ARCHIVAL REPORT

# Stress-Induced Neuroinflammation: Role of the Toll-like Receptor-4 Pathway

Iciar Gárate, Borja Garcia-Bueno, Jose Luis Muñoz Madrigal, Javier Rubén Caso, Luis Alou, Marisa L. Gomez-Lus, Juan Antonio Micó, and Juan Carlos Leza

**Background:** Stressful challenges are associated with variations in immune parameters, including increased innate immunity/inflammation. Among possible mechanisms through which brain monitors peripheral immune responses, toll-like receptors (TLRs) recently emerged as the first line of defense against invading microorganisms. Their expression is modulated in response to pathogens and other environmental stresses.

**Methods:** Taking into account this background, the present study aimed to elucidate whether the toll-like receptor-4 (TLR-4) signaling pathway is activated after repeated restraint/acoustic stress exposure in mice prefrontal cortex (PFC), the potential regulatory mechanism implicated (i.e., bacterial translocation), and its role in conditions of stress-induced neuroinflammation, using a genetic strategy: C3H/HeJ mice with a defective response to lipopolysaccharide stimulation of TLR-4.

**Results:** Stress exposure upregulates TLR-4 pathway in mice PFC. Stress-induced inflammatory nuclear factor κB activation, upregulation of the proinflammatory enzymes nitric oxide synthase and cyclooxygenase type 2, and cellular oxidative/nitrosative damage are reduced when the TLR-4 pathway is defective. Conversely, TLR-4 deficient mice presented higher levels of the anti-inflammatory nuclear factor peroxisome proliferator activated receptor-gamma after stress exposure than control mice. The series of experiments using antibiotic intestinal decontamination also suggest a role for bacterial translocation on TLR-4 activation in PFC after stress exposure.

**Conclusions:** Taken together, all the data presented here suggest a bifunctional role of TLR-4 signaling pathway after stress exposure by triggering neuroinflammation at PFC level and regulating gut barrier function/permeability. Furthermore, our data suggest a possible protective role of antibiotic decontamination in stress-related pathologies presenting increased intestinal permeability (leaky gut) such as depression, showing a potential therapeutic target that deserves further consideration.

**Key Words:** Antibiotic decontamination, bacterial translocation, innate immunity, neuroinflammation, stress, TLR-4 signaling

Certain psychological states and experiences in which the organism is challenged by internal or external stimuli, including stress-related diseases, are associated with variations in the immune system. In separate meta-analysis (1,2), both major depression and stress exposure were significantly associated with alterations in immune parameters. In many cases, both depression and chronic stress exposure have been associated with decreased adaptative/acquired immunity and inflammation, with important general consequences in health (2,3), but in the last decade, it has been demonstrated that after stress exposure or during certain episodes of depression, an innate inflammatory/immune response is strongly activated (4,5). A question of special relevance is how the brain detects the stimuli and orchestrates the proper regulatory responses. Although it has long been considered an immune-privileged organ, this immune status is far from absolute, especially when the blood brain barrier may be affected, as is the case after stress exposure (6,7).

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Received Mar 28, 2012; revised Jun 21, 2012; accepted Jul 6, 2012.

Several mechanisms have been proposed to explain how the brain monitors peripheral immune signals (8): one pathway involves locally produced cytokines activating primary afferent nerves; a second pathway is a humoral pathway in which toll-like receptors (TLRs) expressed on endothelium and macrophage-like cells residing in the circumventricular organs and the choroid plexus respond to circulating pathogen-associated molecular patterns by producing proinflammatory cytokines; a third pathway comprises proinflammatory cytokine transporters at the blood brain barrier; and finally, a fourth pathway involves cytokine receptors located on perivascular macrophages and endothelial cells of brain venules.

Recently, several studies have focused on TLRs and their potential roles in neuropathology (9,10). The discovery that not only immune cells, but also neurons, astroglia, and resident microglia, express a large majority of the 13 already identified TLRs has challenged the way neuroscience explains neuroimmune interactions, re-evaluating the view of the brain as an immune privileged organ.

Toll-like receptors are pattern recognition receptors proposed as main agents of the innate immune response, constituting the first line of defense against invading microorganisms (11). Their expression is modulated rapidly in response to pathogens, a variety of cytokines, and environmental stresses (12,13). Specifically, toll-like receptor-4 (TLR-4) regulates the adrenal response to stress and inflammatory stimuli (14), as well as the brain response to stress (15,16). Toll-like receptor-4 responds predominantly to lipopolysaccharide (LPS) from gram-negative bacteria (17) through its co-receptor, myeloid differentiation protein-2 (MD-2), a requisite for LPS signaling of TLR-4 (18). To achieve specificity of signaling, TLR-4 recruits some other adapter proteins, such as the myeloid differentiation factor 88 (MyD88), during intracellular signal transduction. After various steps in the transduction pathway, the activation of prototypic

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2012.07.005

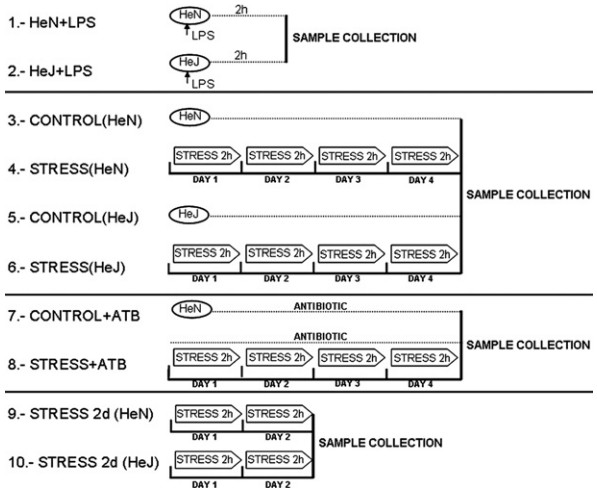


**Figure 1.** Experimental design. ATB, antibiotics; HeJ, C3H/HeJ; HeN, C3H/HeN; LPS, lipopolysaccharide.

inflammatory nuclear transcription factor nuclear factor-kappa B (NF-κB) is produced (19). Nuclear factor-kappa B activation culminates in the production of NF-κB-dependent proinflammatory mediators, such as the enzymes inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase type 2 (COX-2). Interestingly, during the last years, our group and others have described that this cellular pathway is activated in the brain prefrontal cortex (PFC) of rodents submitted to stress exposure, with a key role for NF-κB as a crucial factor in the oxidative/nitrosative damage produced (5,20).

Two major mechanisms have been proposed to activate TLR-4: the first one is related to pathogen-associated molecular patterns released from disrupted cells and extracellular matrix degradation products that may contribute to immune activation after tissue injury (21). The second one comes from some models of stress showing an increase in intestinal permeability and a resultant bacterial translocation to the systemic circulation (22–24). These circulating gram-negative Enterobacteriaceae (i.e., *Escherichia coli*) are a major source of LPS, which can activate brain TLR-4 through multiple pathways, inducing a neuroinflammatory response. This proposed mechanism, known as leaky gut, also takes place in depressive patients and it has been related to the inflammatory pathophysiology of the disease (25,26), as well as in other clinical situations such as chronic fatigue syndrome, a stress-related disorder characterized by increased systemic inflammatory responses (27).

