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ARTICLE

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Risperidone normalizes increased inflammatory parameters and restores anti-inflammatory pathways in a model of neuroinflammation

Karina S. MacDowell[1,2,3], Borja García-Bueno[1,2,3], José L. M. Madrigal[1,2,3], Mara Parellada[2,4], Celso Arango[2,4], Juan A. Micó[2,5] and Juan C. Leza[1,2,3]

[1] *Department of Pharmacology, Faculty of Medicine, Complutense University, Madrid, Spain*
[2] *Centro de Investigación Biomédica en Red de Salud Mental* (*CIBERSAM*)
[3] *Instituto de Investigación Sanitaria Hospital 12 de Octubre* (*I+12*)*, Madrid, Spain*
[4] *Department of Child and Adolescent Psychiatry, Hospital General Universitario Gregorio Marañón and Department of Psychiatry, Faculty of Medicine, Complutense University, Madrid, Spain*
[5] *Department of Pharmacology, Faculty of Medicine, University of Cádiz, Spain*

Abstract

Inflammation, caused by both external and endogenous factors, has been implicated as a main pathophysiological feature of chronic mental illnesses, including schizophrenia. An increase in pro-inflammatory cytokines has been described both in experimental models and in schizophrenia patients. However, not much is known about the effects that antipsychotic drugs have on intra- and intercellular mechanisms controlling inflammation. The aim of the present study was to investigate the possible anti-inflammatory effect of a standard schizophrenia treatment not only at the level of soluble mediators, but also at intra- and intercellular inflammatory pathways. The present study was conducted in a model of mild neuroinflammation using a lipopolysaccharide (LPS) challenge that was not an endotoxaemic dose (0.5 mg/kg i.p.) in young adult rats. Main results: single doses of risperidone (0.3–3.0 mg/kg i.p.) prevented increased inflammatory parameters induced by LPS in brain cortex [expression of inflammatory cytokines, interleukin (IL)-1β and tumour necrosis factor (TNF)-α, activity of the inducible inflammatory enzymes nitric oxide synthase and cyclooxygenase, p38 mitogen-activated protein kinase (MAPK) and inflammatory nuclear transcription factor κB] and restored anti-inflammatory pathways decreased by LPS challenge (deoxyprostaglandins and peroxisome proliferator activated receptor γ). This is the first study demonstrating that risperidone elicits a preventive effect on the anti-inflammatory arm of the homeostatic mechanism controlling inflammation in a model of mild encephalitis in rats. Our findings suggest a possible protective effect of risperidone on brain cells.

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Key words: Antipsychotics, LPS, neuroinflammation, risperidone, schizophrenia.

Introduction

The pathophysiology of schizophrenia is unknown, although several aetiological theories have been proposed for the disease, including developmental or neurodegenerative processes, neurotransmitter abnormalities, immune dysfunction or inflammatory mechanisms.

Address for correspondence: Dr J. C. Leza, Departamento de Farmacología, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain.
Tel.: +34 91 394 1478/64 *Fax*: +34 91 394 1464
Email: jcleza@med.ucm.es

Over the past two decades, inflammation, caused by both external and endogenous factors, has been implicated as a main pathophysiological feature of chronic mental illnesses, including schizophrenia (Lucas *et al.* 2006). A large amount of data supports an inflammatory hypothesis in the pathophysiology of schizophrenia: (1) the demonstration of a pro-inflammatory cytokine profile in plasma, serum, cerebrospinal fluid (CSF) or in post-mortem brain tissue (Drexhage *et al.* 2008; Müller & Schwarz, 2008) of schizophrenia patients; (2) elevated plasma levels of the inflammatory mediator prostaglandin E_2 (PGE_2), the major product of the enzyme cyclooxygenase

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(COX)-2 (Kaiya *et al.* 1989) and increased COX activity (Das & Khan, 1998) were also found in schizophrenia; (3) microglial activation or increased microglial cellular density have also been suggested by post-mortem studies, at least in subpopulations of patients (i.e. suicide victims during acute psychosis; Steiner *et al.* 2008); (4) positron emission tomography studies demonstrated microglial activation in patients within the first 5 yr following the onset of the first psychotic episode (van Berckel *et al.* 2008); (5) the identification of inflammation-related genes up-regulated in brains from schizophrenia patients (Drexhage *et al.* 2010; Saetre *et al.* 2007); (6) COX-2 inhibitors administered as coadjuvant therapies improve some cognitive deficits in patients (Akhondzadeh *et al.* 2007; Müller & Schwarz, 2010); (7) various clinical and experimental studies demonstrated an anti-cytokine effect of some antipsychotics (Arolt *et al.* 2000; Maes *et al.* 1996; Sugino *et al.* 2009).

In line with this, different attempts have been made to evaluate the potential anti-inflammatory effects of different antipsychotics (reviewed in Dean, 2010). Most studies have focused on two aspects: the anti-oxidative effect and the anti-cytokine effect. Taking into account the close relationship between intra- and intercellular biochemical pathways regulating oxidative status and inflammation, many of the studies have been directed towards a possible antioxidant effect. In fact, there is evidence of abnormal activities of antioxidant enzymes in the plasma and CSF in schizophrenia and also a dysregulation of free radical metabolism leading to lipid peroxidation in erythrocytes and platelets (Dietrich-Muszalska *et al.* 2005; Pall *et al.* 1987; Srivastava *et al.* 2001; Yao *et al.* 1998). Such oxidative stress misbalance occurs in acute episodes of schizophrenia (Micó *et al.* 2011) and also probably at other stages of the disorder (Mahadik & Mukherjee, 1996; Mahadik *et al.* 1998).

To date, attempts carried out to elucidate the effects of antipsychotics on antioxidant enzymes and lipid peroxidation, or possible interventions at the oxidative stress level in schizophrenia patients, have led to controversial results, depending on the parameter and the drug evaluated (Huang *et al.* 2010; Padurariu *et al.* 2010). A possible direct, scavenging effect seems to be discarded by *in vivo* studies suggesting that olanzapine and risperidone do not affect the total antioxidant capacity and lipid peroxidation in plasma from healthy volunteers (Dietrich-Muszalska & Kontek, 2010).

As stated earlier, some of the data available about the inflammatory basis of pathogenesis of schizophrenia have been presented on a possible anti-cytokine effect of antipsychotics in clinical and experimental settings (Himmerich *et al.* 2011; Watanabe *et al.* 2010). However, after a large meta-analysis was applied on cytokines in schizophrenia, at the level of the proteins (Potvin *et al.* 2008), no clear conclusions were obtained on the anti-cytokine effect of medication. Recent studies confirm these discrepancies: whereas McNamara *et al.* (2011) demonstrate that risperidone negatively regulates pro-inflammatory signalling cascades through ω-3 fatty acid up-regulation and biosynthesis, Reale *et al.* (2011) found significantly higher levels of constitutive chemokines and cytokines in untreated first-episode schizophrenia patients although no statistically significant differences were detected in serum levels after risperidone, olanzapine or clozapine treatment.

