (Ehlting et al., 2015).

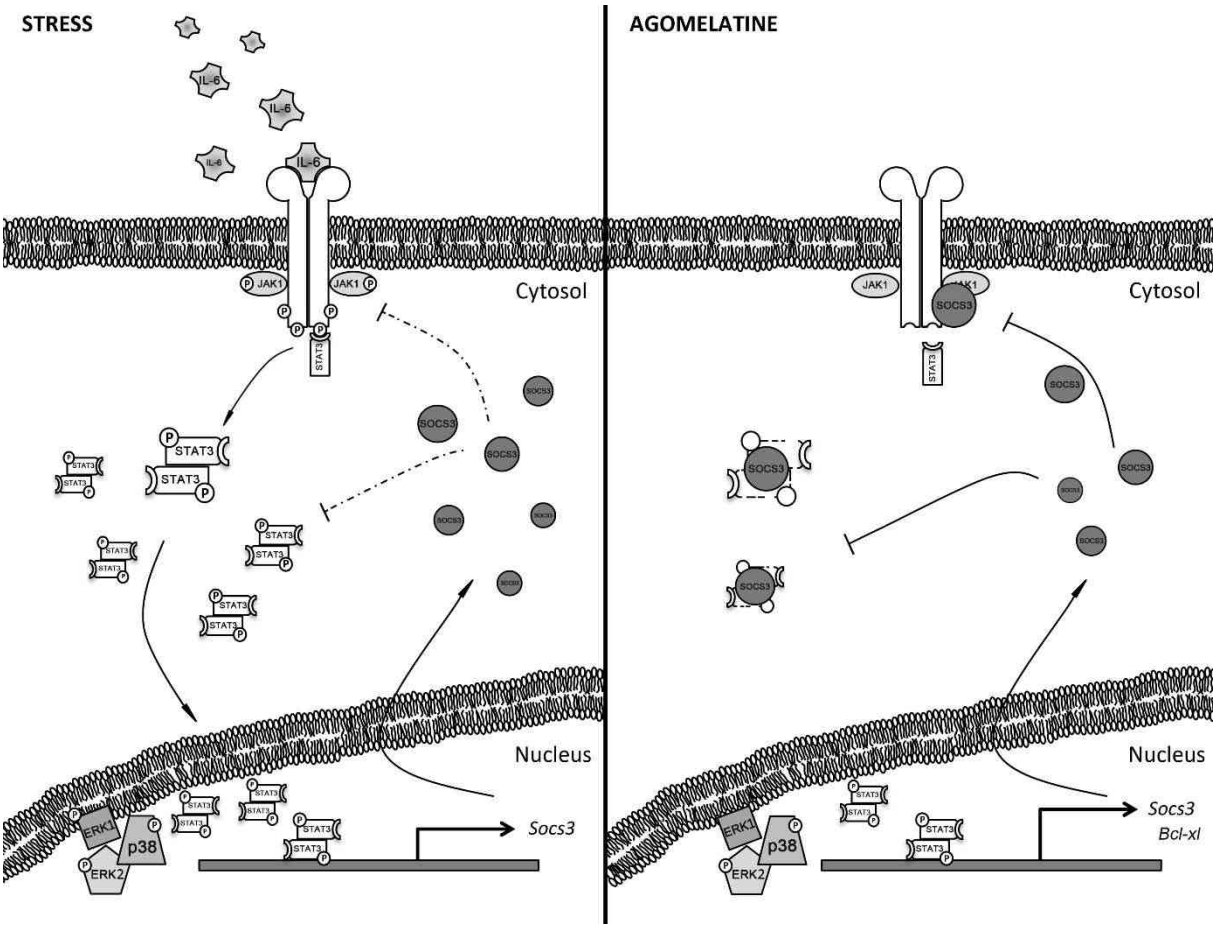

Figure 13. Effect of stress and pharmacological treatment on IL-6 pathway The exposure to chronic mild stress is able to activate the IL-6 pathway in all its parts, with the phosphorylation of JAK1, STAT3 and the promotion of SOCS3 expression. It appears, however that the feedback inhibition activity of SOCS3 is not able to block the effect of stress exposure on the pathway (on the left). On the contrary, the administration of agomelatine is able to induce SOCS3 expression, potentially through the intervention of MAP kinases at nuclear level. In this situation, the antidepressant seems to strengthen the inhibition on this signaling led by SOCS3; moreover, the increased levels of Bcl-xl mRNA in animals that received agomelatine, supports the idea that the pharmacological treatment may have a neuroprotective effect in the prefrontal cortex.

Abbreviations: IL-6: Inteleukin-6; JAK1: Janus Kinase-1; STAT3: signal transducer and activator of transuction3; SOCS3: suppressor of cytokine signaling 3; ERK1/2: extracellular signal-regulated kinase; Bcl-xl: B-cell lymphoma-extra large.

Considering the activity exerted by agomelatine on the activation of the pathway, we may infer that the drug is able to potentiate the feedback inhibition via the up-regulation of SOCS3 gene and protein expression. This idea is strengthened by the analysis of agomelatine *per se*

activity on IL-6 signaling. The chronic administration of the antidepressant induced SOCS3 levels with a gene and protein expression profile similar to the other experimental groups. It remains to be understood how agomelatine treatment is capable of induce STAT3 phosphorylation within the nucleus. We analyzed the phosphorylation site of STAT3 at serine 727 that has been demonstrated to be a regulatory site of activated STAT3. The contribution of this second phosphorylation site is not fully understood, although some groups refer a potentiating effect on STAT3 transcriptional activity, others claim an inhibitory effect on the transcription factor (Breit et al., 2015; Wakahara et al., 2012). In our experimental context, however, this post-translational modification is not modulated in any of the experimental groups, suggesting the intervention of other molecules in the control of $pSTAT3^{Y705}$ levels. At this level we cannot exclude the involvement of regulatory molecules such as the protein inhibitor of activated STAT3 (PIAS3) whose fine modulatory activity has been reported in different transcription factors involved in immune response (Shuai and Liu, 2005; Yagil et al., 2010).

Lastly, to clarify the role of agomelatine on STAT3 transcriptional activity, we analyzed the expression of genes controlled by this transcription factor, namely *Casp1*, *Casp3* and *Bcl-xl*. Interestingly we found that, while the two caspases were not particularly modulated by our experimental paradigm, the antiapoptotic gene *Bcl-xl* showed an increase when the antidepressant was administered. *Bcl-xl* is known to have a pronounced neurotrophic effect and capable of supporting neuronal survival (*Jonas et al, 2014*) and its modulation exerted by the agomelatine, per se and in stress condition, is in line with the reported antiapoptotic properties of different antidepressants (Engel et al., 2013; Kosten et al., 2008; Kubera et al., 2011). Despite the similar effect exerted by agomelatine on STAT3 activation, the modulation of *Bcl-xl*, strengthen the idea that agomelatine has a positive protective activity in the prefrontal cortex of rats exposed or not to chronic stress. This neuroprotective role of the antidepressant may be supported by the activity on SOCS3 in the regulation of stress-induced activation of IL-6 pathway.

Although further studies are demanded to better understand the exact mechanism of action of the pharmacological treatment with agomelatine, the modulation of SOCS3 appears promising in the context of immune modulation exerted by antidepressant drugs, in particular on the fine-tuning of IL-6 signaling.

4.3 Lipopolysaccharide does not affect sucrose intake in stress-resilient rats: potential contribution of microglia.

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Unpublished data

4.3.1 Introduction

Stressful events during life may expose a subject to the development or the exacerbation of major depression, however even if this disease occurs in a significant percentage of stress-exposed subjects, most of them avoid such psychopathology through active coping mechanisms. With these premises, stress resilience has been defined as the process of positive adjustments against stressful events (Walker et al., 2013). It is known that stress exposure strongly influences inflammatory events in the periphery and in the central nervous system (CNS), with an impact on behavioral alterations. The impact of stress on neuroinflammation is mediated by different mechanisms and physiological systems. Peripheral glucocorticoids, pro-inflammatory cytokines and infiltrating immune cells can reach the brain and alter the neuroimmune function, thus leading to the dysregulated production of pro-inflammatory mediators (Wohleb et al., 2016). Microglia -the tissue-resident macrophages that about 10% of the cell population within the brain- play fundamental roles in the control of the homeostasis of the CNS. These cells are not only involved in the regulation of brain inflammatory status, but they also regulate brain development, shaping of brain connections, behavioral and mood under physiological conditions (Tremblay et al., 2011; Wake et al., 2013). Microglia are constantly surveilling the environment and they are extremely reactive to infectious and non infectious inflammatory responses and its dysregulation can lead to the development of neurological and psychiatric disorders (Cronk and Kipnis, 2013; Yirmiya et al., 2015). Specifically, different evidence support the idea of the activation of microglia after stress exposure and its aberrant activation has been associated with long-lasting changes in terms of behavior, cognition and mood (Yirmiya et al., 2015).

On these bases, the purpose of our study was to deepen our knowledge on the molecular mechanisms underpinning stress resilience, with a specific focus on neuroinflammation. We exposed adult male rats to two weeks of chronic mild stress, before being challenged with the bacterial wall component Lipopolysaccharide (LPS, i.p. 250 μg/kg) and sacrificed 24h or 6 days after the immune challenge. Behavioral alterations were monitored through the sucrose consumption test to evaluate the insurgence of anhedonic-like phenotype and to identify stress resilient rats. Moreover, we assessed sucrose intake six days after LPS administration, to evaluate at behavioral level the different susceptibility to an immune challenge of the Stress-Responsive and Stress-Resilient populations. Lastly, we performed molecular analyses

24 hours and six days after LPS administration, to evaluate the short-term impact and the long lasting effects of the immune challenge. More in detail we analyzed the gene expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), toll-like receptor 4 (TLR-4) and markers of microglia activation (CD11b, Iba1, CX3CR1 and its ligand CX3CL1, Arginase1) in the rat dorsal hippocampus, a brain area involved in neuroinflammation-related stress response and in the etiology of depression (Rossetti et al., 2016).

4.3.2. Results

4.3.2.1 Behavioral effects of CMS exposure and subsequent challenge with LPS

Sucrose consumption was evaluated at two time points: after two weeks of stress exposure and after the immune challenge with LPS. As shown in figure 18, at the baseline the animals consumed roughly 11g of 1% sucrose solution. After the first test, the exposure to the chronic mild stress procedure for two weeks was able to induce a strong decrease in sucrose intake, with a significant effect of the stress procedure ($F_{2,48}$= 19,678, *P*<0.001). This test led us to discriminate between a group of animals with decreased sucrose consumption (-9,1 g, *P*<0.001 vs. No Stress; Fig. 18) defined as Responsive and another population of animals that did not show any alteration in the behavior (-9,5g,*P*<0.001 vs. Reactive; Fig. 18). We named this second population as "Resilient".

Six days after LPS administration (Fig. 18B/18C), stress was still effective on reactive animals, indeed this group still consumed less sucrose when compared to Non Stress animals and/or to Resilient rats (stress effect in repeated measures: $F_{5,48}$= 9,313 P<0.001; P<0.001 Responsive vs. No Stress; P<0.001 Responsive vs. Resilient; Fig. 18B).

Moreover, among the groups that received LPS, we observed an interesting statistically significant difference between Non Stress and Resilient rats (P<0.05 vs. LPS; Fig. 18B), thus suggesting that the LPS treatment affects the behavior of control animals, but not the sucrose consumption of stress-resilient rats. More in detail, using the analysis of delta values (Δ sucrose intake at Day 14 - sucrose intake at Day21) between the sucrose consumption of the animals before and after LPS administration, Non Stress/LPS animals showed a significant decrease of sucrose intake when compared to saline-treated rats (-3,9g, P<0.01 vs. Non Stress; Fig. 18C).

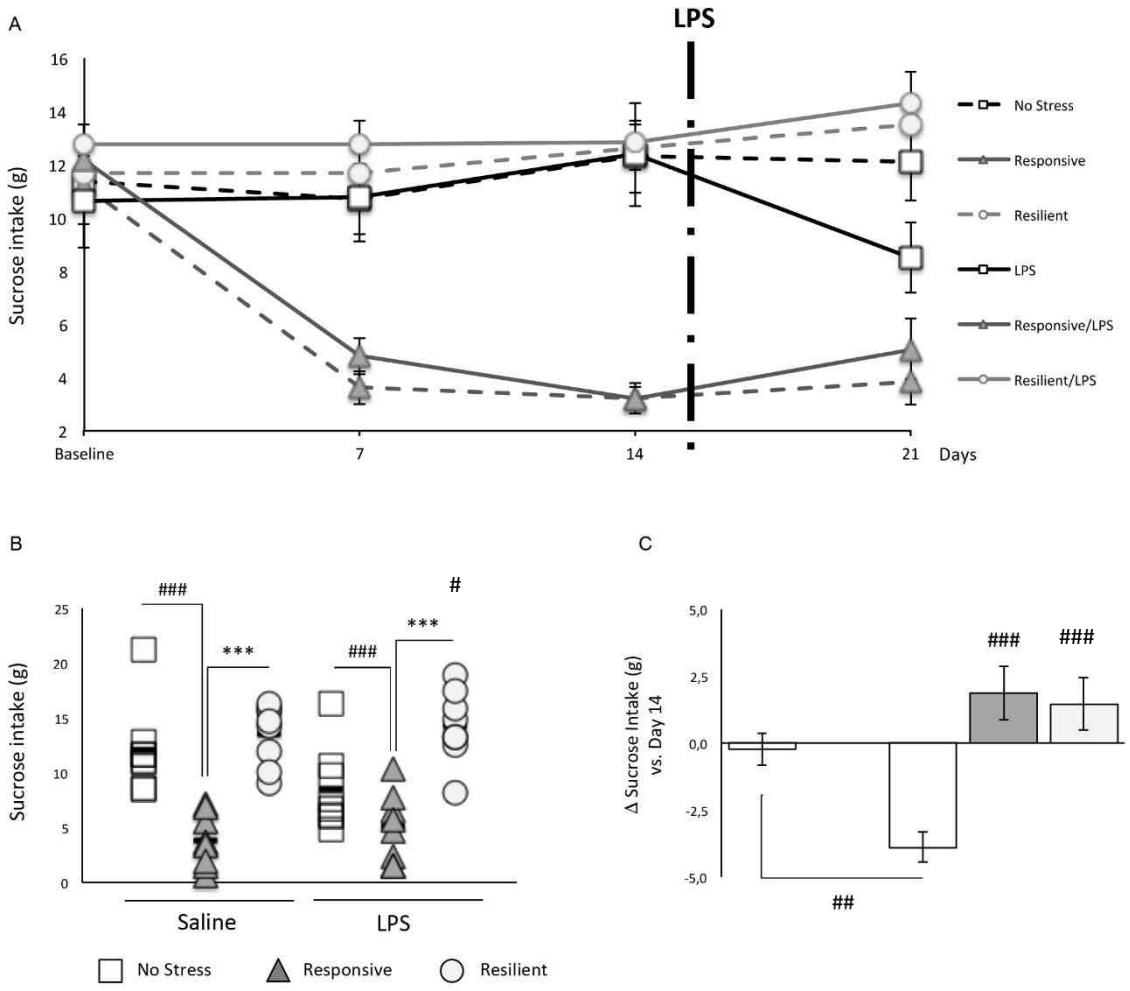

Figure 18. Behavioral analysis after the immune challenge with LPS. The behavioral analyses demonstrated that our CMS paradigm was effective after 1 week of stress exposure, leading to the identification of the Responsive and Resilient populations (A). The analysis at Day 21 (6 days post LPS) demonstrated that the immune challenge did not impair the already decreased sucrose intake in Responsive rats, however was detrimental in Non Stressed animals (B, C). Surprisingly, Resilient rats were able to actively cope against LPS administration, showing no decrease in sucrose intake. #P<0.05, ##P<0.01; ###P<0.001 vs. No Stress or No Stress/LPS; ***P<0.001 vs. Responsive or Responsive/LPS. Repeated measures ANOVA with PLSD test.

4.3.2.2 Gene expression analysis of neuroinflammatory markers after 24 hours from LPS administration in the dorsal hippocampus

In order to evaluate the molecular impact of the immune challenge on the diverse experimental groups, we analyzed the gene expression of different molecules related to neuroinflammation: the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α the marker of microglia activation CD11b and GFAP as an indicator of astrocytes activation.

The analysis of IL-1β gene expression showed a significant effect of LPS administration ($F_{1,40}$= 59,970 *P*<0.05) with an overall increase of the cytokine in all the experimental groups (+341%, *P*<0.001; +522%, *P*<0.001; +416% *P*<0.001 vs. respective controls; Fig. 19A). LPS has an overall effect also on IL-6 mRNA levels ($F_{1,45}$= 4,381, *P*<0.05) with a significant increase in Non Stress animals that received the toxin, when compared to the saline-treated counterpart (+28%, *P*<0.05 vs. No Stress; Fig. 19A) and to stress Responsive rats that received the toxin (-29%, *P*<0.05 vs. Reactive/LPS; Supplementary Fig.2). The pro-inflammatory cytokine TNF-α, on the contrary, showed an effect of stress exposure ($F_{2,45}$= 3,777, *P*<0.05; Fig. 19B) that was reflected in a decreased expression in Responsive/LPS and Resilient/LPS animals; the latter resulted significantly different when compared to No Stress group that received LPS (-49%, *P*<0.01 vs. LPS; Supplementary Fig. 3).

Similar to what we observed for the gene expression of IL-1β, the marker of microglia activation CD11b showed an increased transcription in all the experimental groups (+193%, *P*<0.001; +245%, *P*<0.001; +203% *P*<0.001 vs. respective controls; Fig. 20A), with the general effect of LPS administration ($F_{1,42}$=116,870 *P*<0,001). Lastly, GFAP expression was significantly affected by the immune challenge ($F_{1,46}$= 6,508 *P*<0.05) that resulted in an increase of mRNA levels only in non-stressed animals that received LPS (+48%, P<0.01 vs. No Stress; Fig 20B). The levels of GFAP in animals subjected to CMS were not changed after LPS, probably due to the increased levels of the astrocytic marker in both groups after two weeks of stress (+35, *P*<0.05; +36, *P*<0.05 vs. No Stress respectively; Supplementary Fig. 4).

Considering that 24 hours after LPS administration we observed the major effects on IL-1β and CD11b, we focused our attention on the pathway of TLR-4, and on microglia, the resident macrophagic population within the CNS.

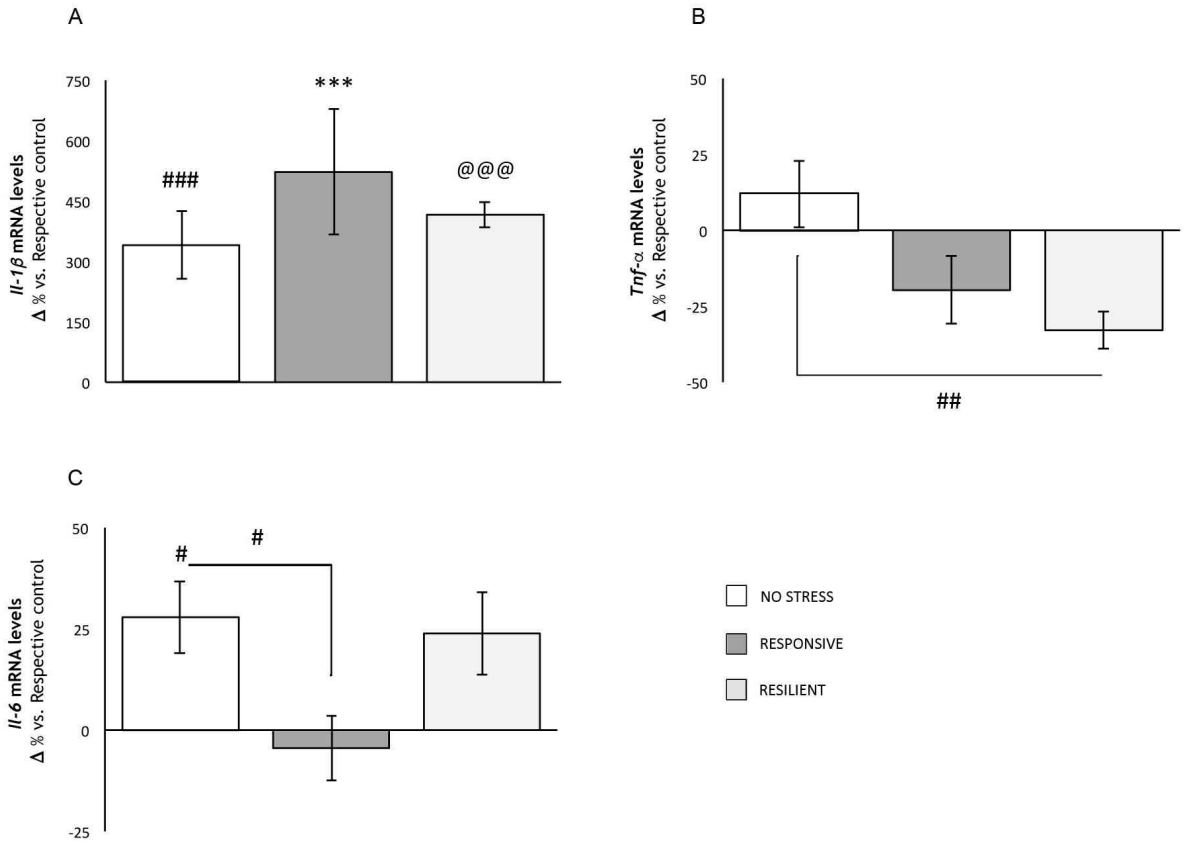

Figure 19. Gene expression analysis of pro-inflammatory cytokines 24 hours after the immune challenge. The mRNA levels of the pro-inflammatory cytokines IL-1β (A), TNF-α (B) and IL-6 (C) were measured in the dorsal hippocampus after 24 hours from LPS administration. The data are expressed as delta values ± SEM between the percentages of saline treated rats (set at 100%) and the LPS treated counterpart. #P<0.05, ##P<0.01, ###P<0.001 vs. Non Stress; ***P<0.001 vs. Responsive; @@@P<0.001 vs. Resilient. Two way ANOVA with Post Hoc LSD

4.3.2.3 Analysis of TLR-4 expression 6 days after LPS administration

TLR-4 gene expression was altered by LPS, as demonstrated by a significant effect of the toxin administration ($F_{1,44}$= 6,875, P<0.05). More in details (Fig. 21A), we found increased mRNA levels of the receptor after LPS in control (+27%, P<0.05 vs. No Stress) and in stress Responsive animals (+31% P<0.01 vs. Responsive) when compared to their saline-treated counterparts). Conversely, no change was found in Resilient animals treated with LPS, probably due to an upregulation of the receptor after stress exposure (+33% P<0.01 vs No Stress; +29% P<0.05 vs. Responsive; Supplementary Fig.5).

The protein level profile of TLR-4, however, was not in line with its gene expression. Indeed, while the total form of TLR-4 was not affected by either stress exposure nor LPS administration, the analysis of the glycosylated form showed a significant effect of LPS ($F_{1,42}$= 12,150 P<0.001). Specifically, we found an upregulation of the activated form of the receptor only in Responsive rats treated with LPS, when compared to the saline treated group (+133%, P<0.001 vs. Responsive; Fig. 21B) and to No Stress animals challenged with LPS (+53%, P<0.05, vs. LPS; Supplementary Fig. 6).

To confirm these results, we analyzed the gene expression of IL-1β, a downstream target of the receptor signaling. We observed that, 6 days after LPS exposure, the levels of this proinflammatory cytokine were upregulated only in Reactive animals exposed to LPS. As shown in figure 21 we found a general effect of stress exposure ($F_{2,41}$= 5,240 P<0.01), and significant differences between LPS-treated Reactive animals and saline treated rats (+50% P<0.01 vs. Reactive; Fig. 21C), LPS group (+73%, P<0.001 vs. LPS) and Resilient animals treated with the toxin (+66%, P<0.01 vs. Resilient/LPS).

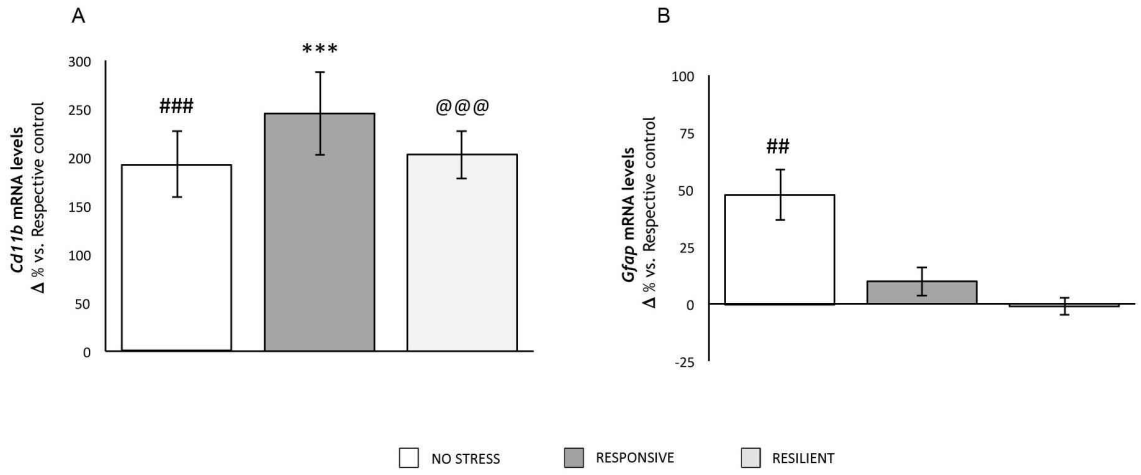

Figure 20. Gene expression analysis of CD11b and GFAP 24 hours after the immune challenge. The mRNA levels of the marker of microglia activation CD11b (A) and the astrocytic marker GFAP (B) were measured in the dorsal hippocampus after 24 hours from LPS administration. The data are expressed as delta values ± SEM between the percentages of saline treated rats (set at 100%) and the LPS treated counterpart. #P<0.05, ##P<0.01, ###P<0.001 vs. Non Stress; ***P<0.001 vs. Responsive; @@@P<0.001 vs. Resilient. Two way ANOVA with Post Hoc LSD

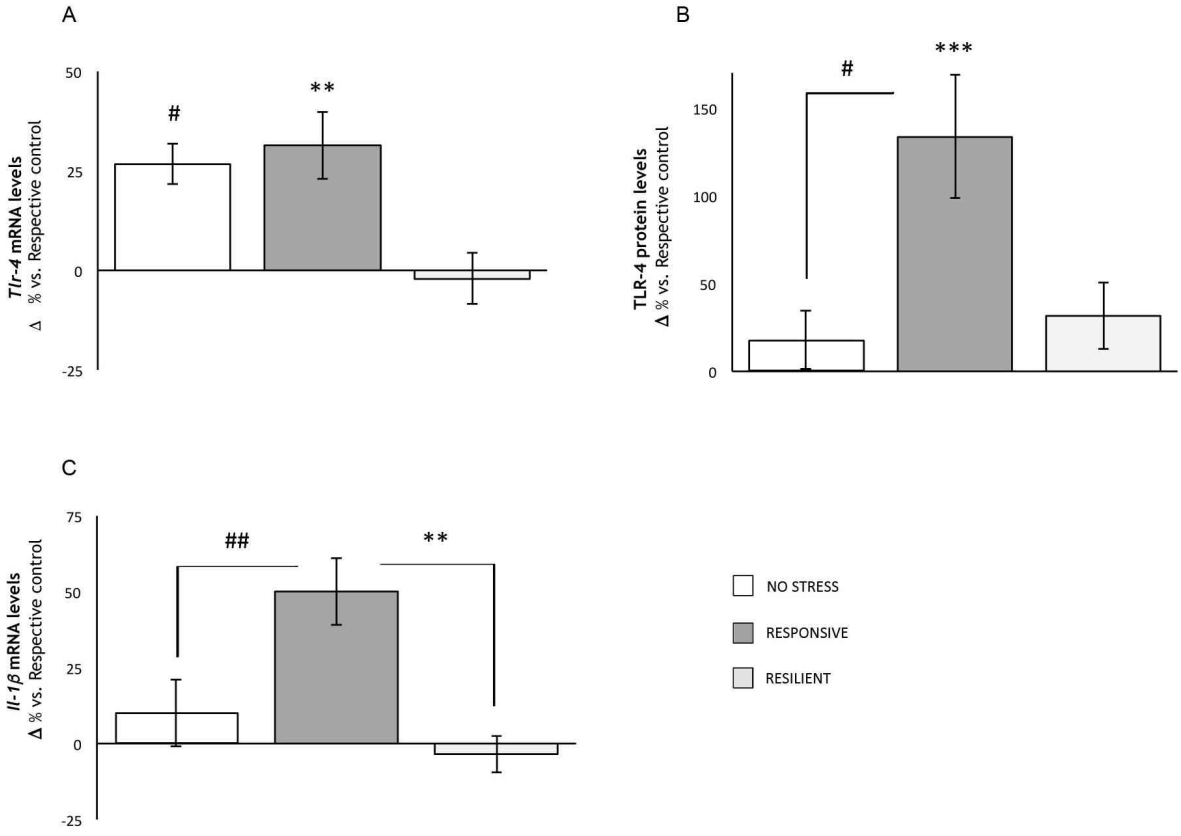

Figure 21. Analyses of the long-term effects of the immune challenge on TLR-4 expression. The mRNA (A) and protein levels (B) of the Toll-like receptor 4 and the mRNA levels of IL-1β (C) were measured in the dorsal hippocampus after 6 days from LPS administration. The data are expressed as delta values ± SEM between the percentages of saline treated rats (set at 100%) and the LPS treated counterpart. #P<0.05, ##P<0.01 vs. Non Stress; **P<0.01, ***P<0.001 vs. Responsive. Two way ANOVA with Post Hoc LSD

4.3.2.4 Molecular characterization of microglia long-term activation after the immune challenge

We firstly investigated the protein levels of IBA-1, a microglia specific protein, marker of cellular activation.

