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

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Abstract

Major depression is a complex disease that originates from the interaction between a genetic background of susceptibility and environmental factors such as stress. At molecular level, it is characterized by dysfunctions of multiple systems including neurotransmitters, hormones, signalling pathways, neurotrophic and neuroplastic molecules and -more recently- inflammatory mediators. Accordingly, in the present study we used the chronic mild stress (CMS) paradigm in the rat to elucidate to what extent brain inflammation may contribute to the development and/or the maintenance of a**n** anhedonic phenotype and how pharmacological intervention may interfere with such behavioral and molecular stress-induced alterations.

To this aim, adult male rats were exposed to CMS for 2 weeks and the cerebral expression of several mediators of the inflammatory system was evaluated in the hippocampus and prefrontal cortex of both stressed and control animals in parallel with the sucrose intake. Next, the animals showed decreased sucrose consumption were exposed to five further weeks of CMS and treated with the antidepressants imipramine or agomelatine, or the antipsychotic lurasidone. Our results demonstrate that only the stressed animals that were characterized by a deficit in sucrose intake showed increased expression of the pro-inflammatory cytokines IL-1β, IL-6 and up-regulation of markers and mediators of microglia activation such as CD11b, CX3CL1 and its receptor CX3CR1 in comparison with stress-resilient animals. Some of these molecular alterations persisted also after longer stress exposure and were modulated, similarly to the behavioral effects of CMS, by chronic pharmacological treatment. These data suggest that neuroinflammation may have a key role in the pathological consequences of stress exposure, thus contributing to the subject's vulnerability for depression.

Keywords: **Chronic mild stress**, **Differential susceptibility**, Sucrose intake, **Cytokines**, **Microglia activation**, Antidepressant.

Chemical compounds studied in this article:

Imipramine: 3-(10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N-dimethylpropan-1-amine;

Agomelatine: N-[2-(7-methoxynaphthalen-1-yl)ethyl]acetamide;

Lurasidone: (3aR,4S,7R,7aS)-2-{(1R,2R)-2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-ylmethyl] cyclohexylmethyl} hexahydro-4,7-methano-2H-isoindole-1,3-dione

1. Introduction

Major depression is a severe psychiatric disorder estimated to become the second leading cause of disability in the world by 2020 [1]. 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 [2-4]. However, even if depression occurs in a significant percentage of stress-exposed subjects, most of them are able to successfully cope with the adverse situation and avoid such psychopathology [5,6]. 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 [7-9]. In particular, it has been reported that depressed subjects exhibit increased levels of inflammatory markers both in the periphery and in brain [10] and several pathologies associated with a moderate grade of inflammation present high co-morbidity with depression [11]. Furthermore, a high percentage of patients with cancer or hepatitis C receiving immunotherapy with interferon-alpha develop major depression [12], 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 [13-15] and similar effects were reported in laboratory animals exposed to different stress paradigms [16-19]. 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 takes into account 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 [20]. Given that, it is thought to be a well-established model of depression and has been widely used to evaluate stress-related molecular mechanisms [21-23].

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 [24] as well as in other animal models of depression [25]. 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 [26,27]. 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 (5HT2c) 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.

2. Methods

General reagents were purchased from Sigma-Aldrich (Milan, Italy) whereas molecular biology reagents were obtained from Applied Biosystem Italia (Monza, Italy), Eurofins MWG-Operon (Ebersberg, Germany) and Bio-Rad Laboratories S.r.l. (Segrate, Italy). Imipramine was purchased by Sigma-Aldrich (Milan, Italy), whereas agomelatine and lurasidone were kindly provided by Servier (Suresnes, France), and by Sumitomo Dainippon Pharma Co. Ltd (Japan) respectively.

2.1 Animals

Adult male Wistar rats (Charles River, Germany) were brought into the laboratory one month before the start of the experiment. Except as described below, 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 $\pm$ 2^{o}C) and humidity (50 $\pm$ 5%) conditions. All procedures used in this study are conformed to the rules and principles of the 2010/63/EU Directive, 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 (n=10 each experimental groups).

2.2 Stress procedure and pharmacological treatment

After a period of adaptation to laboratory and housing conditions, the animals (220$\pm$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. Subsequently, sucrose consumption was monitored, under similar conditions, at weekly intervals throughout the whole experiments. On the basis of their sucrose intake in the final baseline test, animals were divided into two matched groups to be subjected to a chronic mild stress procedure [28] for a period of two (Experiment 1) or seven (Experiment 2) weeks. Each week of the stress regime 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 2h), 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**h** 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 14h preceding each sucrose test, but otherwise food and water were freely available in the home cage. According with these procedures, two separated sets of experiments have been performed:

Experiment 1. Animals were subjected to the stress procedure for two weeks, tested for the sucrose consumption and then killed by decapitation 24h after the final sucrose test. Based on the results of this test, the stressed animals were divided into 2 groups: “stress-reactive” (i.e. showing at least 50% decrease of sucrose consumption) and “stress-non reactive” (i.e. showing small or no decrease of sucrose consumption) to be compared versus un-stressed rats. This experimental design implied three groups **of animals**: unstressed **rats** used as

control group (n=10 animals); stressed animals that showed a decrease in sucrose consumption ("stress-reactive" animals, n=10); stressed animals that were resilient to the CMS ("stress-non reactive", n=10). The brains were removed and the **hippocampus (dorsal and ventral)** and the 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 [29], whereas the ventral hippocampus corresponds to the plates 34-43. **These two hippocampal sub-regions were dissected from the whole brain whereas the** prefrontal cortex (defined as Cg1, Cg3, and IL subregions corresponding to the plates 6-10 according to the atlas of Paxinos and Watson) was dissected from 2-mm-thick slices. The brain specimens were then rapidly frozen in dry ice/isopentane and stored at -80^{o}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 **the** first 2 weeks of stress, both control and stress-reactive groups were further divided into matched subgroups and for the subsequent five weeks they received intraperitoneal injections (i.p.) of vehicle (hydrossi-ethil-cellulose, HEC 1%), imipramine (10 mg/kg daily) or agomelatine (40 mg/kg daily) with a dosage chosen according with previous data [30]. 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 [31,32]. The stress was continued throughout the entire period of drugs administration.

According with this experimental design, the animals were divided into **several** matched subgroups: rats that were left undisturbed and received the appropriate vehicle (i.p or o.s according the respective drug) 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 each); rats that were subjected to the CMS procedure in parallel with pharmacological treatment (STRESS/IMI; STRESS/AGO; STRESS/LUR n=10 each). After five weeks, the treatments were terminated and all 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^{o}C for the further molecular analyses.

2.3 RNA preparation and gene expression analyses

For gene expression analyses, total RNA was isolated from the different brain regions by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l.; Segrate, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were then processed for real-time polymerase chain reaction (PCR) as previously reported [33] to assess mRNA levels of: interleukin-1β (IL-1β); interleukin-6 (IL-6); transforming growth factor-β (TGF-β); integrin alpha M (cluster of differentiation molecule CD11b); chemokine (C-X3-C Motif) ligand 1 (CX3CL1; fractalkine); chemokine receptor 1 (CX3CR1).

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 triplicate as multiplexed reactions with a normalizing internal control (β-actin). Thermal cycling was initiated with incubation at 50°C for 10 min (RNA retrotranscription), and then at 95°C for 5 **minutes** (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 **seconds** to enable the melting process, and then for 30 **seconds** at 60°C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression **versus the control group. Specifically, fold change for each target gene relative to β-actin was determined by the 2-Δ(ΔCT) method, where ΔCT= CT,target - CT,β-actin and Δ(ΔCT) = ΔCT,exp. group - ΔCT,control group and CT is the threshold cycle**. For graphical clarity, the obtained data were then expressed as percentage versus control group, which has been set at 100%. Probe and primer sequences used were purchased from Applied Biosystem Italia and Eurofins MWG-Operon.

