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ORIGINAL INVESTIGATION

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

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ABSTRACT

Objectives: Several studies reported that antidepressant drugs have immune-regulatory effects by acting on specific inflammatory mediators. However, considering the highly complex nature of the inflammatory response, we have adopted an unbiased genome-wide strategy to investigate the immune-regulatory activity of the antidepressant agomelatine in modulating the response to an acute inflammatory challenge.

Methods: Microarray analysis was used to identify genes modulated in the ventral hippocampus of adult rats chronically treated with agomelatine (40 mg/kg, o.s.) before being challenged with a single injection of lipopolysaccharide (LPS; 250 μg/kg, i.p.).

Results: The administration of LPS induced the transcription of 284 genes mainly associated with pathways related to the immune/inflammatory system. Agomelatine modulated pathways not only connected to its antidepressant activity, but was also able to prevent the activation of genes induced by LPS. Further comparisons between gene lists of the diverse experimental groups led to the identification of a few transcripts modulated by LPS on which agomelatine has the larger effect of normalisation. Among them, we found the pro-inflammatory cytokine *Il-1β* and, interestingly, the metabotropic glutamatergic transporter *Grm2*.

Conclusions: These results are useful to better characterise the association between depression and inflammation, revealing new potential targets for pharmacological intervention for depression associated to inflammation.

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Microarray; lipopolysaccharide; agomelatine; antidepressant; neuroinflammation

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%) and 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 & 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 are increased in the blood stream and in the cerebrospinal fluid of depressed patients (Raison et al. 2006; Howren et al. 2009; Dowlati et al. 2010). Moreover, depression often occurs in comorbidity with medical conditions characterised by an inflammatory

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state, such as diabetes, cardiovascular or neurodegenerative disorders (Anisman et al. 2008; Berge & Riise 2015; Reus et al. 2015). In addition, the administration of the cytokine-inducer lipopolysaccharide (LPS) in animal models is able to elicit depressive-like behaviours (Frenois et al. 2007; Zhu et al. 2010; van Heesch et al. 2013), an effect also observed after the central administration of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α (Dantzer et al. 2008; Wu & Lin 2008; Sukoff Rizzo et al. 2012).

On the basis of these observations, 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 (Brunello et al. 2006; Akhondzadeh et al. 2009; Raison et al. 2013). 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 & Raison 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. More in detail, adult male rats were treated with the antidepressant for 21 days or with vehicle and, at the end of the treatment, half of the animals received a single injection of LPS. This experimental approach has been designed based, first, on the hypothesis that the anti-inflammatory properties of antidepressants can, at least in part, be ascribed to the long-term adaptive mechanisms induced by their chronic treatment. Second, because the inflammatory response triggered by LPS is limited to a short temporal window and normalises within hours. The transcriptomic profiles of the different experimental groups were investigated in the hippocampus, based on the role of this brain region in MD pathophysiology (Malykhin et al. 2010; Huang et al. 2013; Malykhin & Coupland 2015). Specifically, we carried out our study in the ventral hippocampus, which is mainly related to stress, emotion and affect (Fanselow & Dong 2010), and it was previously shown to be particularly sensitive to the anti-inflammatory ability of agomelatine on specific pro-inflammatory cytokines (Molteni et al. 2013). With this broader approach we analysed 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.

2. Materials and methods

2.1 Animals

Adult male Sprague-Dawley rats (Charles River, Calco, Italy) weighing 300–350 g were used throughout the experiments. Rats were housed in groups of four per cage under standard conditions (12-h light/dark cycle with food and water ad libitum) and were exposed to daily handling for 1 week before any treatment. 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 minimise animal suffering and to reduce the number of animals used.