The statistical analysis indicated a overall effect of LPS administration ($F_{1,43}$= 15,247 P<0.001) and a stress*LPS interaction ($F_{2,43}$= 6,449 P<0.01). More in details, as depicted in figure 22, we found a statistically significant decrease in IBA-1 in Resilient animals after LPS not only when compared to their controls (-42%, P<0.001; Fig. 22A), but also with respect to LPS group (-26%, P<0.05; Supplementary Fig. 8) and to Responsive animals (-28%, P<0.05; Supplementary Fig.

8). LPS affected also IBA-1 levels in Responsive animals, indeed, even with a lesser extent, this group showed a significant difference from its saline treated counterpart (-18%, $P<0.05$ vs. Responsive; Fig. 22A). Lastly, after stress exposure, we found a difference between both Responsive and Resilient animals compared to No Stress group (+27% $P<0.05$; +31%, $P<0.01$ vs. No Stress respectively; Supplementary Fig. 8).

To strengthen these results, we analyzed the gene expression of different markers of microglia activity. Firstly, we investigated the modulation of CD11b, as an indicator of microglia activation. We found that CD11b expression was affected by LPS ($F_{1,42}$= 12,364 $P<0.001$) and by the interaction of the two experimental variables (Stress*LPS interaction $F_{2,42}$= 11,592 $P<0.001$). The mRNA levels of CD11b resulted upregulated in No Stress group treated with LPS (+24%, $P<0.01$ vs. No Stress; Fig. 22B) and in Responsive animals that received the toxin (+49%, $P<0.001$ vs. Responsive; Fig. 22B), while no effects were observed in Resilient animals. In addition, both LPS and Responsive/LPS groups showed significant differences with respect to Resilient animals (+19%, $P<0.05$; +35%, $P<0.001$ respectively; Fig 22B.). Of note, stress exposure differently impact CD11b expression, indeed Resilient animals showed a significant increase when compared to control animals (+20%, $P<0.05$ vs. No Stress; Supplementary Fig. 9) and to Resilient rats (+26% $P<0.01$ vs. Resilient; Supplementary Fig. 9).

We then analyzed the gene expression of CD68, marker of microglial macrophagic activity. The statistical analysis resulted in an effect of Stress exposure ($F_{2,43}$= 3,327 $P<0.05$) and LPS administration ($F_{1,43}$= 44,594 $P<0.001$). As shown in figure 22 also in this case Resilient rats showed a less pronounced activation of microglia. After LPS both No Stress and Responsive animals showed a massive increase of CD68 expression when compared to their respective controls (+65%, $P< 0.001$ vs. No Stress; +55% $P<0.001$ vs. Responsive; Fig. 22C). LPS was able to induce CD68 transcription also in Resilient rats, even if with a less pronounced effect (+34%, $P<0.05$ vs. Resilient; Fig 22C) as demonstrated by a significant difference also with Responsive animals that received LPS (-41%, $P<0.05$ vs. Responsive; Fig. 22C).

Interestingly the expression profile of this markers followed the effects observed at behavioral level.

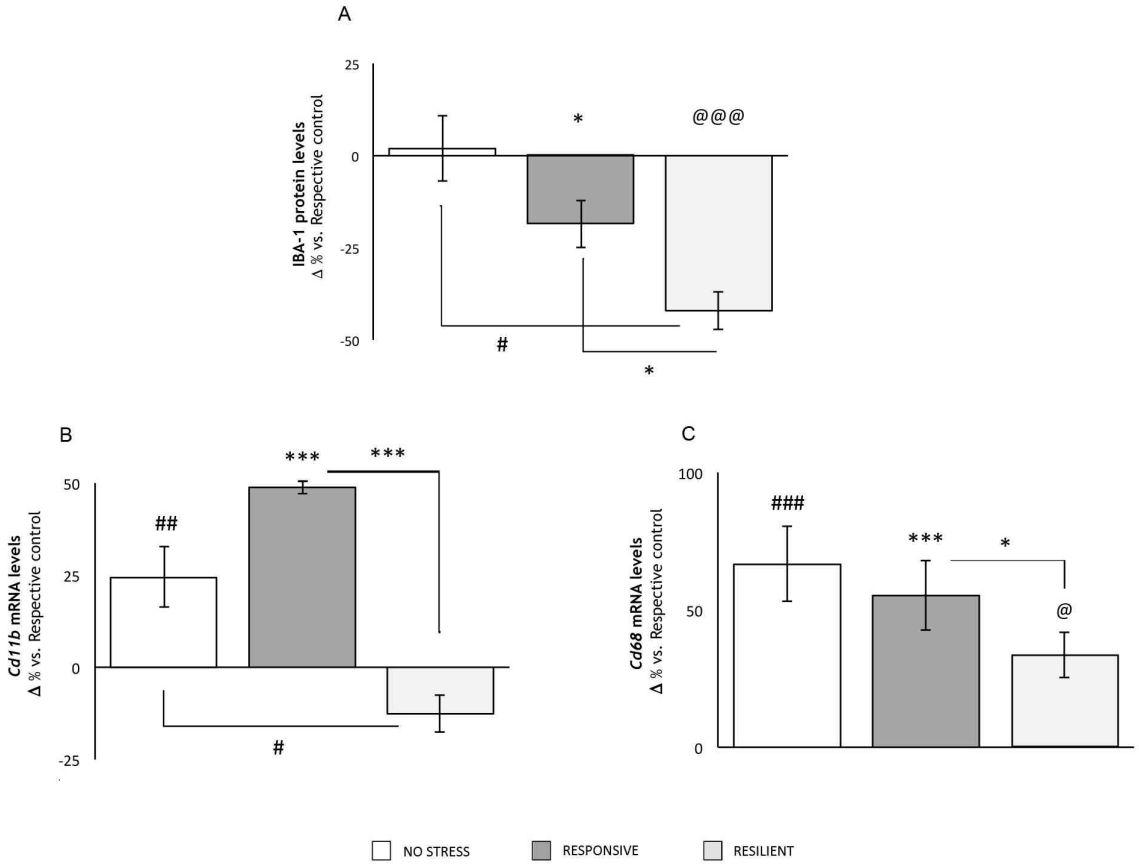

Figure 22 Evaluation of microglia activation after 6 days from the immune challenge. The protein expression of IBA1 (A) and the mRNA levels of CD11b (B) and the marker of phagocytic microglia CD68 (C) were measured in the dorsal hippocampus after 6 days from LPS administration. The data, expressed as delta values ± SEM between the percentages of saline treated rats (set at 100%) and the LPS treated counterpart. #P<0.05, ##P<0.01, ###P<0.001 vs. Non Stress; *P<0.05, ***P<0.001 vs. Responsive; @P<0.05 vs. Resilient. Two way ANOVA with Post Hoc LSD

We then evaluated the involvement of one of the systems known to control the activation of microglia -the so called on/off signaling- by measuring the gene expression of fractalkine (CX3CR1) and its receptor (CX3CR1). Firstly, the analysis of the ligand CX3CL1 showed an effect of LPS administration ($F_{1,43}$= 8,271 P<0.01) that was followed only by a decreased gene expression in Resilient rats subjected to LPS administration, when compared to saline-treated animals (-37%, P<0.05 vs. Resilient; Fig. 23A). However, the mRNA levels of the receptor were affected by both stress ($F_{2,47}$= 9,155 P<0.001) and LPS ($F_{1,47}$= 29,314 P<0.001). In detail, LPS and Responsive/LPS groups showed an increase in the expression of CX3CR1 when compared to their saline-treated counterparts (+27%, P<0.001 vs. No Stress; +15%, P<0.05 vs. Reactive; Fig. 23B). This increase was not present in Resilient animals that received LPS, indeed these animals presented a significant difference in CX3CR1 expression with respect to LPS group (-26%, P<0.01 vs. LPS; Supplementary Fig. 23B)

Lastly, we measured the gene expression of Arginase1, a marker of microglia M2 phenotype. Interestingly we found that Arg1 was affected by stress ($F_{2,26}$=12,764 P<0.001) and immune challenge ($F_{1,46}$= 5,733 P<0.05). Interestingly the comparison between animals that received or not the toxin revealed that: LPS group had increased levels with respect to Resilient/LPS animals (-24% P<0.01 vs. Resilient/LPS; Fig. 23C); Responsive animals that were administered with LPS showed a significant decrease when compared to their saline treated control animals (-28% P<0.01 vs. Responsive; Fig. 23C); the gene expression of Arg1 was significantly more affected than in LPS animals (-20% P<0.05 vs. LPS; Fig. 23C)

Of note, the basal gene expression of Arg1 in Resilient animals was significantly different from the other experimental groups treated with saline (-40%P<0.001 vs. No Stress; -37% P<0.001 vs. Reactive; Supplementary Fig. 13).

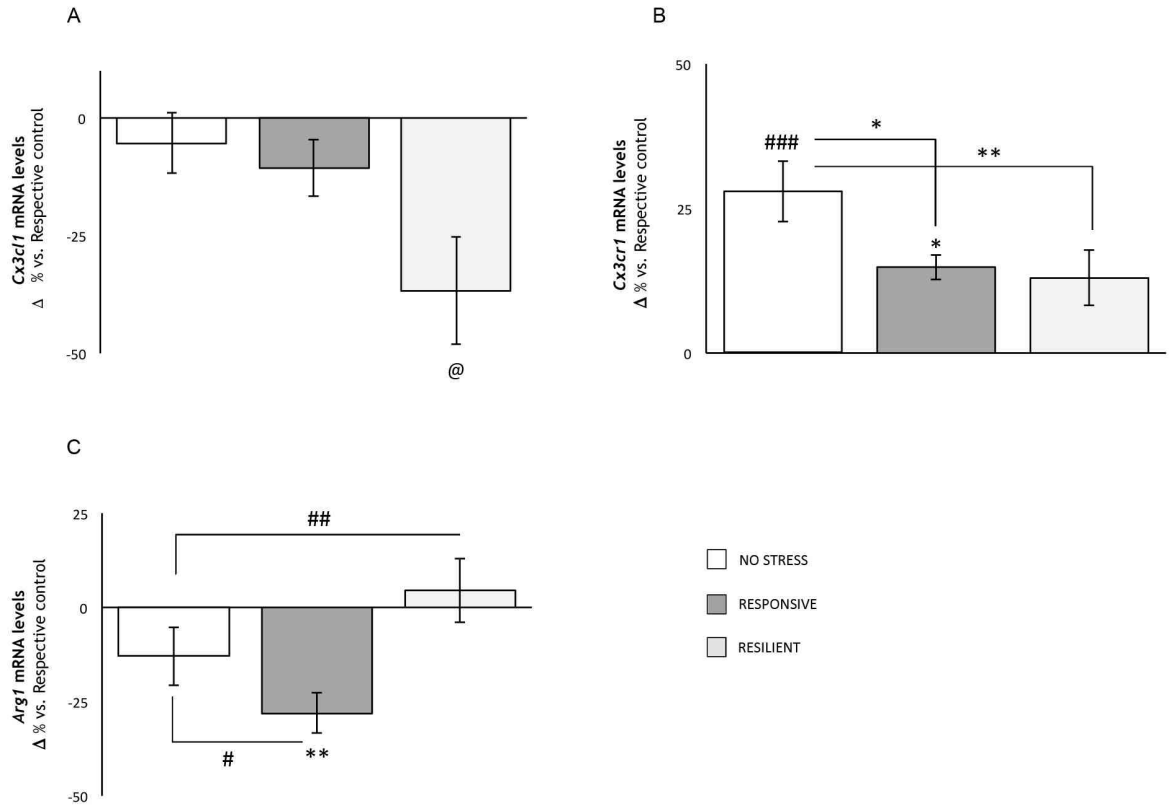

Figure 23 Evaluation of modulators on/off signaling and marker of microglia phenotype after 6 days from the immune challenge. The mRNA levels of CX3CL1 (A), its receptor CX3CR1 and of the M2-phenotype marker Arginase1 (C) were measured in the dorsal hippocampus after 6 days from LPS administration. The data are expressed as delta values ± SEM between the percentages of saline treated rats (set at 100%) and the LPS treated counterpart. #P<0.05, ##P<0.01, ###P<0.01 vs. Non Stress; *P<0.05**P<0.01 vs. Responsive; @P<0.05 vs. Resilient. Two way ANOVA with Post Hoc LSD

4.3.3 Discussion

The aim of this work was to better characterize the molecular mechanisms potentially involved in stress resilience in the context of neuroinflammation. We previously demonstrated that the exposure to two weeks of CMS could induce the upregulation of pro-inflammatory cytokines in the rat brain, with a more pronounced effect in the dorsal hippocampus (Rossetti et al., 2016). More in detail, only animals that showed an altered sucrose intake presented an upregulation of IL-1β and IL-6 mRNA levels.

In the present experiment, we firstly applied a CMS paradigm to identify stress responsive and stress resilient animals. Secondly, we challenged the animals with a low dose of lipopolysaccharide to investigate whether the resilience to stress exposure was related to an increased ability to cope with neuroinflammation. Considering anhedonia as a hallmark symptom of depression, the administration of LPS in rodents has been widely employed to study the effects of inflammation on behavior, with interest on its ability to induce anhedonic-like phenotype (Biesmans et al., 2016).

Interestingly, six days after LPS administration, we found that animals that were resilient to stress exposure, could also better respond to the immune challenge. Indeed, while non-stressed animals and stress-Responsive rats showed a decreased sucrose consumption after LPS administration, stress-Resilient group did not present any behavioral alteration. The susceptibility to LPS in animals not exposed to CMS enlightened the important contribution of the inflammatory system in the insurgence of behavioral alterations, in particular, taking into account that the impairment in sucrose intake persisted for six days after the immune challenge. We cannot exclude that the sucrose intake of stress-Resilient rats was affected by the toxin at an earlier time point; nevertheless, even in this scenario, their response was apparently more rapid with respect to non-stressed animals.

We further investigated these behavioral differences at molecular level, with a specific regard to the dorsal hippocampus. The systemic delivery of LPS can induce a strong immune response, especially within 24 hours from the administration at both central and peripheral level (Biesmans et al., 2016; Dantzer et al., 2008; Molteni et al., 2013). For this reason, we firstly assessed the expression of different markers of neuroinflammation at an early stage. Considering the massive induction of IL-1β and CD11b 24 hours after LPS, we decided to focus

our attention on the gene and protein expression of TLR-4 and on the molecular characterization of microglia activation at later time points.

TLR-4 is a membrane receptor expressed on the surface of diverse cellular types, including cells of the central nervous system (Molteni et al 2016). It has been demonstrated that TLR-4 is induced after chronic stress, with a mechanism that could involve both damage-associated molecular patterns (DAMPs) and bacterial metabolites derived from an altered intestinal permeability (García Bueno et al., 2016). In particular, the activation of its signaling may induce the transcription of different proinflammatory cytokines, among which, IL-1β is strongly involved in stress response (Goshen and Yirmiya, 2009). Interestingly, six days after the immune challenge, LPS administration could unmask the effects of stress exposure on TLR-4 protein levels and on IL-1β gene expression in Responsive animals. This effect is in good agreement with the current literature where TLR-4 has been associated to inflammation-associated stress response within the brain (García Bueno et al., 2016; Gárate et al., 2013; Gárate et al., 2011). The increased activation of TLR-4 and the subsequent upregulation of IL-1β, however, did not seem to be related to the behavioral effects of the immune challenge, indeed, the LPS group did not show an altered expression pattern of the two markers analyzed.

On the contrary, the activation of microglia seems more related to the decrease of sucrose intake observed in LPS and Reactive/LPS groups. Indeed, the analysis of IBA1 -a Calcium binding protein involved in microglia activation and phagocytosis (Hellwig et al., 2016)- revealed a different expression between the groups that showed a different behavioral response to the immune challenge. Accordingly, to the literature reporting an up-regulation of this marker in depression models -among which chronic stress is included (Hinwood et al., 2013; Tynan et al., 2010)- animals that consumed less sucrose presented increased levels of IBA1 when compared to Resilient/LPS animals. Interestingly, this modulation may suggest that Resilient animals have less activated microglia in response to LPS challenge. This hypothesis was sustained by the increased levels of CD11b and CD68 transcripts in Non Stress and Responsive animals treated with LPS.

We then tried to identify the molecular system involved in the diverse activation of microglia analyzing the expression of CX3CL1/Fractalkine and its receptor CX3CR1. These molecules are part of the so-called ON/OFF signaling between neurons and microglia. In particular, fractalkine can be secreted by neurons or be exposed to their cellular surface, to bound its

specific receptor expressed by microglia (Kierdorf and Prinz, 2013). It has been demonstrated that alterations in the homeostasis of this system may induce alterations in synaptic pruning (Paolicelli et al., 2011), impaired brain connectivity and social interactions (Zhan et al., 2014) and protracted depressed like behaviors after LPS exposure (Corona et al., 2010). In our experimental setting, while the ligand CX3CL1 did not show important modulations, the receptor appeared less expressed in Resilient/LPS animals. Considering the role of CX3CR1 in the control of microglia activation this result may appear counterintuitive, however these data are supported by two recent works, in which the knock out of the receptor seems to confer resistance to the detrimental effects of stress (Hellwig et al., 2016; Milior et al., 2016).
To strengthen our hypothesis about the involvement of microglia in the molecular mechanisms of stress resilience, the evaluation of a marker of the M2 phenotype -Arginase1- showed that animals with impaired sucrose intake had decreased levels of this transcript. This data suggests that LPS and Responsive/LPS groups potentially lack the protective role of microglia, thus explaining the more pronounced vulnerability to the immune challenge. T

In conclusion, our data support the idea that neuroinflammation may play a pivotal role in the protective mechanisms underpinning stress resilience. In particular, we found that microglia may be involved in the increased ability of stress-Resilient animals to counteract the effects of CMS and, more interestingly, the consequences of a strong immune challenge such as LPS. These results showed that different systems involved in the control of microglia activation and homeostasis are impaired in animals with impaired sucrose intake, thus suggesting an urgent need to focus on these immune cells to elucidate the molecular mechanisms of stress resilience.

4.4 Genome-wide analysis of LPS-induced inflammatory response in the rat ventral hippocampus: modulatory activity of the antidepressant agomelatine

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Under second revision at The World Journal of Biological Psychiatry

4.4.1 Introduction

It is currently known that conventional pharmacological treatment of Major Depression (MD) -despite the different antidepressants available- has to face several critical issues, such as: a low grade of complete remission (25-30%), a poor response to the treatment in a high percentage of patients and a relapse rate of the 35% within 12 months. In addition, the latency to reach a therapeutic effect, the development of adverse effects and the poor efficacy on cognitive deficits and somatic symptoms, represent critical points for the conventional depression treatments (Connolly and Thase, 2012). All these issues are even worse if we consider that MD affects more than 10% of the general population and it is associated with such a high degree of functional impairment, that it is estimated to become -in the next future- the second leading cause of disability worldwide (Bromet et al., 2011). On these bases, it is crucial to identify new molecular systems and mechanisms involved in the neurobiology of depression, which may represent candidate targets for the development of novel pharmacological interventions. Among the systems that may contribute to the development of depression, a large body of data supports the involvement of the immune/inflammatory system (Dantzer et al., 2008; Haroon et al., 2012; Wohleb et al., 2016). Indeed, the levels of pro-inflammatory mediators such as TNF-α, IL-6 and C-reactive protein (CRP) are increased in the blood stream and in the cerebrospinal fluid of depressed patients (Dowlati et al., 2010; Howren et al., 2009; Raison et al., 2006). Moreover, depression often occurs in comorbidity with medical conditions characterized by an inflammatory state, such as diabetes, cardiovascular or neurodegenerative disorders (Anisman et al., 2008; Berge and Riise, 2015; Réus et al., 2015). In addition, the administration of the cytokine inducer lipopolysaccharide (LPS) in animal models is able to elicit depressive-like behaviors (Frenois et al., 2007; van Heesch et al., 2013; Zhu et al., 2010), an effect also observed after the central administration of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α (Dantzer et al., 2008; Sukoff Rizzo et al., 2012; Wu and Lin, 2008).

On these bases, evidence exists that antidepressant treatments are able to modulate immune/inflammatory systems (Janssen et al., 2010) and that non-steroidal anti-inflammatory drugs or monoclonal antibodies in combination with standard therapy may be beneficial for the therapeutic outcome (Akhondzadeh et al., 2009; Brunello et al., 2006; Raison et al., 2006)). The relevance of these findings is even higher if we take into account that

treatment-resistant depression has been associated with elevated levels of specific inflammatory mediators (Miller et al., 2015; Strawbridge et al., 2015). With all these considerations, by using a candidate-approach analysis, we have already demonstrated that different classes of antidepressants possess anti-inflammatory properties in the chronic mild stress model of depression (Rossetti et al., 2016). Moreover, we showed that the novel antidepressant agomelatine is able to ameliorate the neuroinflammation induced in the rat by an acute inflammatory challenge (Molteni et al., 2013) by acting on specific inflammatory mediators. Conversely, in this study we performed a broader examination of the anti-inflammatory effect of agomelatine by an unbiased genome-wide based approach. Specifically, adult male rats were treated with the antidepressant for 21 days, then a subgroup of animals was challenged with a single injection of LPS at the end of the treatment and they have been sacrificed two hours later to investigate the transcriptomic modulations in the ventral hippocampus, a brain region related to stress, emotion and affect (Fanselow and Dong, 2010). With this broader approach, we analyzed network and pathway alterations in order to better understand the anti-inflammatory properties of agomelatine and identify novel targets for the treatment of depression associated to inflammation.

4.4.2 Results

4.4.2.1 Overall transcriptional effect of chronic treatment with agomelatine and acute administration of lipopolysaccharide

To investigate the overall transcriptional effects of the treatment with agomelatine, LPS, and their combination, we first compared each experimental group (AGO/SAL; VEH/LPS; AGO/LPS) with the control group (VEH/SAL) as common baseline, thus obtaining three lists of genes namely $AGO/SAL_{VEH/SAL}$, $VEH/LPS_{VEH/SAL}$ and AGO/LPS_{VEHSAL}. As shown in figure 14A, we found that agomelatine significantly regulated the expression of 105 genes, with 77 genes (73%) up-regulated and 28 genes (27%) down-regulated. A larger transcriptional effect was observed in animals treated with LPS. Indeed, the inflammatory challenge affected the expression of 284 genes and, out of these, 231 (81%) were up-regulated and the remaining 53 (19%) were down-regulated. Finally, a total of 296 genes were differentially modulated in animals that received both agomelatine and LPS when compared with the control group. Among these, 256 (86%) transcripts were up-regulated, whereas 40 (14%) were down-regulated. Additionally, we analyzed the magnitude of these transcriptional effects finding mild changes in all the experimental groups (Fig. 14B). Specifically, the majority of the genes showed fold-change values (FC) between 1.2 and 1.5 and only a small number of transcripts were regulated between 1.5 and 2 or more than 2-folds with respect to control animals.

Moreover, to investigate the impact of the pretreatment with agomelatine on the effects of the immune challenge we compared the group of animals that received both the antidepressant and LPS (AGO/LPS group) with the animals that received only LPS (VEH/LPS), in order to provide a direct estimate of agomelatine effect in modulating the response to LPS effect. As shown in figure 1C, this analysis resulted in a list of 52 genes, 9 of which were down-regulated (17%) whereas 43 were up-regulated (82%). The magnitude of the modulation of these genes (Fig. 14D) was between 1.2 and 1.5 FC, and only few transcripts exceeded this threshold.

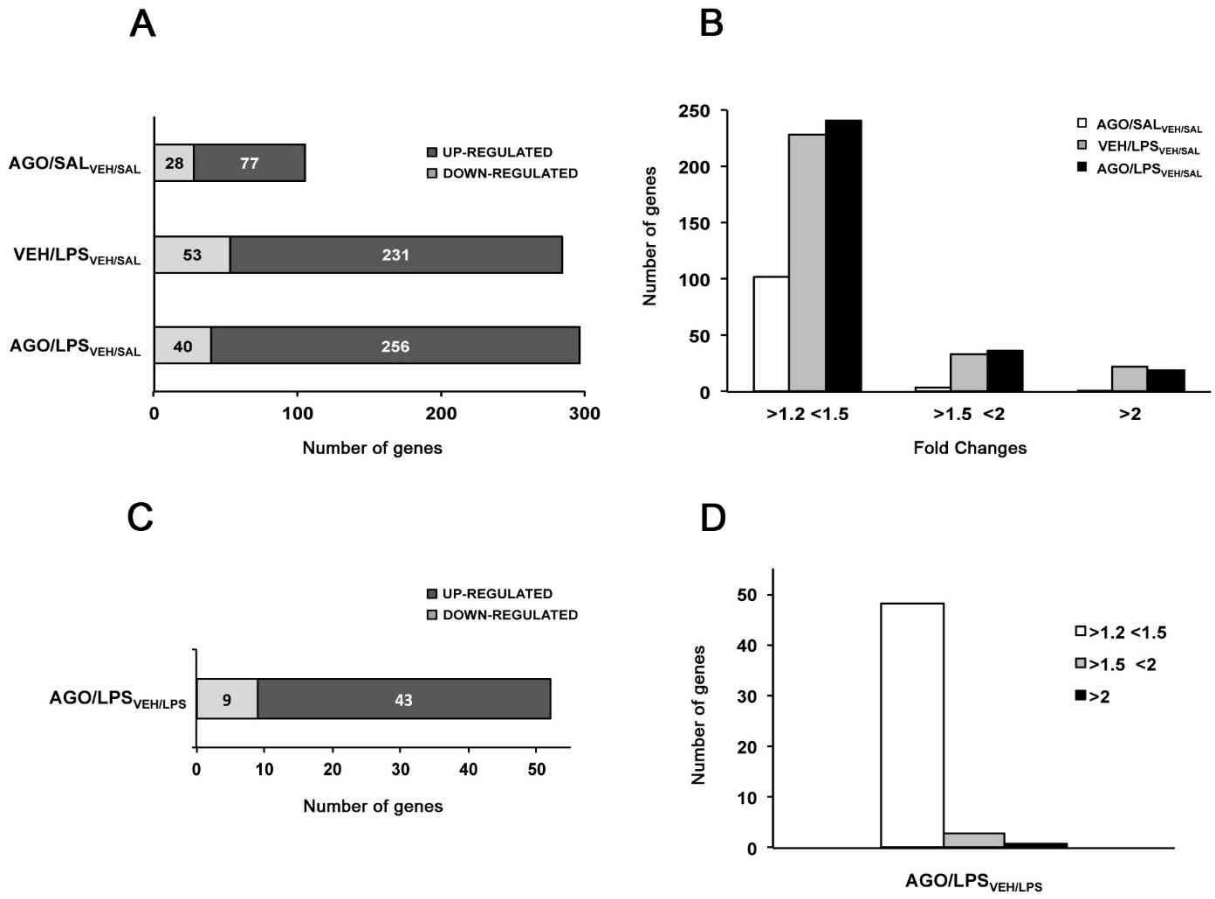


Figure 1. Overall results of microarray analysis (A) Number of genes up-regulated or down-regulated in the ventral hippocampus of rat chronically treated with agomelatine (AGO/SALVEH/SAL), acutely injected with lipopolysaccharide (VEH/LPSVEH/SAL) or receiving both drugs (AGO/LPSVEH/SAL), as compared to the control group. (B) Magnitude of gene expression changes in these experimental groups. (C) Transcriptional effect of the chronic treatment with agomelatine on animals that received only LPS is presented in the AGO/LPSVEH/LPS gene list and magnitude of this modulation (D).