2.4 Western Blot analysis

Dorsal hippocampi from different experimental groups were manually homogenized in a glass-glass potter in ice-cold 0.32M sucrose buffer (pH 7.4) containing 1mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 0.1mM ethylene glycol tetra-acetic acid (EGTA) and 0.1mM phenylmethylsulfonyl fluoride in the presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors. The homogenate was clarified at 1000g for 10 **minutes**. The resulting supernatant was then centrifuged at 13000g for 15 **minutes**. The supernatant was discarded while the pellet (P2), corresponding to the crude membrane fraction, was re-suspended in a buffer (20mM HEPES, 0.1mM dithiothreitol, 0.1mM EGTA) supplemented with protease and phosphatase inhibitors. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories) using bovine serum albumin as the calibration standard. Equal amounts of protein (15 μg) were run under reducing conditions on 8% SDS-PAGE (PolyAcrylamide Gel Electrophoresis) and then electrophoretically

transferred onto nitrocellulose membranes. Unspecific binding sites were blocked for 1 hour in 10% nonfat dry milk in Tris-buffered saline, **membranes were incubated** overnight with CD11b primary antibody (OX-42 mouse monoclonal antibody, Serotec; 1:500 in BSA 5% in Tris-buffered saline) at 4°C and then with secondary antibody (HRP conjugated anti-mouse IgG, 1:500 in 3% nonfat dry milk in Tris-buffered saline) for 1 hour at room temperature. Immunocomplexes were visualized by chemiluminescence using the Western Lightning Plus ECL (PerkinElmer) 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 an internal standard, because its expression is not regulated by the experimental paradigm used.

2.5 Statistical analyses

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

3. Results

3.1 Experiment 1: Expression profiling of inflammatory mediators in stress-reactive and stress-resilient rats

3.1.1 Sucrose consumption test

Consistently with our previous observations [34], approximately 70% of the animals exposed to the CMS paradigm for 2 weeks showed a reduction in sucrose consumption (-4.6 g **vs. CTRL**, $P<0.001$). 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 "reactive" to distinguish them from stressed rats that did not show reduced sucrose intake, **which** were termed as "non-reactive" (Fig. 1). The reduced sucrose intake was not associated with weight loss (data not shown).

3.1.2 Cytokine gene expression analysis

In order to investigate a possible link between the CMS-induced anhedonic phenotype and inflammation, we investigated some critical mediators of the inflammatory response in reactive and non-reactive 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 subregions and in the prefrontal cortex, three brain areas

mainly involved in the pathophysiology of depression. As shown in figure 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 reactive animals (+52%, *P*<0.001 and +27%, *P*<0.05 vs. CTRL respectively), whereas no changes were found in non-reactive 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. CTRL, *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. CTRL, *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. Non-reactive, *P*<0.01). Conversely, TGF-β gene expression was not altered neither in reactive nor in non-reactive rats.

3.1.3 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 [35]. Specifically, we assessed the expression of CD11b, a marker for the activated state of this cellular population [36] 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 state [37]. As shown in figure 3A, we

found that CD11b mRNA levels in the dorsal hippocampus were significantly up-regulated in reactive animals when compared with control animals or non-reactive animals ($F_{2,24}$=6,633 *P*=0.006; +56% **vs.** CTRL, *P*<0.01 and +63% **vs.** non-reactive, *P*<0.001). A similar effect was observed for CX3CL1 ($F_{2,28}$=7.812 *P*=0.002; +37% **vs.** CTRL, *P*<0.001) and its receptor ($F_{2,30}$=5.026 *P*=0.014; +32% vs. CTRL, *P*<0.01; Fig. **4**A and **4**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. CTRL, *P*<0.05; Fig. **4**C) whereas the expression of its receptor was up-regulated ($F_{2,28}$=7.551 *P*=0.003; +32% vs. CTRL, *P*<0.001; Fig. **4**D).

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-reactive animals ($F_{2,30}$=5.226 *P*=0.012; -15% vs. CTRL, *P*<0.01; **-11% vs. non reactive, *P*<0.05**; Fig. **4**E) without concomitant changes of CX3CR1 expression (Fig. **4**F).

3.1.4 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 figure **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 reactive animals when compared to **both** the control group (+95%, *P*<0.001) **and** the non-reactive animals (+92%, *P*<0.001).

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

In order to evaluate if the molecular changes induced by stress in the reactive 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 figure **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. **5**A) and a similar result was also observed for **IL-6** (r=-0.532, *P*<0.01; Fig. **5**B) and CD11b (r=-0.409, *P*<0.05, Fig. **5**C). 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. **5**D, **5**E, **5**F), whereas in the prefrontal cortex (Fig. **5**G, **5**H, **5**I) only the mRNA levels of IL-6 significantly correlated with the intake of sucrose (r=-0.570, *P*<0.01).

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

3.2.1 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 received imipramine (Fig. **6**A) and agomelatine (Fig. **6**B) 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. **6**C) 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, S2, S3.

3.2.2 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 figure 8, the expression of IL-1β was still significantly up-regulated after 7 weeks of CMS, and these changes were normalized by 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. **7**B), whereas imipramine (Fig. **7**A) or lurasidone (Fig. **7**C) 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. **7**C, **7**D, **7**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. **7**G; AGO: $F_{1,36}=2.523$, $P=0.122$, Fig. **7**H; LUR: $F_{1,38}=0.015$, $P=0.905$, Fig. **7**I) with the exception of 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.

3.2.3 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 figure **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. **8**A) and partially restored by agomelatine (Fig. **8**B) and lurasidone treatment (Fig. **8**C).

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. **8**C) while imipramine and agomelatine (Fig. **8**A and **8**B) did not show 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. **8**G; LUR: $F_{1,37}$=21.455, *P*<0.001, Fig. **8**I).

All the percentage of changes of microglia markers and the corresponding *P* values for statistical significance are listed in supplementary Table S5.

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-1β was increased in stress-reactive rats in all the brain regions examined. Moreover, the evidence that pharmacological inhibition [38] or genetic deletion of IL-1β receptor [39] 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 [40]. Moreover, administration of IL-6 in the rat hippocampus increased immobility time in the forced swim test, whereas its inhibition has an opposite effect [41]. 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 [16], 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 [42]). 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 [43]. 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 [44]. Interestingly, a recent study by Milior and colleagues showed that CX3CR1 KO mice do not present an anhedonic-like phenotype after two weeks of stress [45]. 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 [46]. 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 [47, Sayd, 2015 #93] 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 [48,49]. 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 [50] 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 [51].

Interestingly, 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. 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 [52], as well as with the multi-receptor antipsychotic drug lurasidone, which has high affinity for dopamine D2 receptors as well as for 5-HT1A, 5-HT2A and 5-HT7 serotonin receptors [32]. 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 [53] and beneficial effects have been demonstrated with the combined use of anti-inflammatory and antidepressant drugs in animal models of depression [54]. 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 [55].

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-HT_7 receptors [56]. 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 [57].

It has to be noted that the main target of our pharmacological treatment appears to be IL-1β. In fact, stress-induced IL-1β 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 [58], which represents an important link between inflammation and depression [59]. We have previously demonstrated that chronic agomelatine treatment is able to modulate the expression of two of the major enzymes involved in this pathway, namely kynurenine-3-monooxygenase (KMO) and kynurenine aminotransferase (KAT)-II, [60] that, by acting on kynurenine, may switch the pathway toward neurotoxic or neuroprotective arms respectively [58]. 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).

Conclusions

Our results demonstrate that the activation of the inflammatory system in the brain is closely linked with the development of a stress-induced deficit in sucrose consumption and

may be ameliorated by the pharmacological treatment, providing further support to the relationship between stress, inflammation and depression [61,62]. From the translational point of view our findings support the potential role of pro-inflammatory mediators as biomarkers that, when assessed before pharmacological treatment, may help to predict the response of an individual patient to a specific drug [63]. In this context, recent studies have suggested a role for IL-1β as a predictor of the antidepressant response [64]. Considering that the inflammatory response is the result of the interaction between several components, a detailed characterization of the role of specific inflammatory mediators will be important to understand their contribution to specific depressive symptoms in order to develop novel strategies for therapeutic intervention.