2.2 Experimental design and pharmacological treatments

Rats were chronically (21 days) treated by oral gavage with vehicle (VEH; hydroxyethylcellulose 1%, 1 ml/kg) or agomelatine (AGO; 40 mg/kg) at 17:00 h, i.e. 2 h before the dark phase to mimic the evening administration of agomelatine in clinics. This schedule was based on our previous studies (Calabrese et al. 2011; Molteni et al. 2013) in order to have the best condition for its circadian activity on melatonergic receptors (de Bodinat et al. 2010), since the onset of melatonin secretion is during the night phase, independently of the biorhythm of the species (nocturnal or diurnal like) (Pevet 2003). Sixteen hours later, the animals were challenged with LPS (from *E. coli*, serotype 026:B6; 250 μg/kg, i.p.) or saline (SAL). 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). Similarly, the dose of LPS was chosen consistently with our previous studies (Macchi et al. 2013, Molteni et al. 2013) and on the base of several reports showing its capability to reliably induce peripheral and central

inflammatory response and depression-like behaviours (Bluthe et al. 1992; Yirmiya 1996; Konsman et al. 1999).

This experimental design implied four 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). Neither agomelatine nor LPS affected the body weight of the animals.

The animals were sacrificed by decapitation 2 h (11:00 h) post LPS injection, in order to evaluate the effect of agomelatine during the peak of the inflammatory response induced by endotoxin (Dantzer et al. 2008), ventral hippocampus was rapidly dissected, frozen on dry ice and stored at –80 °C for the molecular analyses.

2.3 Total RNA preparation

Total RNA was isolated from ventral hippocampus by single step guanidinium-isothiocyanante/phenol extraction using PureZOL RNA isolation reagent (Bio-Rad Laboratories; Segrate, Italy) according the manufacturer's instructions and quantified by spectrophotometric analysis as previously reported (Rossetti et al. 2016).

2.4 Microarray gene expression analysis

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 synthetised with the Gene Atlas WT Expression Kit (Affymetrix, Santa Clara, CA, USA). The concentration and quality of cRNA and cDNA were determined by measuring its absorbance at 260 nm using NanoDrop Spectrophotometer.

After fragmentation and labelling procedures, 5.5 μg of cDNA were hybridised using Rat Gene 2.1 ST Array Strip. The hybridisation, the fluidics and the imaging were performed on the Affymetrix Gene Atlas instrument according to the manufacturer's protocol.

2.5 Quantitative real-time PCR analysis

Quantitative real-time polymerase chain reaction (PCR) was used to validate microarray results by assessing *Grm2* mRNA levels.

Briefly, an aliquot of each sample was treated with DNAse to avoid DNA contamination and subsequently analysed by TaqManqRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iScript one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in a 384-well format in triplicate as multiplexed reactions with a normalising internal control (*36b4*). Thermal cycling was initiated with incubation at 50 °C for 10 min (RNA retrotranscription) and then at 95° 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. Relative target gene expression was calculated according to the 2(-Delta Delta C(T)) method. Probes and primers sequences used were purchased from Eurofins MWG-Operon.

2.6 Statistical and bioinformatic analyses

For microarray data processing, Affymetrix CEL files were imported into Partek Genomics Suite version 6.6 for data visualisation and statistical testing. All samples passed the criteria for hybridisation controls, labelling 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, normalisation was conducted using Quantiles normalisation (Bolstad et al. 2003) to normalise the distribution of probe intensities among different microarray chips. Subsequently, a summarisation step was conducted using a linear median polish algorithm to integrate probe intensities in order to compute the expression levels for each gene transcript.

After the pre-processing of CEL files for quality control, we aimed to investigate the effect of the immune challenge with LPS and the pharmacological treatment with agomelatine, and their combination. Thus, we first included in the two-way ANOVA the two main independent variables (LPS and agomelatine), allowing us to assess their impact in the whole sample. Subsequently, we applied four contrasts (VEH/LPS vs VEH/SAL; AGO/SAL vs VEH/SAL; AGO/LPS vs VEH/SAL; AGO/LPS vs VEH/LPS) in order to get the transcriptomic profiles in each specific condition of interest. In these comparisons, a filter of a P value of $<.05$ 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 is then used to identify regulation of molecular signalling