Taking into account all this background, the present study aimed to evaluate whether the TLR-4 signaling pathway is activated after repeated stress exposure in mice PFC, the potential regulatory mechanism implicated (i.e., bacterial translocation), and its role in conditions of stress-induced neuroinflammation, using a genetic (TLR-4 deficient mice) strategy.

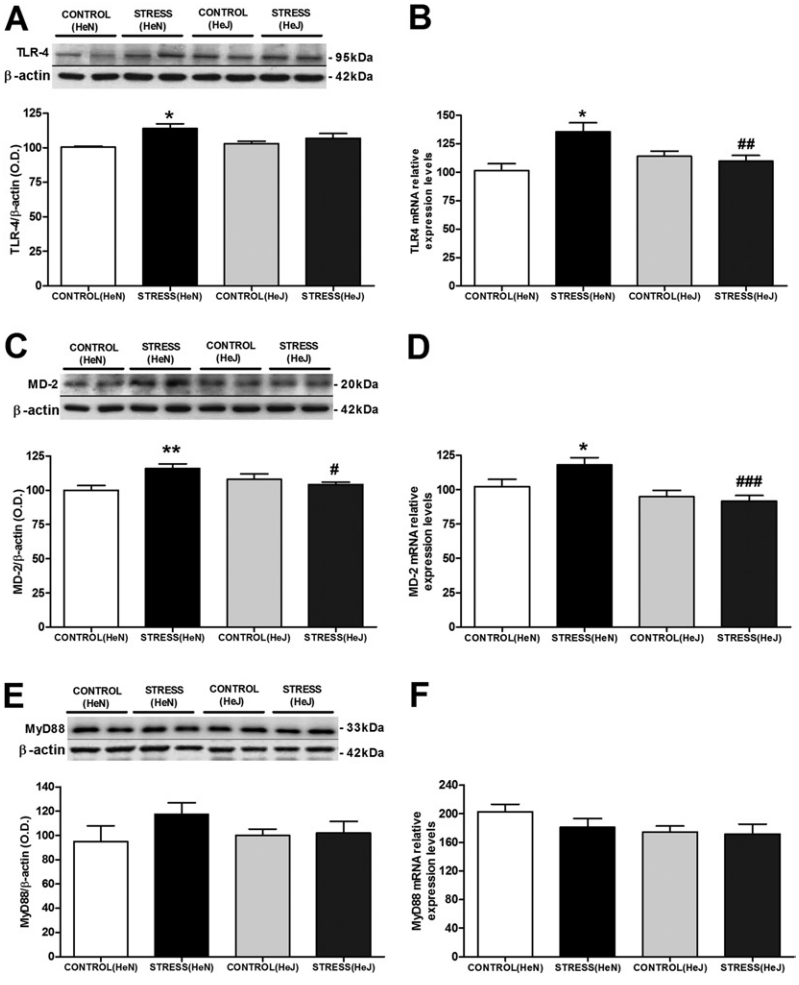


**Figure 2.** Toll-like receptor-4 (TLR-4)/myeloid differentiation protein-2 (MD-2) upregulation is elicited by stress exposure in C3H/HeN (HeN) mice prefrontal cortex (PFC). Protein levels of TLR-4 **(A)**, MD-2 **(C)**, and myeloid differentiation factor 88 (MyD88) **(E)** in brain PFC samples of control and stressed HeN and C3H/HeJ (HeJ) mice. The densitometric data of the respective band of interest are normalized by β-actin (lower band). Data are representative of three experiments. $^{*}p < .05$; $^{**}p < .01$ vs. CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN). Two-way analysis of variance following Bonferroni post hoc test. Messenger RNA (mRNA) relative levels of TLR-4 **(B)**, MD-2 **(D)**, and MyD88 **(F)** in brain PFC samples of control and stressed HeN and HeJ mice. Each mRNA data are normalized by tubulin. Data represent the mean ± SEM ($n = 6$). $^{*}p < .05$ vs. CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN); $^{\#\#}p < .01$ vs. STRESS (HeN); $^{\#\#\#}p < .001$ vs. STRESS (HeN). Two-way analysis of variance following Bonferroni post hoc test. OD, optical density.

## Methods and Materials

### Animals

Adult (14 weeks old) male C3H/HeN (HeN) and C3H/HeJ (HeJ) mice weighing 28 g to 30 g were used (Jackson Laboratories, Bar Harbor, Maine). The C3H/HeJ murine strain has a loss of function point mutation within the coding region of the *TLR-4* gene, which abolishes the response to LPS in these mice (28). The C3H/HeN substrain was used as the control group. Both strains will be described as HeN and HeJ throughout the article.

All the animals were housed in groups ($n = 6$) under standard conditions of temperature and humidity and a 12-hour light/dark cycle (lights on at 08:00 hours) with free access to food and water and were maintained under constant conditions for 7 days before stress. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense following European legislation (2003/65/EC).

### Experimental Design

The experimental design is summarized in Figure 1 (see Supplement 1 for details).

### Intestinal Antibiotic Decontamination

The decontamination protocol used consists of an antibiotic treatment administered from the first day of stress in drinking water, consisting of ciprofloxacin (Fluka-Sigma-Aldrich, Madrid, Spain) 100 mg/L and sucrose 20 g/L, following previous protocols (29). The amount of antibiotic consumed was >75% of initially administrated. In addition to its effects on bacterial replication, antibiotic treatment could produce direct effects on the immune/inflammatory response, representing a potential confound. We checked this possibility by comparing the levels of iNOS and COX-2 in HeN mice with and without antibiotic treatment under control conditions and 3 hours after systemic LPS (500 μg/kg). No major changes were found between them (numerical densitometric data in Table S1 in Supplement 1).