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Figure Legend

Graphical abstract

Adult male rats exposed to 2-weeks of chronic mild stress (CMS) were divided into two subgroups according with the results of sucrose consumption test: a reactive group (characterized by reduced sucrose intake) and a non reactive group (characterized by a sucrose intake similar to control, unstressed animals). Only the reactive rats showed enhanced neuroinflammation with increased cerebral expression of several mediators of the inflammatory response.

The increased neuroinflammation and the reduction of sucrose intake observed in reactive animals persisted after longer stress exposure (5 weeks) and were ameliorated by chronic treatment with different drugs.

Figure 1

Effect of 2 weeks of chronic mild stress (CMS) on sucrose preference. Rats were divided into animals reactive (REACT) and non-reactive (NON REACT) to CMS depending on sucrose intake (*n*=10 each experimental group) and compared to control unstressed rats (CTRL). The data represent the sucrose intake expressed in grams (g) of each animal included in the study. ***$P<0.001$ vs. CTRL (One-way ANOVA with PLSD).

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), ventral hippocampus (D,

E, F) and prefrontal cortex (G, H, I) of stressed (reactive or non-reactive) rats in comparison with unstressed animals (CTRL). The data, expressed as a percentage of CTRL animals (set at 100%), are the mean ± SEM of at least eight independent determinations. **P*<0.05; ***P*<0.01; ****P*<0.001 vs. CTRL; °°*P*<0.01 °°°*P*<0.001 vs. REACT (One-way ANOVA with PLSD).

Figure 3

Effect of 2 weeks of chronic mild stress (CMS) on mRNA and protein levels of the microglia marker CD11b. The gene expression was measured in the dorsal hippocampus (A), ventral hippocampus (B) and prefrontal cortex (C) of stressed (reactive or non-reactive) rats in comparison with unstressed animals (CTRL). Protein levels were assessed by western blot in the dorsal hippocampus (D). Representative Western blot bands of CD11b are shown in panel E.

The data, expressed as a percentage of CTRL animals (set at 100%), are the mean ± SEM of at least eight independent determinations. *****P*<0.01; vs. CTRL; °° ***P***<0.01, vs. REACT (One-way ANOVA with PLSD).**

Figure 4

Effect of 2 weeks of chronic mild stress (CMS) on **the expression of 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), ventral hippocampus (C, D) and prefrontal cortex (E, F) of stressed (reactive or non-reactive) rats in comparison with unstressed animals (CTRL). The data, expressed as a percentage of CTRL animals (set at 100%), are the mean ± SEM of at least eight independent determinations. **P*<0.05; ***P*<0.01; ****P*<0.001 vs. CTRL; °*P*<0.01; vs. REACT (One-way ANOVA with PLSD).

Figure 5

Pearson product–moment correlation (r) between sucrose consumption and gene expression of IL-1β, IL-6 and CD11b **was analyzed** in the dorsal hippocampus (A, B, C), ventral hippocampus (D, E, F) and prefrontal cortex (G, H, I) of unstressed (CTRL), stress reactive and stress non-reactive animals. The statistical significance was assumed with $P<0.05$.

Figure 6

Effect of pharmacological intervention on sucrose intake of animals exposed to 7 weeks of chronic mild stress (CMS). The sucrose intake was measured weekly during the whole experiment in rats ($n=10$ each experimental group) **exposed to CMS and chronically treated 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).

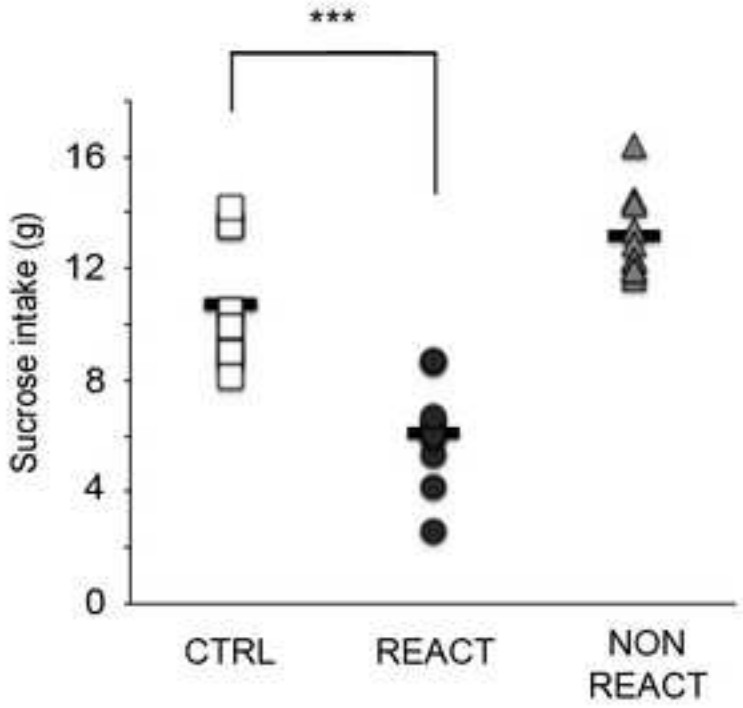
Figure 7

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/Veh (Two-way ANOVA with PLSD).