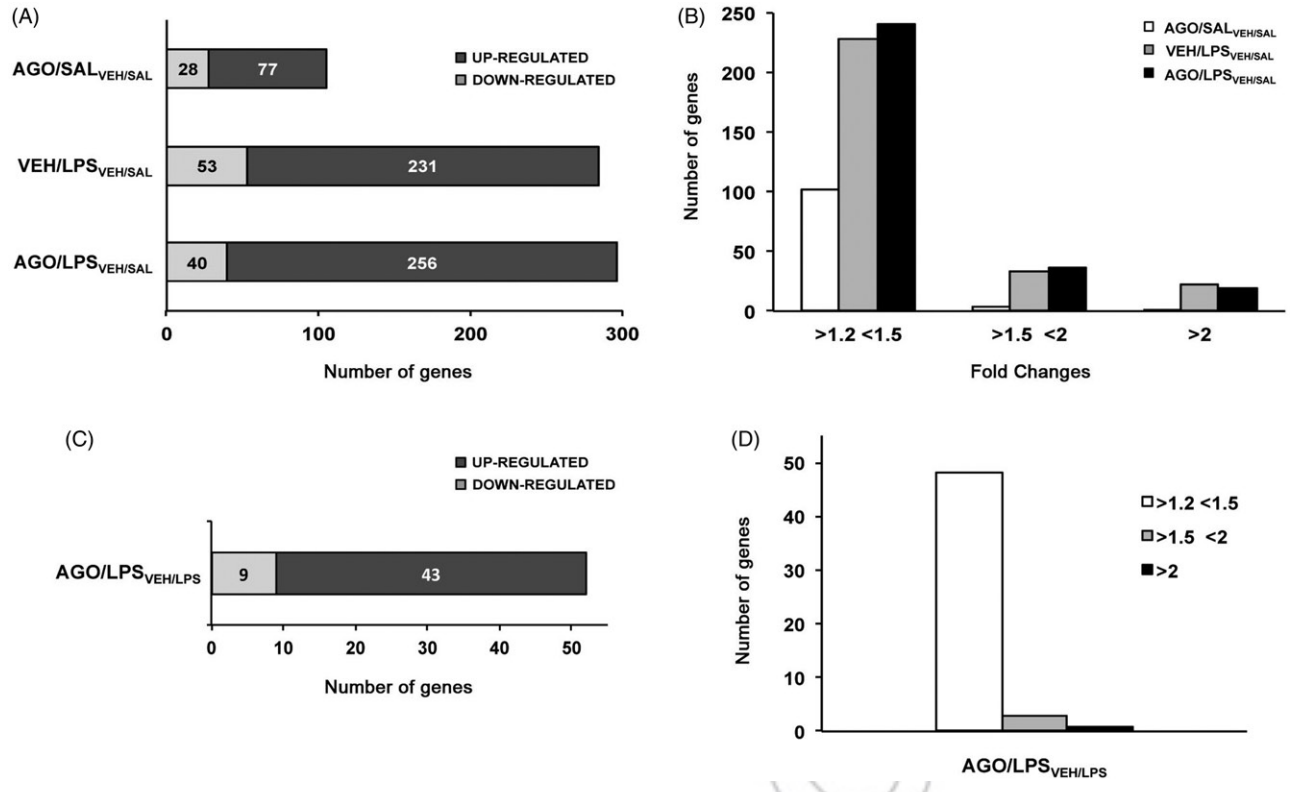


Figure 1. Overall results of microarray analysis. (A) Number of genes up-regulated or down-regulated in the ventral hippocampus of rat chronically treated with agomelatine (AGO/SAL$_{VEH/SAL}$), acutely injected with lipopolysaccharide (VEH/LPS$_{VEH/SAL}$) or receiving both drugs (AGO/LPS$_{VEH/SAL}$), 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/LPS$_{VEH/LPS}$ gene list and magnitude of this modulation (D).

pathways, network and GO terms in each condition. In this case we kept a significance threshold of a log value equal to 1.3 ($P = .05$).

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 analysed by Fisher's Protected Least Significant Difference or single contrast post-hoc test. Significance was assumed for $P < .05$. For graphic clarity, data are presented as means percent ± standard error (SEM) of control group, namely vehicle-pre-treated rats received saline (VEH/SAL).

3. Results

3.1 Overall transcriptional effect of chronic treatment with agomelatine and acute administration of LPS

In order 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 1(A), 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 compared with the control group. Among these, 256 (86%) transcripts were up-regulated, whereas 40 (14%) were down-regulated.

Additionally, we analysed the magnitude of these transcriptional effects finding mild changes in all the experimental groups (Figure 1(B)). 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-fold with respect to control animals.