4.4.2.2Genome-wide effect of the chronic treatment with agomelatine

As previously indicated, a total of 105 genes were differentially expressed in the ventral hippocampus of animals chronically treated with agomelatine with respect to rats that received vehicle. Among these genes, we found -as the most up-regulated- the histone clusters Hist1h4m and Hist2h2ab (FC= +1.66 and +1.36 respectively); the glutathione peroxidase Gpx3 (FC= +1.58); the transcript coding for the fusion protein of fubi and ribosomal protein 30, Fau (FC= +1.48), the Zinc finger protein, Zdhhc22 (FC= +1.40); the Guanine

Nucleotide Binding Protein Gamma13, Gng13 (FC= +1.25). Conversely, the most down-regulated transcripts include the mitochondrial GTPase, Rhot1, with a negative fold-change value of -1.54; the N-Acetyltransferase 8-like or Cml3 (FC= - 1.42), which has a probable N-acetyltransferase activity; the olfactory receptor Olr1513 (FC= -1.34); the Hsp40 homolog Dnajc17 (FC= -1.21). See Supplementary Table S1 for the entire list of genes. Next, in order to capture the diverse and complex mechanisms altered by chronic treatment with agomelatine, we performed a pathway analysis based on the 105 significantly modulated genes using Ingenuity Pathways Analysis software (IPA) identifying 10 pathways that were significantly regulated by the antidepressant. Among these, we found the Rapoport-Luebering shunt of glycolytic pathway, the signaling pathways of phospholipase C and of the chemokine receptor CXCR4 (the entire list of pathways is detailed in Table S2, Supplementary materials).

4.4.2.3 Genome-wide effect of the acute administration of lipopolysaccharide

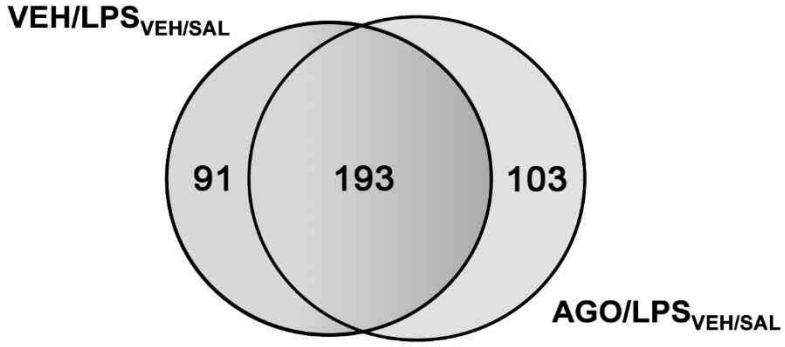
The microarray analysis indicated that 284 genes were differentially expressed between animals injected with LPS and sacrificed 2h later and saline-treated rats. All these genes are listed in TableS3 (Supplementary materials). A large part of these transcripts (81%) was up-regulated by the inflammatory challenge. In particular, Cxcl10 -a chemokine of the CXO subfamily- resulted as the most up-regulated gene, with a +13.06 FC with respect to the control group. As expected, other genes related to the inflammatory response were strongly increased by LPS, including the transcripts coding for: the chemokine Cxcl11 (FC= +4.71); Gbp5, a guanilate binding protein inferred to be involved in IFN-γ cellular response (FC= +4.26); and the interferon-induced protein with tetratricopeptide repeats 3, namely Ifit3 (FC= +4.17). Among the small fraction (19%) of transcripts significantly down-regulated by LPS, we found genes encoding for ion channels, such as the solute carrier family 40 member 1 (Slc40a1) andSlco1a2, namely the solute carrier organic anion transporter family member 1a2 (with a negative FC of -1.65 and -1.56 respectively); the CDC-Like Kinase 2 (Clk2), a protein kinase coding-gene whose targets are involved in the control of the spliceosoma (FC= -1.47); and the transferring receptor (Tfrc) that plays a role in the cellular uptake of iron (FC= -1.44). By using the IPA we identified 100 pathways significantly modulated (listed in Table S4), which, as expected, are mainly related to the inflammatory and cellular response to infections, such as interferon, IL-6 and p38MAPK related signaling.

4.4.2.4 Genome-wide effect of the pretreatment with agomelatine on the inflammatory response induced by LPS

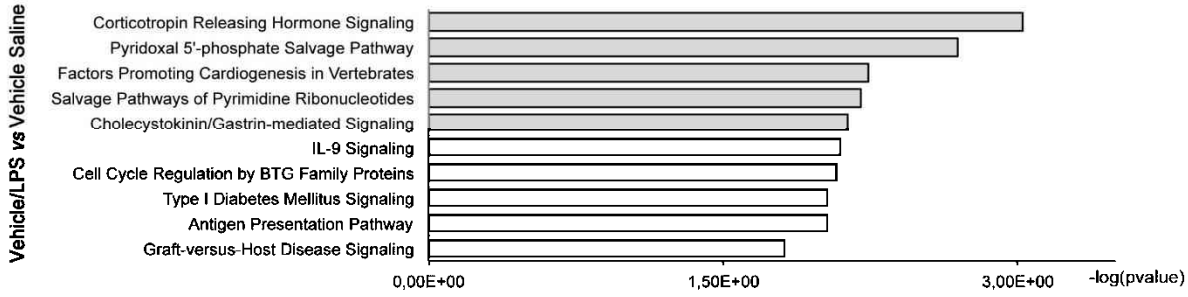
To evaluate the transcriptional impact of the chronic treatment with agomelatine on the LPS-induced inflammatory response, as first step, we compared the list of the 284 genes significantly modulated by LPS treatment (VEH/$LPS_{VEH/SAL}$) with the list of 296 transcripts altered in rats treated with agomelatine and challenged with the endotoxin (AGO/$LPS_{VEH/SAL}$). The resulting Venn diagram (Fig. 15A) identified three subgroups of genes. There were 91 transcripts significantly expressed only in the VEH/$LPS_{VEH/SAL}$ group (Table S5, Supplementary materials), and that were not present at significant level in the list of genes belonging to the AGO/$LPS_{VEH/SAL}$, suggesting that their modulation by the inflammatory challenge was prevented by agomelatine treatment. A comparison of the FC values of these 91 genes in both the experimental groups identified five transcripts whose induction was particularly blunted by the pretreatment with the antidepressant: the chemokine ligand2 (Ccl2, which, as a member of the chemokine family, is involved in the trafficking of immune cells); the major histocompatibility complex, class I, A (RT1-CE1); RAB Interacting Factor or Rabif (a protein involved in the regulation of vesicular transport);the Y box binding protein 1, Ybx1 (a transcription factor that mediates pre-RNA alternative splicing regulation and the transcription of numerous genes); the metabotropic glutamate receptor 2, Grm2 (involved in the regulation of glutamatergic activity). Among the genes with a lower difference in term of FC, we found transcripts strongly related to the inflammatory system, such as interleukin 1β (Il1β); the chemokine (C-X-C motif) ligand 2 (Cxcl2); the suppressor of cytokines signaling (Socs3) and the interleukin 2 gamma subunit (Il2rg). The IPA performed on the 91 genes identified 31 pathways significantly modulated by inflammatory challenge and prevented by agomelatine (Table S6, Supplementary materials), including systems involved in the stress response, such as the corticotropin releasing hormone(CRH) signaling as well as pathways associated with the regulation of specific cytokines (i.e. IL-9 signaling, IL-10 signaling, Role of JAK1 and JAK3 in γc Cytokine Signaling). The top 10 pathways are shown in figures 15B and 15C. Next in the analyses of the Venn diagram of Fig. 15A, 193 transcripts were common between the two lists of genes (Table S7, Supplementary materials), suggesting that their LPS-induced modulation is observed independently from agomelatine treatment. Last, 103 genes were significantly modulated only in animals that received both the pharmacological treatment and the immune challenge (Table S8, Supplementary materials). This list contains

genes that may be linked to the transcriptional impact of agomelatine by itself. In particular, among the top 10 mostly modulated genes in the AGO/LPSVEH/SAL group, we foundHist1h4m, Hist2h2ab (FC= +1.87 and +1.54 respectively), Fau (FC= +1.55) and Dnajc17 (FC= -1.32) that were already present in the list of genes regulated by the antidepressant itself (Table S1). Moreover, we also found genes exclusively modulated by the combination of agomelatine and LPS: CD74 (FC= -1.63) which is associated with class II major histocompatibility complex (MHC) and serves also as receptor of the pro-inflammatory cytokine MIF; the RNA component of the telomerase ribonucleoprotein complex Terc (FC= +1.44); the nueronatin or Nnat (FC= +1.42), involved in the regulation of ion channels during brain development; Acer2 (FC= +1.33) that codifies for the alkaline ceramidase 2, an enzyme responsible for the generation of sphingosine with a role in cell proliferation and survival.

A



B



C

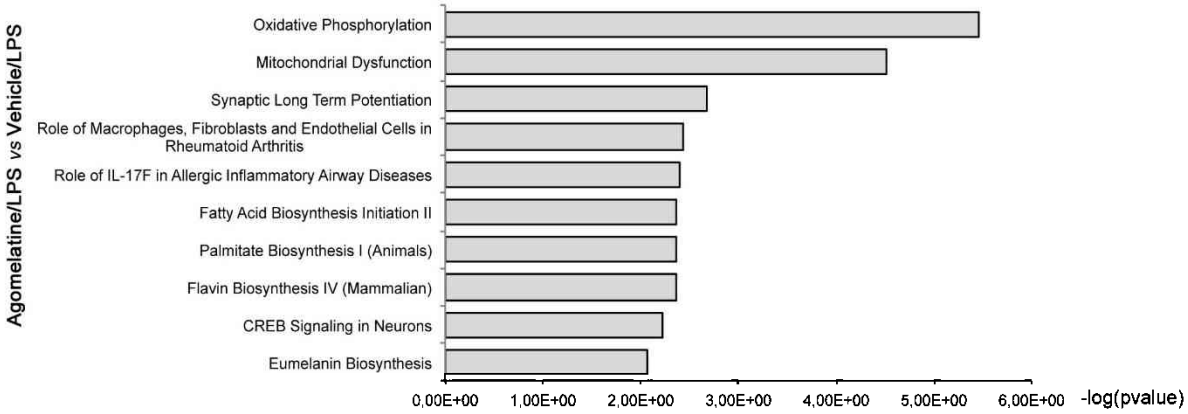
Ingenuity Canonical Pathways	-log(p-value)	Number of genes	Molecules
Corticotropin Releasing Hormone Signaling	3,02E+00	4	CRHR2,PRKCD,NR4A1,MEF2C
Pyridoxal 5'-phosphate Salvage Pathway	2,69E+00	3	CDK18,MAP3K6,PRKCD
Factors Promoting Cardiogenesis in Vertebrates	2,24E+00	3	PRKCD,MEF2C,SMAD1
Salvage Pathways of Pyrimidine Ribonucleotides	2,20E+00	3	CDK18,MAP3K6,PRKCD
Cholecystokinin/Gastrin-mediated Signaling	2,13E+00	3	PRKCD,IL1B,MEF2C
IL-9 Signaling	2,10E+00	2	SOCS3,IL2RG
Cell Cycle Regulation by BTG Family Proteins	2,08E+00	2	BTG2,CCRN4L
Antigen Presentation Pathway	2,03E+00	2	HLA-A,TAP1
Type I Diabetes Mellitus Signaling	2,03E+00	3	SOCS3,HLA-A,IL1B
Graft-versus-Host Disease Signaling	1,81E+00	2	HLA-A,IL1B

Figure 15. Preventive effect of agomelatine: indirect extrapolation of 91 genes modulated by the drug. (A) Venn diagram of the comparison between VEH/$LPS_{VEH/SAL}$ and AGO/$LPS_{VEH/SAL}$. The overlap of

the gene expression changes observed in the animals that received only lipopolysaccharide (VEH/LPS$_{VEH/SAL}$) and those found in the rats pre-treated with agomelatine and then challenged by LPS (AGO/LPS$_{VEH/SAL}$), indicates that 91 genes were altered only in the VEH/LPS$_{VEH/SAL}$ group, 193 genes were modulated by LPS with or without the antidepressant, 103 genes were regulated only when LPS was administered to rats pre-treated with agomelatine. (B) Top 10 canonical pathways most affected by acute injection of lipopolysaccharide in vehicle-pretreated animals. The figure shows the top canonical pathways in terms of -log(*p*-value) identified by Ingenuity Pathway Analysis software among the genes significantly modulated by lipopolysaccharide in rats pre-treated with vehicle. Each pathway is presented in the table (C) with the associated -log(*p*-value), number and name of genes involved.

To further evaluate the impact of agomelatine pretreatment on the inflammatory response induced by LPS, we implemented the previously described comparison focusing on the AGO/LPS$_{VEH/LPS}$ list. This list includes 52 genes (Fig. 14C) and was generated from the AGO/LPS group by using the VEH/LPS group as baseline (see Section 4.1) in order to have a more direct comparison between the animals that received both the treatments and those injected only with LPS. Among the most upregulated genes in this list we found the already mentioned Hist1h4m (FC= +2.04), Fau (FC= +1.95) and Growth Arrest-Specific 5 (Gas5), a long non-coding RNA involved in the regulation of glucocorticoid receptor (FC= +1.81). On the other side, the top down-regulated genes were GH3Domain Containing (Ghdh) with a FC of -1.30 and Grm2 (FC= -1.27). For the complete gene listsee Table S9. The associated IPA generated a list of 33 pathways significantly modulated (Table S10, Supplementary materials). The most altered pathways were associated to oxidative phosphorylation and mitochondrial dysfunction, involving molecules that compose the complex I of NADH dehydrogenase, as well as the long-term potentiation with genes like the Grm2 and the protein kinase C delta (Prkcd) (Fig. 16).

A



B

Ingenuity Canonical Pathways	-log(p-value)	Number of genes	Molecules
Oxidative Phosphorylation	5,46E+00	5	NDUFB4,NDUFA7,NDUFV3,ATP5L,UQCRFS1
Mitochondrial Dysfunction	4,51E+00	5	NDUFB4,NDUFA7,NDUFV3,ATP5L,UQCRFS1
Synaptic Long Term Potentiation	2,68E+00	3	GRM2,PRKCD,ATF4
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2,43E+00	4	MIF,PRKCD,ATF4,IL1B
Role of IL-17F in Allergic Inflammatory Airway Diseases	2,40E+00	2	ATF4,IL1B
Flavin Biosynthesis IV (Mammalian)	2,37E+00	1	RFK
Palmitate Biosynthesis I (Animals)	2,37E+00	1	OXSM
Fatty Acid Biosynthesis Initiation II	2,37E+00	1	OXSM
CREB Signaling in Neurons	2,23E+00	3	GRM2,PRKCD,ATF4
Eumelanin Biosynthesis	2,07E+00	1	MIF

Figure 16. Preventive effect of agomelatine: direct comparison between AGO/LPS and VEH/LPS groups. (A) Top 10 canonical pathways most affected by the acute injection of lipopolysaccharide in agomelatine-pretreated animals. The baseline used in this analysis was the group of animals treated with vehicle and LPS. The figure shows the top canonical pathways in terms of –log(*p*-value) identified by Ingenuity Pathway Analysis software among the genes significantly modulated by lipopolysaccharide in rats received vehicle. Each pathway is presented in the table (C) with the associated –log(*p*-value), number and name of genes involved.

Lastly, with the purpose of narrow the list of genes whose LPS-induced modulation may be prevented by agomelatine, we performed an overlap analysis between the 52 genes belonging to the $AGO/LPS_{VEH/LPS}$ list and the 91 genes, shown respectively in Table S9 and S5, found using VEH/SAL as reference group. The resulting Venn diagram (Fig. 17A) indicates that 9 genes were common between these groups (namely Ybx1, Grm2, Rabif, Lypla1, Tmem93, Fkbpl, Il1β, Tmem60,Prkcd) that represent the transcripts induced by LPS on which the pharmacological pretreatment has the larger effect of normalization. Among these, we focused our attention on the glutamate metabotropic receptor Grm2 and, as shown in figure 17B, the qRT-PCR

analysis confirmed the modulation observed in the microarray study. Indeed, Grm2 mRNA levels were significantly increased by LPS in animals pre-treated with vehicle (+34% p=0.055 vs. VEH/SAL; Fig. 17B) but not in those that received agomelatine (-37% p<0.001 vs. VEH/LPS), as indicated by the significant Drug*LPS interaction ($F_{1,27}=5.718$ $P=0.025$, Two-way ANOVA).

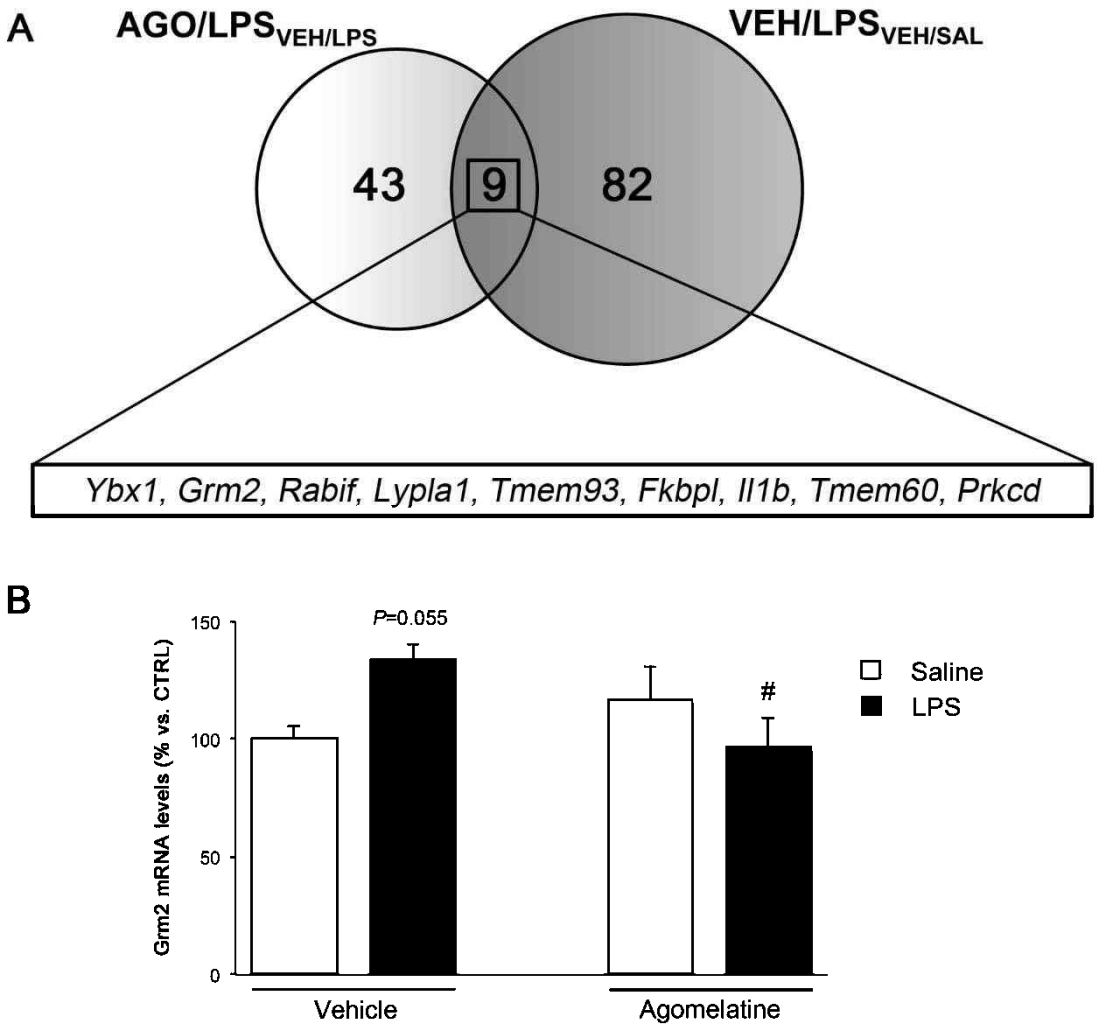


Figure 17. Top 9 genes modulated by agomelatine identified by intersection analysis. (A) Venn diagram of the comparison between AGO/LPS group (with VEH/LPS baseline) and the 91 genes of the $VEH/LPS_{VEH/SAL}$ group whose transcription was prevented by the pretreatment with agomelatine. The overlap between the two groups indicates 9 common genes (listed above in order of absolute fold change value) that should represent the transcripts mostly modulated by the preventive effect of agomelatine on the LPS administration. (B) Analysis by Real time qRT-PCR of the mRNA levels of the metabotropic glutamate receptor 2 (Grm2) in animals treated with vehicle or agomelatine for three weeks and then challenged or not with a single dose of LPS. The data, expressed as a percentage to the control group (vehicle/saline), are the mean ± SEM of independent determinations. p=0.055 vs. vehicle/saline; #p<0.05 vs. vehicle/LPS. Two-way ANOVA with Fisher's PLSD.

4.4.3 Discussion

This study provides novel findings on the transcriptional effect of a chronic treatment with the antidepressant agomelatine and on the ability of this drug to interfere with the response of the brain to an inflammatory challenge. Specifically, by using a genome-wide approach, we identified genes and pathways that may contribute to the therapeutic efficacy of the antidepressant and in particular on its previously demonstrated anti-inflammatory properties (Molteni et al., 2013; Rossetti et al., 2016). The pathway analysis revealed that the administration of agomelatine alone was able to modulate, among the others, two pathways: the signaling of C-X-C chemokine receptor 4 (CXCR4) and phospholipase C (PLC). Chemokines are small molecules that mediate leukocyte mobilization to sites of inflammation in the periphery. Currently, the chemokine family consists of more than 50members with more than 20 G-protein coupled receptors that have also been detected at cerebral level. CXCR4 is the receptor of the very well-studied chemokine CXCL12 (or SDF-1). This signaling pathway is not only important in the immune system, where it has a role in the development of immune cells and neutrophils (Nagasawa, 2014), but it is also fundamental for the regulation of additional non-immune processes, such as neurogenesis and neuronal activity. Indeed, these molecules have a well-defined role in hippocampal development, architecture and function, in the modulation of the GABAergic and glutamatergic activity on serotonergic neurons, and in mechanisms related to neuroprotection such as production and release of different neurotrophic factors (Hanisch and Kettenmann, 2007; Réaux-Le Goazigo et al., 2013; Shyu et al., 2008; Williamson and Bilbo, 2013). Interestingly, it is well known that alterations of these systems are involved in the etiopathology of psychiatric disorders and in particular for depression (Duman and Monteggia, 2006; Sanacora et al., 2012).

Another notable pathway modulated by the chronic administration of agomelatine is the signaling of PLC. Among the PLC isozymes, primary PLCs, PLCβ and PLCγ, are directly triggered by receptor activation. PLCβ isozymes are activated by G protein coupled receptor, whereas PLCγ isozymes are activated by receptor tyrosine kinase (Yang et al., 2013). Different groups have already demonstrated the involvement of the PLC pathway in the therapeutic effect of antidepressants. It has been reported that antidepressants with different synaptic mechanisms are able to increase the phosphorylation of PLCγ through the activation of TrkB, the high affinity receptor for the neurotrophin Brain derived neurotrophic factor (Rantamäki

et al., 2007). Our data add new information as indicate that agomelatine is able to modulate the PLC signaling by acting on a particular G protein, GNG13, which is responsible for the activation of the specific isozyme PLCβ. Interestingly, it has been demonstrated that the signaling of PLCβ may also be activated by the chemokine receptor (Bach et al., 2007) that, as discussed above, is modulated by chronic agomelatine treatment. Moreover, it has been recently reported that a compound able to activate the PLCβ/inositol phosphate 3 pathway has antidepressant properties in a rodent stress-based model of depression, an effect mediated by the BDNF/TrkB signaling (Yang et al., 2013), thus supporting the potential of PLCβ as new pharmacological target. In line with our result, it has been recently demonstrated that TrkB signaling is effectively involved in the antidepressant effect of agomelatine (Boulle et al., 2016). However, beside these pathways involved in the effect of agomelatine per se, we identified genes specifically related to its ability to counteract the inflammatory response. Indeed, by analyzing our data with different approaches, we found that the antidepressant prevented the LPS-induced modulation of several genes. The majority of these genes are related to the inflammatory system such as IL-1β, thus confirming our previous data on the anti-inflammatory properties of agomelatine (Molteni et al., 2013). Other transcripts, belong to pathways related to the synthesis, generation and production of reactive oxygen species, suggesting an anti-oxidant effect of the antidepressant that may be associated with its structural analogy with melatonin, a well-known antioxidant agent (Reiter et al., 2008). By regulating these pathways, agomelatine could counteract the oxidative stress associated to the inflammatory response, an effect in line with its ability to positively modulate energy metabolism and oxidative stress parameters (de Mello et al., 2016). Through different overlap analyses, we further narrowed the list of genes whose LPS-dependent modulation was prevented by the antidepressant, finding 9 transcripts: Ybx1 (a transcription factor that mediates pre-RNA alternative splicing regulation and the transcription of numerous genes); Grm2 (metabotropic glutamate receptor 2); Rabif (member of the family of small GTP-binding proteins that are involved in the regulation of intracellular vesicular transport); Lypla1 (lipophospholipase, a member of the a/b hydrolase superfamily with depalmitoylating activity, involved in the regulation of G-protein signaling); Tmem93 (ECM6, a transmembrane protein present in the endoplasmic reticulum, recently discovered to be involved in cell autophagy); Fkbpl (Fk506 binding protein like, involved in cellular response to stress and homolog of the FKBP protein family); Il1β ; Tmem60 (transmembrane protein 60, at present

no further data are available on this transcript); Pkcd (Protein Kinase Cδ, a family of serine- and threonine-specific protein kinases that can be activated by calcium and the second messenger diacylglycerol).One interesting candidate emerging from our analysis is Grm2, the gene encoding for the presynaptic metabotropic glutamate receptor type 2 (mGluR2) that regulates the glutamatergic homeostasis through an inhibitory tone on glutamate release. The observed LPS-induced up regulation of Grm2 transcription may be due to the activity of NF-kB, a mechanism in line with the literature (Cuccurazzu et al., 2013; Nasca et al., 2013) and with the increased nuclear translocation of this transcription factor following LPS administration (Molteni et al., 2013). Since mGluR2 is also expressed in microglial cells, its increased expression might contribute to the detrimental consequences of microglia activation induced by the inflammatory challenge; this effect may be associated with the capability of this receptor to increase the release of TNF-α, the subsequent activation of neuronal caspase-3 and apoptosis processes (Taylor et al., 2005). In line with this observation, it has been reported that mixed cortical culture with neurons derived from mGlu2 knockout animals are resistant to NMDA toxicity (Corti et al., 2007). Moreover, in a recent gene expression study of a large cohort of postmortem depressed subjects, the increased expression of Grm2 has been proposed as a biomarker of suicide in major depressed patients (Gray et al., 2015). Based on our results, it is feasible to hypothesize that a reduction in LPS-induced increase of Grm2by agomelatine may be part of the anti-inflammatory properties of the drug. In conclusion, in the present study we used an unbiased genome-wide strategy to broaden our view on the immune-regulatory activity of the antidepressant agomelatine.