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*<0.001 vs. Stress/Veh (Two-way ANOVA with PLSD).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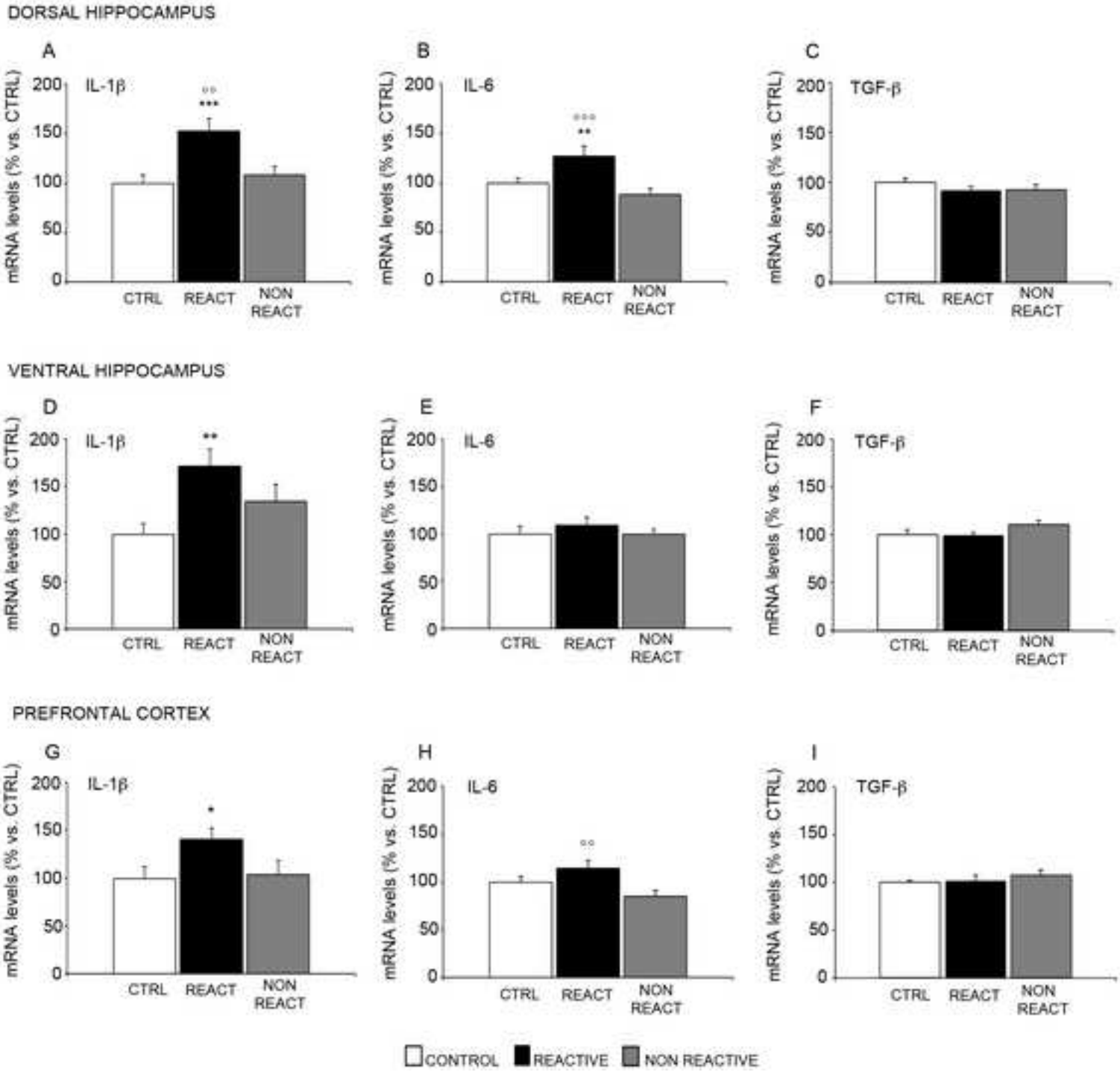
REACT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IL-1β
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TGF-β
VENTRAL HIPPOCAMPUS
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F
TGF-β
PREFRONTAL CORTEX
G
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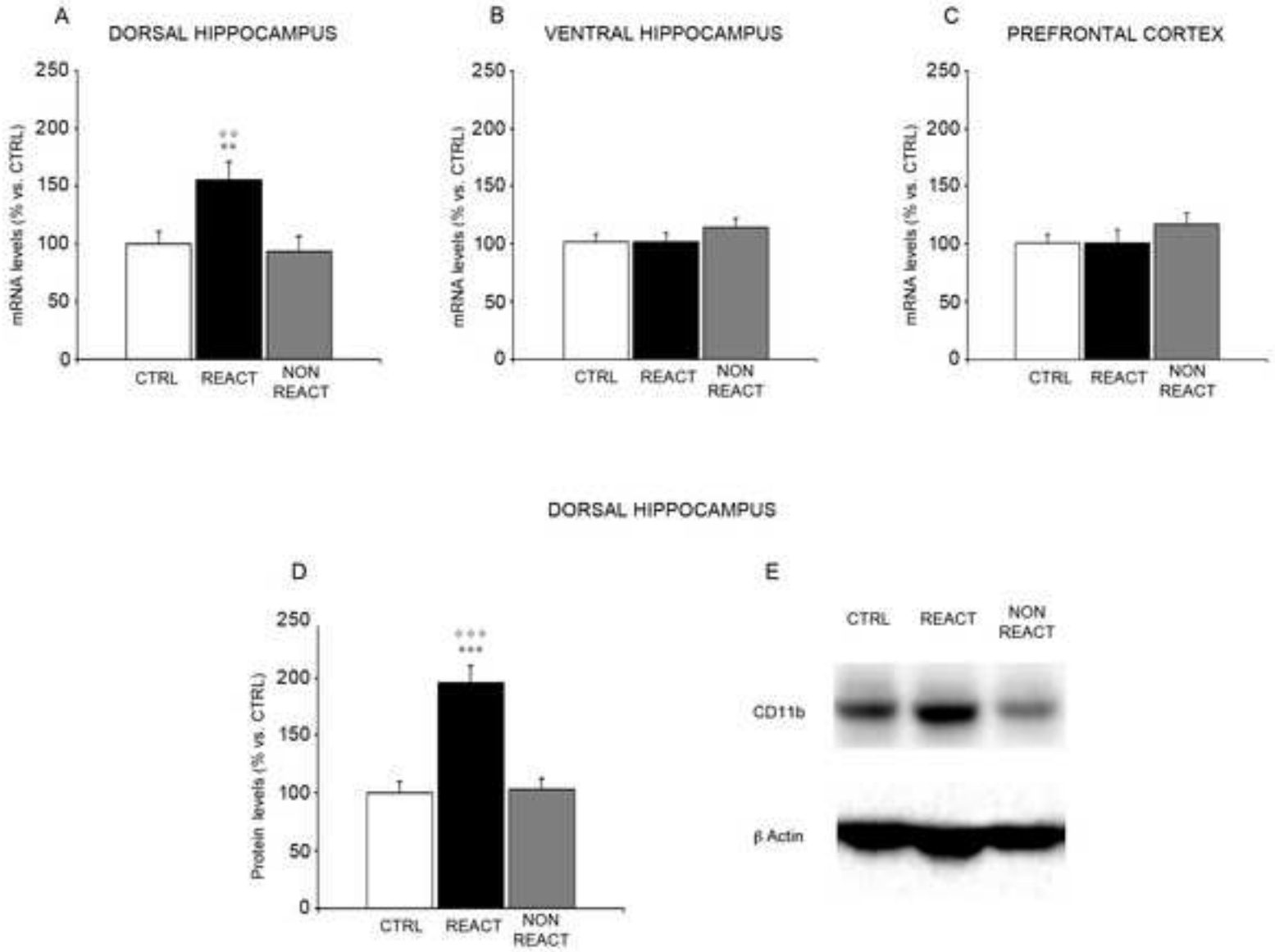
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mRNA levels (% vs. CTRL)
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VENTRAL HIPPOCAMPUS
mRNA levels (% vs. CTRL)
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PREFRONTAL CORTEX
mRNA levels (% vs. CTRL)
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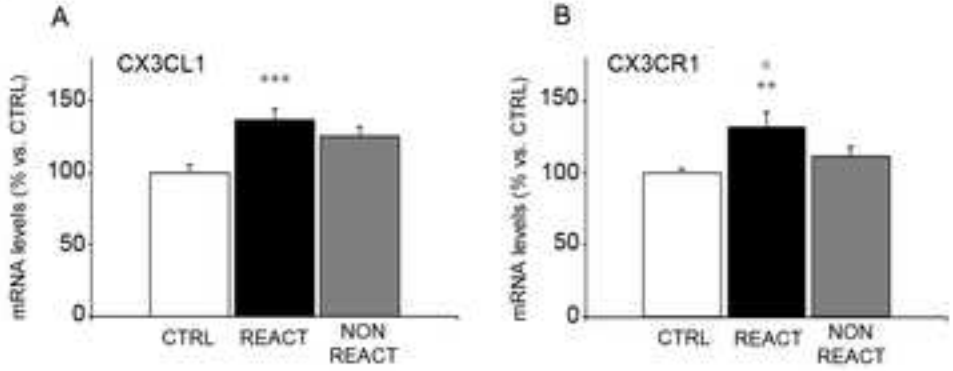
β Actin