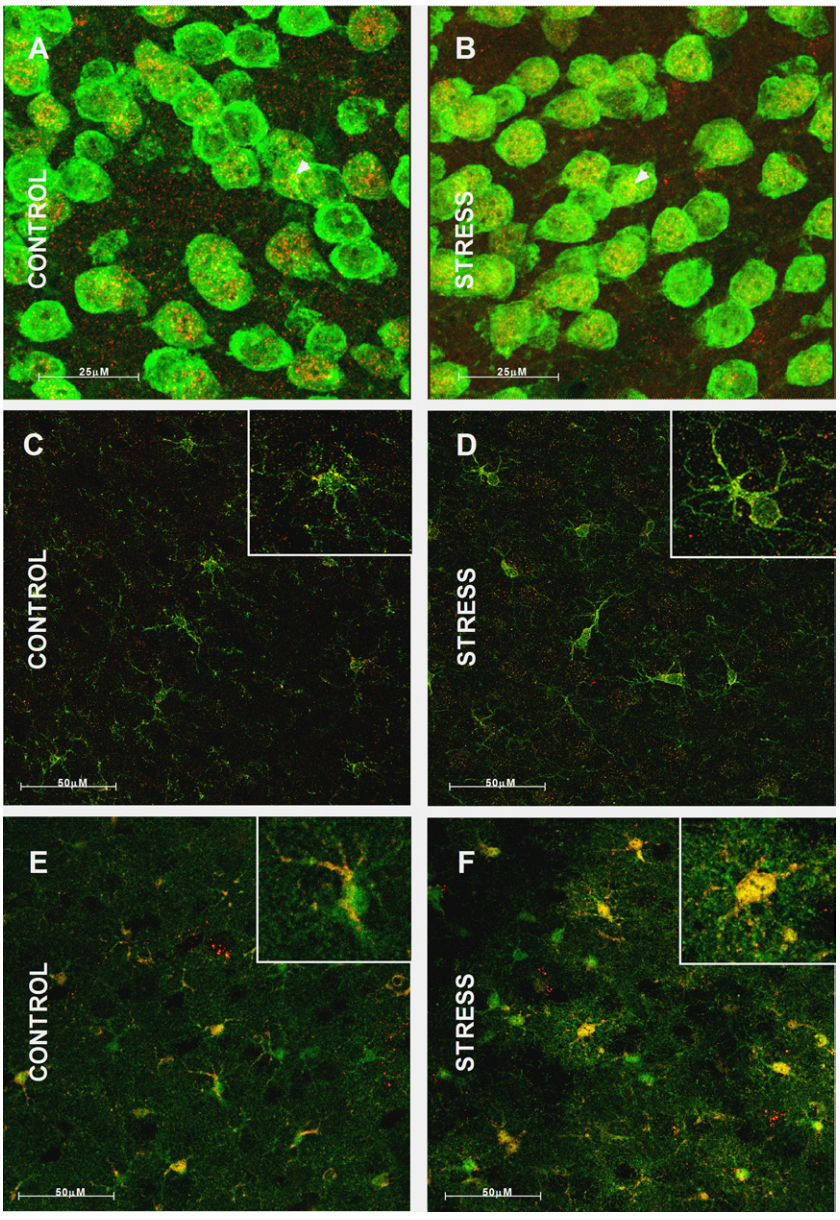


**Figure 3.** Toll-like receptor-4 immunoreactivity in the brain prefrontal cortex of C3H/HeN mice in control and stress conditions. 30 μm-thick sections through the prefrontal cortex of C3H/HeN mice in control **(A, C, E)** and stress **(B, D, F)** conditions. The respective cellular markers appear in green: neurons are identified with neuronal nuclei **(A, B)**, microglia with IBA-1 **(C, D)**, and astroglia with S-100 **(E, F)**. In all cases, toll-like receptor-4 is marked in red. Toll-like receptor-4 localizes to the respective cellular marker with areas of overlap appearing yellow in the merged image (white arrow in **[A, B]**). Scale bar: 25 μm for **(A)** and **(B)**; 50 μm for **(C–F)**.

### Stress Protocol and Tissue Samples

Mice were restrained in 50-mL Falcon conical tubes (BD Biosciences, Franklin Lakes, New Jersey) with modified caps, each with a small hole to accommodate the tail of the mouse. Adequate ventilation was provided by holes drilled into the conical end and at the sides of the tubes. The tubes did not allow forward or backward movements. At the same time, mice were exposed to acoustic stress using an ultrasound bath (2 hours from 13:00 to15:00 hours for 2 or 4 days) (30–32).

Samples were taken immediately after restraint using sodium pentobarbital (320 mg/kg, intraperitoneal; Vetoquinol, Madrid, Spain). Blood for plasma determinations was collected by cardiac puncture and anticoagulated in the presence of trisodium citrate (3.15% wt/vol). After decapitation, the brain was separated from the skull, meninges and blood vessels were removed, and the PFC areas from both hemispheres were excised and frozen at −80°C until assayed. In some groups of animals, a portion of the descending colon was removed and stored at −80°C (see Supplement 1 for more details).

### Preparation of Nuclear Extracts

A modified procedure based on the method of Schreiber *et al.* (33) was used (Supplement 1).

### Bacterial Translocation

Gram negative and positive microbiological cultures preparation, characterization, and quantification are described in detail in Supplement 1.

### Plasma LPS and Lipopolysaccharide Binding Protein Levels

Plasma LPS and lipopolysaccharide binding protein (LBP) levels were determined using commercially available kits following the manufacturer's instructions (Hycult Biotech, Uden, The Netherlands) (see Supplement 1 for more details).

### Immunoglobulin A Determination

Colonic immunoglobulin A (IgA) levels were determined by using a commercially available enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Montgomery, Texas) (see Supplement 1 for details).

### Western Blot Analysis

To determine the expression levels of TLR-4 MD-2 and MyD88, the enzymes iNOS and COX-2, brain prefrontal cortices were used. The same protocol was used to determine the protein levels of the intestinal epithelial cell-derived chemokine CCL28 in colonic samples. Finally, in the case of the NF-κB subunit p65 and peroxisome proliferator-activated receptor γ (PPARγ), analyses were carried out in nuclear extracts from PFC samples (see previous point). Detailed information about sample preparation, protocol, and primary and secondary antibodies used in Supplement 1.

### Real Time-Polymerase Chain Reaction Analysis

Total cytoplasmic RNA was prepared from samples of PFC using TRIZOL reagent (Invitrogen, Grand Island, New York); aliquots were converted to complementary DNA using random hexamer primers. Quantitative changes in messenger RNA (mRNA) levels were estimated by real time-polymerase chain reaction (see Supplement 1 for details).

### Perfusion and Histology

At the end of the last session of stress, mice were anesthetized and perfused via the ascending aorta with 4% paraformaldehyde in .1 mol/L phosphate buffered saline, pH 7.4, and the brains were removed, overnight postfixed, and cryoprotected in 15% sucrose during 24 hours. Regularly spaced series of 30 μm-thick coronal sections were collected in cryoprotectant solution and stored at −20°C until processing.