All these findings emphasize the need for the design of research studies focused and refined to mechanistically elucidate possible anti-inflammatory effects of antipsychotics. Furthermore, recent studies have identified an imbalance between pro- and anti-inflammatory mechanisms in schizophrenia patients (Martínez Gras *et al.* 2011). We hypothesized a possible anti-inflammatory effect of a standard schizophrenia treatment, not only at the level of soluble mediators but also at intra- and intercellular inflammatory pathways. In an attempt to explore this, the present study was conducted in a standardized model of mild neuroinflammation using a lipopolysaccharide (LPS) challenge that was not an endotoxaemic dose (0.5 mg/kg i.p.) in young adult rats.

Method

Animals

Seventy-six adult (aged 12 wk) male Wistar Hannover rats (HsdHan:Wist, from Harlan Ibérica), weighing 225–250 g were used. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense in accordance with European legislation (DC 86/609/CEE; 2003/65/CE). The rats were housed individually with standard temperature and humidity conditions and in a 12-h light/dark cycle (lights on at 08:00 hours) with free access to food and water. All the animals were maintained under constant conditions for 7 d prior to the experiment.

Drug administration and experimental design

Risperidone free base (MW 410) was kindly donated by Janssen Cork (Belgium). LPS (*Escherichia coli* serotype 0111:B4, ref. L2630) and others chemicals were purchased from Sigma-Aldrich (Spain) or as indicated.

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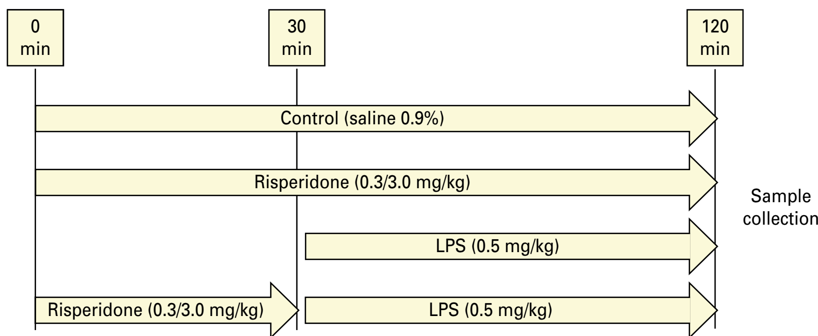

Fig. 1. Experimental design.

LPS was dissolved in 0.9% saline and risperidone in a saline solution with 0.1% Tween 20 by sonication for 1 min. The number of subjects used was as follows: control, saline ($n=14$); 0.3 mg/kg risperidone ($n=10$); 3.0 mg/kg risperidone ($n=10$); 0.5 mg/kg LPS ($n=14$); 0.3 mg/kg risperidone+0.5 mg/kg LPS ($n=14$); 3.0 mg/kg risperidone+0.5 mg/kg LPS ($n=14$). The rats were injected i.p. with 2 ml/kg saline or risperidone appropriate for each group. Thirty minutes later, LPS was injected i.p. and animals were killed 90 min after LPS using sodium pentobarbital (320 mg/kg i.p.) (Fig. 1).

The timing and doses of risperidone were chosen on the basis of previous *in vivo* studies in young adult Wistar rats for central and peripheral determinations (i.e. plasma pharmacokinetic/pharmacodynamic relationship and steady state for D_2 receptor occupancy; half-life; Dremencov *et al.* 2007*a*, *b*; Naiker *et al.* 2006; Olsen *et al.* 2008).

Preparation of biological samples

Blood for plasma determinations was collected by cardiac puncture and anticoagulated in the presence of trisodium citrate (3.15% (wt/vol), 1 vol citrate/9 vol blood). Plasma was obtained from blood samples by centrifuging them at 402 ***g*** for 10 min at 4 °C immediately after sample collection. All plasma samples were stored at −40 °C before assay. Peripheral mononuclear blood cells (PMBC) were prepared for some determinations: the rest of the sample discarded after plasma removal was 1:2 diluted in culture medium (RPMI 1640; Lonza, Belgium) and a gradient with Ficoll-Paque® (GE Healthcare, Sweden) by centrifugation (500 ***g*** for 40 min, 4 °C). PBMC layer was carefully aspired and resuspended in RPMI and centrifuged (1116 ***g*** for 10 min). The supernatant was removed and the mononuclear cell-enriched pellet was resuspended in RPMI and stored at −80 °C until processing. After decapitation, the brain was removed from the skull and after careful removal of the meninges and blood vessels, the prefrontal cortical areas from both brain hemispheres were excised and frozen at −80 °C until assayed.

Determinations of transcription factors, its inhibitory subunits or nuclear receptors were carried out in cytosolic and/or nuclear extracts from brain tissue or PMBC (transcription factors and nuclear receptors in nuclei, inhibitory subunits of transcription factors in cytosol). For preparation of cytosolic fraction and nuclear extracts, a modified procedure based on Schreiber *et al.* (1989) was used. In short, samples (brain frontal cortex or PMBC) were homogenized in 300 μl buffer [10 mmol/l *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (pH 7.9), 1 mmol/l EDTA, 1 mmol/l EGTA, 10 mmol/l KCl, 1 mmol/l dithiothreitol, 0.5 mmol/l phenylmethylsulfonyl fluoride, 0.1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml Na-p-tosyll-lysine-chloromethyl ketone, 5 mmol/l NaF, 1 mmol/l $NaVO_4$, 0.5 mol/l sucrose and 10 mmol/l Na_2MoO_4]. After 15 min, Nonidet P-40 (Roche, Germany) was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s and nuclei were collected by centrifugation at 8000 ***g*** for 5 min. Supernatants were considered as the cytosolic fraction. The pellets were resuspended in 100 ml buffer supplemented with 20% glycerol and 0.4 mol/l KCl and gently shaken for 30 min at 4 °C. Nuclear protein extracts were obtained by centrifugation at 13 000 ***g*** for 5 min and aliquots of the supernatant were stored at −80 °C. All steps of the fractionation were carried out at 4 °C. As an analysis of purity of cytosolic and nuclear extracts, these were Western blot (WB) assayed against IκBα, sp-1 or β-actin (in cytosol: 83 ± 4; 19 ± 5; 98 ± 1% of total optical density (OD) signal, respectively; in nuclei: 16 ± 9; 81 ± 7; 99 ± 1% of total OD signal, respectively).

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Plasma measurements

Nitrites (NO_2^-) levels

As the stable metabolites of the free radical nitric oxide (NO•), NO_x^- were measured by using the Griess method (Green *et al.* 1982). Briefly, in an acidic solution with 1% sulphanilamide and 0.1% NEDA, nitrites convert into a pink compound that is photometrically calculated at 540 nm in a microplate reader (Synergy 2; BioTek, USA) (Salter *et al.* 1996).

Tissue (brain prefrontal cortex) or PMBC measurements

Brain prefrontal cortex was chosen because of its high levels of pro-inflammatory [inducible isoforms of nitric oxide synthase and cyclooxygenase, iNOS and COX-2 respectively and nuclear transcription factor (NF)-κB] and anti-inflammatory [peroxisome proliferator activator receptor (PPAR)-γ] mediators and its susceptibility to the neuroinflammatory process elicited by inflammatory/immune challenge, such as LPS (reviewed in García-Bueno *et al.* 2008*a*, *b*). Furthermore, some cognitive deficits and alterations in the neuronal circuitry of the prefrontal cortex, including dopamine neurotransmission disturbances, have been described in schizophrenia (Volk & Lewis, 2010).

Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid test for malondialdehyde (MDA) following the method described by Das & Ratty (1987) with some modifications. Samples were sonicated in 10 vol 50 mmol/l phosphate buffer and deproteinized with 40% trichloroacetic acid and 5 mol/l HCl, followed by the addition of 2% (wt/vol) thiobarbituric acid in 0.5 mol/l NaOH. The reaction mixture was heated in a water bath at 90 °C for 15 min and centrifuged at 11 752 *g* for 10 min. The pink chromogen was measured at 532 nm in a microplate reader (Synergy 2; BioTek). The results were expressed as nmol/mg protein.

WB analysis

After determining and adjusting protein levels, homogenates of prefrontal cortex tissue, once centrifuged (11 752 *g*, 10 min at 4 °C) were mixed with Laemmli sample buffer (Bio-Rad, USA) (SDS 10%, distilled H_2O, 50% glycerol, 1 M Tris HCl, pH 6.8, dithiothreitol and Bromophenol Blue) with β-mercaptoethanol (50 μl/ml Laemmli) and 20 μl (1 μg/μl) were loaded into an electrophoresis gel. Once separated on the basis of molecular weight, proteins from the gels were blotted onto a nitrocellulose membrane (Amersham Ibérica, Spain) with a semi-dry transfer system (Bio-Rad) and were incubated with specific antibodies: (1) rabbit polyclonal PPAR-γ in a dilution of 1:1000 in TBS tween (sc-7196; Santa Cruz Biotechnology, USA); (2) rabbit polyclonal IκBα in a dilution of 1:1000 in TBS tween (sc-371; Santa Cruz Biotechnology); (3) rabbit polyclonal NF-κB p65 and rabbit polyclonal NF-κB p50, both diluted 1:800 in BSA 1% (sc-109; Santa Cruz Biotechnology); (4) phosphorylated *p38* rabbit polyclonal antibody in a dilution of 1:1000 in TBS tween (sc-17852R; Santa Cruz Biotechnology); (5) iNOS rabbit polyclonal antibody dilution of 1:1000 in 2.5% BSA in TBS tween (sc-650; Santa Cruz Biotechnology); (6) COX-2 goat polyclonal antibody dilution of 1:1000 in 2.5% BSA in TBS tween (sc-1747; Santa Cruz Biotechnology); (7) lipocalin-prostaglandin D_2 synthase (L-PGDs) rabbit polyclonal antibody in a dilution of 1:1500 in BSA 2.5% (16003; Cayman Chemical Europe, Estonia); (8) m-PGES-1 mouse monoclonal antibody in a dilution of 1:1000 in TBS tween (10004350; Cayman Chemical Europe, Estonia); (9) β-actin mouse monoclonal in a dilution of 1:10 000 (Clone AC-15; Sigma, Spain).

Proteins were recognized by the respective horseradish peroxidase-linked secondary antibodies (in a dilution of 1:2000 in TBS Tween) and visualized on X-ray film by chemiluminescence following manufacturer's instructions. Autoradiographs were quantified by densitometry (program Image J, NIH) and several time expositions were analysed to ensure the linearity of the band intensities. In all the WB analyses, the housekeeping gene β-actin was used as a loading control (the blots are shown in the respective figures).

mRNA analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Spain); aliquots were converted to cDNA using random hexamer primers. Quantitative changes in mRNA levels were estimated by real time (RT) quantitative polymerase chain reaction (Q-PCR) using the following cycling conditions: 35 cycles of denaturation at 95 °C for 10 s, annealing at 58–61 °C for 15 s, depending on the specific set of primers for IL-1β, TNF-α, lipocalin-prostaglandin D_2 synthase (L-PGDS), m-PGES-1, COX-2, iNOS, NF-κB, IκBα, PPAR-γ, GADPH and tubulin (Table 1) and extension at 72 °C for 20 s. Reactions were carried out in the presence of SYBR Green (1:10 000 dilution of stock solution from Molecular Probes, USA), carried out in a 20 μl reaction in a Corbett Rotor-Gene (Corbett Research,

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Table 1. Specific primers for real-time polymerase chain reaction

	Forward primers (5′–3′)	Reverse primers (5′–3′)
IL-1β	ACCTGCTAGTGTGTGATGTTCCCA	AGGTGGAGAGCTTTCAGCTCACAT
TNF-α	CTGGCCAATGGCATGGATCTCAAA	ATGAAATGGCAAATCGGCTGACGG
L-PGDS	CTTGGGTCTCTTGGGATTTCCA	GTTGTAGGTGTACTGTCCGGGAAC
m-PGES-1	GGTGAAGCAAATGTTCCCAGCTCA	TTTAGCGGTTGGTCAAAGCCCATC
COX-2	CTTCGGGAGCACAACAGAG	GCGGATGCCAGTGATAGAG
iNOS	GGACCACCTCTATCAGGAA	CCTCATGATAACGTTTCTGGC
NF-κB	CATGCGTTTCCGTTACAAGTGCGA	TGGGTGCGTCTTAGTGGTATCTGT
IκBα	TGGCCTTCCTCAACTTCCAGAACA	TCAGGATCACAGCCAGCTTTCAGA
PPAR-γ	ACCAACTTCGGAATCAGCTC	AGGCTCTACTTTGATCGCAC
Tubulin	CCCTCGCCATGGTAAATACAT	ACTGGATGGTACGCTTGGTCT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

IL, Interleukin; TNF-α, tumour necrosis factor α; L-PGDS, prostaglandin D_2 synthase; m-PGES-1, microsomal prostaglandin E synthase; COX-2, cyclooxygenase 2; NF-κB, nuclear transcription factor κB; PPAR-γ, peroxisome proliferator activator receptor γ.