DORSAL HIPPOCAMPUS



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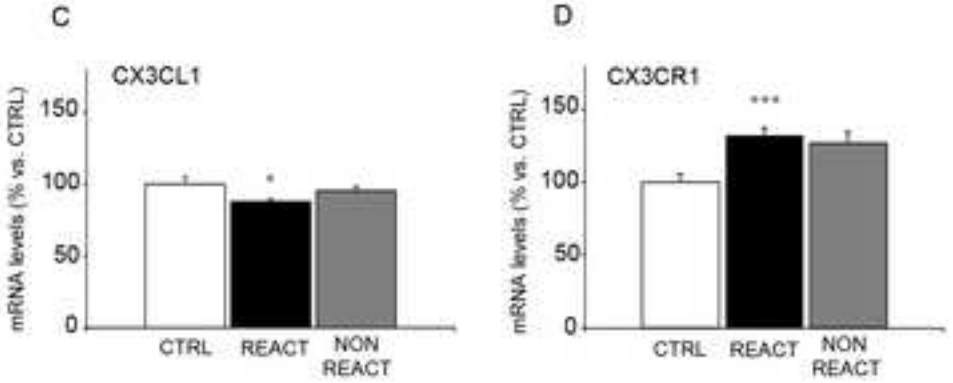
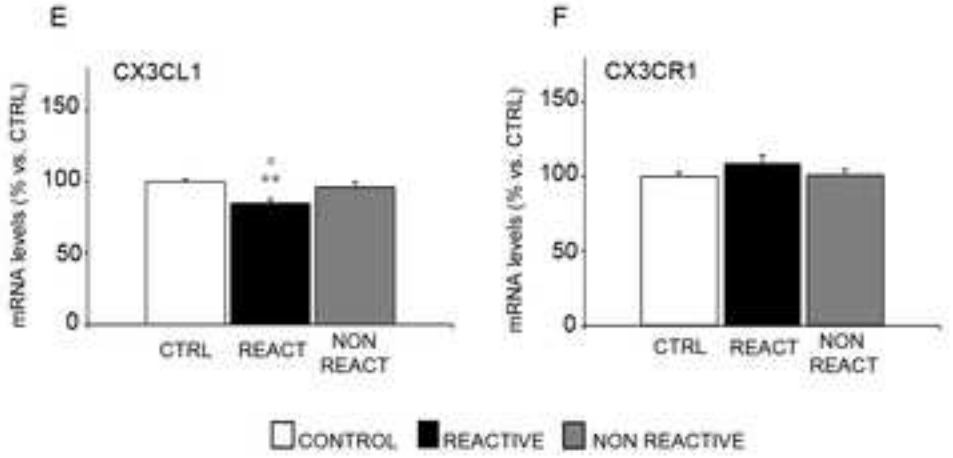