Although further studies are needed to better investigate the modulatory activity of agomelatine and other antidepressants on the transcripts and pathways identified in our study, the information emerging from these results are useful to better understand the mechanisms of action of agomelatine and to identify novel targets for pharmacological intervention as well as to characterize the mechanisms involved in the association between depression and inflammation.

4.5 Different response to lipopolysaccharide in male and female BDNF heterozygous mice: gender and genotype Interaction

Rossetti A.C., Paladini M.S., Trepci A., Gass P., Riva M.A., Molteni R.

Unpublished data

4.5.1 Introduction

Among the multiple systems affected in depressive state, neurotrophins play a crucial role. Indeed, Brain derived neurotrophic factor (BDNF) alterations are present in depressed subjects as well as in animal model of depression, and antidepressant drugs are able to ameliorate such defects (Calabrese et al., 2011; Molteni et al., 2010).

In the context of neuroinflammation, the literature shows several examples of the detrimental effects of inflammation on BDNF homeostasis. More in detail, it has been demonstrated that the administration of the cytokine inducer LPS lead to the reduction of BDNF expression in vivo (Raetz and Whitfield, 2002). Moreover, in a recent study, Chapman and co-workers showed that an inflammatory challenge can affect the expression of BDNF transcripts in the hippocampus, suggesting a functional interaction between inflammation and the activity of the neurotrophin (Chapman et al., 2012). In addition, microglial cells, which represent one of the critical component of the inflammatory response, express BDNF mRNA, secrete the neurotrophin following stimulation, and have their functions regulated by BDNF (Trang et al., 2011).

With these premises, the aim of this study was to investigate the mutual influence between BDNF and inflammatory system by evaluating the inflammatory response in animals characterized by deficit in BDNF system. In particular, we induced an inflammatory response by acute injection of LPS in BDNF heterozygous mutant mice After 24 hours from the immune challenge animals, male and female, were tested with the open field test and with the forced swim test, to assess the insurgence of locomotor dysfunctions, anxiety-like and depressive-like behaviors. Mediators of the inflammatory response were evaluated in the hippocampus and frontal lobe to investigate the molecular impact of LPS in a condition of BDNF impaired function.

4.5.2. Results

4.5.2.1. Effects of LPS administration on locomotion, anxiety-like and depressed-like behaviors in wild-type and BDNF heterozygous mice

4.5.2.1 Locomotor activity

The total distance that male mice treated with LPS moved during the OF was reduced when compared to the locomotion of saline-treated mice. As a confirmation, the statistical analysis revealed that LPS administration had significant effect ($F_{1,23}$= 74.485 *P*<0.001; Fig. 24A). Male mice that received or not the LPS injection spent more time moving in the beginning of the test than in the last part. As shown in figure 24 repeated measurement ANOVA revealed that the timing of the test ($F_{1,19}$= 6,603 P=0,019) and treatment ($F_{(1,19)}$=74.458 *P*<0.001) had significant effects.

All male mice moved faster in the first 5 minutes of the test and the velocity of mice treated with LPS was slower than mice treated with saline. A repeated measurement ANOVA revealed that factor time ($F_{1,19}$= 6.534 *P*=0.019) and treatment ($F_{1,19}$= 73.876 *P*<0.001) were significant (Fig. 24B). The analysis at the later time point revealed that only treatment had a significant effect in the velocity of the mice ($F_{1,19}$= 73.859 *P*<0.001; Fig. 24B). No significant effects were found in the direct comparisons between groups.

The total distance moved by female mice treated with LPS, was decreased with respect to the groups of mice treated with saline in the last time point of the test. Indeed, the treatment with the toxin affected significantly the distance traveled ($F_{1,20}$= 7.566 *P*=0.012; Fig. 25A). Also the timing of the test was significant in terms of locomotion in female mice, with animals moving less in the second trial of the OF ($F_{1,20}$= 15.356 *P*=0.001; Fig. 25A)

Female mice treated with LPS had an overall reduced velocity than mice treated with saline. If we compare the two sessions of the OF, treatment ($F_{1,20}$= 7.561 *P*=0.012) and time ($F_{1,20}$= 15.341 *P*=0.001) had significant effects on the velocity of the female mice (Fig. 25B).No significant effects were found in the direct comparisons between groups.

4.5.2.2 Anxiety-like behavior

A two-way ANOVA, analyzed at the last time point revealed that the time male mice spent in the center of the arena was affected by genotype in a significant way ($F_{1,23}$= 3.238 *P*=0.088). Interestingly we observed also a significant genotype*treatment interaction ($F_{1,23}$= 4.589 *P*=0,045; Fig. 24C) with a tendency toward decrease in wild type animals that received LPS. In

addition, repeated measurement ANOVA revealed that time ($F_{1,19}$= 8.830 *P*=0.008), genotype ($F_{1,19}$= 3.239 *P*=0.088) and the interaction treatment*genotype ($F_{1,19}$= 4.588 *P*=0.045) affected the performance of the animals when considering the two session of the OF (Fig. 24CA).

In male mice, the two-way ANOVA revealed that not treatment but genotype affected significantly the behavior of the mice ($F_{1,23}$= 3.418 *P*=0.080; Fig. 24D). In addition, if we compared the first 5 minutes of the OF with the second trial, only the mutant mice treated with LPS did not have an increase in the distance to walls, while the other groups stayed farther away from the external walls. Indeed, we found not only an effect of time ($F_{1,19}$= 16.448 *P*=0.001) and genotype ($F_{1,19}$=3.418 *P*=0.080), but also an interaction between the two variables (time*genotype $F_{1,19}$=4.361*P*=0.050; 24D).

LPS treated female mice generally tended to spend less time in the center, however the statistical analysis didn't reveal any significant effect. The mutant mice treated with LPS did not have differences of the time they stayed in the center during all the period of the test. A repeated measurement ANOVA revealed that the behavior changed over time ($F_{1,20}$= 12.041 *P*=0.002) and mice reacted differently dependently on the treatment (interaction time*treatment: $F_{1,20}$= 5.445 *P*=0.030; Fig. 25C).

Mice of all experimental groups moved closer to walls in the first half of the behavioral test, than in the second half. A repeated measurement ANOVA revealed that this change was affected significantly by two factors, time ($F_{1,20}$= 13.503 *P*=0.002) and treatment ($F_{1,20}$= 6.069 *P*=0.023), which showed also a significant interaction (time*treatment: $F_{1,20}$= 7.856 *P*=0.011) (Fig. 3.19.A). The analysis we did in the last time point of the test, the two way ANOVA revealed that only treatment affected this behavior (factor treatment: $F_{(1,20)}$=6.071 *P*=0.023; Fig. 25D).

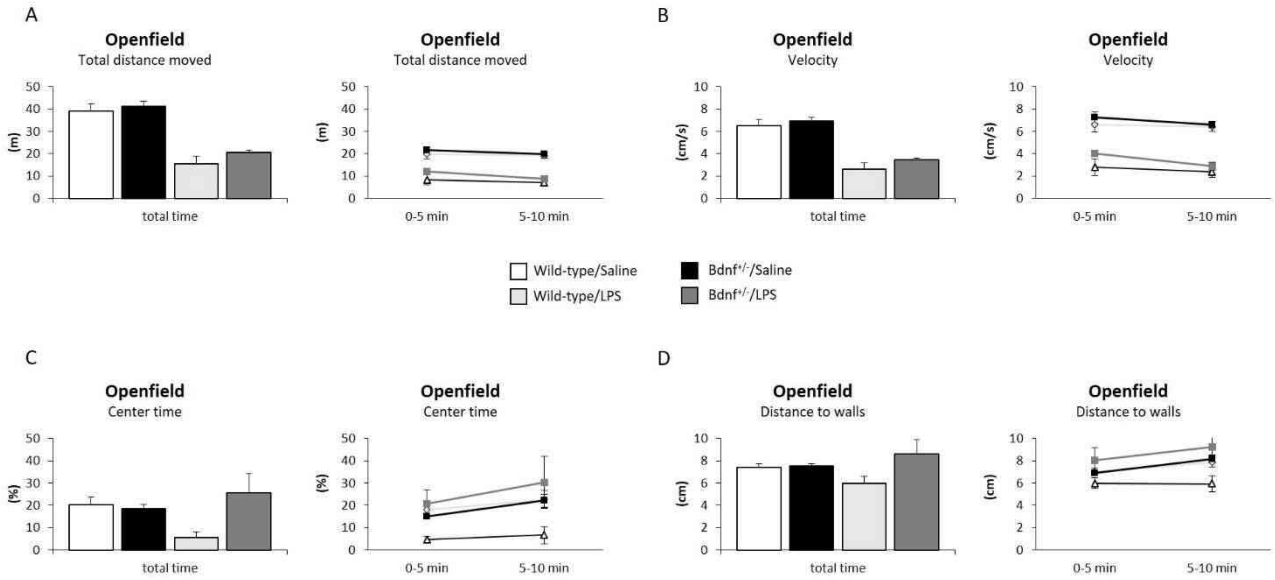


Figure 24 Effect of LPS on locomotion and anxiety like behavior on wild type or BDNF$^{+/-}$ male mice
Evaluation of the behavioral effect of LPS administration in the open field arena. Here we present the total distance travedel (A) and the velocity (B) as parameters of locomotion. The time spent in the center of the arena (C) and the distance from the walls were used as indicators of anxiety-like phenotype. Each measurment is presented as difference between groups at the later time point (bar graphs) and in the different session of the test (line graps).

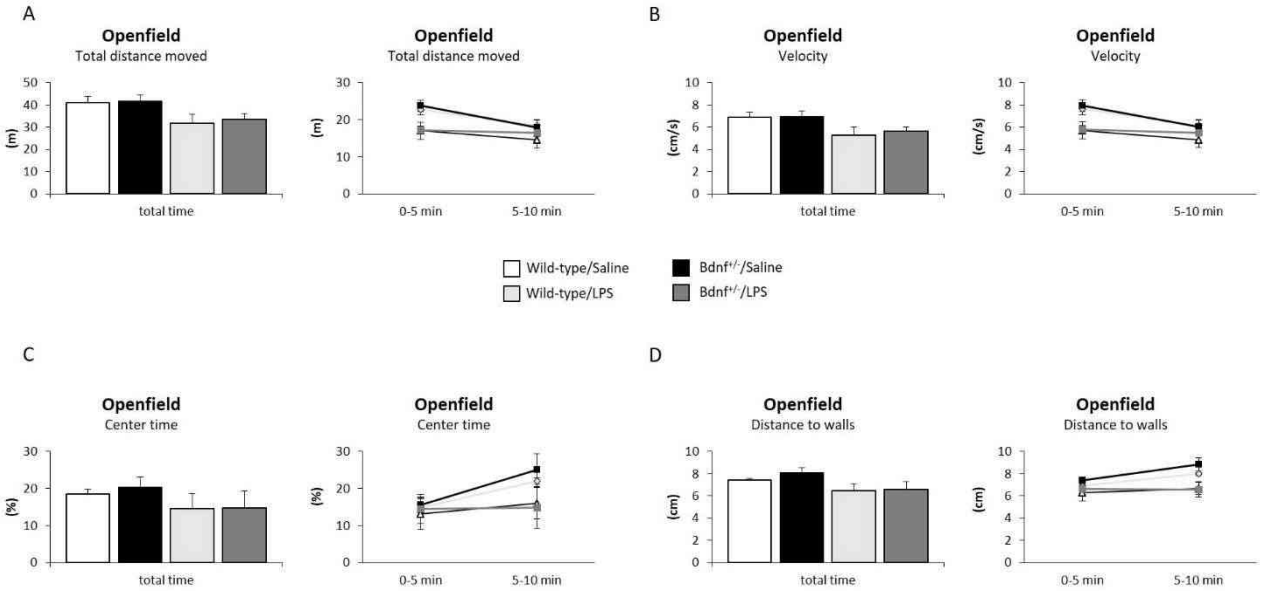


Figure 25 Effect of LPS on locomotion and anxiety like behavior on wild type or BDNF$^{+/-}$female mice
Evaluation of the behavioral effect of LPS administration in the open field arena. Here we present the total distance travedel (A) and the velocity (B) as parameters of locomotion. The time spent in the center of the arena (C) and the distance from the walls were used as indicators of anxiety-like phenotype. Each measurment is presented as difference between groups at the later time point (bar graphs) and in the different session of the test (line graps).

4.5.2.3Depressive-like behavior

All male mice had comparable latencies to start floating: genotype and treatment did not show any significant effect (Fig. 26 A, B). When the immobility time was analyzed in 2-min time segments, a repeated measurement ANOVA revealed that there was only the factor time significant ($F_{2,38}$=108.314 *P*<0.001), whereas treatment and genotype did not influence the immobility time (Fig. 26 B). No specific differences between groups were observed.

The latency to start floating was approximately 60s in all female mice tested; genotype and LPS administration had no significant effects (Fig. 26D). Concerning the total immobility time, we found a general effect of LPS treatment ($F_{1,20}$=5.519 *P*=0.029). Female BDNF heterozygous mice showed different reaction to LPS than wild type mice treated with the toxin as suggested by a treatment*genotype interaction ($F_{1,20}$=6.310 *P*=0.021). When analyzing the immobility in 2-min time segment, we observed significant effects of time ($F_{2,40}$= 150.152*P*<0.001), treatment($F_{1,20}$=5.519 *P*=0.029) and an interaction between the two variables treatment*genotype ($F_{1,20}$=6.310 *P*=0.021; Fig. 26E). No specific differences between groups were observed.

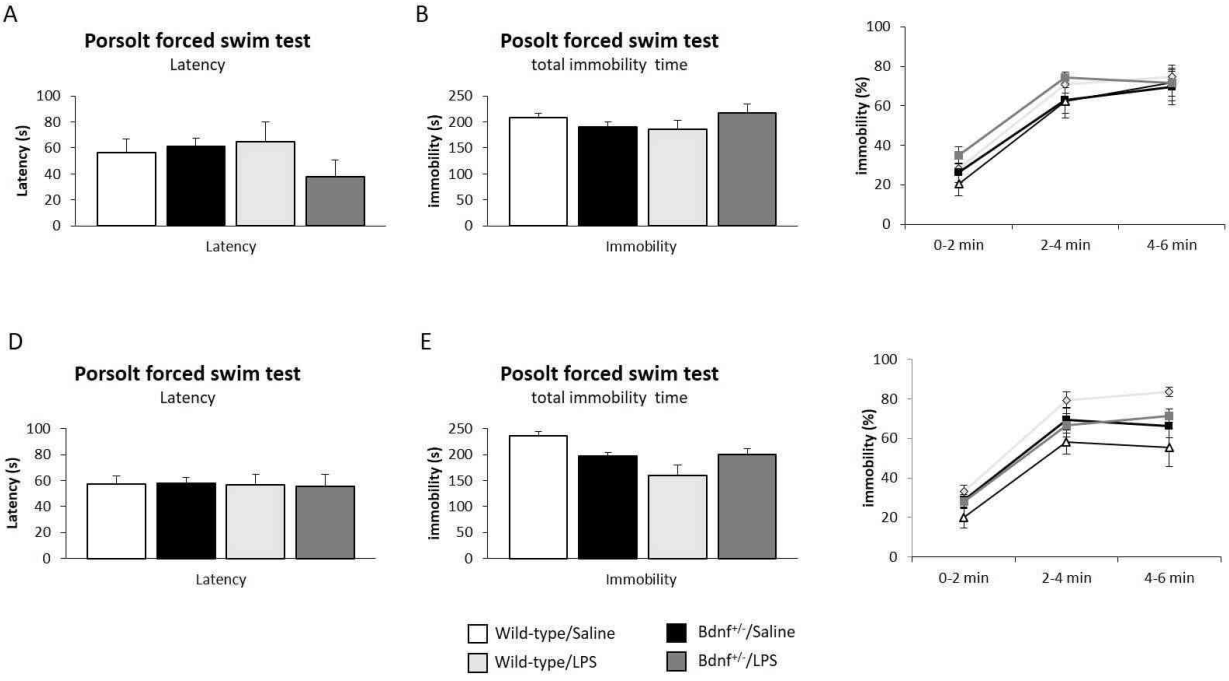


Figure 26 Effect of LPS ondepressive-like behavior on wild type or BDNF$^{+/-}$ mice Evaluation of the behavioral effect of LPS administration Forced Swim test. Here we present the latency of the first freezing and the total immobility time in male (A, B) and females (C, D) mice. Total immobility time is presented as difference between groups at the later time point (bar graphs) and in the different session of the test (line graps).

4.5.2.4 Molecular effects of LPS administration on mediators of the immune/inflammatory system in the hippocampus and in the frontal lobe of wild-type and BDNF heterozygous mice

Although the LPS administration did not have a marked impact on the behavior of wild-type and mutant animals, we evaluated if the inflammatory challenge could unmask differences in the inflammatory response at molecular level in the different groups. To this aim, we analyzed the gene expression of pro-inflammatory cytokines and markers of microglia activation in the hippocampus and in the frontal lobe of wild-type and mutant mice.

Gene expression analysis of the pro-inflammatory cytokine IL-1β

We first measured IL-1β gene expression and, as shown in figure 27A, its mRNA levels were significantly modulated by LPS administration in the hippocampus of both wild-type and heterozygous male mice ($F_{1,19}$=179.8 *P*=0.001). Specifically, the inflammatory challenge markedly increased the pro-inflammatory cytokine without differences between the two genotypes (WT/LPS +465% vs. WT/SAL, *P*<0.001; +/d/LPS +571% vs. +/d/SAL, *P*<0.001).

Conversely, a different profile was observed in the hippocampus of female mice, where the significant effect of the LPS injection ($F_{1,18}$=14.17, *P*=0.002) was restricted to the mutant animals, as indicated by ANOVA ($F_{1,18}$=5.89, *P*=0.029; LPS*Genotype interaction: $F_{1,18}$=10.85, *P*<0.005). Indeed, as shown in figure 27B, IL-1β mRNA levels were significantly induced by LPS only in the heterozygous mice (+/d/LPS +324% vs. +/d/SAL, *P*<0.001) with no changes in wild-type animals (+/d LPS +262% vs. WT/LPS, *P*<0.01). Moreover, it has to be noted that the magnitude of the cytokine induction in female mice was lower with respect to male animals although its basal expression was similar.

In the frontal lobe of male mice was similar to what observed in the hippocampus. More in details, LPS administration significantly increased the gene expression of the pro-inflammatory cytokine ($F_{1,22}$=45.49, *P*=0.001) in both wild-type (WT/LPS +309% vs. WT/SAL, *P*<0.001) and heterozygous male mice (+/d/LPS +324% vs. +/d/SAL, *P*<0.001) without differences between the two experimental groups (Fig. 27C). Conversely, no changes in IL-1β expression were found in the frontal lobe of female mice exposed to LPS, neither in wild type nor in heterozygous animals (Fig. 27D).

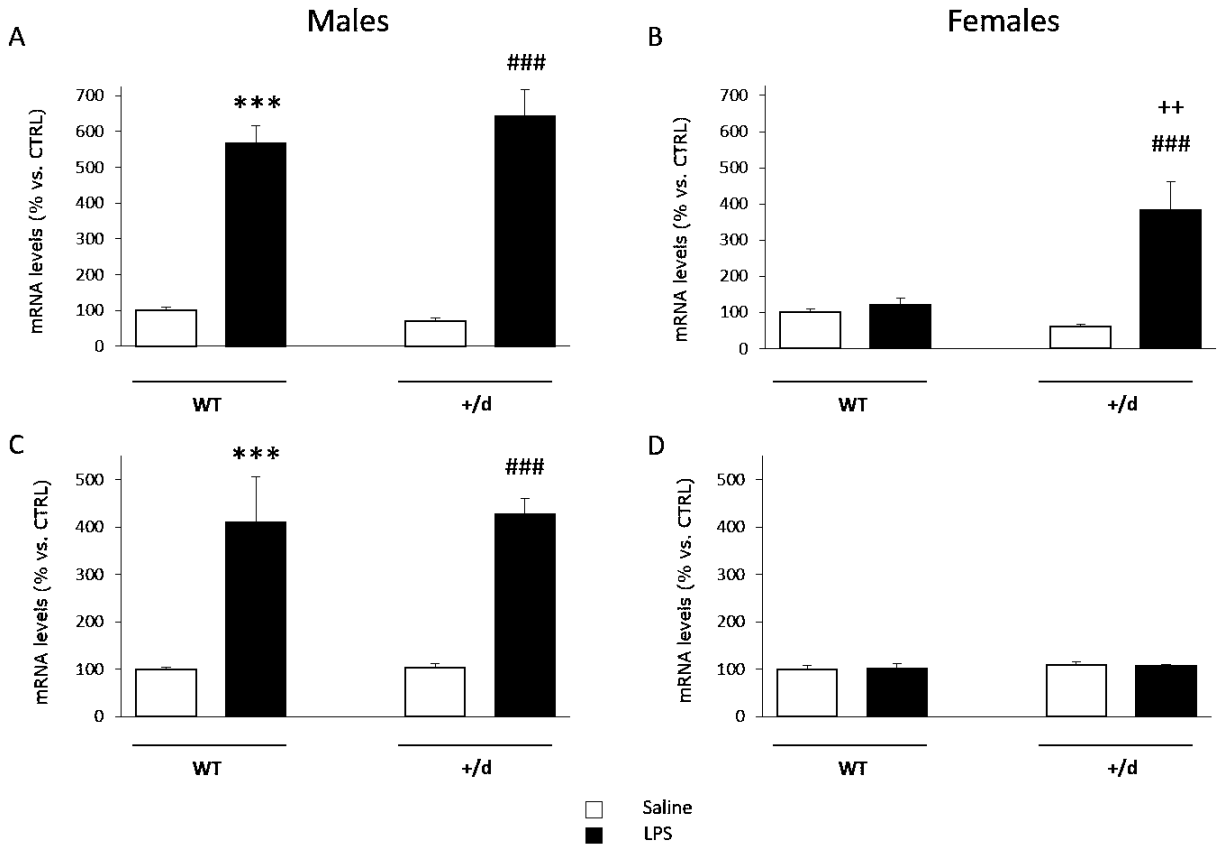


Figure 27. Gene expression analysis of IL-1β in the hippocampus and in frontal lobe. The mRNA levels of the pro-inflammatory cytokine IL-1β were measured in the hippocampus and in the frontal lobe of males (A,C) and females (B,D) wild-type (WT) and BDNF heterozygous (+/d) mice 24 h after a single injection of lipopolisaccharyde (LPS, 400 μg/kg i.p.) in comparison with mice treated with saline (SAL). The data, expressed as a percentage of the saline-injected wild-type mice (CTRL, set at 100%), represent the mean ± SEM of at least 6 independent determinations. ***P<0.001 vs. CTRL; $^{###}P$<0.001 vs. BDNF +/d; ^{++}P<0.01 vs. WT/LPS (Two-way ANOVA with PLSD).

Gene expression analysis of the pro-inflammatory cytokine TNF-α

Similarly, to what observed in this brain region for IL-1β, the mRNA levels of TNF-α were significantly up-regulated by the LPS treatment in male mice ($F_{1,20}$=42.58, *P*=0.001), an effect independent by the genotype. In fact, the inflammatory challenge strongly induced the expression of TNF-α in both wild-type (WT/LPS +1287% vs. WT/SAL, *P*<0.001) and mutant mice (+/d/LPS +1142% vs. +/d/SAL, *P*<0.001) without any statistical difference (Fig. 28A).

On the contrary, the increase of TNF-α gene expression by LPS was limited to the mutant animals in female mice with a general effect of LPS ($F_{1,18}$=6.74, *P*=0.021), genotype ($F_{1,18}$=10.7, *P*=0.006) and an interaction between LPS*Genotype ($F_{1,18}$=4.95, *P*=0.043), This increase was less pronounced with respect to that observed in male mice (+/d/LPS +186% vs. +/d/SAL, *P*<0.01; +/d/LPS +164% vs. WT/LPS ***P*<0.01; Fig. 28B).

In the frontal lobe, the gene expression profile of TNF-α was qualitatively identical to what observed in the hippocampus although the effect of the inflammatory challenge was lower. As shown in figure 28C, we found a significant increase of its mRNA levels after LPS injection ($F_{1,22}$ =42.43, *P*=0.001) in both wild-type (WT/LPS +544% vs. WT/SAL, *P*<0.001) and mutant male mice (+/d/LPS +678% vs. +/d/SAL, *P*<0.001).

A slight but significant modulation of TNF-αby LPS ($F_{1,21}$=4.45, *P*=0.05) was also specifically observed in heterozygous female mice (+/d/LPS +55 vs. +/d/SAL, *P*<0.01; Fig. 28D).

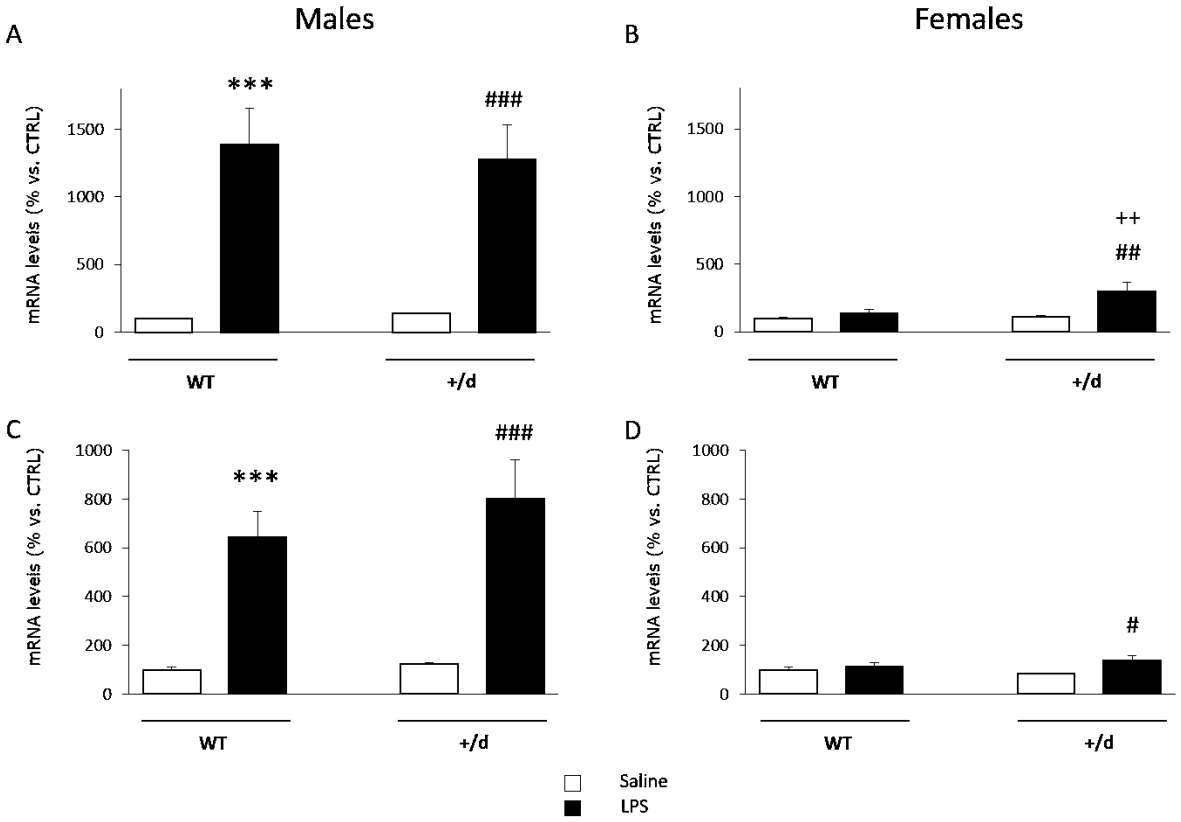


Figure 28. Gene expression analysis of TNF-α in the hippocampus and in the frontal lobe. The mRNA levels of the pro-inflammatory cytokine TNF-α were measured in the hippocampus and in the frontal lobe of males (A, C) and females (B, D) wild-type (WT) and BDNF heterozygous (+/d) mice 24 h after a single injection of lipopolysaccharide (LPS, 400 μg/kg i.p.) in comparison with mice treated with saline (SAL). The data, expressed as a percentage of the saline-injected wild-type mice (CTRL, set at 100%), represent the mean ± SEM of at least 6 independent determinations. ***P<0.001 vs. CTRL; $^{###}P$<0.001 vs. BDNF +/d; $^{#}P$<0.05, $^{##}P$<0.01 vs. BDNF +/d; ^{++}P<0.01 vs. WT/LPS (Two-way ANOVA with PLSD).