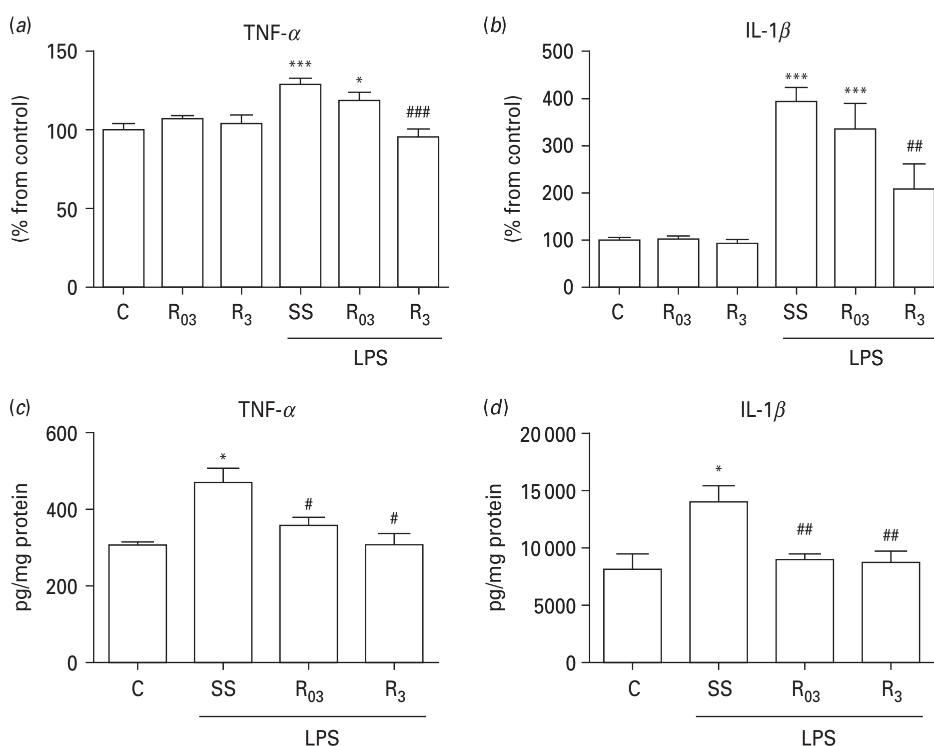

Fig. 2. Risperidone anti-inflammatory effects in brain I. Pro-inflammatory cytokines. Quantitative polymerase chain reaction (Q-PCR) analysis of the pro-inflammatory cytokines tumour necrosis factor (TNF)-α (*a*) and interleukin (IL)-1β (*b*) mRNA and levels of TNF-α (*c*) and IL-1β (*d*) in the prefrontal cortex of control (C), 0.3 mg/kg risperidone (R_{03}), 3 mg/kg risperidone (R_3), saline solution + lipopolysaccharide (LPS) (SS), 0.3 mg/kg risperidone (R_{03}) + LPS, 3 mg/kg risperidone (R_3) + LPS. Data are normalized by tubulin and GAPDH. * $p<0.05$ *vs.* C, *** $p<0.001$ *vs.* C; ## $p<0.01$ *vs.* LPS, ### $p<0.001$ *vs.* LPS (one-way ANOVA followed by Newman–Keuls *post-hoc* test). Data represent mean ± S.E.M.

Australia). Relative mRNA concentrations were calculated from the take-off point of reactions using the included software, GADPH and tubulin levels were used to normalize data.

Nitrites (NO_2^-) levels were also assessed as previously described in plasma samples.

IL-1β and TNF-α levels were detected using a specific enzyme-linked immunosorbent assay (ELISA)

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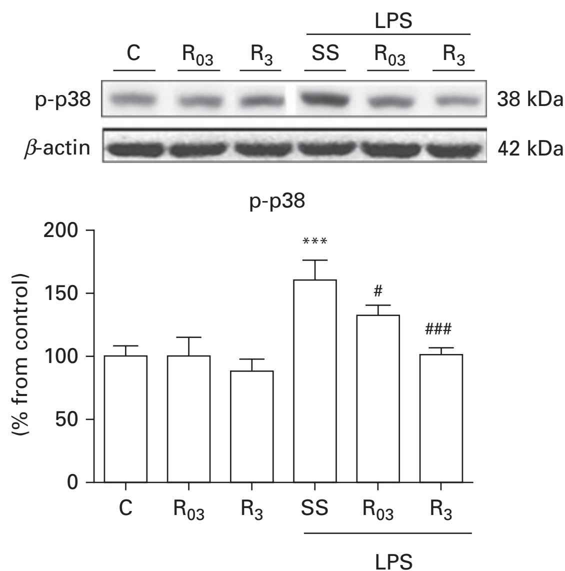

Fig. 3. Risperidone anti-inflammatory effects in brain II. Phosphorylated p38 mitogen-activated protein kinase (MAPK). Phosphorylated p38 MAPK Western blot and densitometric analysis in the prefrontal cortex of control (C), 0.3 mg/kg risperidone (R_{03}), 3 mg/kg risperidone (R_3), saline solution + lipopolysaccharide (LPS) (SS), 0.3 mg/kg risperidone (R_{03}) + LPS, 3 mg/kg risperidone (R_3) + LPS. *** $p < 0.001$ *vs.* C; # $p < 0.05$ *vs.* LPS, ### $p < 0.001$ *vs.* LPS (one-way analysis of variance followed by Newman–Keuls *post-hoc* test). Data represent mean ± S.E.M.

for rat tissue carried out according to the manufacturer's instructions (RayBiotech Inc., USA). The minimum detectable dose of TNF-α and IL-1β was <25 and 80 pg/ml respectively. Absorbance in each well was measured using a spectrophotometric plate reader (Synergy 2; BioTek) at 450 nm.

PGE_2 and 15d-PGJ_2 levels were measured by enzyme immunoassay (EIA) using reagents in kit form (Prostaglandin E_2 EIA Kit-Monoclonal; Cayman Chemical) and 15-deoxy-D12,14-prostaglandin J_2 ELISA Kit (DRG Diagnostics, Germany), respectively. Samples were sonicated in 400 μl homogenization buffer (0.1 M phosphate buffer, pH, 7.4, 1 mM EDTA and 10 mM indomethacin) and purified in 4 volume ethanol for 5 min at 4 °C. Samples were then centrifugated at 3000 *g* for 10 min and acidified with glacial acetic acid (pH 3.5). Prostaglandin compound was then extracted using SPE (C-18) columns (Waters, USA) rinsed with methanol and water. After the application of samples, columns were washed with water and hexane and PGE_2 and 15d-PGJ_2 were eluted with ethyl acetate. Samples were then evaporated to dryness under nitrogen and resuspended in EIA buffer. Levels of PGE_2 and 15d-PGJ_2 were measured in a 96-well plate and read at 405 nm following manufacturer's instructions (Synergy 2; BioTek). The sensitivity of the assay for PGE_2 was 15 pg/ml; intra- and interassay coefficients of variation were 6.6 and 15.5%, respectively, at 62.5 pg/ml and for 15d-PGJ_2 the sensitivity was 36.8 pg/ml; intra- and interassay coefficient of variations were 7.4 and 13%, respectively, at 4.4 pg/ml.

Protein assay

Protein levels were measured using the Bradford method based on the principle of protein-dye binding (Bradford, 1976).

Statistical analyses

Data in text and figures are expressed as mean ± S.E.M. For multiple comparisons, a one-way analysis of variance (ANOVA) followed by the Newman–Keuls *post-hoc* test to compare all pairs of means between groups was made. A p value <0.05 was considered statistically significant.

Results

Effects of risperidone on LPS-induced increase in the levels of proinflammatory cytokines in frontal cortex

Administration of LPS induced an increase in the mRNA levels of the two main proinflammatory cytokines, TNF-α and IL-1β, in brain frontal cortex (Fig. 2*a, b*) when compared with levels in control animals [control TNF-α: 0.8 ± 0.06 arbitrary units (AU); control IL-1β: 0.53 ± 0.03 AU]. Risperidone, only at the higher dose tested, decreased LPS-induced TNF-α and IL-1β mRNA increase in prefrontal cortex (Fig. 2*a, b*). Furthermore, the levels of both proinflammatory cytokines were increased after LPS in prefrontal cortex and, in both cases, risperidone, at the two doses tested, decreased levels in parallel with the effects on mRNAs (Fig. 2*c, d*).