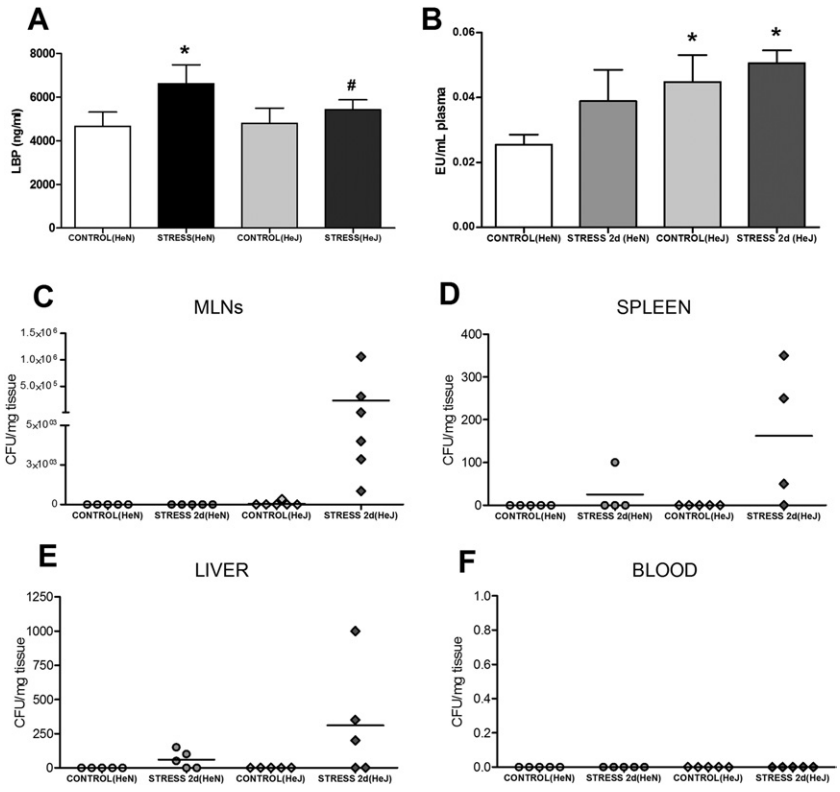


**Figure 4.** Bacterial translocation after repeated stress exposure in C3H/HeN (HeN) and C3H/HeJ (HeJ) mice. **(A)** Lipopolysaccharide binding protein (LBP) plasma levels of control and 4 days (2 hours/day) stressed HeN and HeJ mice. Data represent the mean ± SEM of six animals per group. $^{*}p < .05$ vs. CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN). Two-way analysis of variance following Bonferroni post hoc test. **(B)** Lipopolysaccharide plasma levels of control and stressed HeN and HeJ mice. Data represent the mean ± SEM ($n = 6$). $^{*}p < .05$ vs. CONTROL (HeN). Two-way analysis of variance following Bonferroni post hoc test. Bacteria colony-forming units (CFUs) per mg of tissue detected in mesenteric lymph nodes (MLNs) **(C)**, spleen **(D)**, liver **(E)**, and blood **(F)** in control and stressed HeN and HeJ mice. The data represent the CFU found in each animal. EU, endotoxin units.

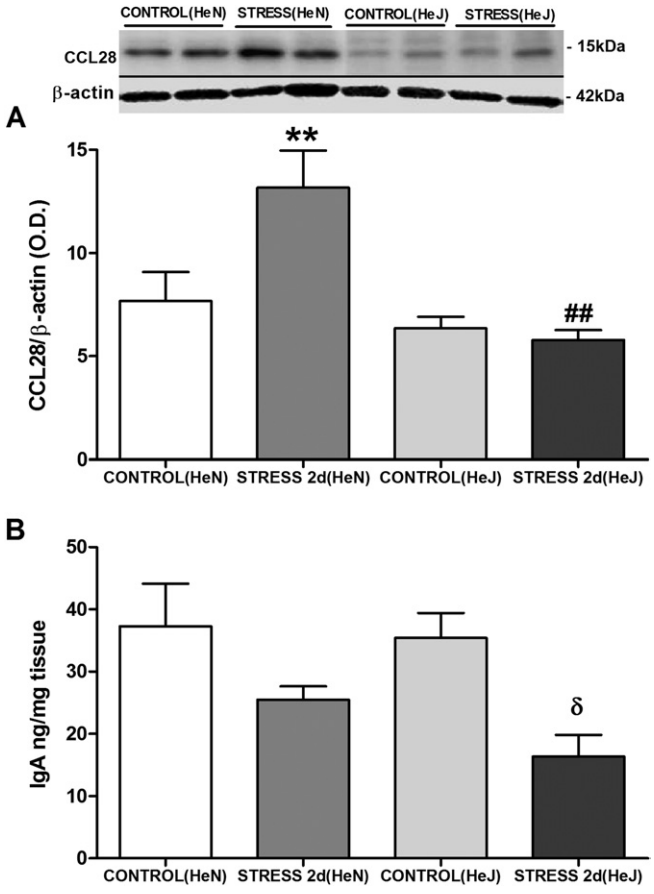


**Figure 5.** Intestinal levels of CCL28 and immunoglobulin A (IgA) decrease after 2 days of stress in C3H/HeJ (HeJ) animals. **(A)** Western blot of CCL28 in colonic samples of control and 2 days stressed C3H/HeN (HeN) and HeJ mice. The densitometric data of the band of interest are normalized by β-actin (lower band) and represent three experiments. $^{**}p < .01$ vs. CONTROL (HeN); $^{\#\#}p < .01$ vs. STRESS (HeN). Two-way analysis of variance following Bonferroni post hoc test. **(B)** Immunoglobulin A levels quantification in colonic samples of control and 2 days stressed HeN and HeJ mice. Data represent the mean ± SEM of six animals per group. $^{\delta}p < .05$ vs. CONTROL (HeJ). Two-way analysis of variance following Bonferroni post hoc test. OD, optical density.

### Immunohistochemistry

To identify the cell type(s) displaying TLR-4-like immunoreactivity, a dual immunofluorescence protocol was used (see Supplement 1 for protocol details).

### NF-κB Transcription Factor Assay

Nuclear factor-kappa B transcription factor activity was determined on nuclear extracts by using an enzyme-linked immunosorbent assay-based kit (Cayman Chemicals, Tallin, Estonia) (see Supplement 1 for sequence details).

### Lipid Peroxidation

Lipid peroxidation was measured by the thiobarbituric acid test (34), with some modifications (see Supplement 1 for details).

### Plasma Corticosterone

Plasma was obtained from blood samples by centrifuging the sample at 1.000*g* for 15 minutes immediately after stress. All plasma samples were stored at −20°C before assay. Corticosterone was measured by the radioimmunoassay kit Coat-a-Count (Siemens, Los Angeles, California) (see Supplement 1 for details).

### Protein Assay

Protein levels were measured using Bradford method (35).

### Chemicals

Unless otherwise stated, the chemicals were from Sigma, Madrid, Spain.

### Statistical Analyses

Data in text, tables, and figures are expressed as mean ± SEM. For multiple comparisons of the antibiotic groups, a one-way analysis of variance followed by the Newman-Keuls post hoc test was made. A $p$ value $<.05$ was considered statistically significant.

All data derived of the experiments with TLR-4 deficient mice (HeJ) were analyzed with a two-way analysis of variance followed by Bonferroni post hoc test, considering as first factor the presence/absence of stress or LPS and as second factor the mice strain (HeN vs. HeJ). A $p$ value $<.05$ was considered statistically significant.

## Results

### Proinflammatory Enzymes in the PFC of HeN and HeJ After LPS

To verify whether systemic LPS upregulates iNOS and COX-2 in the PFC of mice via TLR-4 activation, .1mg/kg of LPS was intraperitoneal injected to HeN and HeJ mice. Two hours later, both parameters were significantly increased in HeN, but not in HeJ, mice (densitometric data in Table S2 in Supplement 1).