Gene expression analysis of the pro-inflammatory cytokine IL-6

In male mice, LPS administration significantly affected also the expression of IL-6 ($F_{1,19}$=42,84, *P*=0.001). However, differently from the other pro-inflammatory cytokines examined, the inflammatory challenge decreased its mRNA levels, an effect observed in both the genotypes (WT/LPS -56% vs. WT/SAL, *P*<0.001; +/d/LPS -65% vs. +/d/SAL, *P*<0.001; Fig. 29A).

On the contrary, we did not observe any significant change in female mice (Fig. 29B).

Similarly to what observed in the hippocampus of male mice, the expression of IL-6 was significantly down-regulated by LPS also in the frontal lobe ($F_{1,19}$=123.9, *P*=0.001) of both wild-type (WT/LPS -77% vs. WT/SAL, *P*<0.001) and heterozygous (+/d /LPS -66% vs. +/d /SAL, *P*<0.001) male animals (Fig. 29C), an effect even greater in this brain region.

Once again, female mice did not show any significant change in the expression of IL-6 in all the experimental groups (Fig. 29D).

Gene expression analysis of the marker of microglia activation CD11b

We found that the mRNA levels of CD11b were significantly affected by the inflammatory challenge ($F_{1,23}$= 48.93, *P*=0.001) and by the genotype ($F_{1,23}$= 5.05, *P*=0.037) in the hippocampus of male mice. As shown in 30A, its expression was increased in both wild-type (WT/LPS +27% vs. WT/SAL, *P*<0.01) and BDNF heterozygous mice (+/d/LPS +60% vs. +/d/SAL, *P*<0.001), an effect significantly higher in the mutant animals (+/d/LPS +31% vs. WT/LPS, *P*<0.01) as indicated by the LPS*Genotype interaction ($F_{1,23}$=6.85, *P*=0.017).

In female mice, CD11b gene expression was up-regulated by LPS ($F_{1,23}$= 13.06 *P*=0.002) only in mutant animals (+/d/LPS +44% vs. +/d/SAL, *P*<0.001; Fig. 30B)

In the frontal lobe, we did not observe any significant modulation of the expression of CD11b by LPS or by the genotype, neither in male nor in female mice (Fig. 30C, D).

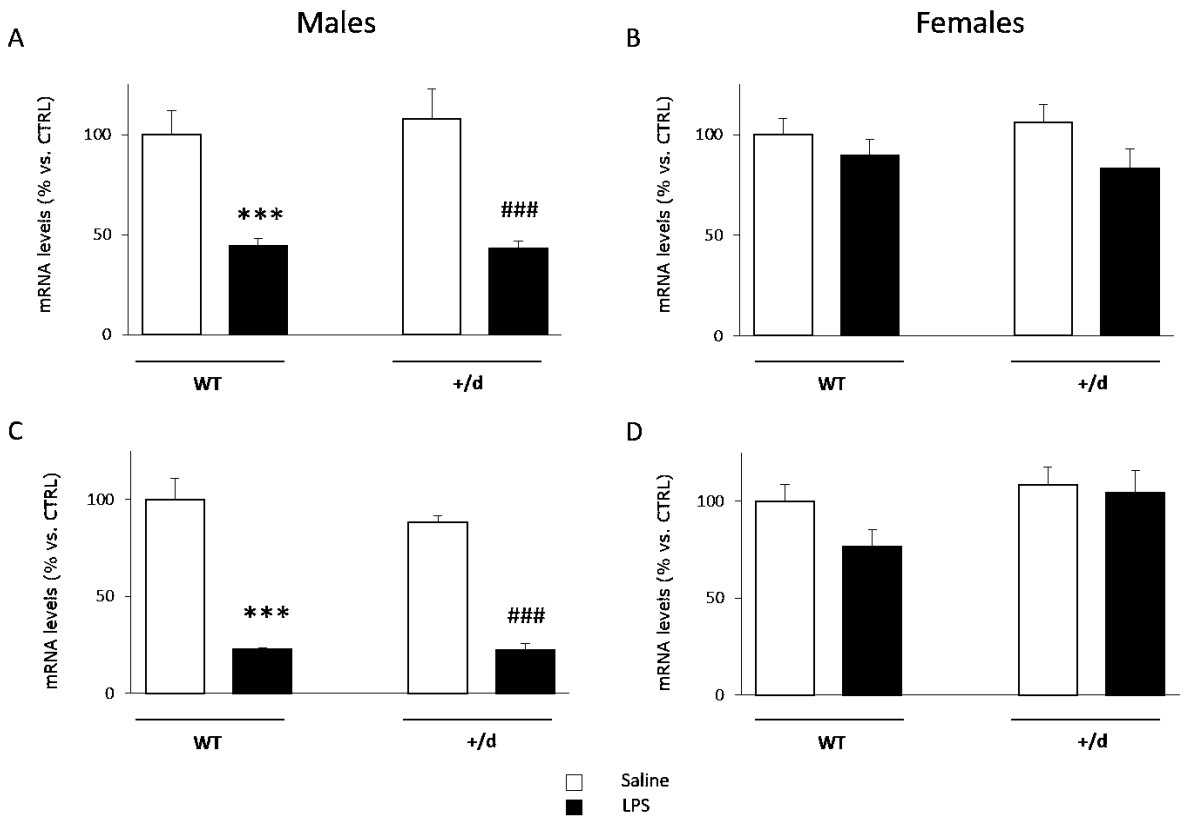


Figure 29. Gene expression analysis of IL-6 in the hippocampus and in the frontal lobe. The mRNA levels of the pro-inflammatory cytokine IL-6 were measured in the hippocampus and in the frontal lobe of males (A, C) and females (B, C) wild-type (WT) and BDNF heterozygous (+/d) mice 24 h after a single injection of lipopolysaccharide (LPS, 400 μg/kg i.p.) in comparison with mice treated with saline (SAL). The data, expressed as a percentage of the saline-injected wild-type mice (CTRL, set at 100%), represent the mean ± SEM of at least 6 independent determinations. ***P<0.001 vs. CTRL; $^{###}P$<0.001 vs. BDNF +/d; ^{++}P<0.01 vs. WT/LPS (Two-way ANOVA with PLSD).

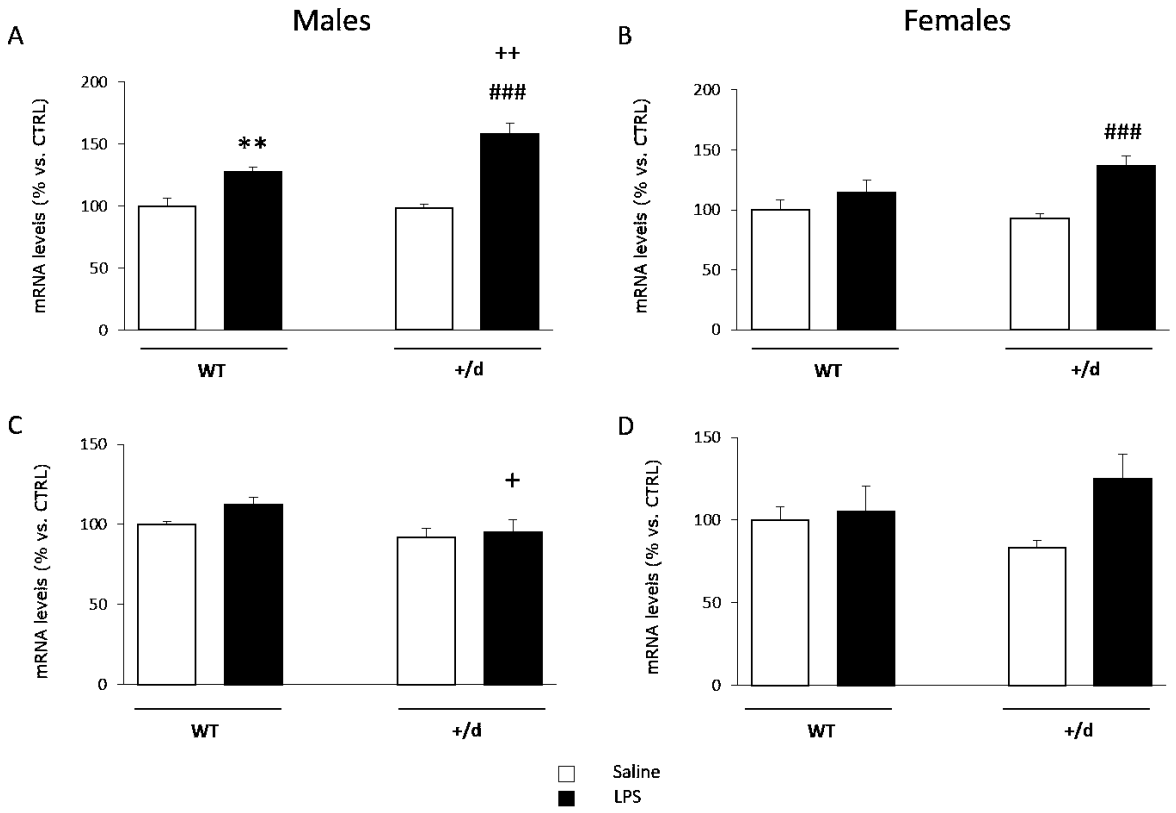


Figure 30. Gene expression analysis of CD11b in the hippocampus and in the frontal lobe. The mRNA levels of the pro-inflammatory cytokine CD11b were measured in the hippocampus and in the frontal lobe of males (A, C) and females (B, C) wild-type (WT) and BDNF heterozygous (+/d) mice 24 h after a single injection of lipopolysaccharide (LPS, 400 μg/kg i.p.) in comparison with mice treated with saline (SAL). The data, expressed as a percentage of the saline-injected wild-type mice (CTRL, set at 100%), represent the mean ± SEM of at least 6 independent determinations. $^{**}P<0.01$ vs. CTRL; $^{\#\#\#}P<0.001$ vs. BDNF +/d; $^{++}P<0.01$ vs. WT/LPS (Two-way ANOVA with PLSD).

Gene expression analysis fractalkine (CX3CL1) and its receptor (CX3CR1)

Despite a significant genotype effect ($F_{1,22}$=8.72, *P*=0.009), the gene expression of fractalkine in the hippocampus of male mice was not strongly modulated by our experimental paradigm. In fact, as shown in figure 31A, the basal level of CX3CL1 was significantly higher only in BDNF heterozygous mice with respect to control mice (+/d/SAL +36% vs. WT/SAL, *P*<0.01).

Conversely, LPS significantly reduced the mRNA levels of CX3CL1 in female mice ($F_{1,23}$=5.07, *P*=0.036), an effect observed only in only in wild-type animals (WT/LPS -16% vs. WT/SAL, *P*<0.05; Fig. 31B).

Despite the slight modulation of fractalkine, the inflammatory challenge shown a significant effect on its receptor in the hippocampus of male mice ($F_{1,22}$=34.17, *P*=0.001). In fact, the mRNA levels of CX3CR1 were significantly increased by LPS in both wild-type (WT/LPS +50% vs. WT/SAL, *P*<0.001) and BDNF heterozygous mice (+/d/LPS +45% vs. +/d/SAL, *P*<0.001; Fig 32A).

In female mice**,** the CX3CR1 mRNA levels were up-regulated following the treatment ($F_{1,24}$=5.54 *P*=0.029) only in heterozygous mice (+/d/LPS +22% vs. +/d/SAL, *P*<0.05; Fig 32B).

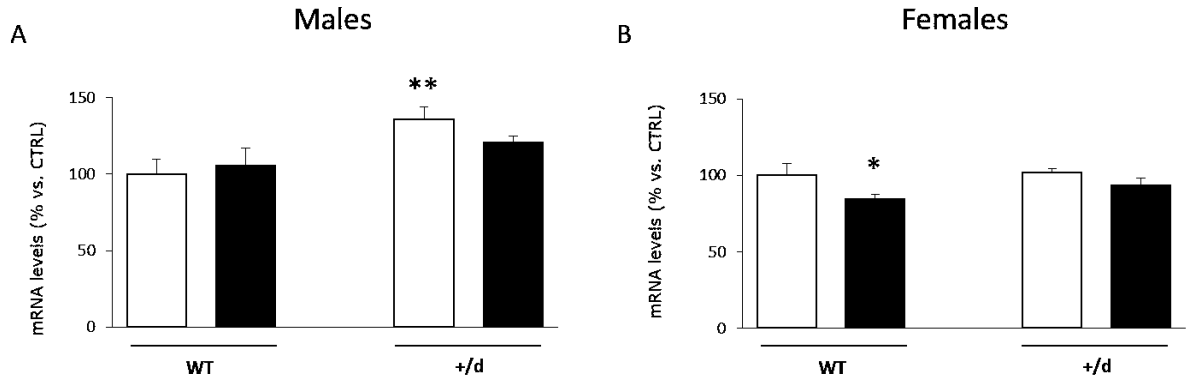


Figure 31. Gene expression analysis of CX3CL1 in the hippocampus. The mRNA levels of the pro-inflammatory cytokine CX3CL1 were measured in the hippocampus of male (A,) and female (B) wild-type (WT) and BDNF heterozygous (+/d) mice 24 h after a single injection of lipopolysaccharide (LPS, 400 μg/kg i.p.) in comparison with mice treated with saline (SAL). The data, expressed as a percentage of the saline-injected wild-type mice (CTRL, set at 100%), represent the mean ± SEM of at least 6 independent determinations. ***P*<0.01 vs. CTRL; **P*<0.05 vs. CTRL (Two-way ANOVA with PLSD).

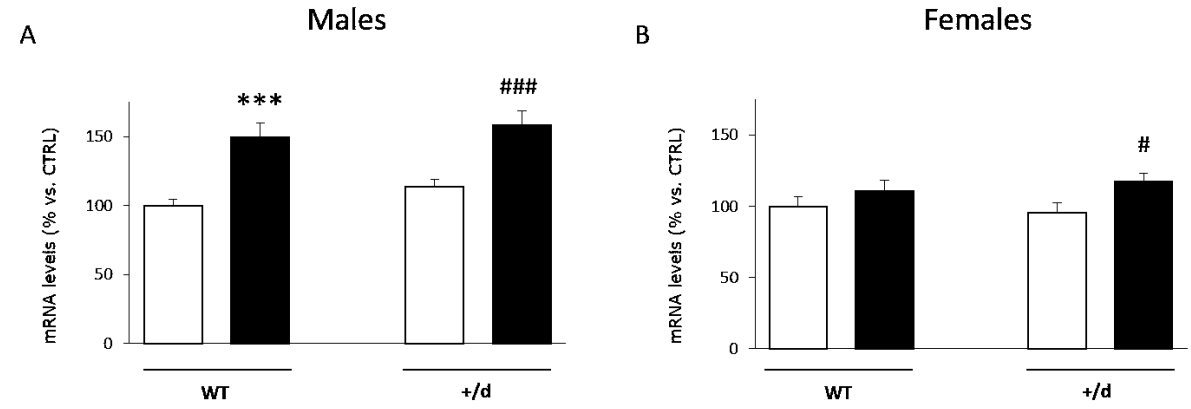


Figure 32. Gene expression analysis of CX3CR1 in the hippocampus. The mRNA levels of the pro-inflammatory cytokine CX3CR1 were measured in the hippocampus of male (A,) and female (B) wild-type (WT) and BDNF heterozygous (+/d) mice 24 h after a single injection of lipopolysaccharide (LPS, 400 μg/kg i.p.) in comparison with mice treated with saline (SAL). The data, expressed as a percentage of the saline-injected wild-type mice (CTRL, set at 100%), represent the mean ± SEM of at least 6 independent determinations. ****P*<0.001 vs. CTRL; #*P*<0.05, ###P<0.001 vs. BDNF +/d (two-way ANOVA with PLSD).

4.5.3 Discussion

The results of this study clearly indicate that a single systemic injection of LPS in mice induces a differential inflammatory response in male versus female animals and that this effect is influenced by BDNF expression and/or function. Specifically, we found a marked inflammatory response characterized by up-regulation of pro-inflammatory cytokines and microglia activation in male rats, an effect independent by the genotype. Conversely, a lower LPS impact was selectively observed in heterozygous animals without alteration in the wild-type. It has to be noted that the influence of a gender*genotype interaction on LPS response depends also by the brain region considered.

The differential response to the inflammatory challenge has been observed for several mediators of the immune/inflammatory systems such as pro-inflammatory cytokines, anti-inflammatory cytokines and marker for microglia activation, whereas only trends to modulation were observed at behavioral level. To this regard, it has been reported that the acute administration of LPS in rodents may induce 24 hours later a depressive phenotype characterized by anhedonia and behavioral despair (Frenois et al., 2007). However, in our experiment, we did not observe such a phenotype by using the Porsolt test and similarly we did not find an anxious phenotype by testing the LPS-received animals in the open field. Although the lack of behavioral effect in our experiment, that may be due to the different dosage of LPS used, the inflammatory challenge may be a useful tool to unmask -at molecular level- differences between the experimental groups. Indeed, we found a differential modulation of the inflammatory system in male and female LPS-treated animals. Indeed, in line with the literature, female seems to be “protected” to the inflammatory challenge, and one possible explanation is that the involvement of the hormonal status in which estrogens may play a key role. It is well documented that estrogens inhibit inflammatory response within the brain (Arevalo et al., 2012) and it has been recently demonstrated that estrogens deficiency induced in female rats by ovariectomy is associated with depressive-like phenotype and increased levels of inflammatory mediators at cerebral levels (Xu et al., 2015). The involvement of these hormones is particularly interesting since It is well known that women may be more susceptible to develop depressive disorders than men and that the effect of estrogen on mood have well established. For example, it has been reported that the reduction of circulating estrogens during menopause is associated with the insurgence of mood

disorders in women (Freeman, 2010). Moreover, several data indicate that estrogen may have a protective role in different harmful conditions. For example, estrogens both protect against the detrimental effects of repeated stress in females, and prevents the stress-induced impairments when administered to males (Wei et al., 2014). This suggests that the stress hormone corticosterone and estrogen interact leading to a fine tuning of functional plasticity. In this context, it is interesting to note that local brain synthesis of estrogen from endogenous cholesterol, through the action of neuronal aromatases, could play a role in the modulation of neurotransmission in response to repeated stress. Indeed, it was shown that the inhibition of aromatase in female rats resulted in the loss of protection against neural and behavioral consequences of chronic stress, thus suggesting that central estrogen production is necessary for the protective action of estrogen.

Another possibility is the involvement of Toll-like receptor-4, which mediated the inflammatory action of LPS. Among the cells that express TLR-4 there are astrocytes. These cells show sex differences in number, differentiation and function and since are involved in the response to injury and inflammation, they may participate in the generation of sex differences in the response of the brain to LPS. In line with this hypothesis, LPS-induced pro-inflammatory cytokine up-regulation was higher in astrocytes derived from male or androgenized females in comparison to astrocytes derived from control or vehicle- injected female rats (Santos-Galindo et al., 2011).

However, we did not find changes on the basal expression of TLR-4 between male and female wild-type animals. Nevertheless, beside the mechanisms underlying the gender effect, we have to consider its interaction with the genotype. Specifically, the lack of 50% of BDNF in female seems to “consent” the inflammatory response and further studies are demanded to investigate how BDNF system may interact with estrogens.

5. Summary and conclusions

In conclusion, the results obtained during my Ph.D. add new preclinical evidence about the association between stress-related disorders -such as major depression- and alterations in the inflammatory system within the brain. By using different approaches, i.e. rats exposed to chronic stress or treated with lipopolysaccharide, antidepressant treatment, mice with partial deletion of the neurotrophin Brain-derived neurotrophic factor, we strengthened the idea of a direct involvement of neuroinflammation in behavioral alterations associated to psychopathology and brought to new insights on the molecular effects of antidepressant drugs in the context of modulation of the inflammatory response.

Indeed, in the chronic mild stress study, we demonstrated that only the anhedonic-like phenotype correlates with neuroinflammation, in terms of increased expression of pro-inflammatory mediators such as IL-1β, IL-6 and the marker of microglial activation CD11b. These effects suggest a direct involvement of that neuroinflammation in the development of the depressive-like behavior, rather than being an adaptive response to of stress exposure. This idea has been supported by our data on LPS administration in animals exposed to CMS: on one side the susceptibility to LPS in animals not exposed to CMS enlightened the important contribution of the inflammatory system in the insurgence of behavioral alterations, in particular taking into account that the impairment in sucrose intake persisted for six days after the immune challenge. On the other, the apparent resistance to the LPS-induced neuroinflammation in stress-Resilient rats may suggest that inhibition of the inflammatory response may be one crucial mechanism underpinning stress resiliency. More in details, we pointed out that microglia is crucial for the development of altered behavior in stress-vulnerable animals challenged with the cytokine inducer. Lipopolysaccharide, indeed, was a crucial tool to unmask the molecular differences between stress-Resilient rats and animals with decreased sucrose consumption, leading to the finding that the prolonged activation of microglia after the immune challenge seems strictly related to the decrease of sucrose intake. We hypothesize that the long-lasting behavioral effects of LPS may be due to alterations in microglia cross-talk with neurons (through the on/off signaling) or to an impaired M1/M2 polarization of these cells, thus enlightening the role of microglia in modulating stress resilience.

Among the molecules examined, we found that IL-1β -as the mostly up-regulated cytokine analyzed- may possibly play a pivotal role in the insurgence of depressive-like phenotype, especially in the dorsal hippocampus. Indeed, this cytokine has been also the main target of chronic pharmacological treatment of the drugs administered to animals exposed to CMS. In this context, we found that drugs characterized by different mechanisms of action were able to normalize the decrease of sucrose intake and ameliorate the neuroinflammatory signature observed in CMS rats. In fact, an overall dampening of stress-induced neuroinflammation was observed following chronic treatment with the tricyclic antidepressant imipramine, with the antidepressant agomelatine and with the antipsychotic lurasidone, thus suggesting that the regulation of the immune response within the brain may contribute to the therapeutic activity of these drugs.

With the aim of elucidating the molecular targets of the modulation of inflammation within brain areas involved in major depression, we focused our attention of the feedback inhibition system of the IL-6 signaling as a potential target of antidepressant drugs. Considering that IL-6 was mainly modulated by agomelatine, we investigated the impact of chronic administration of the drug on stressed animals. Our laboratory already demonstrated that agomelatine possesses anti-inflammatory properties, but its specific mechanisms of action in an inflammatory context are still elusive. Considering the observed effects of agomelatine activity on the IL-6 pathway, we propose that the antidepressant may be able to potentiate the feedback inhibition via the up-regulation of SOCS3 gene and protein expression. Although further studies are demanded to better understand the exact mechanism of action of how agomelatine acts on this system, the modulation of SOCS3 appears promising in the context of immune modulation exerted by antidepressant drugs, in particular on the fine-tuning of IL-6 signaling.

Another aspect that we analyzed in the context of molecular activity of antidepressant drugs on neuroinflammation was the genome-wide study on agomelatine activity. We found interesting data about the potential involvement of the pathway linked to chemokine receptor CXCR4 in the basal activity of agomelatine and on the anti-oxidant effect of the drug in animals challenged with lipopolysaccharide. In details, we found that the drug was able to modulate pathways related to synthesis, generation and production of reactive oxygen species. By regulating these pathways, agomelatine could potentially contribute to the modulation of the

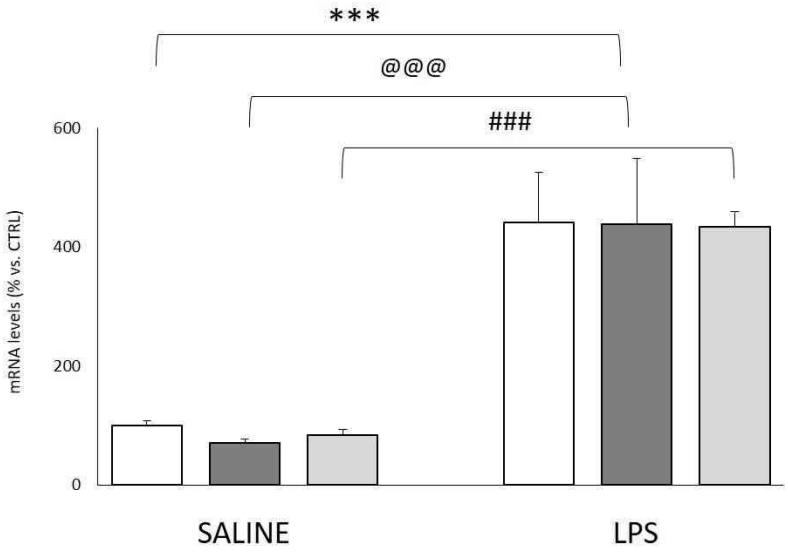
oxidative stress associated to the inflammatory response. The diverse comparisons that we made between the list of modulated genes in the different experimental groups led us to the identification of nine transcripts, potentially involved in the anti-inflammatory and protective activity of agomelatine. Among them it was interesting to find the transcript of IL-1β, thus confirming the data obtained so far on the pro-inflammatory cytokine. Moreover, the microarray study enlightened the metabotropic glutamate receptor 2 (Grm2) as a potential pharmacological target of antidepressant in the context of neuroinflammation. This result, in light of the data presented in this work, appears promising considering that this receptor may be involved in the control of microglia activation.

Lastly, another important point addressed during my Ph.D. was to investigate the potential mutual influence between alteration of inflammatory system and molecular systems known to be involved in depression pathophysiology. In this context, considering the recognized importance of BDNF in the etiology of major depression and on the therapeutic activity of psychotropic drugs, we evaluated the possible interaction between this neurotrophin and the immune response. Indeed, it is known that this complex disorder affects multiple systems i.e. molecules involved in neurotransmission, hormones and mediators of neuronal plasticity and among them the neurotrophin brain-derived neurotrophic factor (BDNF) plays a crucial role. BDNF levels are reduced in depressed subjects and its modulation represents a key step in long-term adaptive changes brought about by antidepressant drugs. In addition, microglial cells, which represents one of the critical component of the inflammatory response express BDNF mRNA, secrete the neurotrophin following stimulation and their function are regulated by BDNF. On this basis, the aim of this study was to establish if BDNF dysfunctions were associated with alteration of the inflammatory system and if inflammatory response was exacerbated under condition of impaired BDNF function. In line with the literature, we found that a single systemic injection of LPS in mice induced a differential inflammatory response in male versus female mice. However, interestingly this effect is influenced by BDNF expression and/or function. Specifically, we found a marked inflammatory response characterized by up-regulation of pro-inflammatory cytokines and microglia activation in male rats, an effect independent by the genotype. Conversely, a lower LPS impact was selectively observed in heterozygous animals without alteration in the wild-type. One possible explanation is that the differential inflammatory response observed in the two different genders is driven by the

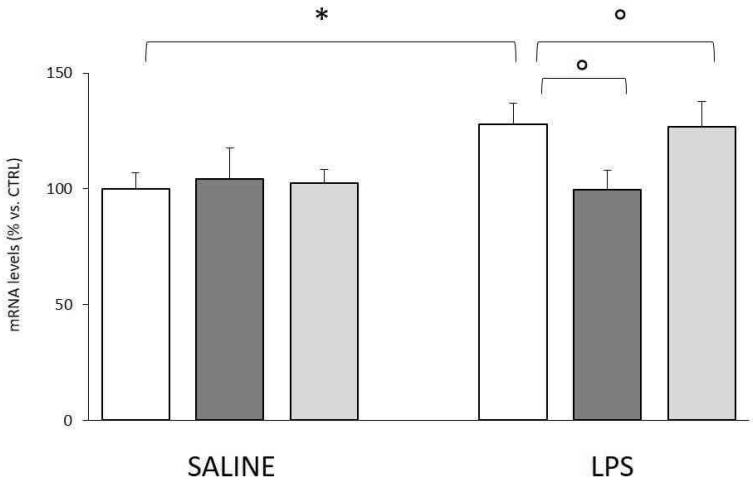
hormonal status in which estrogens may play a key role. Specifically, the lack of 50% of BDNF seems to “consent” the inflammatory response and further studies are demanded to investigate how BDNF system may interact with estrogens.