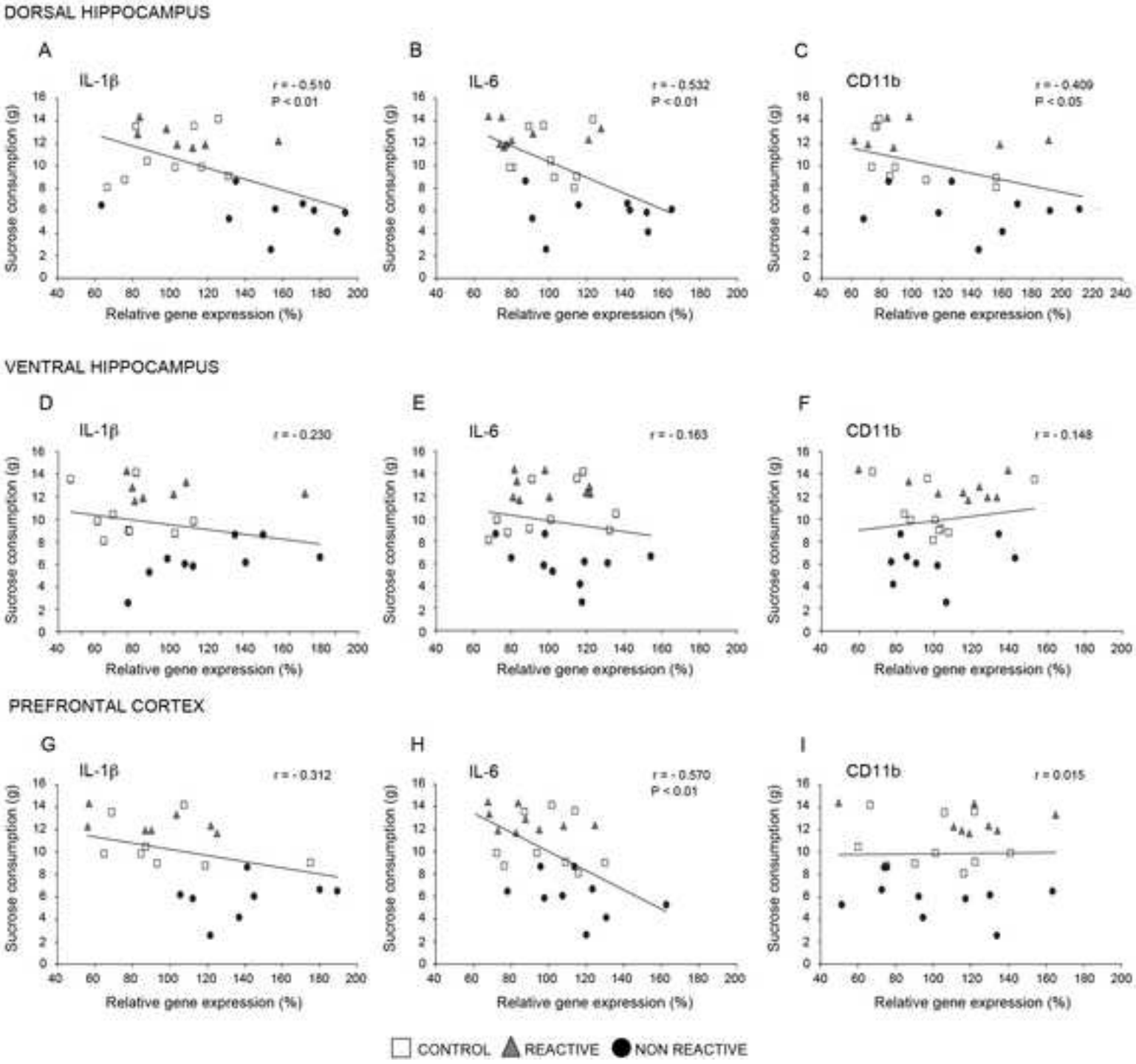
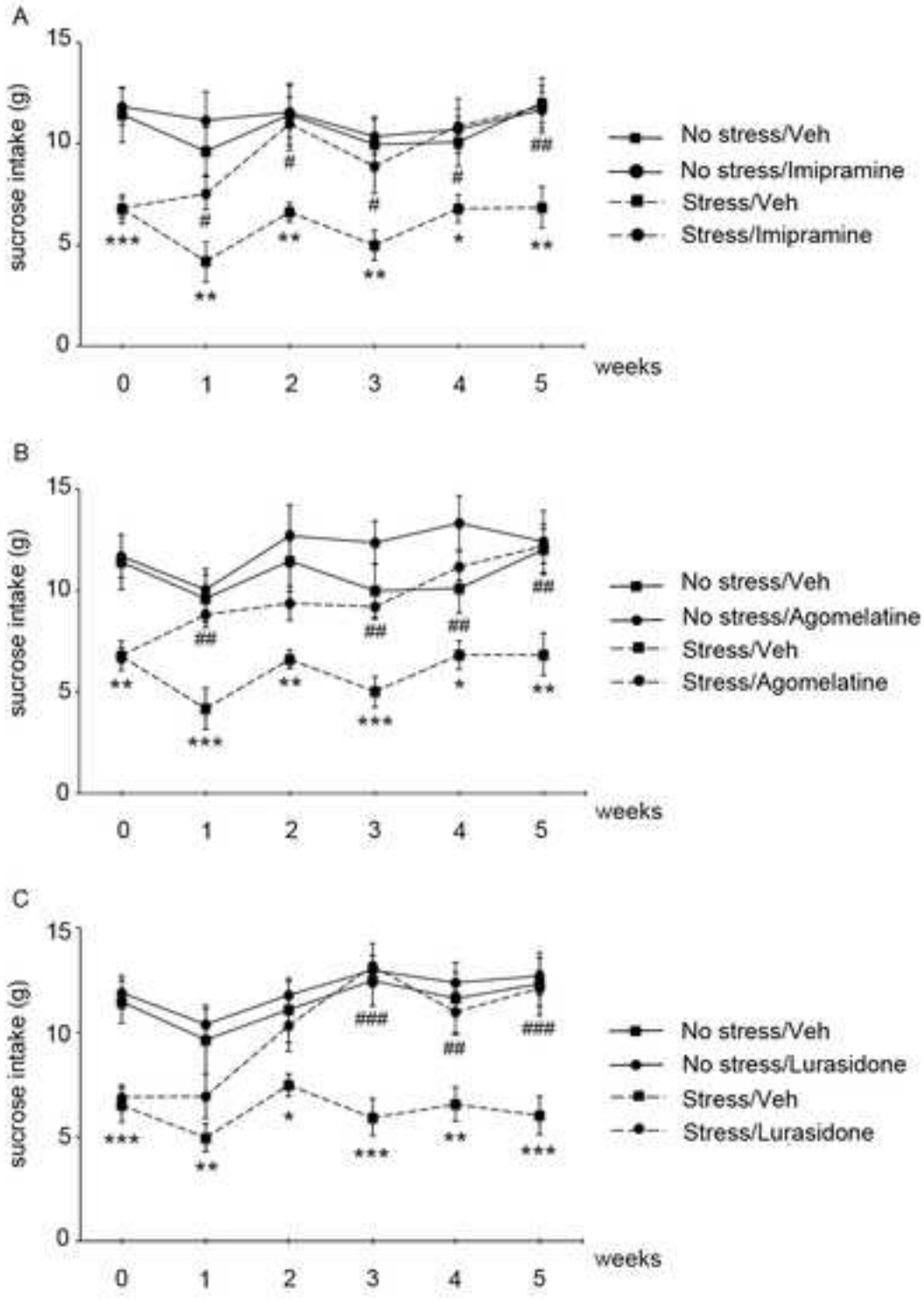
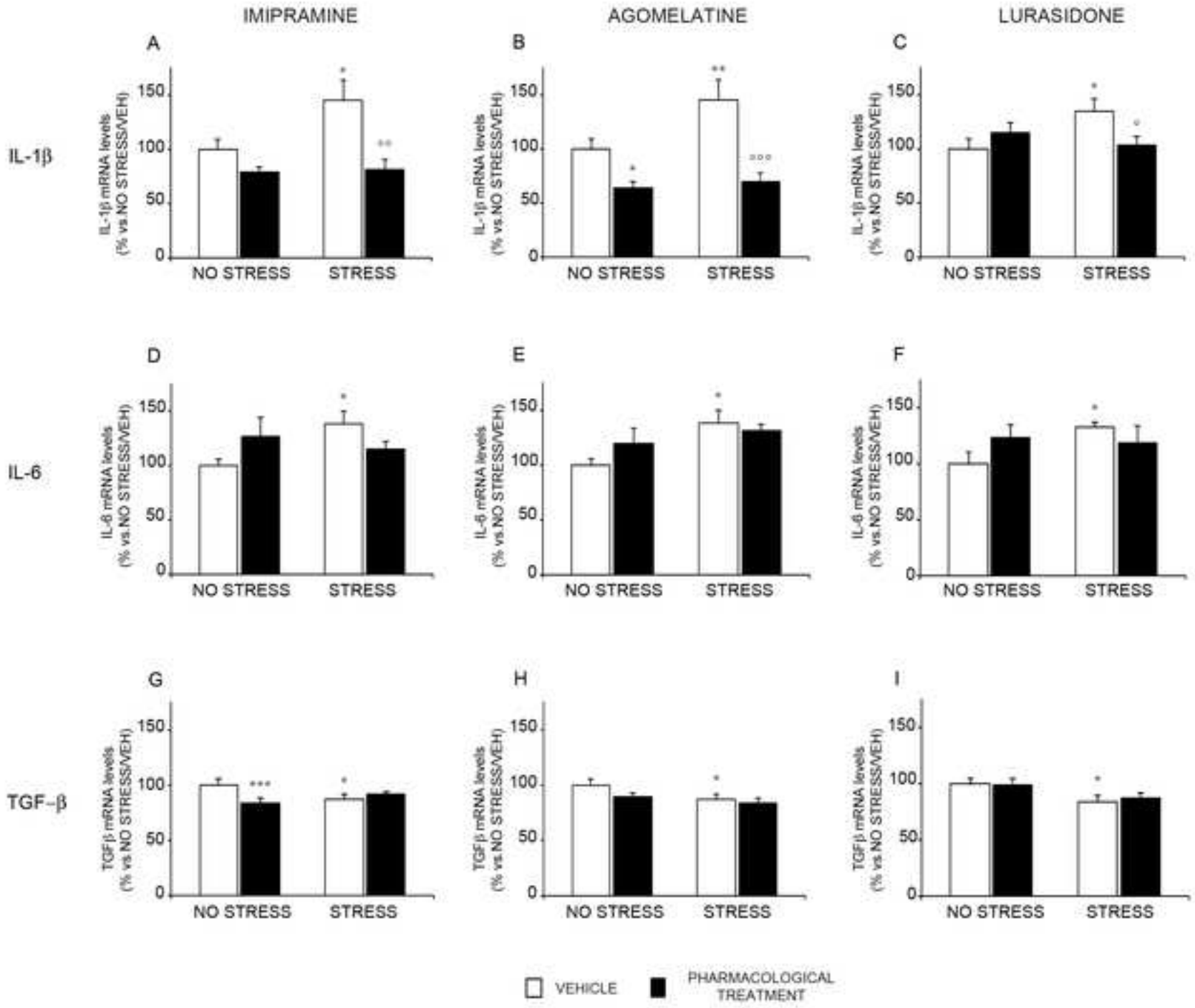