Moreover, to investigate the impact of the pre-treatment 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 1(C), this analysis resulted in a list of 52 genes, nine of which were down-regulated (17%), whereas 43 were up-regulated (82%). The magnitude of the modulation of these genes (Figure 1(D)) was between 1.2 and 1.5 FC, and only few transcripts exceeded this threshold.

3.2 Genome-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 with 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 gamma-13, *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 homologue 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 signalling pathways of phospholipase C and of the chemokine receptor CXCR4 (the entire list of pathways is detailed in Table S2, Supplementary materials).

3.3 Genome-wide effect of the acute administration of LPS

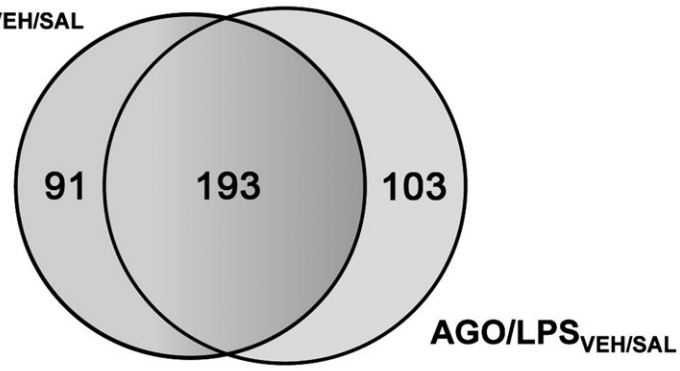
The microarray analysis indicated that 284 genes were differentially expressed between animals injected with LPS and sacrificed 2 h later and saline-treated rats. All these genes are listed in Table S3 (Supplementary materials). A large part of these transcripts (81%) was up-regulated by the inflammatory 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)* and *Slco1a2,* 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 transferrin 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 p38 MAPK related signalling.

3.4 Genome-wide effect of the pre-treatment with agomelatine on the inflammatory response induced by LPS

In order 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 (Figure 2(A)) 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 pre-treatment 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

(A)



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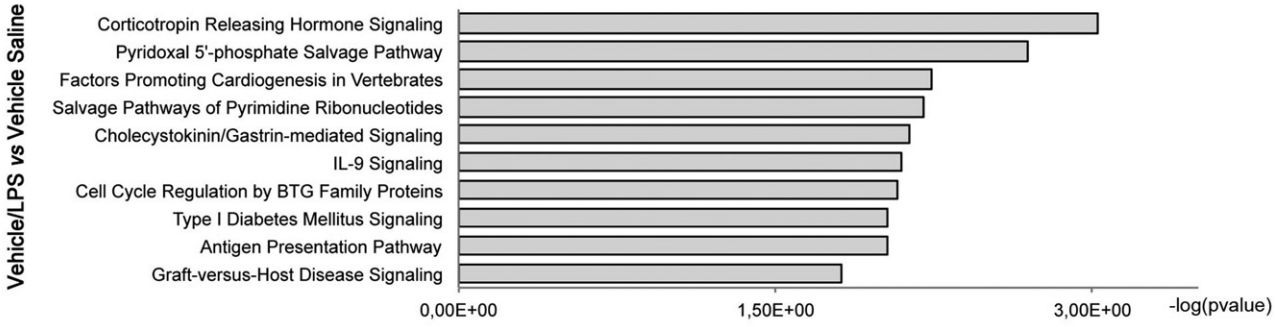


(C)

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 2. 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 ten canonical pathways most affected by acute injection of lipopolysaccharide in vehicle pre-treated 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.

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 signalling (*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) signalling as well as pathways associated with the regulation of specific cytokines (i.e. IL-9 signalling, IL-10 signalling, Role of JAK1 and JAK3 in γc Cytokine Signalling). The top 10 pathways are shown in Figures 2(B,C).

Next in the analyses of the Venn diagram of Figure 2(A), 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 $AGO/LPS_{VEH/SAL}$ group, we found *Hist1h4m*, *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.

To further evaluate the impact of agomelatine pretreatment on the inflammatory response induced by LPS, we implemented the previously described comparison focussing on the $AGO/LPS_{VEH/LPS}$ list. This list includes 52 genes (Figure 1(C)) 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 up-regulated 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 GH3 Domain Containing (*Ghdh*) with a FC of –1.30 and *Grm2* (FC $=-1.27$). For the complete gene list see 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*) (Figure 3).