Effects of risperidone on LPS-induced increase in the levels of phosphorylated p38 MAPK in frontal cortex

MAPK pathways transduce a large variety of external signals, leading to a wide range of cellular responses, including inflammation via NF-κB activation (see below). Administration of LPS induced an increase in the expression of phosphorylated p38 MAPK in brain frontal cortex [$p < 0.001$ *vs.* control (0.7 ± 0.069 AU)] (Fig. 3). Risperidone, at the two doses tested,

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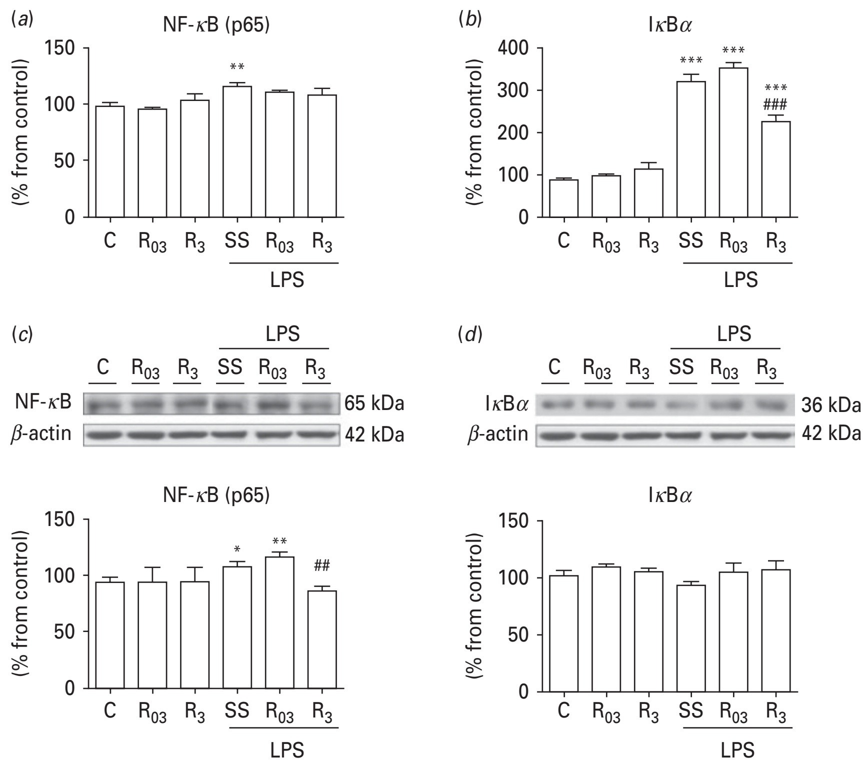

Fig. 4. Risperidone anti-inflammatory effects in brain III. Nuclear transcription factor (NF)-κB and IκBα. (*a*) NF-κB pro-inflammatory subunit p65 mRNA levels; (*b*) NF-κB inhibitory protein IκBα mRNA levels; (*c*) NF-κB (p65) Western blot and densitometric analysis; (*d*) NF-κB inhibitory protein IκBα Western blot and densitometric analysis in the prefrontal cortex of control (C), 0.3 mg/kg risperidone (R_{03}), 3 mg/kg risperidone (R_3), saline solution + lipopolysaccharide (LPS) (SS), 0.3 mg/kg risperidone (R_{03}) + LPS, 3 mg/kg risperidone (R_3) + LPS. * $p<0.05$ *vs.* C, ** $p<0.01$ *vs.* C, *** $p<0.001$ *vs.* C; ## $p<0.01$ *vs.* LPS, ### $p<0.001$ *vs.* LPS (one-way analysis of variance followed by Newman–Keuls *post-hoc* test). Data represent mean ± S.E.M.

decreased LPS-induced p38 MAPK increase in prefrontal cortex.

Effects of risperidone on brain NF-κB changes induced by LPS challenge

The transcription factor NF-κB is a key regulator of pro-inflammatory and oxidative/nitrosative mediators both in the brain and periphery by inducing the expression of inflammatory genes, including some cytokines. We also tested the possible involvement of risperidone at the level of NF-κB expression. RT-PCR studies of NF-κB (p65) mRNA revealed an increase in the LPS group when compared to control (0.89 ± 0.04 AU). WB studies mimicked the same pattern between control and stressed groups (control 0.69 ± 0.04 AU) at protein level (Fig. 4*a, c*). p65 mRNA up-regulation was not affected by risperidone (Fig. 4*a*), but the higher dose of the atypical antipsychotic completely inhibited LPS-dependent p65 up-regulation at protein level (Fig. 4*c*). NF-κB p50 subunit expression was also determined. Although LPS increased the expression of this subunit (193.13 ± 18.1, $p<0.05$ *vs.* control, 100 ± 9.9% from control value, AU), none of the doses of risperidone used modified the effects of LPS (112.97 ± 55.4; 217.49 ± 62.23, $p>0.05$ *vs.* control). NF-κB activity can be additionally confirmed at the level of its inhibitory protein (IκBα). RT-PCR revealed an increase of IκBα mRNA in brain after LPS challenge when compared to control (0.86 ± 0.04 AU), with a preventive effect of risperidone (3 mg/kg) (Fig. 4*b*). Protein expression studies did not show changes between all groups of animals (Fig. 4*d*).

Effects of risperidone on inflammatory and oxidative/nitrosative markers induced by LPS challenge in brain

Two main enzymatic sources involved in the accumulation of oxidative/nitrosative and inflammatory mediators after LPS exposure are iNOS and COX-2. We decided to explore the effects of risperidone on

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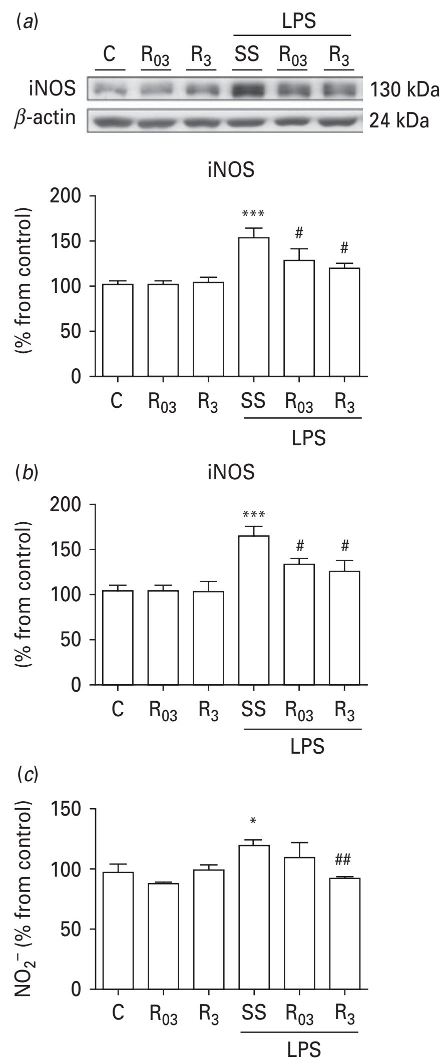

Fig. 5. Risperidone anti-inflammatory effects in brain IV. iNOS and NO_x levels. (*a*) Pro-inflammatory enzyme iNOS Western blot and densitometric analysis; (*b*) pro-inflammatory enzyme iNOS mRNA levels; (*c*) brain levels of nitrites in the prefrontal cortex of control (C), 0.3 mg/kg risperidone (R_{03}), 3 mg/kg risperidone (R_3), saline solution + lipopolysaccharide (LPS) (SS), 0.3 mg/kg risperidone (R_{03}) + LPS, 3 mg/kg risperidone (R_3) + LPS. * $p<0.05$ *vs.* C, *** $p<0.001$ *vs.* C; # $p<0.05$ *vs.* LPS, ## $p<0.01$ *vs.* LPS (one-way analysis of variance followed by Newman–Keuls *post-hoc* test). Data represent mean ± S.E.M.

the expression and/or activity of these enzymes. Both doses of risperidone decreased the LPS-induced increase of iNOS protein and mRNA in cerebral cortex (Fig. 5*a*, *b*) (control values: expression: 0.91 ± 0.08 AU; mRNA: 0.7 ± 0.05 AU). The concentration of nitrites increased after LPS (control value: 0.00123 ± 0.00015192 μM/mg protein) and the higher dose of risperidone prevented this increase (Fig. 5*c*).