### TLR-4 Expression and Signaling in the Brain PFC of HeN and HeJ Mice Submitted to Stress

To elucidate whether TLR-4 signaling pathway is activated in mice brain PFC after stress exposure, we studied the expression at mRNA and protein level of TLR-4, MD-2, and MyD88.

Stress exposure increased both TLR-4 and MD-2 at protein and mRNA level in HeN mice (Figure 2A–D). Conversely, MyD88 expression remained unaffected (Figure 2E, F). As expected, stress exposure in HeJ mice did not change TLR-4, MD-2, or MyD88 expression when compared with control status (Figure 2A–F).

### Cellular Types Displaying TLR-4 Immunoreactivity in the PFC of HeN Mice

Afterward, a qualitative approach trying to identify the cellular types where TLR-4 is expressed in PFC was made. A detailed examination of the images indicates that TLR-4 is expressed in neurons (Figure 3A, B), microglia (Figure 3C, D), and astroglia (Figure 3E, F), both in control and after stress exposure conditions. Toll-like receptor-4 immunoreactivity in neurons is uniformly distributed in the neuronal soma. In microglia, TLR-4 is more associated to the cellular membrane and processes. In the case of astroglia, TLR-4 immunostaining is present both in astrocyte somata and processes. In addition, no major differences in TLR-4 expression or cellular distribution were found between control and stress conditions in HeN mice in the three cellular types studied (see detailed Figures S1 through S3 in Supplement 1).

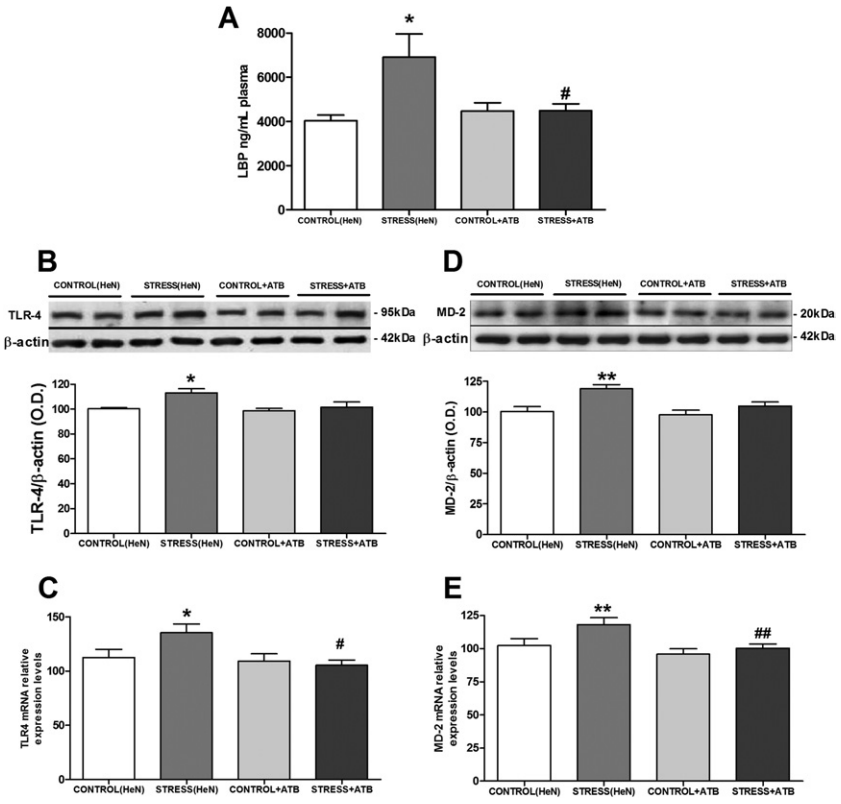


**Figure 6.** Intestinal decontamination prevents stress-induced increase in plasma lipopolysaccharide binding protein (LBP) and toll-like receptor-4 (TLR-4) signaling pathway activation. **(A)** Lipopolysaccharide binding protein plasma levels of control and 4 days stressed C3H/HeN (HeN) mice with or without antibiotic (ATB). Data represent the mean ± SEM ($n = 6$). $^{*}p < .05$ vs. CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN). Two-way analysis of variance (ANOVA) following Bonferroni post hoc test. Protein (Western blot) of TLR-4 **(B)**, myeloid differentiation protein-2 (MD-2) **(D)** in brain prefrontal cortex samples of control and stressed HeN mice with or without antibiotic. The densitometric data are normalized by β-actin (lower band) and are representative of three experiments. $^{*}p < .05$ vs. CONTROL (HeN); $^{**}p < .01$ vs. CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN), $^{\#\#}p < .01$ vs. STRESS (HeN). One-way ANOVA following Newman-Keuls post hoc test. Toll-like receptor-4 **(C)** and MD-2 **(E)** respective messenger RNAs (mRNAs) relative levels in brain cortex control and stressed HeN mice with or without antibiotic. Each mRNA data are normalized by tubulin. Data represent the mean ± SEM ($n = 6$). $^{*}p < .05$ vs. CONTROL (HeN); $^{**}p < .01$ vs. CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN); $^{\#\#}p < .01$ vs. STRESS (HeN). One-way ANOVA following Newman-Keuls post hoc test. OD, optical density.

### Possible Regulatory Mechanisms of TLR-4 Activation in Brain PFC After Stress

To clarify the origin of the stress-induced activation of the TLR-4 pathway, we studied LPS and LBP levels in plasma, as well as bacterial translocation in mesenteric lymphatic nodes (MLNs), liver, and spleen. Stress exposure increased plasma LBP and significant differences were found between both stressed mice strains (Figure 4A). Neither plasma LPS levels nor bacterial translocation showed significant differences after 4 days of stress exposure in both stressed strains of mice (data not shown).

Since there is a marked intestinal inflammation and an increase in bacterial translocation in acute stress protocols that tend to disappear in chronic conditions (24) and taking into account that LBP and TLR-4 were increased after 4 days of stress exposure, we decided to study the possibility that bacterial translocation and intestinal dysfunction took place before the fourth day of stress. To verify this possibility, we exposed the mice to only 2 days of stress.

In this case, LPS did not change after stress exposure in HeN mice, although a trend to increase exists (Figure 4B). However, HeJ mice showed increased levels both in control and stress conditions (Figure 4B).

A further analysis of viable microorganisms was conducted. After 2 days of stress, bacteria were found in MLNs of one control and all of the stressed HeJ mice and in the spleen and liver of both groups of stressed animals (Figure 4C–E). However, there were no bacteria in the blood of any group of mice (Figure 4F). The qualitative analysis identified the gram positive bacterial strains *Staphylococcus aureus*, coagulase-negative *Staphylococcus*, *Bacillus sp,* and *Lactobacillus sp* after stress exposure in HeN mice, while in HeJ mice, the strains of translocated bacteria were *Staphylococcus aureus*, *Bacillus sp*, *Lactobacillus sp*, *Streptomyces sp*, and the gram negative *Proteus mirabilis*. All these bacterial strains are part of the resident intestinal microbiota of C3H mice.