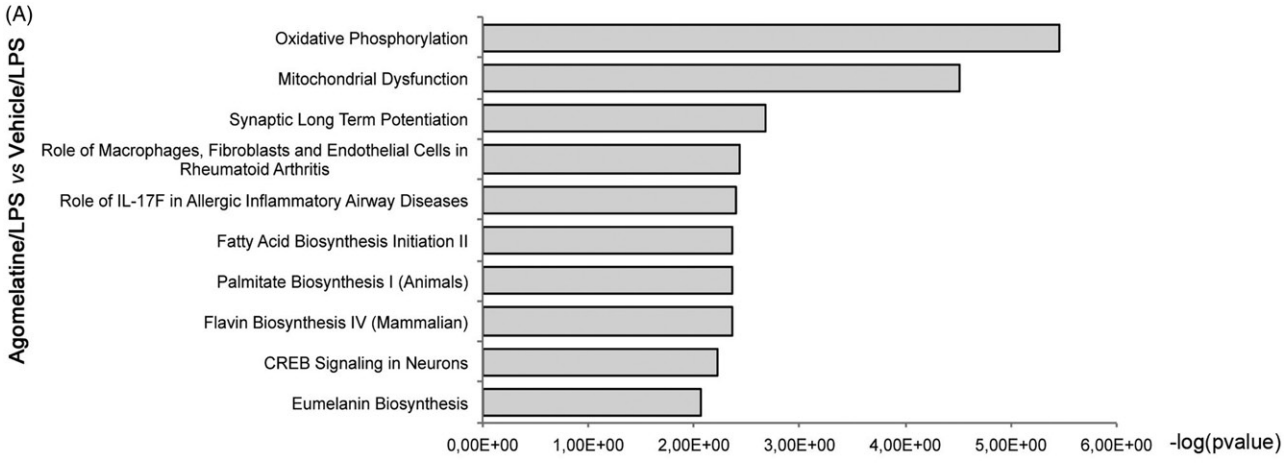
Lastly, with the purpose of narrow the list of genes whose LPS-induced modulation may be prevented by agomelatine, we performed an overlap analysis between the 52 genes belonging to the $AGO/LPS_{VEH/LPS}$ list and the 91 genes, shown respectively in Table S9 and S5, found using VEH/SAL as reference group. The resulting Venn diagram (Figure 4(A)) indicates that nine 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 pre-treatment has the larger effect of normalisation. Among these, we focussed our attention on the glutamate metabotropic receptor Grm2 and, as shown in Figure 4(B), the qRT-PCR analysis confirmed the modulation observed in the microarray study. Indeed, Grm2 mRNA levels were significantly increased by LPS in animals pretreated with vehicle (+34% $P=.055$ vs VEH/SAL) but not in those that received agomelatine (–37% $P<.001$ vs VEH/LPS), as indicated by the significant Drug × LPS interaction ($F_{1,2\ 7}=5.718$, $P=.025$, two-way ANOVA).

4. 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 others, two pathways: the signalling of C-X-C chemokine receptor 4 (CXCR4) and phospholipase C (PLC). Chemokines are small molecules that mediate leukocyte mobilisation to sites of inflammation in the periphery. Currently, the chemokine family consists of more than 50 members with more than 20 G-protein-coupled receptors that have also been detected at a cerebral level (Bajetto et al. 2001). CXCR4 is the receptor of the very well-studied chemokine CXCL12 (or SDF-1). This signalling 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

(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 3. Preventive effect of agomelatine: direct comparison between AGO/LPS and VEH/LPS groups. (A) Top ten canonical pathways most affected by the acute injection of lipopolysaccharide in agomelatine pre-treated 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 (B) with the associated –log(*P* value), number and name of genes involved.

serotonergic neurons, and in mechanisms related to neuroprotection such as production and release of different neurotrophic factors (Shyu et al. 2008; Heinisch & Kirby 2010; Reaux-Le Goazigo et al. 2013; Williamson & 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 & Monteggia 2006; Sanacora et al. 2012).