Similarly, COX-2 protein (Fig. 6*a*) and mRNA (Fig. 6*b*) were increased after LPS challenge (control values: expression: 0.79 ± 0.05 AU; mRNA: 0.9 ± 0.05 AU). Risperidone prevented mRNA expression at the two doses tested and protein expression after administration of 3 mg/kg (Fig. 6*a*, *b*). As an inducible isoform, when compared to its constitutive isoform, increased COX-2 activity accounts for higher formation of sub-products (endoperoxides). However, the final products depend on specific enzymes. The microsomal prostaglandin E synthase 1 (m-PGES-1) is the specific enzymatic source of PGE_2, the main _inflammatory prostaglandin in the brain. The study of m-PGES-1 revealed similar changes after LPS and the preventive effect of risperidone (Fig. 6*c*, *d*) control values: expression: 0.78 ± 0.04 AU; mRNA: 0.76 ± 0.06 AU). Finally, risperidone, at both doses tested, prevented LPS-induced increase in PGE_2 concentration in brain cortex (Fig. 6*e*) (control values: 60.32 ± 5.4 pg/ml).

Lipid peroxidation is the final consequence of oxidative/nitrosative attack to lipidic components of a cell. In order to test possible preventive effects of risperidone at this level, we first checked LPS increase in brain level of the lipid peroxidation marker MDA (control value: 1.76 ± 0.1527 nmol/mg protein). Risperidone reduced LPS mediated increase in MDA levels in the brain at 3 mg/kg (Fig. 7).

Effects of risperidone on cellular anti-inflammatory counterbalancing mechanisms in brain cortex after LPS administration

Inflammation is a natural defence response of the organism finely regulated by inter- and intracellular counterbalancing systems. One of them, widely described in the brain after iNOS and COX2 hyperactivity (García-Bueno *et al.* 2008*b*), is the activation of the anti-inflammatory pathway mediated by deoxyprostaglandins (deoxyPGs). We decided to explore this possibility by studying the expression of the specific enzymatic source of deoxyPGs such as L-PGDS and the nuclear target of deoxyPGs, PPAR-γ. Risperidone prevented the LPS-induced decrease in L-PGDS in brain at the protein level (Fig. 8*a*) and increased its mRNA (Fig. 8*b*). On the other hand, although the dose of LPS used in the study did not decrease the main anti-inflammatory deoxyPG, 15-deoxy-PGJ_2, risperidone, at 3 mg/kg, increased its brain levels when compared to control and LPS animals (Fig. 8*c*). Finally, in order to assess the possibility that risperidone acts at the final effector level of the

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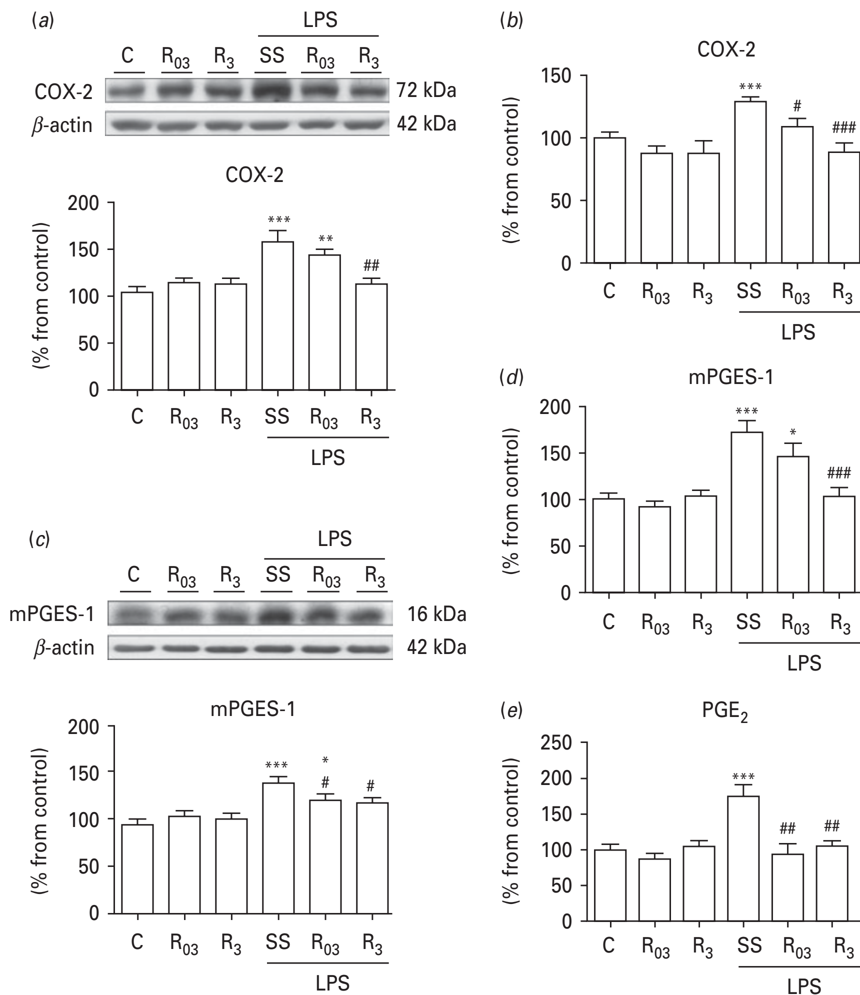

Fig. 6. Risperidone anti-inflammatory effects in brain V. Cyclooxigenase 2 (COX-2), microsomal prostaglandin E synthase 1 (mPGES-1) and prostaglandin E_2 (PGE_2) levels. (*a*) Pro-inflammatory enzyme COX-2 Western blot and densitometric analysis; (*b*) pro-inflammatory enzyme COX-2 mRNA levels; (*c*) pro-inflammatory enzyme mPGES-1 Western blot and densitometric analysis; (*d*) anti-inflammatory enzyme lipocalin-prostaglandin D_2 synthase (L-PGDS) mRNA levels; (*e*) pro-inflammatory PGE_2 levels (index of COX-2 activity) in prefrontal cortex homogenates of control (C), 0.3 mg/kg risperidone (R_{03}), 3 mg/kg risperidone (R_3), saline solution + lipopolysaccharide (LPS) (SS), 0.3 mg/kg risperidone (R_{03}) + LPS, 3 mg/kg risperidone (R_3) + LPS. * $p<0.05$ *vs.* C, ** $p<0.01$ *vs.* C, *** $p<0.001$ *vs.* C; # $p<0.05$ *vs.* LPS, ## $p<0.01$ *vs.* LPS, ### $p<0.001$ *vs.* LPS (one-way analysis of variance followed by Newman–Keuls *post-hoc* test). Data represent mean ± S.E.M.

counterbalancing anti-inflammatory mechanism, we studied protein expression and mRNA of the nuclear target of deoxyPGs. Indeed, LPS decreased protein levels of PPAR-γ and risperidone prevented LPS effects at this level (Fig. 8*d*). As occurred with 15-deoxy-PGJ_2, risperidone also increased PPAR-γ mRNA when compared to control and LPS animals (Fig. 8*e*) (control values: L-PGDS expression: 0.79 ± 0.03 AU; mRNA: 0.79 ± 0.06 AU; 15d-PGJ_2: 76.33 ± 4.3 units; PPAR-γ expression: 1.12 ± 0.06 AU; mRNA: 0.79 ± 0.07 AU).