To clarify whether bacterial translocation observed after 2 days of stress exposure was related to intestinal inflammation, we decided to study some parameters related in this organ. We quantified the chemokine CCL28, also known as mucosae-associated epithelial chemokine, which regulates the migration of Immunoglobulin-expressing cells. Immunoglobulin A represents a first line of defense mechanism against pathogens and a decrease in its amount in the colon contributes to bacterial translocation. CCL28 levels were increased after 2 days of stress in HeN but not in HeJ mice (Figure 5A). In addition, HeJ mice showed lower levels than HeN mice after stress exposure (Figure 5A). Furthermore, IgA levels decreased in both groups after stress exposure, but only the HeJ group reached significant differences compared with its control (Figure 5B). No differences were found between strains after stress exposure (Figure 5B).

### Intestinal Decontamination on Stress-Induced TLR-4 Activation in HeN Mice

Antibiotic decontamination decreased stress-induced LBP levels in plasma (Figure 6A). There was no bacterial translocation to MLNs, spleen, liver, or blood of HeN mice treated with antibiotic (data not shown).

The blocking effect of bacterial decontamination in HeN animals is extended to stress-induced TLR-4 (Figure 6B, C) and MD-2 (Figure 6D, E) respective upregulation in brain PFC. Antibiotic administration before stress exposure did not modify MyD88 levels (data not shown).

### Role of TLR-4 in Stress-Induced Neuroinflammation and Oxidative Damage in Mice Brain PFC

The nuclear factor-kappa B p65 subunit is increased in nuclear extracts of stressed HeN animals (Figure 7A). However, stress expo-

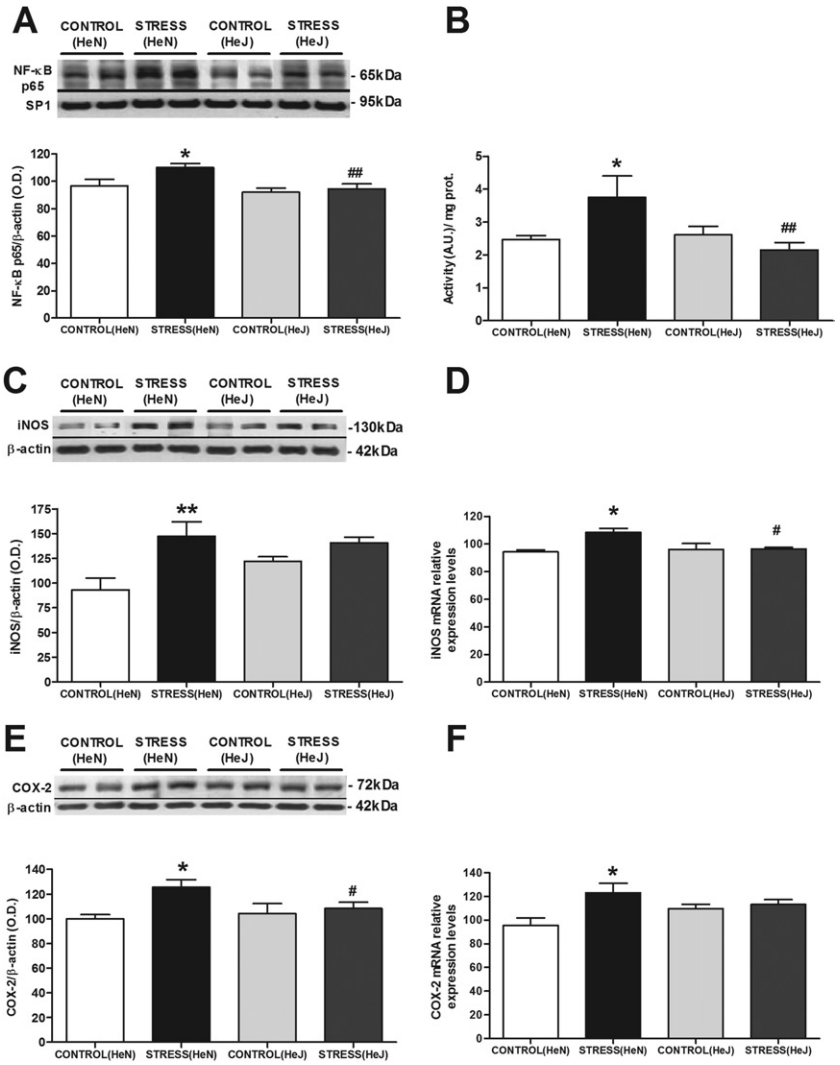


**Figure 7.** Lack of toll-like receptor-4 prevents stress-induced nuclear factor-kappa B (NF-κB) expression and activation and cyclooxygenase type 2 (COX-2) and inducible nitric oxide synthase (iNOS) upregulation. **(A)** Western blot detection of NF-κB p65 subunit and densitometric analysis of the band of interest in brain prefrontal cortex nuclear extract samples of control and stressed C3H/HeN (HeN) and C3H/HeJ (HeJ) mice. The densitometric data are normalized by SP1 (lower band) and are representative of three experiments. $^{*}p < .05$ vs. CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN). Two-way analysis of variance (ANOVA) following Bonferroni post hoc test. **(B)** Transcriptional activity of NF-κB p65 subunit in nuclear extracts from control and stressed HeN and HeJ mice. The data represent the mean ± SEM ($n = 6$). $^{*}p < .05$ vs. corresponding CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN). Two-way ANOVA following Bonferroni post hoc test. Western Blot detection of the proinflammatory enzymes iNOS **(C)** and COX-2 **(E)** and densitometric analysis of the respective bands of interest in brain cortex samples of control and stressed HeN and HeJ mice. The densitometric data are normalized by β-actin (lower band) and are representative of three experiments. $^{*}p < .05$; $^{**}p < .01$ vs. CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN). Two-way ANOVA following Bonferroni post hoc test. Inducible nitric oxide synthase **(D)** and COX-2 **(F)** messenger RNAs (mRNAs) relative levels in brain cortex of control and stressed HeN and HeJ mice. Each mRNA data are normalized by tubulin. Data represent the mean ± SEM ($n = 6$). $^{*}p < .05$ vs. CONTROL (HeN); $^{\#}p < .05$ vs. STRESS (HeN); $^{\#\#}p < .01$ vs STRESS (HeN). Two-way ANOVA following Bonferroni post hoc test. A.U., arbitrary units; OD, optical density.

sure did not change p65 nuclear levels in HeJ mice (Figure 7A). Complementary studies on p65 transcriptional activity mimic the same pattern in both strains (Figure 7B).

Stress exposure also caused an increase in the expression of COX-2 and iNOS at protein (Figure 7C–E) and mRNA levels (Figure 7D–F). These effects were absent in HeJ stressed mice (Figure 7C–F).