In conclusion, the results obtained during my PhD thesis strongly sustain the involvement of neuroinflammation in the insurgence of depressive like phenotype and on the activity of diverse antidepressant drugs. In addition, we support the idea of a dramatic role of microglia in the regulation of stress response, in particular in term of resilience. This aspect definitely needs to be pursued especially in terms of pharmacological research of new potential targets for the treatment of major depression and stress-related disorders.

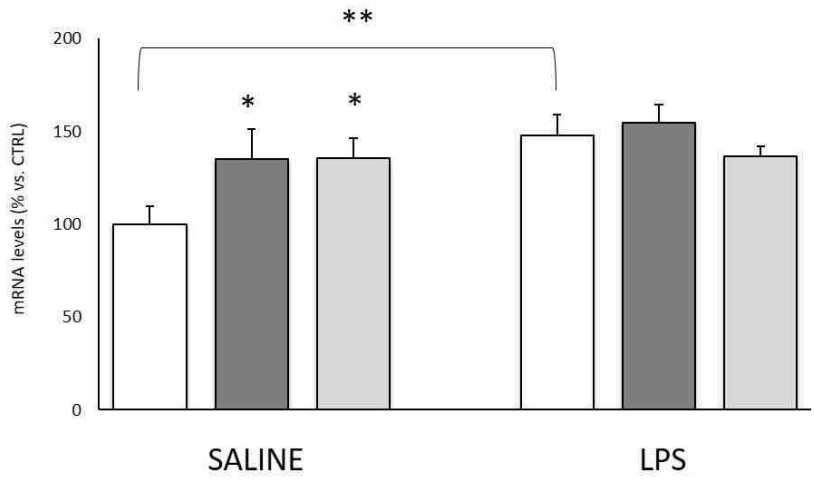
6. Appendix - Supplementary figures



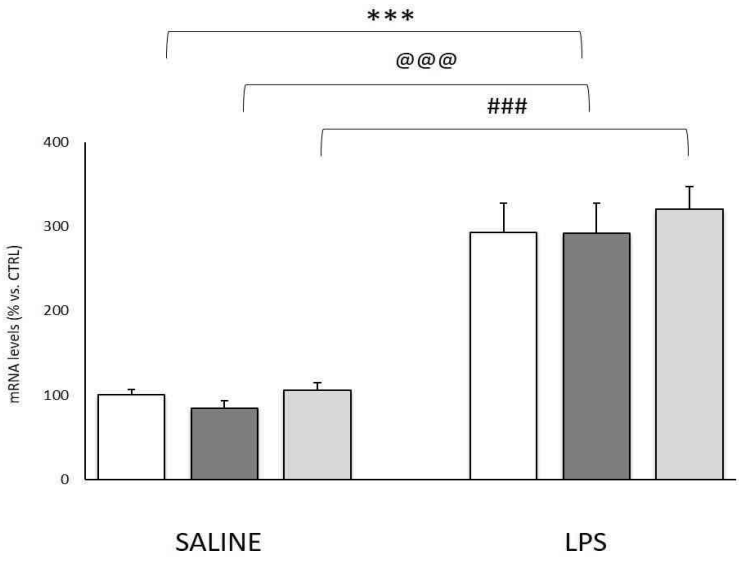
Supplementary figure 1. Gene expression analysis of IL-1β in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of IL-1 β were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (****P*<0.001 vs. CTRL; @@@*P*<0.001 vs RESPONSIVE; ###*P*<0.001 vs. RESILIENT; Two-way ANOVA with post hoc LSD).



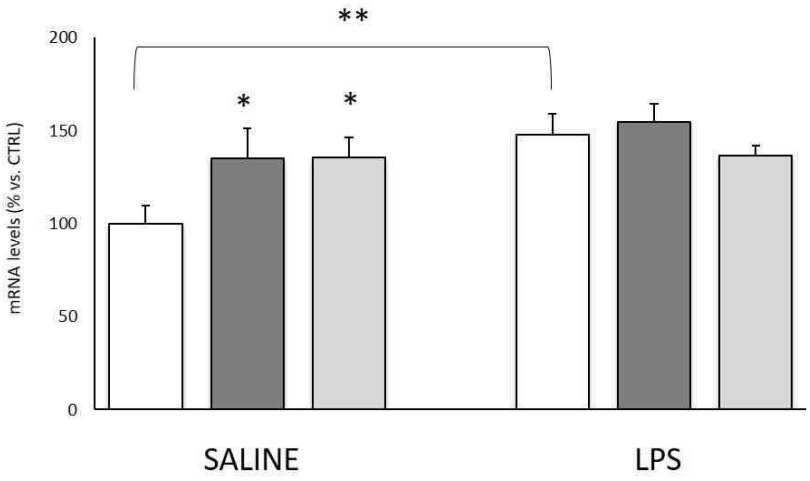
Supplementary figure 2. Gene expression analysis of IL-6 in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of IL-6 were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (*P<0.05 vs. CTRL; °P<0.05 vs. LPS; Two-way ANOVA with post hoc LSD).



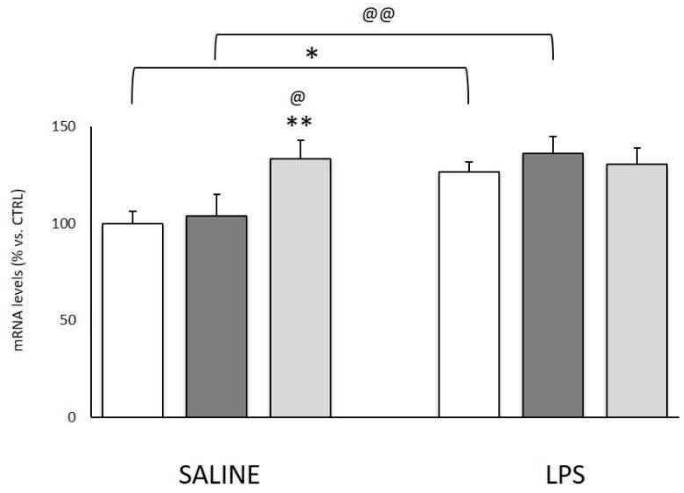
Supplementary figure 3. Gene expression analysis of TNF-a in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of *TNF-a* were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (**P*<0.05, **P<0.01 vs. CTRL; Two-way ANOVA with post hoc LSD).



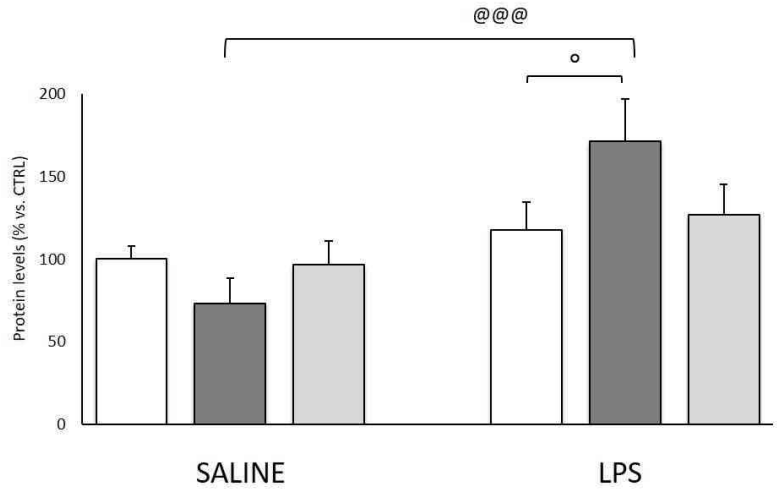
Supplementary figure 4. Gene expression analysis of CD11b in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of CD11b were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (***P<0.001 vs. CTRL; @@@P<0.001 vs RESPONSIVE; ##P<0.001 vs. RESILIENT; Two-way ANOVA with post hoc LSD).



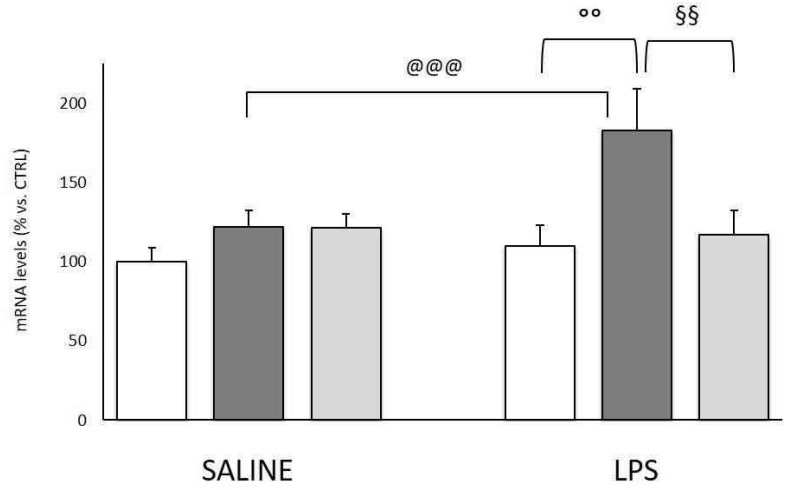
Supplementary figure 5 Gene expression analysis of GFAP in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of GFAP were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (**P*<0.05, ***P*<0.01 vs. CTRL; Two-way ANOVA with post hoc LSD).



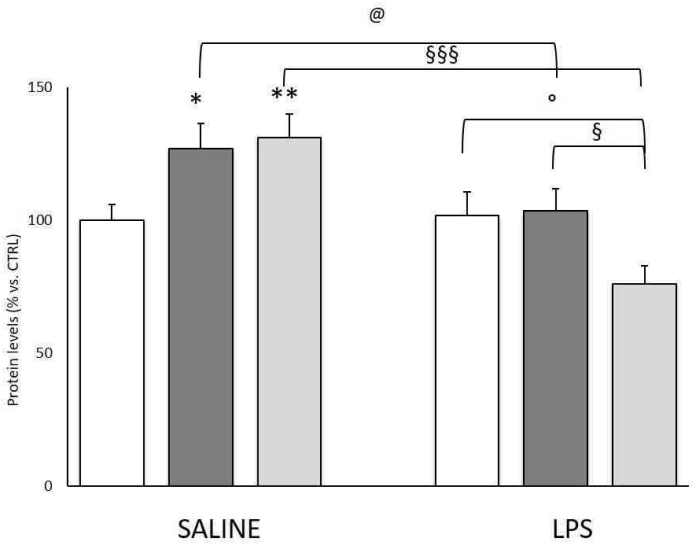
Supplementary figure 6. Gene expression analysis of TLR-4 in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of TLR-4 were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (*P<0.05, **P<0.01 vs. CTRL; @P<0.05, @@P<0.01 vs RESPONSIVE; Two-way ANOVA with post hoc LSD).



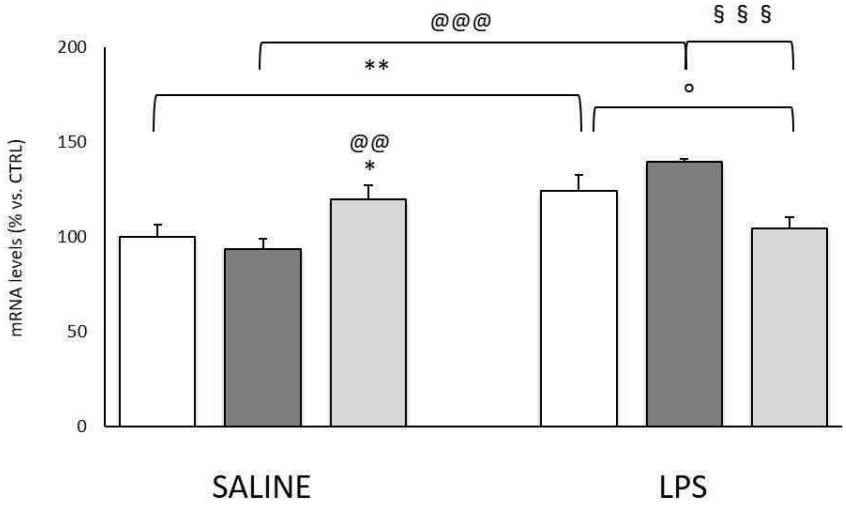
Supplementary figure 7. Protein levels analysis of TLR-4 in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The protein levels of TLR-4 were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (@@@P<0.001 vs RESPONSIVE; °P<0.05 vs. LPS; Two-way ANOVA with post hoc LSD).



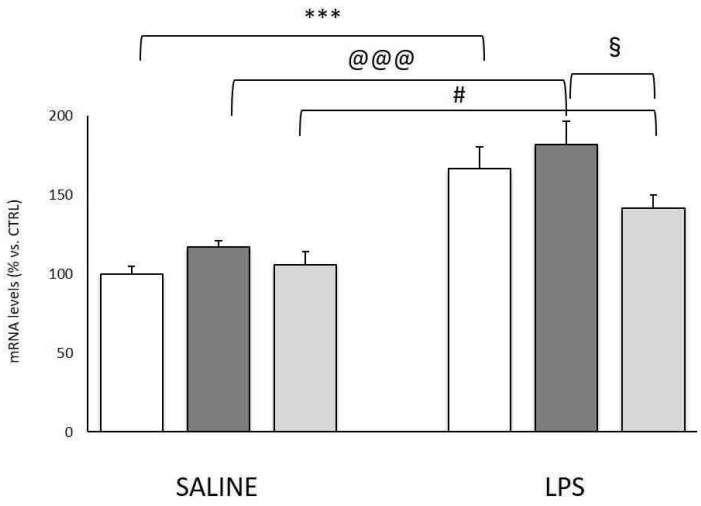
Supplementary figure 8. Gene expression analysis of IL-1β in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of IL-1 β were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (@@@*P*<0.001 vs RESPONSIVE; °°*P*<0.01 vs. LPS; §§P<0.01 vs. RESILIENT/LPS Two-way ANOVA with post hoc LSD).



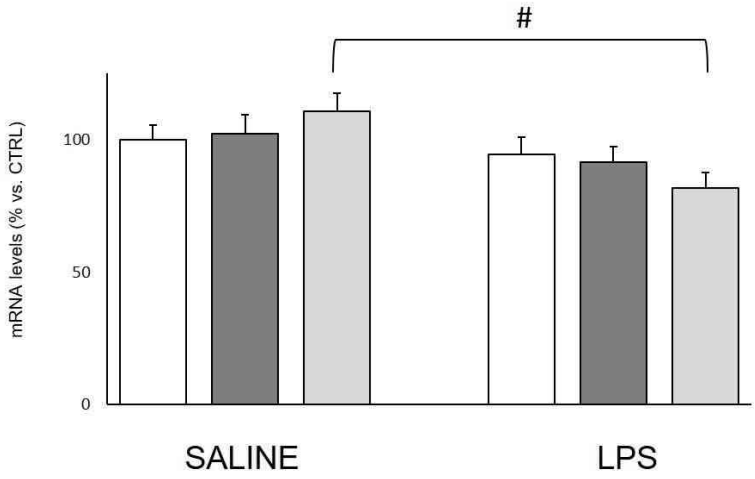
Supplementary figure 9. Protein levels analysis of IBA1 in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The protein levels of IBA1 were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (*P<0.05, **P<0.01 vs CTRL; @P<0.05 vs RESPONSIVE; °P<0.05 vs. LPS; §P<0.05, §§§P<0.001 vs. RESILIENT Two-way ANOVA with post hoc LSD)



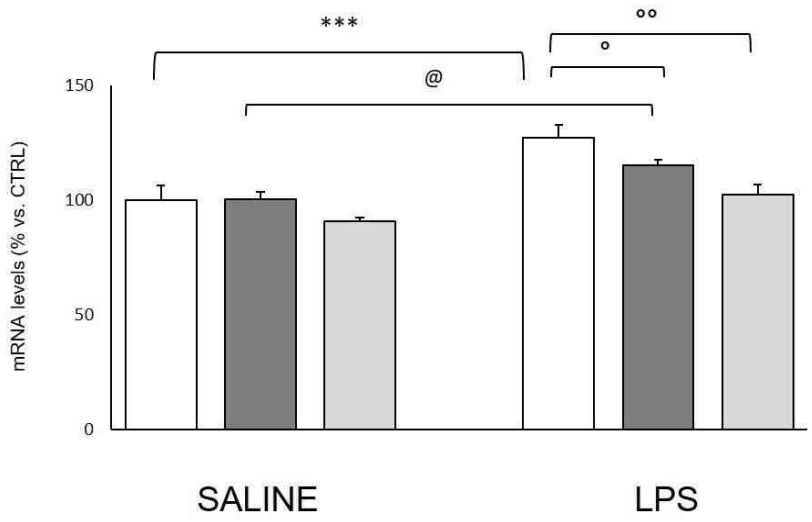
Supplementary figure 10. Gene expression analysis of CD11b in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of CD11b were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (**P<0.01 vs. CTRL; @@P<0.01, @@@P<0.001 vs RESPONSIVE; °P<0.05 vs. LPS; §§§P<0.001 vs. RESILIENT Two-way ANOVA with post hoc LSD.



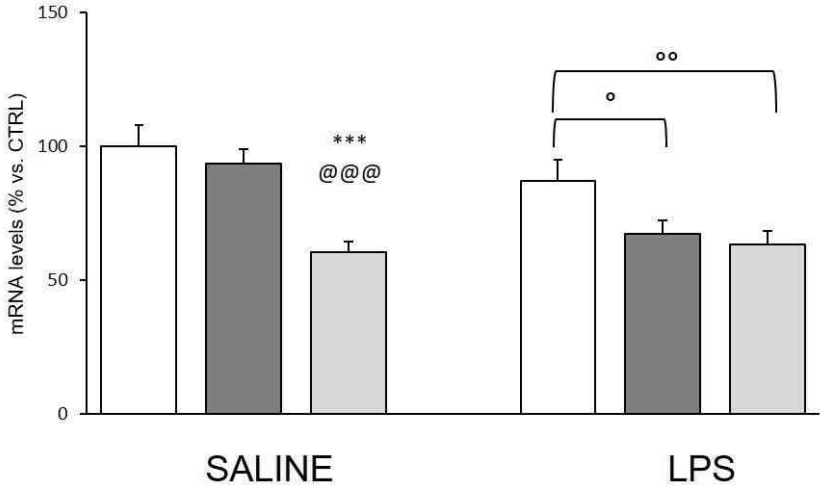
Supplementary figure 11. Gene expression analysis of CD68 in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of CD68 were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (***P<0.001 vs. CTRL; @@@P<0.001 vs RESPONSIVE; §P<0.05 vs. RESILIENT; Two-way ANOVA with post hoc LSD).



Supplementary figure 12. Gene expression analysis of CX3CL1 in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of CX3CL1 were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (§P<0.05 vs. RESILIENT; Two-way ANOVA with post hoc LSD).



Supplementary figure 13. Gene expression analysis of CX3CR1 in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of CX3CR1 were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (***P<0.001 vs. CTRL; @P<0.05 vs. RESPONSIVE; °P<0.05, °°P<0.01 vs. LPS; Two-way ANOVA with post hoc LSD).



Supplementary figure14. Gene expression analysis of Arg1 in dorsal hippocampus of animals exposed to chronic stress and subsequent administration of LPS. The mRNA levels of Arg1 were measured in the rat dorsal hippocampus of animals exposed or not to chronic stress and treated with saline or LPS. Data are expressed as percentages with respect to saline-treated non stressed animals and they represent the average ± SEM. (***P<0.001 vs. CTRL; @@@P<0.001 vs. RESPONSIVE; °P<0.05, °°P<0.01 vs. LPS; Two-way ANOVA with post hoc LSD).

Supplementary tables

Supplementary Table 1 Summary of the effects of chronic mild stress (CMS) and treatment with imipramine on sucrose preference. Animals were exposed for 7 weeks to CMS procedure that was combined during the last 5 weeks with intraperitoneal injections (i.p.) of vehicle (hydroxyethylcellulose, HEC 1%) or imipramine (10 mg/kg daily). The table shows the weekly sucrose intake (g) and the corresponding *P* value obtained by Two-way ANOVA and PLSD test. The "baseline" values refer to the sucrose intake of the animals before the stress procedure.

Drug	Week	Experimental Group	Sucrose Intake (g)	P-Value Vs. CTRL	P-Value Vs. CMS
IMIPRAMINE	BASELINE	CTRL	10,2	-	-
		Imipramine	11,2	-	-
		CMS	11,1	-	-
		CMS+IMI	12,4	-	-
	0	CTRL	11,4	-	-
		Imipramine	11,8	0,733	-
		CMS	6,8	**0,001 *** **	-
		CMS+IMI	6,8	-	0,982
	1	CTRL	9,6	-	-
		Imipramine	11,2	0,329	-
		CMS	4,2	**0,002 ****	-
		CMS+ IMI	7,5	-	**0,041 #**
	2	CTRL	11,4	-	-
		Imipramine	11,6	0,940	-
		CMS	6,6	**0,008 ****	-
		CMS+ IMI	11	-	**0,014 #**
	3	CTRL	10	-	-
		Imipramine	10,4	0,817	-
		CMS	5	**0,003 ****	-
		CMS+IMI	8,9	-	**0,016 #**
	4	CTRL	10,1	-	-
		Imipramine	10,7	0,691	-
		CMS	6,8	**0,039 ***	-
		CMS+ IMI	10,9	-	**0,012 #**
	5	CTRL	12	-	-
		Imipramine	11,7	0,834	-
		CMS	6,9	**0,002 ****	-
		CMS+ IMI	11,8	-	**0,002 ##**

Supplementary Table 2 Summary of the effects of chronic mild stress (CMS) and treatment with agomelatine on sucrose preference. Animals were exposed for 7 weeks to CMS procedure that was combined during the last 5 weeks with intraperitoneal injections (i.p.) of vehicle (hydroxyethylcellulose, HEC 1%) or agomelatine (40 mg/kg daily). The table shows the weekly sucrose intake (g) and the corresponding *P* value obtained by Two-way ANOVA and PLSD test. The "baseline" values refer to the sucrose intake of the animals before the stress procedure.

Drug	Week	Experimental Group	Sucrose Intake (g)	P-Value Vs. CTRL	P-Value Vs. CMS
AGOMELATINE	BASELINE	CTRL	10,2	-	-
		Agomelatine	11,2	-	-
		CMS	11,1	-	-
		CMS+ AGO	11,6	-	-
	0	CTRL	11,4	-	-
		Agomelatine	11,78	0,832	-
		CMS	6,8	**0,002 ****	-
		CMS+ AGO	6,8	-	1,000
	1	CTRL	9,6	-	-
		Agomelatine	10	0,758	-
		CMS	4,2	**0,000 *****	-
		CMS+AGO	8,8	-	**0,002 ##**
	2	CTRL	11,4	-	-
		Agomelatine	12,7	0,463	-
		CMS	6,6	**0,006 ****	-
		CMS+ AGO	9,4	-	0,105
	3	CTRL	10	-	-
		Agomelatine	12,4	0,093	-
		CMS	5	**0,001 *****	-
		CMS+ AGO	9,2	-	**0,004 ##**
	4	CTRL	10,1	-	-
		Agomelatine	13,3	0,033	-
		CMS	6,8	**0,029 ***	**0,004 ##**
		CMS+AGO	11,2	-	-
	5	CTRL	12	-	-
		Agomelatine	12,4	0,814	-
		CMS	6,9	**0,004 ****	-
		CMS+ AGO	12,2	-	**0,003 ##**

Supplementary Table 3 Summary of the effects of chronic mild stress (CMS) and treatment with lurasidone on sucrose preference. Animals were exposed for 7 weeks to CMS procedure that was combined during the last 5 weeks with oral administration (by gavage) of vehicle (hydroxyethylcellulose, HEC 1%) or lurasidone (3 mg/kg daily). The table shows the weekly sucrose intake (g) and the corresponding *P* value obtained by Two-way ANOVA and PLSD test. The "baseline" values refer to the sucrose intake of the animals before the stress procedure

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Drug	Week	Experimental Group	Sucrose Intake (g)	P-Value Vs. CTRL	P-Value Vs. CMS
LURASIDONE	BASELINE	CTRL	11,3	-	-
		Lurasidone	11,9	-	-
		CMS	11,7	-	-
		CMS+ LUR	12	-	-
	0	CTRL	11,4	-	-
		Lurasidone	11,9	0,704	-
		CMS	6,5	**0,000 *** **	-
		CMS+ LUR	6,9	-	0,737
	1	CTRL	9,6	-	-
		Lurasidone	10,4	0,655	-
		CMS	5	**0,005 ** **	-
		CMS+ LUR	6,9		-0,220
	2	CTRL	11,1	-	-
		Lurasidone	11,8	0,661	-
		CMS	7,5	**0,024 ***	-
		CMS+LUR	10,3	-	0,075
	3	CTRL	12,5	-	-
		Lurasidone	13	0,714	-
		CMS	5,9	**0,000 *** **	-
		CMS+ LUR	13,2	-	**0,000 ###**
	4	CTRL	11,6	-	-
		Lurasidone	12,4	0,64	-
		CMS	6,6	**0,003 ** **	-
		CMS+ LUR	11	-	**0,01 ** **
	5	CTRL	12,3	-	-
		Lurasidone	12,7	0,816	-
		CMS	6	**0,000 *** **	-
		CMS+LUR	12,1	-	**0,000 ###**

Supplementary Table 4 Summary of the effects of chronic mild stress (CMS) and pharmacological treatment on the mRNA levels of the pro-inflammatory cytokines IL-1β and IL-6 and the anti-inflammatory cytokine TGF-β in the rat dorsal hippocampus. Animals were exposed for 7 weeks to CMS procedure that was combined during the last 5 weeks with intraperitoneal injections (i.p.) of vehicle (hydroxyethylcellulose, HEC 1%) or imipramine (10 mg/kg daily) or agomelatine (40 mg/kg daily). Another groups of animals received oral administration (by gavage) of vehicle (HEC 1%) or lurasidone (3 mg/kg daily). 24 hours after the last drug administration rats were killed by decapitation and the dorsal hippocampus was rapidly dissected for the molecular analyses. The table shows the percentage of change for each inflammatory protein and the corresponding *P* value obtained by Two-way ANOVA and PLSD test.

Drug	Gene (mRNA)	Experimental Group	% change (of CTRL)	P-Value Vs. CTRL	P-Value Vs. CMS	Figure
IMIPRAMINE	IL-1β	CTRL	0	-	-	Fig. 7A
		Imipramine	-21	0,261	-	
		CMS	+45	**0,033 ***	-	
		CMS+IMI	-19	-	**0,002 ##**	
AGOMELATINE		CTRL	0	-	-	Fig. 7D
		Agomelatine	-36	**0,014 ***	-	
		CMS	+45	**0,007 ****	-	
		CMS+AGO	-31	-	**0,000 ###**	
LURASIDONE		CTRL	0	-	-	Fig. 7G
		Lurasidone	+15	0,245	-	
		CMS	+35	**0,016 ***	-	
		CMS+LUR	+3	-	**0,34 #**	
IMIPRAMINE	IL-6	CTRL	0	-	-	Fig. 7B
		Imipramine	+26	0,119	-	
		CMS	+38	**0,018 ***	-	
		CMS+IMI	+15	-	0,191	
AGOMELATINE		CTRL	0	-	-	Fig. 7E
		Agomelatine	+119	0,214	-	
		CMS	+138	**0,012 ***	-	
		CMS+AGO	+131	-	0,641	
LURASIDONE		CTRL	0	-	-	Fig. 7H
		Lurasidone	+23	0,137	-	
		CMS	+32	**0,042 ***	-	
		CMS+LUR	+18	-	0,374	
IMIPRAMINE	TGF-β	CTRL	0	-	-	Fig. 7C
		Imipramine	-16	**0,001 *****	-	
		CMS	-13	**0,021 ****	-	
		CMS+IMI	-9	-	0,26	
AGOMELATINE		CTRL	0	-	-	Fig. 7F
		Agomelatine	-11	0,073	-	
		CMS	-13	**0,043 ***	-	
		CMS+AGO	-16	-	0,666	
LURASIDONE		CTRL	0	-	-	Fig. 7I
		Lurasidone	-1	0,822	-	
		CMS	-16	**0,021 ***	-	
		CMS+LUR	-13	-	0,705	

Supplementary Table 5 Summary of the effects of chronic mild stress (CMS) and pharmacological treatment on the mRNA levels of CD11b, marker of microglia activation, fractalkine (CX3CL1) and its receptor (CX3CR1) as regulators of neuron-microglia cross-talk in the rat dorsal hippocampus. Animals were exposed for 7 weeks to CMS procedure that was combined during the last 5 weeks with intraperitoneal injections (i.p.) of vehicle (hydroxyethyl cellulose, HEC 1%) or imipramine (10 mg/kg daily) or agomelatine (40 mg/kg daily). Another groups of animals received oral administration (by gavage) of vehicle (HEC 1%) or lurasidone (3 mg/kg daily). 24 hours after the last drug administration rats were killed by decapitation and the dorsal hippocampus was rapidly dissected for the molecular analyses. The table shows the percentage of change for each inflammatory protein and the corresponding *P* value obtained by Two-way ANOVA and PLSD test.