Effects of risperidone on peripheral markers of LPS-induced oxidative/nitrosative and inflammatory stress

In order to explore possible anti-inflammatory systemic effects of risperidone, some markers were studied in plasma or cytosolic and/or nuclear extracts of PMBC. At the doses used here, LPS challenge increased iNOS expression, decreased PPAR-γ expression in PMBC as well as increased NO_2^- in plasma (not shown). The administration of risperidone at

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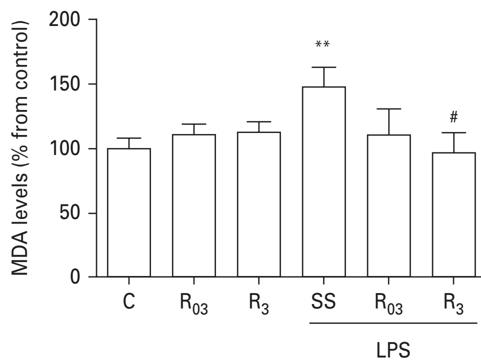

Fig. 7. Risperidone effects of lipid peroxidation levels in the prefrontal cortex of control (C), 0.3 mg/kg risperidone (R_{03}), 3 mg/kg risperidone (R_3), saline solution + lipopolysaccharide (LPS) (SS), 0.3 mg/kg risperidone (R_{03}) + LPS, 3 mg/kg risperidone (R_3) + LPS. * $p<0.05$ *vs.* C; # $p<0.05$ *vs.* LPS (one-way analysis of variance followed by Newman–Keuls *post-hoc* test). Data represent mean ± S.E.M.

3 mg/kg decreased LPS-induced nitrite levels and prevented PPAR-γ decrease in plasma and PMBC respectively (Table 2).

Discussion

The results of the present study, carried out in an *in vivo* model of central and systemic inflammation, indicate that the atypical antipsychotic risperidone elicits a preventive anti-inflammatory effect. In particular, the two doses tested prevent brain activation (mRNA and protein expression) of two of the main inflammatory and oxido-nitrosative enzymatic sources, iNOS and COX-2 as well as the LPS-induced increase in inflammatory cytokines, although only the higher dose studied prevents lipid peroxidation in brain. More interestingly, this atypical antipsychotic increases an anti-inflammatory counterbalancing mechanism (L-PGDS/15d-PGJ_2/PPAR-γ) in a specific experimental model of mild neuroinflammation.

Most of the current data on the effects of antipsychotics on inflammation have been carried out in *in vitro* models or are restricted to their anti-cytokine or anti-oxidative effects. In addition, several of the experimental studies carried out use LPS at endotoxaemic or lethal doses and it is difficult to extrapolate the results obtained to neuropathologies related to mild inflammation, as can be the case of schizophrenia (Dean, 2010; Müller & Schwartz, 2010). Indeed, septic shock is a prevalent condition that often causes disturbances in cognition, mood and behaviour, particularly due to central actions of the inflammatory cytokines. In these studies, several antipsychotics have demonstrated anti-inflammatory effects, mainly related to their anti-cytokine action (Gerard *et al.* 1993; Howard *et al.* 1993; Robertson *et al.* 2006). A crucial difference with the data presented here is that the experimental approach used is a mild stimulus (0.5 mg/kg, 90 min) that elicits an inflammatory response in the brain as well as systemically, which accurately resembles the mild encephalitis hypothesis of schizophrenia, as described by Bechter (2004).

It has been recently reported that atypical antipsychotics have anti-inflammatory effects in animal models of inflammation restricted to serum cytokine levels (Sugino *et al.* 2009). In this study, clozapine is more potent than haloperidol and also when compared to atypical antipsychotics such as olanzapine and risperidone. Other anti-inflammatory effects of atypical antipsychotics are associated with suppression of microglial activation induced by cytokines or chemokines in *in vitro* studies (Bian *et al.* 2008; Hou *et al.* 2006) or to the decrease of cytokine levels induced by inflammatory stimulus (Sugino *et al.* 2009). In addition, a counterbalancing effect of clozapine on anti-inflammatory cytokines, based on the effect of IL-10, decreasing the production of IL-1, IL-6 and TNF-α *in vitro* and *in vivo* has been reported (Gerard *et al.* 1993; Howard *et al.* 1993; Robertson *et al.* 2006).

Although not consistently (Potvin *et al.* 2008), single studies have demonstrated that atypical (clozapine, olanzapine, risperidone) and typical (haloperidol) antipsychotics modulate the production of pro-inflammatory cytokines (TNF-α, IL-6, IL-4, interferon-γ; Kato *et al.* 2007; Leykin *et al.* 1997; Maes *et al.* 1996) and/or cytokine receptors (IL-1RA, soluble IL-2, IL-6 receptors; Müller *et al.* 1997; Song *et al.* 2000) and their normalization correlates with improvement of psychotic symptoms (Monji *et al.* 2009). The possibility that these effects are related to the inhibition of microglial activation observed in post-mortem tissue and *in vitro* studies (Kato *et al.* 2007, 2011) and/or with dopamine receptor affinity deserves further study.

In addition to cytokines, pathophysiology of inflammation has recently focused on the homeostatic, double-faceted role of this defence response. Inflammation is a complex set of coordinated mechanisms governed by the interaction of multiple specific mediators (cytokines, chemokines, prostaglandins, etc.) released by different types of immune cells. In spite of the presence of the brain–blood barrier, the brain responds to peripheral inflammatory stimuli, mounting a local inflammatory response called neuroinflammation, generating hypothalamic-pituitary-adrenal axis

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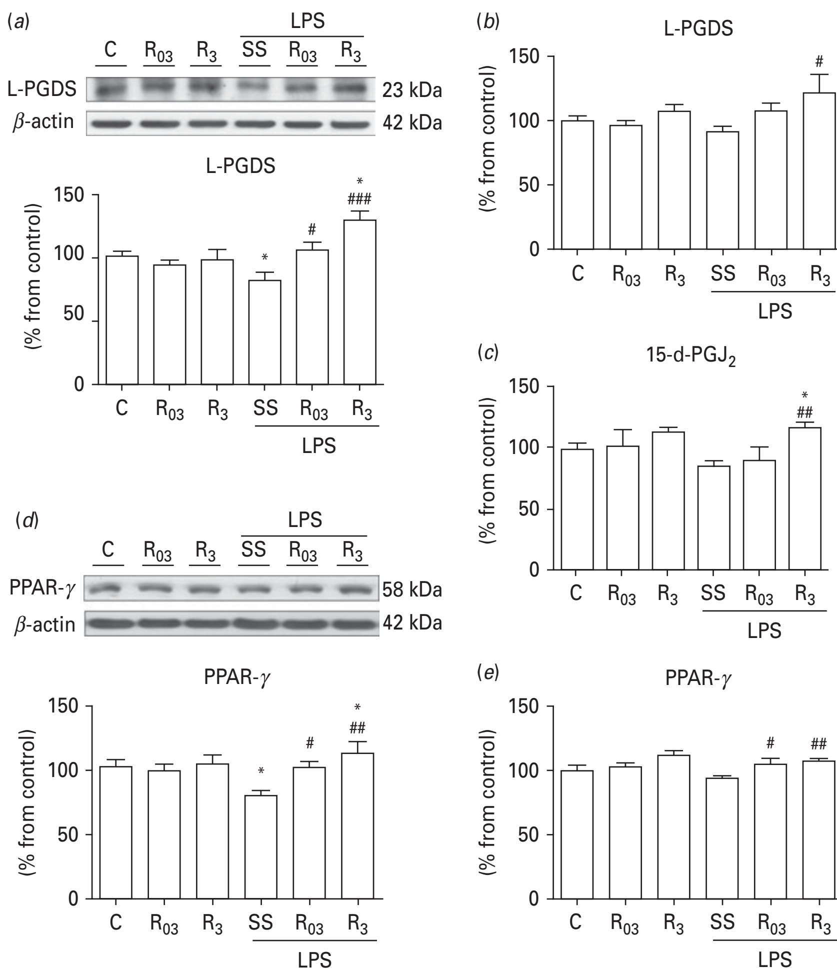