In addition, interleukin-6 was also measured by polymerase chain reaction as a specific proinflammatory marker. In HeN mice, there is an increase of this cytokine after stress, which is missing in HeJ mice (Figure 8A).

As a final index of stress-induced cellular damage, we measured the accumulation of malondialdehyde in the brain PFC. C3H/HeN animals presented a malondialdehyde overaccumulation after stress exposure not present in stressed HeJ mice (Figure 8B).

Peroxisome proliferator-activated receptor γ is regulated by stress exposure (36) and also by TLR-4 activation in other experimental settings (37). We explore the possible effect of TLR-4 deficiency on this anti-inflammatory mediator produced in our model. Western blot and real time-polymerase chain reaction studies showed increased levels of PPARγ in PFC samples of HeJ stressed mice compared with HeN stressed group (Figure 8C, D).

### Intestinal Decontamination Effect on Stress-Induced Neuroinflammation in HeN Mice

Antibiotic treatment blocked stress-induced activation of NF-κB in the PFC of HeN mice (Figure 9A, B). The anti-inflammatory effects of antibiotic treatment were extended to stress-induced iNOS and COX-2 respective upregulation in the PFC of HeN mice (Figure 9C, D).

### Role of TLR-4 in the Hypothalamic-Pituitary-Adrenal Axis Activation Elicited by Stress Exposure

Stress increased plasma corticosterone levels in HeN mice after 2 or 4 days of exposure, as expected. Interestingly, TLR-4 deficient individuals only present significant differences to their control subjects after 2 days of stress exposure. In addition, HeN and HeJ mice respective plasma corticosterone levels after both times of stress exposure were very similar, at least at the moment of blood extraction (see Table 1 for numerical data).

## Discussion

The present work demonstrates that repeated restraint/acoustic stress exposure upregulates TLR-4 pathway and suggests a key role for TLR-4 in the neuroinflammation produced in the brain PFC

(Figure 10). We have demonstrated that stress-induced NF-κB activation, iNOS and COX-2 upregulation, and cellular oxidative/nitrosative damage are reduced when the TLR-4 pathway is defective. Conversely, TLR-4 deficient mice presented higher levels of PPARγ after stress exposure. The battery of experiments using antibiotic intestinal decontamination also suggests a role of bacterial translocation on TLR-4 signaling pathway activation in the PFC after stress exposure, as well as implicates TLR-4 in the regulation of the gut barrier dysfunction and bacterial translocation produced.

Increasing evidence has been accumulated regarding the role of TLRs in the pathophysiology of several neurological/neurogenerative diseases (38–40). The results presented here support the role of TLR-4 as an important regulatory factor in the physiological stress response and open the possibility for its pharmacological modulation to minimize oxidative and inflammatory damage in the brain after stress and in stress-related pathologies. However, TLRs have multiple physiological functions, such as the restoration of central nervous system homeostasis (41), the proper resolution of the inflammatory process (42), or the trigger of acute phase responses (43). Further investigation is necessary to address if inhibition of TLR-4 might be beneficial or harmful in different pathological scenarios.

Regarding TLR-4 signaling pathway regulation, our results suggest that bacterial translocation is responsible, at least in part, for the activation of TLR-4 in the PFC after stress exposure. Of special interest is the fact that HeJ mice presented higher levels of plasma LPS after 2 hours of stress exposure than HeN mice. In addition, HeJ animals also presented more colonies of living bacteria in the MLNs, liver, and spleen (gram negative bacteria included) and lower intestinal protection afforded by IgA. These findings suggest that TLR-4 deficiency aggravates stress-induced intestinal dysfunction and subsequent bacterial translocation. In agreement with the results reported here, a proper TLR-4 signaling is required for intestinal response to epithelial injury and to restrict bacterial translocation in a murine model of acute colitis (44). A role for TLR-4 increasing the gut production of IgA has also been reported, showing that this effect was mediated by TLR-induced expression of the chemokine CCL28, which promoted the recruitment of B cells into the lamina propria (45). Indeed, HeJ mice are more prone to the development of gram negative sepsis (28).

Toll-like receptor-4 increased expression induced by different protocols of psychological stress in rodents, such as chronic mild stress or social disruption, has also been demonstrated in the brain PFC and in splenic macrophages, respectively (16,46). It is worth noting that TLR-4 can be regulated by other stress mediators, such as heat shock proteins (47) and the excitatory amino-acid glutamate, which regulates TLR-4 expression in an *N*-methyl-D-aspartate receptor-related mechanism after LPS (48). Concerning other stress