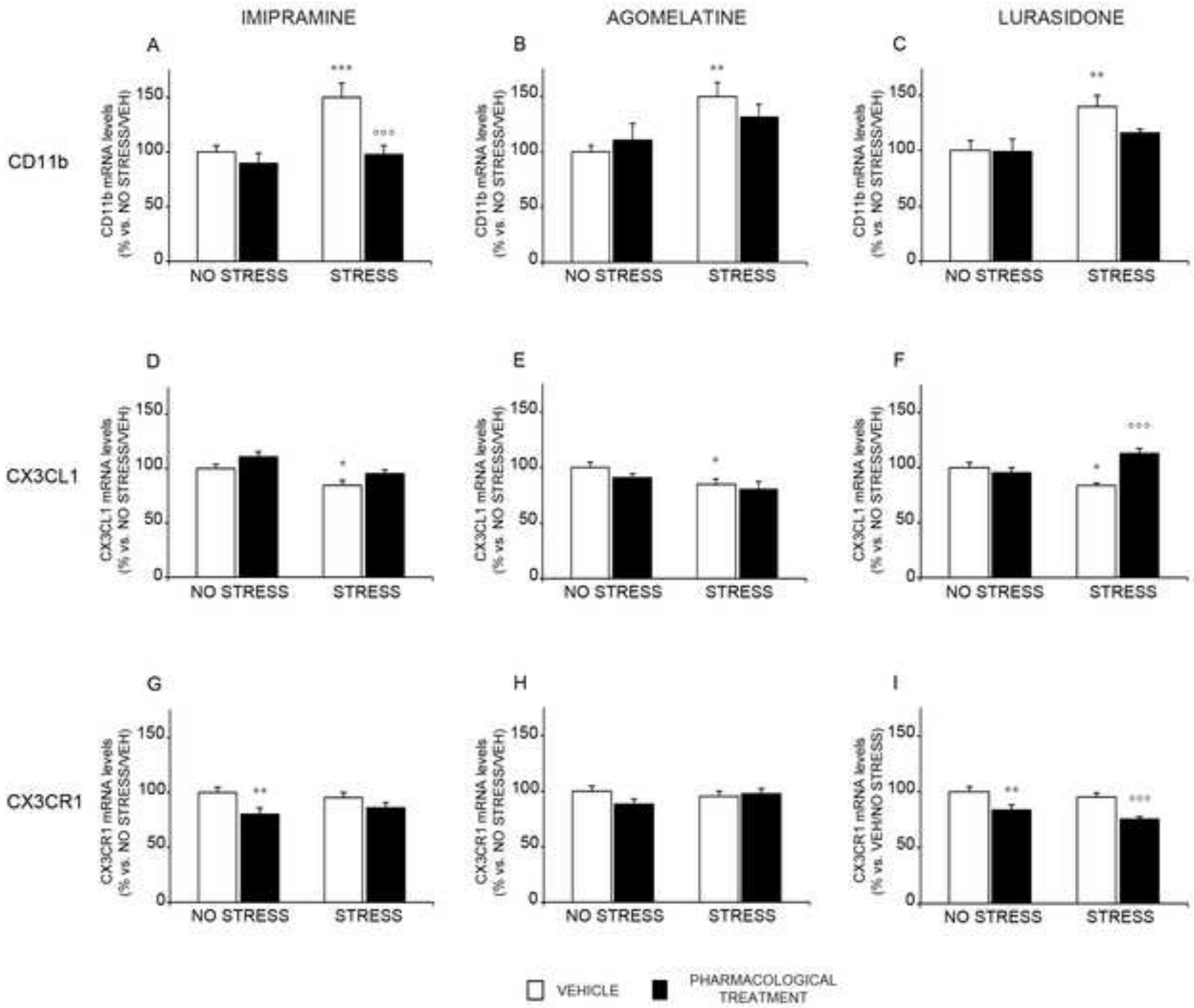
PREFRONTAL CORTEX




DORSAL HIPPOCAMPUS
A
IL-1β
r = - 0.510
P < 0.01
B
IL-6
r = - 0.532
P < 0.01
C
CD11b
r = - 0.409
P < 0.05
VENTRAL HIPPOCAMPUS
D
IL-1β
r = - 0.230
E
IL-6
r = - 0.163
F
CD11b
r = - 0.148
PREFRONTAL CORTEX
G
IL-1β
r = - 0.312
H
IL-6
r = - 0.570
P < 0.01
I
CD11b
r = 0.015
Sucrose consumption (g)
Relative gene expression (%)
CONTROL
REACTIVE
NON REACTIVE


A
sucrose intake (g)
15
10
5
0
weeks
0
1
2
3
4
5
No stress/Veh
No stress/Imipramine
Stress/Veh
Stress/Imipramine
B
sucrose intake (g)
No stress/Veh
No stress/Agomelatine
Stress/Veh
Stress/Agomelatine
C
sucrose intake (g)
No stress/Veh
No stress/Lurasidone
Stress/Veh
Stress/Lurasidone


IMIPRAMINE
AGOMELATINE
LURASIDONE
IL-1β
IL-6
TGF-β
A
B
C
D
E
F
G
H
I
IL-1β mRNA levels (% vs.NO STRESS/VEH)
IL-6 mRNA levels (% vs.NO STRESS/VEH)
TGFβ mRNA levels (% vs.NO STRESS/VEH)
150
100
50
0
NO STRESS
STRESS
VEHICLE
PHARMACOLOGICAL TREATMENT


IMIPRAMINE
AGOMELATINE
LURASIDONE
A
B
C
D
E
F
G
H
I
CD11b
CX3CL1
CX3CR1
CD11b mRNA levels (% vs. NO STRESS/VEH)
CX3CL1 mRNA levels (% vs. NO STRESS/VEH)
CX3CR1 mRNA levels (% vs. NO STRESS/VEH)
CX3CR1 mRNA levels (% vs. VEH/NO STRESS)
0
50
100
150
NO STRESS
STRESS
VEHICLE
PHARMACOLOGICAL TREATMENT

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.

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. 5A
		Imipramine	-21	0,261	**-**	
		CMS	+45	**0,033 ***	-	
		CMS+IMI	-19	-	**0,002 ##**	
AGOMELATINE		CTRL	0	-	-	Fig. 5D
		Agomelatine	-36	**0,014 ***	**-**	
		CMS	+45	**0,007 ****	-	
		CMS+AGO	-31	**-**	**0,000 ###**	
LURASIDONE		CTRL	0	-	-	Fig. 5G
		Lurasidone	+15	0,245	-	
		CMS	+35	**0,016 ***	-	
		CMS+LUR	+3	-	**0,34 #**	
IMIPRAMINE	IL-6	CTRL	0	-	-	Fig. 5B
		Imipramine	+26	0,119	-	
		CMS	+38	**0,018 ***	-	
		CMS+IMI	+15	-	0,191	
AGOMELATINE		CTRL	0	-	-	Fig. 5E
		Agomelatine	+119	0,214	-	
		CMS	+138	**0,012 ***	-	
		CMS+AGO	+131	**-**	0,641	
LURASIDONE		CTRL	0	-	-	Fig. 5H
		Lurasidone	+23	0,137	-	
		CMS	+32	**0,042 ***	-	
		CMS+LUR	+18	-	0,374	
IMIPRAMINE	TGF-β	CTRL	0	-	-	Fig. 5C
		Imipramine	-16	**0,001 *****	-	
		CMS	-13	**0,021 ****	-	
		CMS+IMI	-9	-	0,26	
AGOMELATINE		CTRL	0	-	-	Fig. 5F
		Agomelatine	-11	0,073	-	
		CMS	-13	**0,043 ***	-	
		CMS+AGO	-16	**-**	0,666	
LURASIDONE		CTRL	0	-	-	Fig. 5I
		Lurasidone	-1	0,822	**-**	
		CMS	-16	**0,021 ***	-	
		CMS+LUR	-13	-	0,705	

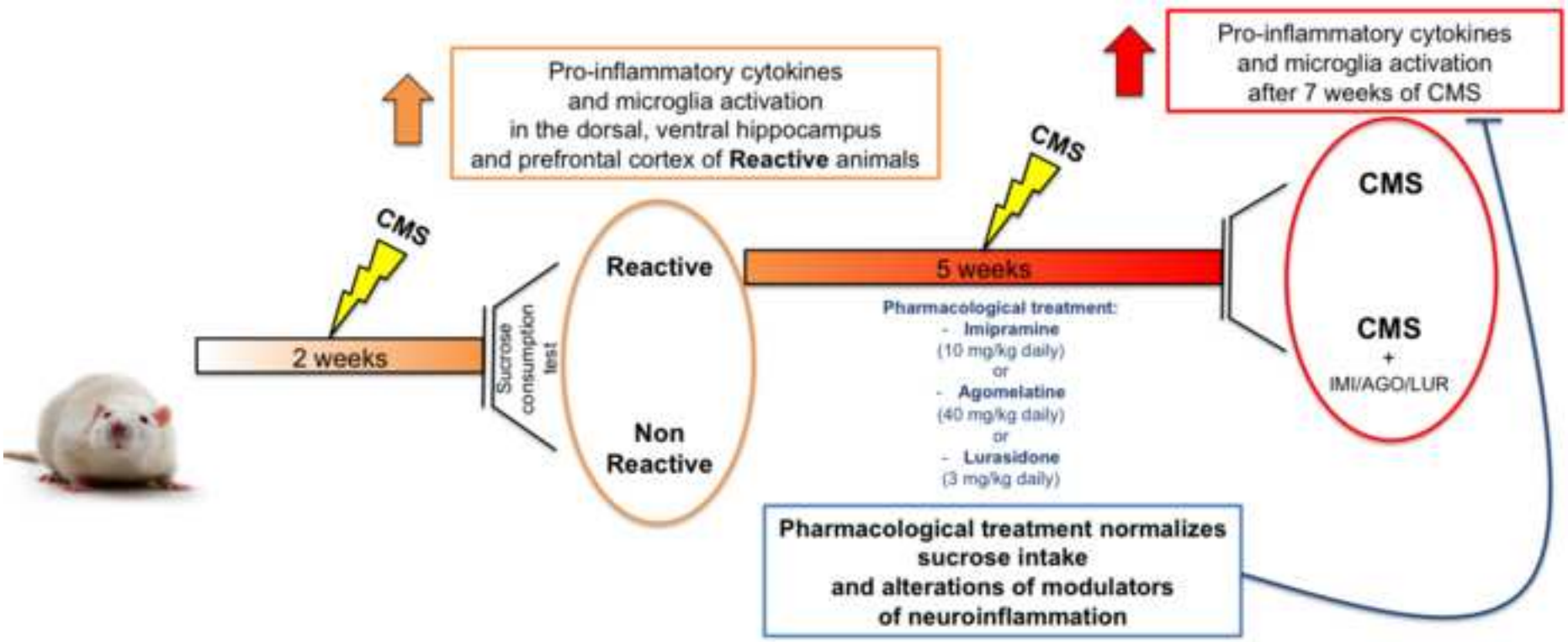
Supplementary Table 5

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 (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.