Fig. 8. Risperidone anti-inflammatory effects in brain VI. Lipocalin-prostaglandin D_2 synthase (L-PGDS), 15d-PGJ2 and peroxisome proliferator activator receptor γ (PPAR-γ). (*a*) Pro-inflammatory enzyme L-PGDS Western blot and densitometric analysis; (*b*) anti-inflammatory enzyme L-PGDS mRNA levels; (*c*) anti-inflammatory 15d-PGJ$_2$ levels; (*d*) Western blot and densitometric analysis of the nuclear receptor PPAR-γ; (*e*) nuclear receptor PPAR-γ mRNA levels in the prefrontal cortex homogenates of control (C), 0.3 mg/kg risperidone (R_{03}), 3 mg/kg risperidone (R_3), saline solution + lipopolysaccharide (LPS) (SS), 0.3 mg/kg risperidone (R_{03}) + LPS, 3 mg/kg risperidone (R_3) + LPS. * $p<0.05$ *vs.* C; $^{\#}$ $p<0.05$ *vs.* LPS, $^{\#\#}$ $p<0.01$ *vs.* LPS, $^{\#\#\#}$ $p<0.001$ *vs.* LPS (one-way analysis of variance followed by Newman–Keuls *post-hoc* test). Data represent mean ± S.E.M.

activation and other acute phase responses, including lethargy, somnolence, fever and anorexia, referred to collectively as 'sickness behaviour', aimed to maintain organic homeostasis threatened by injury or infection (reviewed in Allan & Rothwell, 2003). Thus, the inflammatory process is a protective mechanism, conserved during evolution in all types of organisms. However, when it is excessive in intensity (over-expression or over-activity of its mediators) and time (inefficient resolution), it becomes harmful and can exacerbate numerous diseases. There is extensive evidence that excessive inflammation within the CNS contributes to many acute and chronic degenerative disorders (Parkinson's disease, Alzheimer's disease, etc.; reviewed in González-Scarano & Baltuch, 1999). Also, there is a crescent perception about its role in some psychiatric diseases (i.e. depression, post-traumatic stress disorder and schizophrenia; Dantzer *et al.* 2008; Hanson & Gottesman, 2005). In fact, schizophrenia patients showed activation of the key inflammatory factor NF-κB, which may play a pivotal role in the disease through its interaction with pro-inflammatory cytokines (Martínez Gras *et al.* 2011; Song *et al.* 2009). The results presented here about the preventive effects of risperidone on LPS-induced increases in inflammatory MAPK and NF-κB pathways

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Table 2. Risperidone anti-inflammatory effects in peripheral blood

	LPS	R_3	*p* value
iNOS (AU)	1.33±0.06	1.02±0.13	0.09
COX-2 (AU)	1.315±0.03	1±0.14	0.0504
m-PGES-1 (AU)	0.885±0.048	0.776±0.055	0.2
L-PGDS (AU)	0.8±0.036	0.9±0.05	0.16
PPARγ (AU)	1.205±0.034	1.495±0.015	0.005
NO_2^- (μM/ml plasma)	17.73±2.71	9.46±3.41	0.032

AU, Arbitrary units.
Enzymatic protein expression in peripheral mononuclear blood cells and NO^-_x levels in plasma after lipopolysaccharide (LPS) administration. Effects of 3 mg/kg risperidone (R_3). Protein data are normalized by tubulin and GAPDH LPS *vs.* R_3 (Student's *t* test).
Data represent mean±S.E.M.

support a multi-target anti-inflammatory effect of the drug.

In the last few years, a rich database has been developed substantiating the capacity of endogenous counterbalancing mechanisms taking place when an inflammatory or immune stimulus appears (Serhan *et al.* 2007). One of these mechanisms is the activation of peroxisome proliferator activated receptors (Kapadia & Vemuganti, 2008). These receptors constitute a subfamily included in the superfamily of nuclear hormone receptors (Houseknecht *et al.* 2002) and are expressed in the great majority of brain and peripheral immune cells (Heneka & Landreth, 2007). They act as ligand-dependent transcription factors, binding to DNA in specific regions and regulating the expression of genes that are related to lipid and glucose metabolism, inflammatory processes and cellular differentiation (Kapadia & Vemuganti, 2008).

Interestingly, several COX-derived products, such as deoxyPGs (D and J series, especially 15d-PGJ_2) act as endogenous anti-inflammatory agents by targeting PPAR-γ in immune responsive cells in the brain and its periphery (Forman *et al.* 1997). Indeed, PPAR-γ acting as a ligand-dependent transcription factor is capable of decreasing the expression and activity of some inflammatory factors, such as NF-κB, in various experimental settings (García-Bueno *et al.* 2007). Thus, the PPAR-γ pathway may act as a mediator of 'central neurogenic neuroprotection', providing protection in neuroinflammatory conditions (Galea *et al.* 2003). Interestingly, its pharmacological stimulation prevents inflammation, oxidative/nitrosative damage and excitotoxicity in the brain in different experimental settings (García-Bueno *et al.* 2005, 2008*a*, *b*).

Such integrative views have been recently demonstrated to occur in peripheral blood cells from schizophrenia patients. Martínez-Gras *et al.* (2011) identified an imbalance of intracellular pro- and anti-inflammatory regulating mechanisms, with a decrease in deoxyPGs and PPAR-γ in patients. The presence of such an imbalance strengthens the data presented here and emphasizes the hypothesis of a failure in homeostatic mechanisms in psychosis (both in brain and periphery), with important consequences at diagnostic and therapeutical levels.

In summary, the results described here suggest a systemic and central anti-inflammatory effect of a single dose of risperidone in a model of neuroinflammation *in vivo*. More interestingly, this atypical antipsychotic prevents the decrease of the anti-inflammatory, counterbalancing mechanisms after LPS challenge. To our knowledge, the data presented here constitute the first description of such anti-inflammatory effects of risperidone, the use of an acute protocol being the main limitation in this study. The chronic effects of risperidone, the use of related long-acting derivatives and comparative studies with other antipsychotics will be needed.

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Statement of Interest

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