Drug	Gene (mRNA)	Experimental Group	% change (of CTRL)	P-Value Vs. CTRL	P-Value Vs. CMS	Figure
IMIPRAMINE	CD11b	CTRL	0	-	**-**	Fig. 8A
		Imipramine	-11	0,351	**-**	
		CMS	+50	**0,000 *** **	-	
		CMS+IMI	-3	-	**0,000 ###**	
AGOMELATINE		CTRL	0	-	**-**	Fig. 8D
		Agomelatine	+10	0,547	**-**	
		CMS	+50	**0,005 ** **	-	
		CMS+AGO	+31	-	0,263	
LURASIDONE		CTRL	0	-	**-**	Fig. 8G
		Lurasidone	-1	0,969	**-**	
		CMS	+40	**0,007 ** **	-	
		CMS+LUR	+16	-	0,097	
IMIPRAMINE	CX3CL1	CTRL	0	-	-	Fig. 8B
		Imipramine	+11	0,099	**-**	
		CMS	-15	**0,028 ***	-	
		CMS+IMI	-5	-	0,104	
AGOMELATINE		CTRL	0	-	-	Fig. 8E
		Agomelatine	-10	0,148	-	
		CMS	-15	**0,039 ***	-	
		CMS+AGO	-20	**-**	0,475	
LURASIDONE		CTRL	0	-	-	Fig. 8H
		Lurasidone	-5	0,385	-	
		CMS	-16	**0,012 ***	-	
		CMS+LUR	+12	-	**0,000 ###**	
IMIPRAMINE	CX3CR1	CTRL	0	-	-	Fig. 8C
		Imipramine	-20	**0,005 ** **	**-**	
		CMS	-5	0,483	-	
		CMS+IMI	-14	**-**	0,167	
AGOMELATINE		CTRL	0	-	-	Fig. 8F
		Agomelatine	-12	0,079	-	
		CMS	-5	0,482	-	
		CMS+AGO	-3	-	0,831	
LURASIDONE		CTRL	0	-	-	Fig. 8I
		Lurasidone	-17	**0,005 ** **	**-**	
		CMS	-5	0,401	-	
		CMS+LUR	-25	**-**	**0,001 ###**	

Supplementary Table 6 List of the 105 genes differentially expressed in the ventral hippocampus of animals chronically treated with agomelatine with respect to the rats that received only the vehicle (fold-change cut-off: ±1.2; p<0.05 here presented as -log(p Value)).

Gene Symbol	p-value (AGO/SAL vs. VEH/SAL)	Fold-Change (AGO/SAL vs. VEH/SAL)
Hist1h4m	0,010	1,66
Gpx3	0,008	1,58
Fau	0,018	1,48
Zdhhc22	0,009	1,40
Npas4	0,021	1,38
Krtap4-3	0,005	1,38
Hist2h2ab	0,047	1,36
Romo1	0,000	1,36
Fstl4	0,002	1,36
Slc35c2	0,014	1,34
Tnxb	0,029	1,33
Rpl36a-ps4	0,000	1,32
Ndufv3	0,000	1,32
B3gat3	0,002	1,30
Ervfrd-1	0,003	1,30
Slc2a5	0,003	1,29
Rtbdn	0,001	1,28
Ndufa7	0,007	1,28
Prokr2	0,004	1,27
Tmem14c	0,007	1,27
Myl12b	0,000	1,26
Rnf208	0,028	1,26
Gng13	0,003	1,25
Kcna3	0,004	1,25
Olr1332	0,045	1,25
Mgp	0,046	1,24
Ubfd1	0,001	1,24
Panx2	0,001	1,24
Ppia	0,002	1,24
Fam43a	0,017	1,24
Cln6	0,000	1,23
Pitx1	0,009	1,23
Jph4	0,000	1,23
Samd4b	0,001	1,23
Zfp580	0,016	1,23
Fam100a	0,000	1,23
Cort	0,040	1,23
Wiz	0,000	1,23

Fam57b	0,001	1,23
Kcnk9	0,005	1,23
Rgma	0,012	1,22
Sh2d3c	0,000	1,22
Adam19	0,004	1,22
Qars	0,002	1,22
Mef2d	0,000	1,22
Gpr137	0,010	1,22
Pom121	0,006	1,22
Sh2b3	0,004	1,22
Wnk2	0,005	1,21
Slc39a13	0,004	1,21
Mark4	0,040	1,21
Mrc2	0,000	1,21
Wdsub1	0,005	1,21
Tox2	0,000	1,21
Slpil2	0,008	1,21
Syn3	0,000	1,21
Commd9	0,023	1,21
Olr1736	0,017	1,21
Grik3	0,010	1,21
Stra6	0,022	1,21
Babam1	0,008	1,21
Fam189b	0,002	1,21
Slc25a28	0,019	1,21
Dlgap3	0,003	1,21
Sec61g	0,008	1,21
Zfp688	0,003	1,20
Cic	0,000	1,20
Brpf3	0,037	1,20
St6gal2	0,019	1,20
Taar7e	0,044	1,20
Pvrl1	0,049	1,20
Zmiz2	0,000	1,20
Prkcdbp	0,022	1,20
Mdk	0,031	1,20
Arid1a	0,001	1,20
Trim47	0,001	1,20
Lgi2	0,018	1,20
Ttc5	0,043	-1,20
Fam82a2	0,039	-1,20
Atg5	0,031	-1,20
Tmed9	0,007	-1,21
Gnpnat1	0,021	-1,21
Agl	0,008	-1,21
Olr204	0,007	-1,21

Dnajc17	0,012	-1,21
Rpl3	0,009	-1,22
Exosc3	0,039	-1,22
Hspd1	0,001	-1,22
Minpp1	0,024	-1,22
Pcdhb12	0,007	-1,23
Dbndd2	0,030	-1,23
Uprt	0,044	-1,24
Vamp3	0,007	-1,24
Isoc1	0,047	-1,25
Olr1590	0,027	-1,25
Vom2r57	0,021	-1,27
Sec11a	0,047	-1,29
Nt5c3	0,016	-1,29
Olr1237	0,046	-1,29
Eid1	0,006	-1,30
Timmdc1	0,002	-1,31
Clk2	0,002	-1,31
Olr1513	0,048	-1,34
Cml3	0,012	-1,42
Rhot1	0,002	-1,54

Supplementary Table 7 List of the 10 significantly modulated pathways after chronic treatment with agomelatine. This list has been obtained from the genes presented in Supplementary Table 1, using the Ingenuity Pathways Analysis software (IPA). The clusterization of the genes for each pathway is presented in the last column ($p<0.05$, here presented as -log(p Value)).

Comparison	Canonical Pathway (Ingenuity)	-log (p Value)	Ratio	Genes involved
Agomelatine/Saline *vs* **Vehicle/Saline**	Cardiac Hypertrophy Signaling	1,79E+00	1,79E-02	RHOT1,MEF2D,GNG13,MYL12A
	Rapoport-Luebering Glycolytic Shunt	1,76E+00	2,50E-01	MINPP1
	Phospholipase C Signaling	1,70E+00	1,67E-02	RHOT1,MEF2D,GNG13,MYL12A
	UDP-N-acetyl-D-glucosamine Biosynthesis II	1,59E+00	1,67E-01	GNPNAT1
	CXCR4 Signaling	1,55E+00	1,97E-02	RHOT1,GNG13,MYL12A
	Glycoaminoglycan-protein Linkage Region Biosynthesis	1,52E+00	1,43E-01	B3GAT3
	UDP-N-acetyl-D-galactosamine Biosynthesis II	1,42E+00	1,11E-01	GNPNAT1
	RhoGDI Signaling	1,41E+00	1,73E-02	RHOT1,GNG13,MYL12A
	Glycogen Degradation II	1,37E+00	1,00E-01	AGL
	Thrombin Signaling	1,30E+00	1,57E-02	RHOT1,GNG13,MYL12A

Supplementary Table 8 List of the 284 genes differentially expressed in the ventral hippocampus of animals that received a single injection of Lipopolysaccharide with respect to rats that received only the vehicle (fold-change cut-off: ±1.2; p<0.05).

Comparison	Gene Symbol	p-value (VEH/LPS *vs.* VEH/SAL)	Fold-Change (VEH/LPS *vs.* VEH/SAL)
Vehicle/LPS *vs.* **Vehicle/Saline**	Cxcl10	0,000	13,07
	Cxcl11	0,000	4,71
	Gbp5	0,000	4,26
	Ifit3	0,000	4,17
	Zfp36	0,000	3,77
	Osmr	0,000	3,27
	Rsad2	0,000	3,18
	Birc3	0,000	2,87
	Nfkbia	0,000	2,74
	Pdk4	0,000	2,64
	Ifit2	0,000	2,56
	Mt1a	0,000	2,55
	Sgk1	0,000	2,53
	Rgs16	0,000	2,47
	Irf1	0,000	2,46
	Ptges	0,000	2,45
	Apold1	0,000	2,28
	Dusp1	0,000	2,26
	Ch25h	0,000	2,21
	Gpd1	0,000	2,10
	Ifit1	0,001	2,05
	Vcam1	0,000	2,04
	Icam1	0,000	1,98
	Ccl2	0,013	1,96
	Gbp2	0,000	1,93
	Plat	0,000	1,80
	Aspa	0,001	1,76
	Tgm2	0,000	1,76
	Herc6	0,000	1,74
	Lcn2	0,001	1,74
	Cdkn1a	0,000	1,72
	Ier3	0,000	1,72
	Oasl	0,000	1,72
	Atf3	0,000	1,66
	Gadd45g	0,000	1,66
	Tuba1c	0,009	1,65

	Tnfrsf11a	0,000	1,65
	Nuak2	0,000	1,64
	Slc2a1	0,000	1,61
	Cxcl16	0,003	1,61
	Adamts1	0,000	1,61
	Errfi1	0,000	1,60
	Nfkb2	0,000	1,59
	Tinagl1	0,000	1,59
	Pla2g3	0,000	1,58
	Gpatch4	0,000	1,56
	Il6r	0,000	1,56
	Hif3a	0,000	1,56
	Usp18	0,000	1,54
	Ifitm3	0,000	1,54
	Apcdd1	0,000	1,53
	Trim16	0,000	1,52
	Oas1b	0,000	1,52
	Bcl6b	0,000	1,52
	Per1	0,000	1,50
	Bcl3	0,000	1,48
	Ddit4	0,002	1,47
	Kdm6b	0,000	1,46
	Angptl4	0,000	1,46
	Cxcl9	0,000	1,46
	Cnksr3	0,000	1,46
	Cp	0,000	1,45
	Gpx3	0,025	1,45
	Upp1	0,000	1,45
	Selp	0,000	1,45
	Irak2	0,000	1,45
	Cryab	0,008	1,45
	Mt2A	0,000	1,45
	Fam43a	0,000	1,45
	Gpr4	0,000	1,44
	Lfng	0,000	1,44
	Pate4	0,000	1,43
	Il4ra	0,000	1,42
	Fn1	0,001	1,42
	Plekhf1	0,004	1,42
	Rin3	0,000	1,42
	Cd274	0,000	1,42
	Hbb-b1	0,001	1,42
	Stra6	0,000	1,42

	Arrdc2	0,000	1,41
	RT1-CE1	0,012	1,41
	Olig2	0,003	1,41
	Adamts9	0,000	1,40
	Arid5a	0,000	1,40
	Esam	0,000	1,40
	Parp9	0,001	1,39
	Zc3h12a	0,000	1,39
	Prr5	0,000	1,39
	Rnd1	0,000	1,39
	Tnfrsf1b	0,001	1,39
	Rpl37a-ps1	0,017	1,39
	Sp140	0,000	1,38
	RT1-CE4	0,001	1,38
	Zfp189	0,000	1,38
	Sik1	0,000	1,37
	Samd9l	0,003	1,37
	Nfkbiz	0,000	1,37
	Pla1a	0,000	1,37
	Arrdc3	0,000	1,37
	PVR	0,002	1,37
	Prkd2	0,002	1,37
	Fstl4	0,002	1,36
	Ripk2	0,003	1,36
	Socs1	0,000	1,35
	Pim1	0,001	1,35
	Cxcl1	0,005	1,35
	Apol3	0,000	1,35
	Prokr2	0,001	1,34
	Chchd1	0,038	1,34
	F2rl1	0,000	1,34
	Oas1a	0,001	1,34
	Usp54	0,001	1,34
	Grrp1	0,000	1,33
	Nt5e	0,003	1,33
	Pnpla2	0,000	1,33
	Gpr31	0,000	1,32
	Bhlhe40	0,000	1,31
	Ncl	0,012	1,31
	Map3k8	0,000	1,31
	Ralgds	0,000	1,31
	Oasl2	0,007	1,31
	Csrnp1	0,000	1,30

	Nfil3	0,000	1,30
	Tagln2	0,006	1,30
	Igtp	0,000	1,30
	Olig1	0,018	1,29
	Serpine1	0,000	1,29
	Isg20	0,018	1,29
	Tmem88	0,000	1,29
	Slc25a13	0,000	1,29
	Map3k6	0,001	1,29
	Csf1	0,005	1,29
	Nfe2l2	0,007	1,29
	Ifih1	0,000	1,28
	Zdhhc22	0,045	1,28
	Akap2	0,000	1,28
	Plxnd1	0,004	1,28
	Litaf	0,007	1,28
	Cmpk2	0,000	1,27
	Dtx3l	0,000	1,27
	Golga7b	0,019	1,27
	Lipe	0,000	1,27
	Cdc37l1	0,001	1,27
	Tsc22d3	0,007	1,27
	Rapgef3	0,001	1,27
	Il1b	0,008	1,27
	Smad1	0,000	1,27
	Irf7	0,002	1,27
	Zc3hav1	0,000	1,26
	Cflar	0,003	1,26
	Timp1	0,002	1,26
	Tap1	0,000	1,26
	Cd59	0,035	1,26
	Ddx58	0,003	1,26
	Tgfb1	0,003	1,25
	Slc35c2	0,048	1,25
	Tbx3	0,000	1,25
	Ndufb8	0,041	1,25
	Socs3	0,001	1,25
	Cables1	0,004	1,25
	Rnf125	0,002	1,25
	Tfcp2l1	0,000	1,25
	Lonrf3	0,000	1,25
	Ptp4a3	0,003	1,25
	Flnb	0,000	1,25

	Klf9	0,000	1,25
	Cspg4	0,001	1,25
	Mfsd2a	0,000	1,25
	Hcn4	0,001	1,25
	Usp30	0,005	1,24
	Ccnl1	0,002	1,24
	Smarcd2	0,000	1,24
	Grm2	0,046	1,24
	Dusp10	0,017	1,24
	Gpt2	0,010	1,24
	Rnf208	0,037	1,24
	Pvrl1	0,026	1,24
	Npsr1	0,001	1,24
	Tmem119	0,004	1,24
	Hist2h4	0,003	1,24
	Cdk18	0,016	1,23
	Sema7a	0,006	1,23
	Ceacam1	0,008	1,23
	Bag3	0,001	1,23
	Fam176a	0,002	1,23
	Spsb1	0,004	1,23
	Plekhh1	0,018	1,23
	Sla	0,008	1,23
	Sbno2	0,000	1,23
	Tle3	0,001	1,23
	Chrna4	0,017	1,22
	Mertk	0,003	1,22
	Rasd2	0,019	1,22
	Klf15	0,002	1,22
	Zfp64	0,028	1,22
	Mx2	0,028	1,22
	Zbtb16	0,004	1,22
	Tceb2	0,030	1,22
	Lrrc8a	0,004	1,22
	Dusp5	0,003	1,22
	Ifngr1	0,008	1,22
	Trex1	0,001	1,22
	Dlc1	0,001	1,22
	Mef2c	0,045	1,22
	Tns1	0,008	1,22
	Nr4a1	0,009	1,22
	RT1-M3-1	0,007	1,22
	Lrig3	0,001	1,21

	Crhr2	0,007	1,21
	Ppia	0,004	1,21
	Brpf3	0,034	1,21
	Ccrn4l	0,001	1,21
	Hbegf	0,038	1,21
	Rpl36a-ps4	0,005	1,21
	Prkcd	0,037	1,21
	Gja4	0,013	1,21
	Pmaip1	0,001	1,21
	Cxcl2	0,017	1,21
	Btg2	0,011	1,21
	Zfp704	0,031	1,21
	Mrf	0,002	1,20
	Fzd9	0,027	1,20
	Slfn2	0,004	1,20
	Pitpnc1	0,005	1,20
	Ecel1	0,041	1,20
	Gadd45b	0,001	1,20
	Il2rg	0,004	1,20
	Eya1	0,000	1,20
	Lgi3	0,006	1,20
	Pric285	0,006	1,20
	Clic1	0,006	1,20
	Gnpnat1	0,024	-1,20
	Sugt1	0,039	-1,20
	Vof16	0,005	-1,20
	Rasgrp3	0,033	-1,20
	Ces2a	0,006	-1,20
	Ppp6c	0,005	-1,21
	Tmc7	0,002	-1,21
	Ghitm	0,033	-1,21
	Tmem93	0,016	-1,21
	Timmdc1	0,021	-1,21
	Fkbpl	0,020	-1,21
	Tmem60	0,023	-1,21
	Set	0,017	-1,21
	Agps	0,037	-1,21
	Olr663	0,016	-1,21
	Ybx1	0,034	-1,22
	Spry2	0,002	-1,22
	Egfl7	0,010	-1,22
	Chi3l1	0,008	-1,23
	C1H6orf35	0,011	-1,23

	Tmem33	0,011	-1,23
	Aldh1a1	0,006	-1,23
	Lef1	0,000	-1,23
	Lypla1	0,026	-1,24
	Trpc6	0,040	-1,24
	Ocln	0,000	-1,24
	Nudt19	0,027	-1,24
	Abcg2	0,004	-1,25
	Rpl27a	0,012	-1,25
	Tek	0,001	-1,25
	Mars2	0,035	-1,25
	Olr1590	0,027	-1,25
	Tbpl1	0,018	-1,25
	Exosc3	0,021	-1,26
	Nt5c3	0,026	-1,26
	Cyyr1	0,003	-1,27
	Slc7a1	0,000	-1,27
	Prom1	0,001	-1,30
	Hist2h2be	0,003	-1,30
	Tnfrsf11b	0,008	-1,31
	Rasl11a	0,000	-1,33
	Sox2	0,002	-1,38
	Rabif	0,001	-1,39
	Slco1c1	0,000	-1,39
	Rhot1	0,011	-1,39
	Rbm12b	0,004	-1,41
	Hes5	0,002	-1,42
	Npas4	0,014	-1,42
	Gpr34	0,010	-1,42
	Tfrc	0,001	-1,44
	Clk2	0,000	-1,47
	Slco1a2	0,000	-1,56
	Slc40a1	0,000	-1,65

Supplementary Table 9 List of the 100 significantly modulated pathways after a single injection of Lipopolysaccharide. This list has been obtained from the 284 genes presented in Supplementary Table 3, using the Ingenuity Pathways Analysis software (IPA). The clusterization of the genes for each pathway is presented in the last column (p<0.05 here presented as -log(p Value)).

Comparison	Canonical Pathway (Ingenuity)	-log (p Value)	Ratio	Genes involved
Vehicle/LPS *vs.* **Vehicle/Saline**	Interferon Signaling	8,06E+00	2,35E-01	IFIT3,SOCS1,IFITM3,OAS1,MX1,IFNGR1,TAP1,IRF1
	Type I Diabetes Mellitus Signaling	6,77E+00	1,00E-01	HLA-G,SOCS1,SOCS3,NFKBIA,HLA-A,IL1B,IFNGR1,NFKB2,TNFRSF1B,IRF1,TNFRSF11B
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	6,58E+00	7,11E-02	VCAM1,ICAM1,FN1,IL6R,IFNGR1,NFKB2,CXCL3,CSF1,TGFB1,TIMP1,IL1B,SERPINE1,TNFRSF1B,TNFRSF11B
	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	6,49E+00	5,70E-02	SOCS1,SOCS3,VCAM1,ICAM1,FN1,IL6R,FZD9,NFKBIA,F2RL1,CSF1,TGFB1,PRKCD,IL1B,LEF1,TNFRSF1B,TNFRSF11B,IRAK2
	HMGB1 Signaling	6,39E+00	9,17E-02	VCAM1,ICAM1,RHOT1,TGFB1,IL1B,IFNGR1,NFKB2,TNFRSF1B,SERPINE1,TNFRSF11B,PLAT
	Granulocyte Adhesion and Diapedesis	6,32E+00	7,34E-02	CXCL10,CXCL16,CXCL3,VCAM1,CXCL11,ICAM1,SELP,Ccl2,IL1B,CXCL2,TNFRSF1B,Cxcl9,TNFRSF11B
	Acute Phase Response Signaling	5,72E+00	7,10E-02	SOCS1,SOCS3,FN1,NFKBIA,IL6R,IL1B,CP,OSMR,NFKB2,TNFRSF1B,SERPINE1,TNFRSF11B
	Agranulocyte Adhesion and Diapedesis	5,22E+00	6,35E-02	CXCL10,CXCL3,CXCL16,CXCL11,VCAM1,ICAM1,FN1,SELP,Ccl2,IL1B,CXCL2,Cxcl9
	Salvage Pathways of Pyrimidine Ribonucleotides	4,52E+00	8,42E-02	CDK18,CMPK2,MAP3K6,PIM1,SGK1,PRKCD,UPP1,MAP3K8
	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	4,42E+00	7,09E-02	IFIH1,OAS1,IRF7,TGFB1,PRKCD,Oas1b,IL1B,NFKB2,RIPK2
	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	3,95E+00	5,56E-02	NFKBIA,MAP3K6,RHOT1,PRKCD,IFNGR1,MAP3K8,NFKB2,TNFRSF1B,IRF1,TNFRSF11B

IL-6 Signaling	3,91E+00	6,90E-02	SOCS1,SOCS3,NFKBIA,IL6R,IL1B,NFKB2,TNFRSF1B,TNFRSF11B
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	3,90E+00	5,02E-02	NFKBIA,CSF1,TGFB1,IL1B,FZD9,LEF1,TNFRSF1B,TNFRSF11A,BIRC3,SMAD1,TNFRSF11B
p38 MAPK Signaling	3,88E+00	6,84E-02	DUSP1,TGFB1,DUSP10,PLA2G3,IL1B,MEF2C,TNFRSF1B,IRAK2
Pyridoxal 5'-phosphate Salvage Pathway	3,78E+00	9,38E-02	CDK18,MAP3K6,PIM1,SGK1,PRKCD,MAP3K8
Death Receptor Signaling	3,75E+00	7,61E-02	NFKBIA,ZC3HAV1,NFKB2,CFLAR,TNFRSF1B,BIRC3,PARP9
Atherosclerosis Signaling	3,73E+00	6,50E-02	VCAM1,ICAM1,SELP,CSF1,TGFB1,PLA2G3,IL1B,NFKB2
iNOS Signaling	3,62E+00	1,14E-01	NFKBIA,IFNGR1,NFKB2,IRF1,IRAK2
Hepatic Cholestasis	3,61E+00	5,56E-02	SLCO1C1,NFKBIA,TGFB1,PRKCD,IL1B,NFKB2,TNFRSF1B,TNFRSF11B,IRAK2
Role of JAK family kinases in IL-6-type Cytokine Signaling	3,57E+00	1,60E-01	SOCS1,SOCS3,IL6R,OSMR
Molecular Mechanisms of Cancer	3,56E+00	3,84E-02	FZD9,RAPGEF3,NFKB2,NFKBIA,RABIF,RHOT1,TGFB1,PRKCD,CDKN1A,LEF1,CFLAR,SMAD1,BIRC3,RALGDS
T Helper Cell Differentiation	3,53E+00	8,45E-02	IL2RG,TGFB1,IL6R,IFNGR1,TNFRSF1B,TNFRSF11B
TNFR2 Signaling	3,32E+00	1,38E-01	NFKBIA,NFKB2,TNFRSF1B,BIRC3
Glucocorticoid Receptor Signaling	3,25E+00	4,21E-02	CXCL3,VCAM1,ICAM1,NFKBIA,DUSP1,TGFB1,SGK1,CDKN1A,SMARCD2,IL1B,SERPINE1
Type II Diabetes Mellitus Signaling	3,11E+00	5,98E-02	SOCS1,SOCS3,NFKBIA,PRKCD,NFKB2,TNFRSF1B,TNFRSF11B
IL-9 Signaling	3,05E+00	1,18E-01	SOCS3,IL2RG,BCL3,NFKB2
RANK Signaling in Osteoclasts	3,03E+00	6,82E-02	NFKBIA,MAP3K6,MAP3K8,NFKB2,TNFRSF11A,BIRC3
IL-17A Signaling in Fibroblasts	3,00E+00	1,14E-01	NFKBIA,LCN2,NFKB2,NFKBIZ
Induction of Apoptosis by HIV1	2,99E+00	8,33E-02	NFKBIA,NFKB2,TNFRSF1B,BIRC3,TNFRSF11B
Factors Promoting Cardiogenesis in Vertebrates	2,93E+00	6,52E-02	TGFB1,PRKCD,FZD9,LEF1,MEF2C,SMAD1
Activation of IRF by Cytosolic Pattern Recognition Receptors	2,87E+00	7,81E-02	IFIH1,IRF7,NFKBIA,NFKB2,IFIT2
Erythropoietin Signaling	2,78E+00	7,46E-02	SOCS1,SOCS3,NFKBIA,PRKCD,NFKB2
GADD45 Signaling	2,76E+00	1,58E-01	GADD45B,GADD45G,CDKN1A

Antigen Presentation Pathway	1,93E+00	8,11E-02	HLA-G,HLA-A,TAP1
Pancreatic Adenocarcinoma Signaling	1,93E+00	4,72E-02	TGFB1,CDKN1A,HBEGF,NFKB2,RALGDS
Phospholipase C Signaling	1,92E+00	3,35E-02	TGM2,RHOT1,PRKCD,PLA2G3,MEF2C,RAPGEF3,NFKB2,RALGDS
PEDF Signaling	1,89E+00	5,63E-02	NFKBIA,NFKB2,CFLAR,PNPLA2
JAK/Stat Signaling	1,87E+00	5,56E-02	SOCS1,SOCS3,CDKN1A,NFKB2
Prolactin Signaling	1,85E+00	5,48E-02	SOCS1,SOCS3,PRKCD,IRF1
Role of PKR in Interferon Induction and Antiviral Response	1,84E+00	7,50E-02	NFKBIA,NFKB2,IRF1
Toll-like Receptor Signaling	1,83E+00	5,41E-02	NFKBIA,IL1B,NFKB2,IRAK2
MIF Regulation of Innate Immunity	1,81E+00	7,32E-02	NFKBIA,PLA2G3,NFKB2
TREM1 Signaling	1,81E+00	5,33E-02	CXCL3,ICAM1,IL1B,NFKB2
Protein Ubiquitination Pathway	1,76E+00	3,14E-02	CRYAB,USP18,HLA-A,USP54,USP30,SUGT1,BIRC3,TAP1
Role of IL-17F in Allergic Inflammatory Airway Diseases	1,73E+00	6,82E-02	CXCL10,IL1B,NFKB2
Prostate Cancer Signaling	1,68E+00	4,88E-02	NFKBIA,CDKN1A,LEF1,NFKB2
Graft-versus-Host Disease Signaling	1,63E+00	6,25E-02	HLA-G,HLA-A,IL1B
TNFR1 Signaling	1,61E+00	6,12E-02	NFKBIA,NFKB2,BIRC3
Alanine Degradation III	1,59E+00	5,00E-01	GPT2
Alanine Biosynthesis II	1,59E+00	5,00E-01	GPT2
TGF-β Signaling	1,59E+00	4,60E-02	IRF7,TGFB1,SERPINE1,SMAD1
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	1,58E+00	4,55E-02	CSF1,TGFB1,IL1B,NFKB2
Virus Entry via Endocytic Pathways	1,56E+00	4,49E-02	FLNB,HLA-A,PRKCD,TFRC
OX40 Signaling Pathway	1,56E+00	4,49E-02	HLA-G,NFKBIA,HLA-A,NFKB2
Apoptosis Signaling	1,56E+00	4,49E-02	NFKBIA,NFKB2,TNFRSF1B,BIRC3
Human Embryonic Stem Cell Pluripotency	1,54E+00	3,73E-02	SOX2,TGFB1,FZD9,LEF1,SMAD1
Communication between Innate and Adaptive Immune Cells	1,53E+00	4,40E-02	CXCL10,HLA-G,HLA-A,IL1B