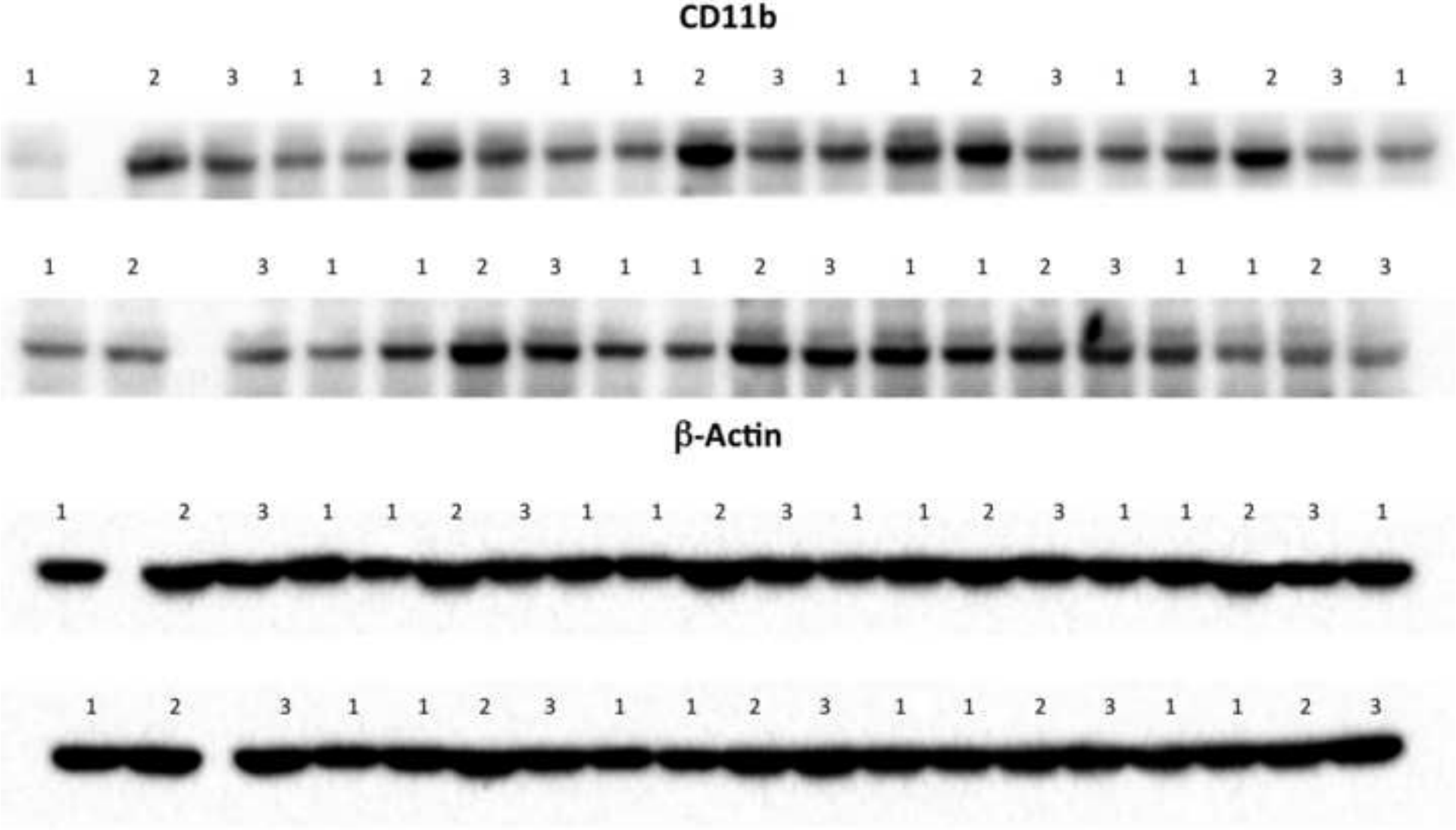
The table shows the percentage of change for each inflammatory protein and the corresponding *P* value obtained by Two-way ANOVA and PLSD test.

Drug	Gene (mRNA)	Experimental Group	% change (of CTRL)	P-Value Vs. CTRL	P-Value Vs. CMS	Figure
IMIPRAMINE	CD11b	CTRL	0	-	**-**	Fig. 6A
		Imipramine	-11	0,351	**-**	
		CMS	+50	**0,000 *** **	-	
		CMS+IMI	-3	-	**0,000 ###**	
AGOMELATINE		CTRL	0	-	**-**	Fig. 6D
		Agomelatine	+10	0,547	**-**	
		CMS	+50	**0,005 ** **	-	
		CMS+AGO	+31	-	0,263	
LURASIDONE		CTRL	0	-	**-**	Fig. 6G
		Lurasidone	-1	0,969	**-**	
		CMS	+40	**0,007 ** **	-	
		CMS+LUR	+16	-	0,097	
IMIPRAMINE	CX3CL1	CTRL	0	-	-	Fig. 6B
		Imipramine	+11	0,099	**-**	
		CMS	-15	**0,028 ***	-	
		CMS+IMI	-5	-	0,104	
AGOMELATINE		CTRL	0	-	-	Fig. 6E
		Agomelatine	-10	0,148	-	
		CMS	-15	**0,039 ***	-	
		CMS+AGO	-20	**-**	0,475	
LURASIDONE		CTRL	0	-	-	Fig. 6H
		Lurasidone	-5	0,385	-	
		CMS	-16	**0,012 ***	-	
		CMS+LUR	+12	-	**0,000 ###**	
IMIPRAMINE	CX3CR1	CTRL	0	-	-	Fig. 6C
		Imipramine	-20	**0,005 ** **	**-**	
		CMS	-5	0,483	-	
		CMS+IMI	-14	**-**	0,167	
AGOMELATINE		CTRL	0	-	-	Fig. 6F
		Agomelatine	-12	0,079	-	
		CMS	-5	0,482	-	
		CMS+AGO	-3	-	0,831	
LURASIDONE		CTRL	0	-	-	Fig. 6I
		Lurasidone	-17	**0,005 ** **	**-**	
		CMS	-5	0,401	-	
		CMS+LUR	-25	**-**	**0,001 ###**	

***Graphical Abstract**



Western Blot analysis of CD11b in the dorsal hippocampus of rats exposed to two weeks of CMS



Legend: 1= CTRL; 2= Reactive; 3= Non Reactive

Conflict of interest

The Author GR has received compensation as speaker/consultant for Servier, Janssen, Otsuka. The Author MAR has received compensation as speaker/consultant for Servier, Eli Lilly, Lundbeck, Sumitomo DainippomPharma Co. Ltd and Sunovion. MAR has also received research grants from Sunovion. The other Authors declare no financial interest or potential conflict of interest. This research has been supported by the Institute de Researches InternationalesServier (IRIS), Suresnes, France. IRIS had no further role in study design, in the collection, analysis and interpretation of the data, in the writing the manuscript, and in the decision to submit it for publication.