Another notable pathway modulated by the chronic administration of agomelatine is the signalling 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 (Rantamaki et al. 2007). Our data add new information as indicate that agomelatine is able to modulate the PLC signalling 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 signalling 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 signalling (Jiang et al. 2015), thus supporting the potential of PLCβ as new pharmacological target. In line with our result, it has been recently demonstrated that TrkB signalling is effectively involved in the antidepressant effect of agomelatine (Boulle et al. 2016).

However, besides these pathways involved in the effect of agomelatine per se, we identified genes specifically related to its ability to counteract the

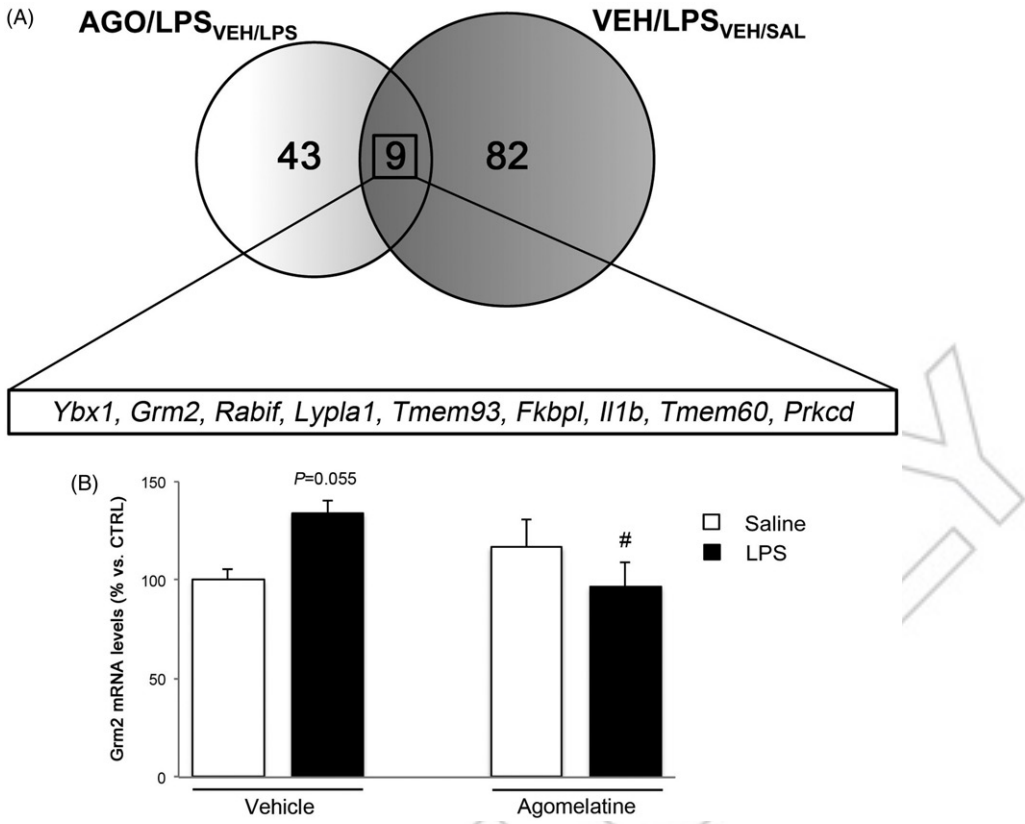


Figure 4. Top nine genes modulated by agomelatine identified by intersection analysis. (A) Venn diagram of the comparison between AGO/LPS group (with VEH/LPS baseline) and the 91 genes of the VEH/LPS$_{VEH/SAL}$ group whose transcription was prevented by the pre-treatment with agomelatine. The overlap between the two groups indicates nine 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 3 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. $P = .055$ vs vehicle/saline; #$P < .05$ vs vehicle/LPS. Two-way ANOVA with Fisher's PLSD.

inflammatory response. Indeed, by analysing our data with different approaches, we found that the LPS-induced modulation of several genes was prevented by the antidepressant. 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. 2015).

Through different overlap analyses, we further narrowed the list of genes whose LPS-dependent modulation was prevented by the antidepressant, finding nine 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 signalling); *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 homologue 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 post-mortem 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 hypothesise that a reduction in LPS-induced increase of *Grm2* by 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 characterise the mechanisms involved in the association between depression and inflammation.

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