Semaphorin Signaling in Neurons	1,52E+00	5,66E-02	RND1,RHOT1,SEMA7A
Lymphotoxin β Receptor Signaling	1,50E+00	5,56E-02	VCAM1,NFKBIA,NFKB2
Role of IL-17A in Arthritis	1,50E+00	5,56E-02	CXCL3,NFKBIA,NFKB2
Mouse Embryonic Stem Cell Pluripotency	1,47E+00	4,21E-02	SOX2,FZD9,LEF1,SMAD1
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	1,46E+00	8,70E-02	LCN2,IL1B
Antioxidant Action of Vitamin C	1,44E+00	4,12E-02	NFKBIA,SLC2A1,PLA2G3,NFKB2
Triacylglycerol Degradation	1,43E+00	8,33E-02	LIPE,PNPLA2
Glycerol-3-phosphate Shuttle	1,42E+00	3,33E-01	GPD1
Glioblastoma Multiforme Signaling	1,40E+00	3,42E-02	RHOT1,PRKCD,CDKN1A,FZD9,LEF1
IL-17A Signaling in Gastric Cells	1,39E+00	8,00E-02	CXCL10,CXCL11
Cholecystokinin/Gastrin-mediated Signaling	1,39E+00	3,96E-02	RHOT1,PRKCD,IL1B,MEF2C
Gαq Signaling	1,39E+00	3,40E-02	NFKBIA,RHOT1,PRKCD,RGS16,NFKB2
Leukocyte Extravasation Signaling	1,37E+00	3,03E-02	VCAM1,ICAM1,TIMP1,PRKCD,RAPGEF3,DLC1
HGF Signaling	1,34E+00	3,81E-02	MAP3K6,PRKCD,CDKN1A,MAP3K8
IL-15 Production	1,33E+00	7,41E-02	NFKB2,IRF1
ERK5 Signaling	1,33E+00	4,76E-02	SGK1,MAP3K8,MEF2C
Role of JAK1 and JAK3 in γc Cytokine Signaling	1,33E+00	4,76E-02	SOCS1,SOCS3,IL2RG
G-Protein Coupled Receptor Signaling	1,33E+00	2,73E-02	GRM2,NFKBIA,DUSP1,RGS16,MAP3K8,RAPGEF3,NFKB2
NGF Signaling	1,31E+00	3,74E-02	MAP3K6,PRKCD,MAP3K8,NFKB2
IL-17A Signaling in Airway Cells	1,31E+00	4,69E-02	CXCL3,NFKBIA,NFKB2
CD40 Signaling	1,30E+00	4,62E-02	ICAM1,NFKBIA,NFKB2

RAR Activation	2,70E+00	4,55E-02	ALDH1A1,DUSP1,TGFB1,PRKCD,SMARCD2,NFKB2,SMAD1,ZBTB16
Aryl Hydrocarbon Receptor Signaling	2,66E+00	5,00E-02	TGM2,ALDH1A1,TGFB1,CDKN1A,IL1B,NFKB2,NFE2L2
STAT3 Pathway	2,61E+00	6,85E-02	SOCS1,SOCS3,PIM1,CDKN1A,TNFRSF11A
Role of RIG1-like Receptors in Antiviral Innate Immunity	2,59E+00	8,89E-02	IFIH1,IRF7,NFKBIA,NFKB2
Role of JAK1, JAK2 and TYK2 in Interferon Signaling	2,46E+00	1,25E-01	SOCS1,IFNGR1,NFKB2
Protein Kinase A Signaling	2,37E+00	3,11E-02	AKAP2,FLNB,DUSP5,NFKBIA,DUSP1,TGFB1,PRKCD,DUSP10,LIPE,LEF1,NFKB2,EYA1
CD27 Signaling in Lymphocytes	2,36E+00	7,69E-02	NFKBIA,MAP3K6,MAP3K8,NFKB2
Pathogenesis of Multiple Sclerosis	2,26E+00	2,22E-01	CXCL10,CXCL11
Crosstalk between Dendritic Cells and Natural Killer Cells	2,24E+00	5,62E-02	HLA-G,IL2RG,HLA-A,NFKB2,TNFRSF1B
Tight Junction Signaling	2,24E+00	4,19E-02	TGFB1,PVRL1,NFKB2,CNKSR3,TNFRSF1B,TNFRSF11B,OCLN
ATM Signaling	2,16E+00	6,78E-02	GADD45B,NFKBIA,GADD45G,CDKN1A
NF-κB Signaling	2,16E+00	4,05E-02	NFKBIA,IL1B,MAP3K8,NFKB2,TNFRSF1B,TNFRSF11A,TNFRSF11B
PPAR Signaling	2,14E+00	5,32E-02	NFKBIA,IL1B,NFKB2,TNFRSF1B,TNFRSF11B
IL-12 Signaling and Production in Macrophages	2,10E+00	4,44E-02	TGFB1,PRKCD,IFNGR1,MAP3K8,NFKB2,IRF1
PPARα/RXRα Activation	2,08E+00	3,91E-02	GPD1,NFKBIA,TGFB1,IL1B,BCL3,MEF2C,NFKB2
Dendritic Cell Maturation	2,08E+00	3,91E-02	ICAM1,NFKBIA,HLA-A,IL1B,NFKB2,TNFRSF1B,TNFRSF11B
MIF-mediated Glucocorticoid Regulation	2,07E+00	9,09E-02	NFKBIA,PLA2G3,NFKB2
Retinoic acid Mediated Apoptosis Signaling	2,04E+00	6,25E-02	ZC3HAV1,CFLAR,PARP9,IRF1
TWEAK Signaling	2,03E+00	8,82E-02	NFKBIA,NFKB2,BIRC3
IL-8 Signaling	2,03E+00	3,83E-02	VCAM1,ICAM1,RHOT1,PRKCD,HBEGF,TEK,IRAK2
IL-10 Signaling	1,95E+00	5,88E-02	SOCS3,NFKBIA,IL1B,NFKB2
Colorectal Cancer Metastasis Signaling	1,95E+00	3,39E-02	RHOT1,TGFB1,IL6R,FZD9,LEF1,IFNGR1,NFKB2,RALGDS

Supplementary Table 10 List of the 91 genes resulted from the overlap analysis between the 284 genes significantly modulated by the LPS treatment (Supplementary Table 3) and the list of 296 transcripts altered in rats treated with agomelatine and challenged with the endotoxin. They represent the genes whose transcription is prevented by agomelatine: the fold change value is shown in the two experimental groups (fold-change cut-off: ±1.2).

Gene Symbol	Fold Change value	
	VehLPS *vs*. VehSal	**AgoLPS *vs*. VehSal**
Ccl2	1,96	1,49
RT1-CE1	1,41	1,12
Rabif	-1,39	-1,10
Ybx1	-1,22	1,06
Grm2	1,24	-1,02
Lypla1	-1,24	1,00
Gpr34	-1,42	-1,19
Tmem93	-1,21	1,03
Il1b	1,27	1,04
Fkbpl	-1,21	1,01
Hbb-b1	1,42	1,20
Tmem60	-1,21	-1,00
Prkcd	1,21	1,00
Tap1	1,26	1,06
Rbm12b	-1,41	-1,21
Agps	-1,21	-1,02
Cryab	1,45	1,26
Tmem33	-1,23	-1,04
Ecel1	1,20	1,04
Gpx3	1,45	1,29
Plekhh1	1,23	1,07
Rasd2	1,22	1,07
Zfp64	1,22	1,07
Set	-1,21	-1,07
Npsr1	1,24	1,09
Nudt19	-1,24	-1,10
Chi3l1	-1,23	-1,09
Hcn4	1,25	1,11
Smad1	1,27	1,13
Eya1	1,20	1,07
Akap2	1,28	1,16
Pvrl1	1,24	1,12
Hist2h2be	-1,30	-1,18
Zdhhc22	1,28	1,17
Dusp5	1,22	1,11
Ppp6c	-1,21	-1,09

Olr663	-1,21	-1,10
Mars2	-1,25	-1,14
Mef2c	1,22	1,11
Isg20	1,29	1,18
Ncl	1,31	1,20
Map3k6	1,29	1,18
Trpc6	-1,24	-1,13
C1H6orf35	-1,23	-1,12
Ptp4a3	1,25	1,15
Tbpl1	-1,25	-1,15
Tfcp2l1	1,25	1,16
Nt5c3	-1,26	-1,17
Zfp704	1,21	1,11
Socs3	1,25	1,16
Ghitm	-1,21	-1,12
Cspg4	1,25	1,16
Clic1	1,20	1,12
Lgi3	1,20	1,12
Pitpnc1	1,20	1,12
Mrf	1,20	1,13
Cd59	1,26	1,18
Sugt1	-1,20	-1,13
Crhr2	1,21	1,14
Nr4a1	1,22	1,15
Fam176a	1,23	1,16
Hbegf	1,21	1,14
Cdk18	1,23	1,17
Gnpnat1	-1,20	-1,14
Rnf208	1,24	1,18
Rpl27a	-1,25	-1,19
Exosc3	-1,26	-1,20
Tle3	1,23	1,17
Mertk	1,22	1,17
Rnf125	1,25	1,20
Chchd1	1,34	1,29
Dlc1	1,22	1,17
Pric285	1,20	1,15
Lrrc8a	1,22	1,17
Tmc7	-1,21	-1,16
Ccrn4l	1,21	1,17
Gja4	1,21	1,17
Bag3	1,23	1,19
Lrig3	1,21	1,18
Gadd45b	1,20	1,17
Rasgrp3	-1,20	-1,17
Ndufb8	1,25	1,22

Slc35c2	1,25	1,22
Brpf3	1,21	1,18
Pmaip1	1,21	1,17
Spsb1	1,23	1,20
Egfl7	-1,22	-1,20
Timmdc1	-1,21	-1,18
Cxcl2	1,21	1,19
Btg2	1,21	1,20
Il2rg	1,20	1,19

Supplementary Table 11 List of the 31 significantly modulated pathways obtained from the genes presented in Supplementary Table 5, using the Ingenuity Pathways Analysis software (IPA). The clusterization of the genes for each pathway is presented in the last column ($p<0.05$ here presented as -log(p Value)).

Comparison	Canonical Pathway (Ingenuity)	-log (p Value)	Ratio	Genes involved
Vehicle/LPS *vs.* Vehicle/Saline (genes from Table S5)	Corticotropin Releasing Hormone Signaling	3,02E+00	3,60E-02	CRHR2,PRKCD,NR4A1,MEF2C
	Pyridoxal 5'-phosphate Salvage Pathway	2,69E+00	4,69E-02	CDK18,MAP3K6,PRKCD
	Factors Promoting Cardiogenesis in Vertebrates	2,24E+00	3,26E-02	PRKCD,MEF2C,SMAD1
	Salvage Pathways of Pyrimidine Ribonucleotides	2,20E+00	3,16E-02	CDK18,MAP3K6,PRKCD
	Cholecystokinin/Gastrin-mediated Signaling	2,13E+00	2,97E-02	PRKCD,IL1B,MEF2C
	IL-9 Signaling	2,10E+00	5,88E-02	SOCS3,IL2RG
	Cell Cycle Regulation by BTG Family Proteins	2,08E+00	5,71E-02	BTG2,CCRN4L
	Antigen Presentation Pathway	2,03E+00	5,41E-02	HLA-A,TAP1
	Type I Diabetes Mellitus Signaling	2,03E+00	2,73E-02	SOCS3,HLA-A,IL1B
	Graft-versus-Host Disease Signaling	1,81E+00	4,17E-02	HLA-A,IL1B
	Primary Immunodeficiency Signaling	1,75E+00	3,85E-02	IL2RG,TAP1
	Protein Ubiquitination Pathway	1,74E+00	1,57E-02	CRYAB,HLA-A,SUGT1,TAP1
	UDP-N-acetyl-D-glucosamine Biosynthesis II	1,63E+00	1,67E-01	GNPNAT1
	Role of JAK1 and JAK3 in γc Cytokine Signaling	1,59E+00	3,17E-02	SOCS3,IL2RG
	Calcium-induced T Lymphocyte Apoptosis	1,58E+00	3,12E-02	PRKCD,NR4A1
	Erythropoietin Signaling	1,54E+00	2,99E-02	SOCS3,PRKCD
	IL-10 Signaling	1,53E+00	2,94E-02	SOCS3,IL1B
	Growth Hormone Signaling	1,52E+00	2,90E-02	SOCS3,PRKCD

Airway Pathology in Chronic Obstructive Pulmonary Disease	1,51E+00	1,25E-01	CXCL3
Granulocyte Adhesion and Diapedesis	1,49E+00	1,69E-02	CXCL3,Ccl2,IL1B
Prolactin Signaling	1,47E+00	2,74E-02	SOCS3,PRKCD
UDP-N-acetyl-D-galactosamine Biosynthesis II	1,46E+00	1,11E-01	GNPNAT1
TREM1 Signaling	1,45E+00	2,67E-02	CXCL3,IL1B
Agranulocyte Adhesion and Diapedesis	1,41E+00	1,59E-02	CXCL3,Ccl2,IL1B
ErbB Signaling	1,35E+00	2,33E-02	PRKCD,HBEGF
Neuregulin Signaling	1,33E+00	2,27E-02	PRKCD,HBEGF
G Beta Gamma Signaling	1,33E+00	2,27E-02	PRKCD,HBEGF
Virus Entry via Endocytic Pathways	1,32E+00	2,25E-02	HLA-A,PRKCD
Crosstalk between Dendritic Cells and Natural Killer Cells	1,32E+00	2,25E-02	IL2RG,HLA-A
Communication between Innate and Adaptive Immune Cells	1,30E+00	2,20E-02	HLA-A,IL1B
Role of IL-17A in Psoriasis	1,30E+00	7,69E-02	CXCL3

Supplementary Table 12 List of the 193 genes resulted from the overlap analysis between the 284 genes significantly modulated by the LPS treatment (Supplementary Table 3) and the list of 296 transcripts altered in rats treated with agomelatine and challenged with the endotoxin. They represent the transcripts modulated by the LPS with or without the administration of agomelatine. The fold change value is shown in the two experimental groups (fold-change cut-off: ±1.2).

Gene Symbol	Fold Change value	
	VehLPS *vs.* VehSal	**AgoLPS *vs.* VehSal**
Cxcl10	13,07	12,51
Cxcl11	4,71	4,46
Gbp5	4,26	3,97
Ifit3	4,17	4,28
Zfp36	3,77	3,97
Osmr	3,27	2,99
Rsad2	3,18	3,31
Birc3	2,87	2,68
Nfkbia	2,74	2,66
Pdk4	2,64	2,77
Ifit2	2,56	2,69
Mt1a	2,55	2,40
Sgk1	2,53	2,51
Rgs16	2,47	2,39
Irf1	2,46	2,17
Ptges	2,45	2,01
Apold1	2,28	2,23
Dusp1	2,26	2,26
Ch25h	2,21	1,96
Gpd1	2,10	1,97
Ifit1	2,05	1,78
Vcam1	2,04	1,85
Icam1	1,98	2,12
Gbp2	1,93	1,91
Plat	1,80	1,69
Aspa	1,76	1,88
Tgm2	1,76	1,53
Herc6	1,74	1,80
Lcn2	1,74	2,11
Cdkn1a	1,72	1,68
Ier3	1,72	1,67
Oasl	1,72	1,64
Atf3	1,66	1,57

Gadd45g	1,66	1,53
Tuba1c	1,65	1,51
Tnfrsf11a	1,65	1,45
Nuak2	1,64	1,66
Slc2a1	1,61	1,61
Cxcl16	1,61	1,53
Adamts1	1,61	1,45
Errfi1	1,60	1,67
Nfkb2	1,59	1,63
Tinagl1	1,59	1,67
Pla2g3	1,58	1,43
Gpatch4	1,56	1,40
Il6r	1,56	1,48
Hif3a	1,56	1,57
Usp18	1,54	1,60
Ifitm3	1,54	1,75
Apcdd1	1,53	1,65
Trim16	1,52	1,39
Oas1b	1,52	1,37
Bcl6b	1,52	1,48
Per1	1,50	1,55
Bcl3	1,48	1,43
Ddit4	1,47	1,73
Angptl4	1,46	1,48
Cxcl9	1,46	1,71
Cnksr3	1,46	1,46
Cp	1,45	1,25
Upp1	1,45	1,44
Selp	1,45	1,25
Irak2	1,45	1,43
Mt2A	1,45	1,47
Fam43a	1,45	1,32
Gpr4	1,44	1,61
Lfng	1,44	1,40
Pate4	1,43	1,48
Il4ra	1,42	1,43
Kdm6b	1,42	1,42
Fn1	1,42	1,34
Plekhf1	1,42	1,40
Rin3	1,42	1,44
Cd274	1,42	1,43
Stra6	1,42	1,30
Arrdc2	1,41	1,36
Olig2	1,41	1,46
Adamts9	1,40	1,47
Arid5a	1,40	1,44

Esam	1,40	1,58
Parp9	1,39	1,33
Zc3h12a	1,39	1,38
Prr5	1,39	1,42
Rnd1	1,39	1,41
Tnfrsf1b	1,39	1,22
Rpl37a-ps1	1,39	1,31
Sp140	1,38	1,32
RT1-CE4	1,38	1,21
Zfp189	1,38	1,38
Sik1	1,37	1,36
Samd9l	1,37	1,48
Nfkbiz	1,37	1,30
Pla1a	1,37	1,36
Arrdc3	1,37	1,37
PVR	1,37	1,28
Prkd2	1,37	1,39
Fstl4	1,36	1,21
Ripk2	1,36	1,48
Socs1	1,35	1,38
Pim1	1,35	1,37
Cxcl1	1,35	1,34
Apol3	1,35	1,39
Prokr2	1,34	1,32
F2rl1	1,34	1,49
Oas1a	1,34	1,38
Usp54	1,34	1,25
Grrp1	1,33	1,31
Nt5e	1,33	1,33
Pnpla2	1,33	1,45
Gpr31	1,32	1,22
Bhlhe40	1,31	1,33
Map3k8	1,31	1,46
Ralgds	1,31	1,27
Oasl2	1,31	1,40
Csrnp1	1,30	1,28
Nfil3	1,30	1,26
Tagln2	1,30	1,30
Igtp	1,30	1,26
Olig1	1,29	1,36
Serpine1	1,29	1,27
Tmem88	1,29	1,21
Slc25a13	1,29	1,23
Csf1	1,29	1,35
Nfe2l2	1,29	1,23
Ifih1	1,28	1,31

Plxnd1	1,28	1,23
Litaf	1,28	1,38
Cmpk2	1,27	1,31
Dtx3l	1,27	1,20
Golga7b	1,27	1,26
Lipe	1,27	1,26
Cdc37l1	1,27	1,26
Tsc22d3	1,27	1,46
Rapgef3	1,27	1,23
Irf7	1,27	1,32
Zc3hav1	1,26	1,27
Cflar	1,26	1,30
Timp1	1,26	1,22
Ddx58	1,26	1,24
Tgfb1	1,25	1,23
Tbx3	1,25	1,21
Cables1	1,25	1,29
Lonrf3	1,25	1,30
Flnb	1,25	1,25
Klf9	1,25	1,27
Mfsd2a	1,25	1,26
Usp30	1,24	1,21
Ccnl1	1,24	1,24
Smarcd2	1,24	1,32
Dusp10	1,24	1,21
Gpt2	1,24	1,21
Tmem119	1,24	1,21
Hist2h4	1,24	1,39
Sema7a	1,23	1,20
Ceacam1	1,23	1,24
Sla	1,23	1,22
Sbno2	1,23	1,20
Chrna4	1,22	1,22
Klf15	1,22	1,30
Mx2	1,22	1,27
Zbtb16	1,22	1,23
Tceb2	1,22	1,22
Ifngr1	1,22	1,27
Trex1	1,22	1,32
Tns1	1,22	1,21
RT1-M3-1	1,22	1,21
Ppia	1,21	1,29
Rpl36a-ps4	1,21	1,33
Fzd9	1,20	1,23
Slfn2	1,20	1,23
Vof16	-1,20	-1,28

Ces2a	-1,20	-1,22
Spry2	-1,22	-1,22
Aldh1a1	-1,23	-1,24
Lef1	-1,23	-1,23
Ocln	-1,24	-1,24
Abcg2	-1,25	-1,26
Tek	-1,25	-1,35
Olr1590	-1,25	-1,29
Cyyr1	-1,27	-1,32
Slc7a1	-1,27	-1,21
Prom1	-1,30	-1,32
Tnfrsf11b	-1,31	-1,31
Rasl11a	-1,33	-1,35
Sox2	-1,38	-1,32
Slco1c1	-1,39	-1,31
Rhot1	-1,39	-1,32
Hes5	-1,42	-1,34
Npas4	-1,42	-1,49
Tfrc	-1,44	-1,52
Clk2	-1,47	-1,25
Slco1a2	-1,56	-1,42
Slc40a1	-1,65	-1,58

Supplementary Table 14 List of the 52 genes obtained from the comparison between the AGO/LPS group, previously analyzed with respect to the animals treated with the vehicle and now directly compared to the rats received LPS (fold-change cut-off: ±1.2; p<0.05 here presented as -log(p Value)).

Comparison	Gene Symbol	p-value (AGO/LPS *vs.* VEH/LPS)	Fold-Change (AGO/LPS *vs.* VEH/LPS)
Agomelatine/LPS *vs.* **Vehicle/LPS**	Hist1h4m	0,001	2,04
	Fau	0,000	1,96
	Gas5	0,022	1,81
	Rn5-8s	0,016	1,75
	Hist2h2ab	0,019	1,45
	Prelp	0,007	1,40
	Rmrp	0,005	1,38
	Cstb	0,014	1,35
	Dmrtc1b	0,013	1,31
	Atp5l	0,008	1,31
	Pou3f1	0,003	1,31
	Mif	0,006	1,30
	Hmgn2	0,050	1,29
	Ybx1	0,009	1,28
	Sec61g	0,001	1,27
	Uqcrfs1	0,003	1,26
	Morf4l1	0,044	1,26
	Rabif	0,011	1,26
	Olr397	0,005	1,25
	Snhg4	0,016	1,25
	Ndufb4	0,008	1,24
	Leprotl1	0,004	1,24
	S100a16	0,032	1,24
	Psme1	0,023	1,24
	Lypla1	0,024	1,24
	Tmem93	0,007	1,24
	Rpl18a	0,003	1,23
	Ndufa7	0,018	1,23
	Fkbpl	0,013	1,23
	Ndufv3	0,000	1,23
	Vgf	0,005	1,23
	Atf4	0,030	1,23
	Rfk	0,018	1,23
	Cln6	0,000	1,23

	Romo1	0,007	1,22
	Ptges3	0,031	1,22
	Myl12b	0,002	1,22
	Actr6	0,023	1,22
	Trub2	0,032	1,22
	Tmem60	0,024	1,21
	Rab27b	0,030	1,21
	Rabl5	0,046	1,21
	Nhp2l1	0,023	1,20
	Prkcd	0,040	-1,20
	Ppfibp1	0,025	-1,21
	Il1b	0,022	-1,22
	Dus2l	0,045	-1,22
	Olr75	0,009	-1,22
	Oxsm	0,019	-1,24
	Grm2	0,029	-1,27
	Ghdc	0,009	-1,31
	RT1-CE15	0,027	-1,33

Supplementary Table 15 List of the 33 significantly modulated pathways obtained from the genes presented in Supplementary Table 9 using, the Ingenuity Pathways Analysis software (IPA). The clusterization of the genes for each pathway is presented in the last column ($p<0.05$ here presented as -log(p Value)).

Comparison	Canonical Pathway (Ingenuity)	-log (p Value)	Ratio	Genes involved
Agomelatine/LPS *vs.* **Vehicle/LPS**	Oxidative Phosphorylation	5,46E+00	4,59E-02	NDUFB4,NDUFA7,NDUFV3,ATP5L,UQCRFS1
	Mitochondrial Dysfunction	4,51E+00	2,92E-02	NDUFB4,NDUFA7,NDUFV3,ATP5L,UQCRFS1
	Synaptic Long Term Potentiation	2,68E+00	2,52E-02	GRM2,PRKCD,ATF4
	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2,43E+00	1,34E-02	MIF,PRKCD,ATF4,IL1B
	Role of IL-17F in Allergic Inflammatory Airway Diseases	2,40E+00	4,55E-02	ATF4,IL1B
	Flavin Biosynthesis IV (Mammalian)	2,37E+00	5,00E-01	RFK
	Palmitate Biosynthesis I (Animals)	2,37E+00	5,00E-01	OXSM
	Fatty Acid Biosynthesis Initiation II	2,37E+00	5,00E-01	OXSM
	CREB Signaling in Neurons	2,23E+00	1,75E-02	GRM2,PRKCD,ATF4
	Eumelanin Biosynthesis	2,07E+00	2,50E-01	MIF
	Huntington's Disease Signaling	1,88E+00	1,30E-02	PSME1,PRKCD,ATF4
	Phospholipase C Signaling	1,84E+00	1,26E-02	PRKCD,ATF4,MYL12A
	Prostanoid Biosynthesis	1,72E+00	1,11E-01	PTGES3
	Neuropathic Pain Signaling In Dorsal Horn Neurons	1,71E+00	2,00E-02	GRM2,PRKCD

Cholecystokinin/Gastrin-mediated Signaling	1,70E+00	1,98E-02	PRKCD,IL1B
Xenobiotic Metabolism Signaling	1,70E+00	1,11E-02	PRKCD,IL1B,PTGES3
NGF Signaling	1,66E+00	1,87E-02	PRKCD,ATF4
Corticotropin Releasing Hormone Signaling	1,63E+00	1,80E-02	PRKCD,ATF4
p38 MAPK Signaling	1,59E+00	1,71E-02	ATF4,IL1B
P2Y Purigenic Receptor Signaling Pathway	1,57E+00	1,68E-02	PRKCD,ATF4
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	1,52E+00	1,57E-02	PRKCD,IL1B
GNRH Signaling	1,51E+00	1,55E-02	PRKCD,ATF4
Aryl Hydrocarbon Receptor Signaling	1,44E+00	1,43E-02	IL1B,PTGES3
Synaptic Long Term Depression	1,44E+00	1,42E-02	GRM2,PRKCD
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	1,42E+00	5,56E-02	IL1B
CXCR4 Signaling	1,38E+00	1,32E-02	PRKCD,MYL12A
Endoplasmic Reticulum Stress Pathway	1,36E+00	4,76E-02	ATF4
Polyamine Regulation in Colon Cancer	1,34E+00	4,55E-02	PSME1
Dopamine-DARPP32 Feedback in cAMP Signaling	1,33E+00	1,24E-02	PRKCD,ATF4
Hepatic Cholestasis	1,33E+00	1,23E-02	PRKCD,IL1B
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	1,32E+00	4,35E-02	IL1B
Protein Kinase A Signaling	1,31E+00	7,77E-03	PRKCD,ATF4,MYL12A
Cdc42 Signaling	1,30E+00	1,20E-02	RT1-A3 (includes others),MYL12A

7. Bibliography

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