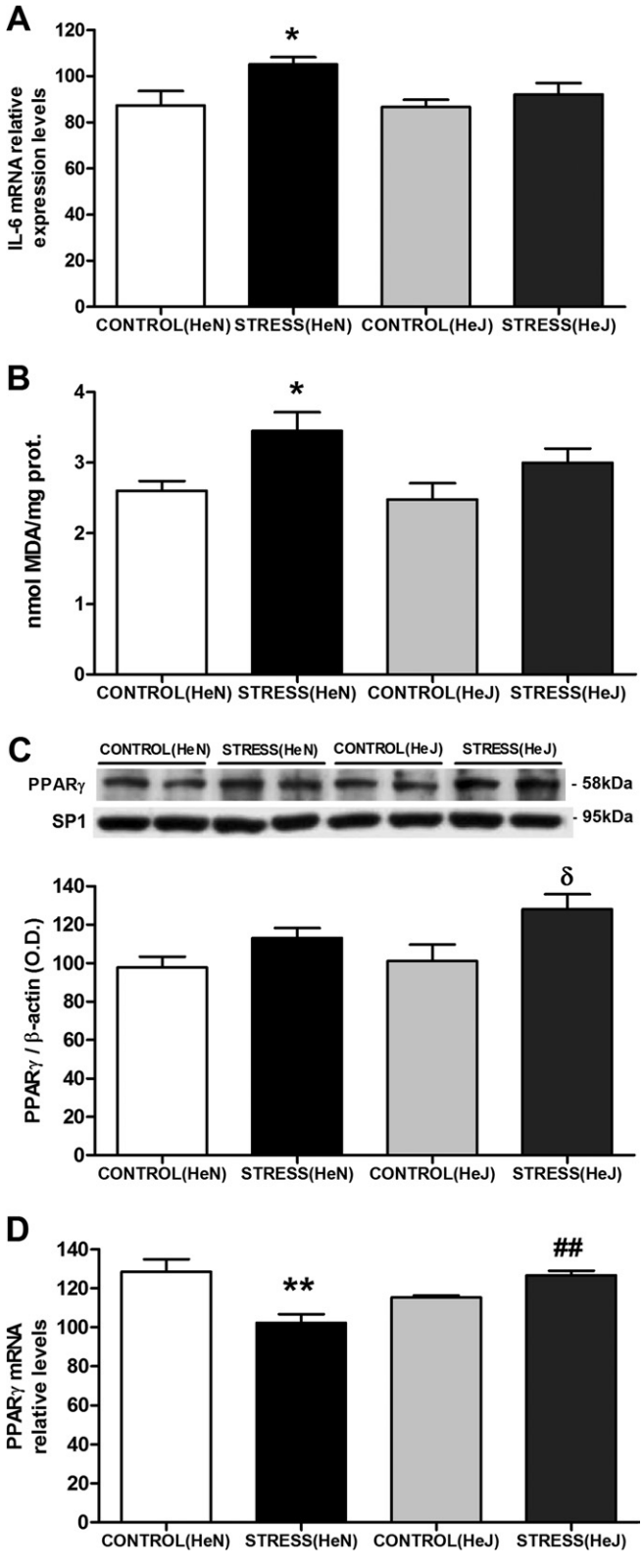


**Figure 8.** Toll-like receptor-4 deficiency prevents stress-induced interleukin-6 (IL-6) upregulation and peroxisome proliferator-activated receptor γ (PPARγ) downregulation in C3H/HeN (HeN) mice. **(A)** Interleukin-6 messenger RNA (mRNA) relative levels in brain cortex of control and stressed HeN and C3H/HeJ (HeJ) mice. Data represent the mean ± SEM ($n = 6$). $^{*}p < .05$ vs. CONTROL (HeN); two-way analysis of variance (ANOVA) following Bonferroni post hoc test. **(B)** Malondialdehyde (MDA) levels in prefrontal cortex homogenates of control and stressed HeN and HeJ mice. Data represent the mean ± SEM ($n = 6$). $^{*}p < .05$ vs. CONTROL (HeN); two-way ANOVA following Bonferroni post hoc test. **(C)** Peroxisome proliferator-activated receptor γ protein levels in brain prefrontal cortex nuclear extracts samples of control and stressed HeN and HeJ mice. The densitometric data are normalized by SP1 (lower band) and are representative of three experiments. $^{\delta}p < .05$ vs. CONTROL (HeJ). Two-way ANOVA following Bonferroni post hoc test. **(D)** Peroxisome proliferator-activated receptor γ mRNA relative levels in brain cortex of control and stressed HeN and HeJ mice. Messenger RNA data are normalized by tubulin. Data represent the mean ± SEM ($n = 6$). $^{**}p < .01$ vs. CONTROL (HeN); $^{\#\#}p < .01$ vs. STRESS (HeN). Two-way ANOVA following Bonferroni post hoc test. OD, optical density.

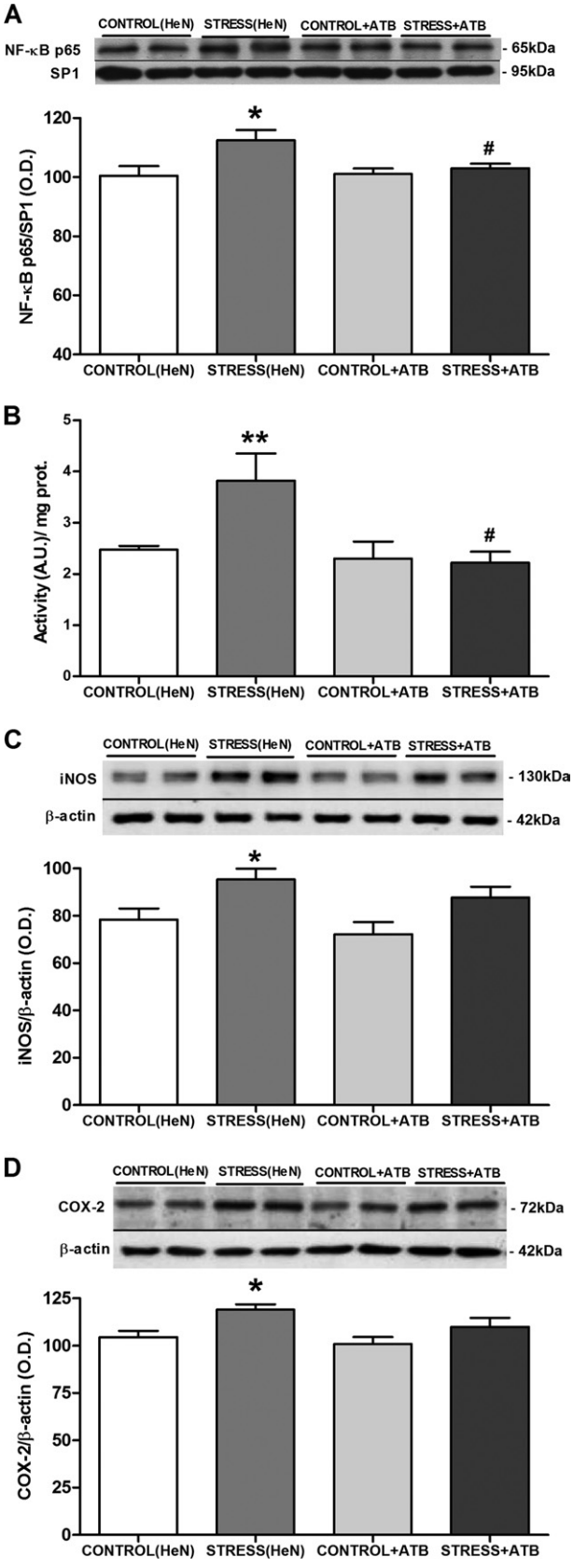


**Table 1.** Plasma Corticosterone Levels (ng/mL) Under Basal and Stress Conditions in HeN and HeJ Mice

| | HeN Mice | HeJ Mice |
|---|---|---|
| Control | 74.89 ± 14.41 | 118.8 ± 22.72 |
| Stress 2 Days | 219.3 ± 52.06[a] | 238.8 ± 46.02[b] |
| Stress 4 Days | 157.6 ± 18.27[a] | 155.9 ± 23.4 |

Data represent the mean ± SEM of six mice per group.
HeJ, C3H/HeJ; HeN, C3H/HeN.
[a] $p < .05$ vs. control (HeN).
[b] $p < .05$ vs. control (HeJ). Two-way analysis of variance following Bonferroni post hoc test.

pathways, such as the sympathetic nervous system, TLR-4 is regulated by the $\beta_2$-adrenergic receptor in macrophages (49,50). Indeed, it is possible that different stressors could differentially influence TLR-4-driven innate immune response by means of nonexclusive mechanisms.

An issue of special interest is how LPS signal gets into the brain from the periphery, particularly in brain areas far removed from circumventricular organs, such as the PFC. Our results agree with previous studies showing TLR-4 expression in murine primary cortical neurons (51), astrocytes (40), and microglia (52) under inflammatory conditions. However, further quantitative immunohistochemical studies are needed to identify the nature of the cellular types implicated in the upregulation of TLR-4 elicited by stress exposure and the potential function of TLR-4 present in the PFC regulating the hypothalamic-pituitary-adrenal (HPA) axis response to LPS or other immune/inflammatory challenges. In addition to the well known expression of TLR-4 in the periphery, our results suggest that it can regulate the indigenous flora and bacterial translocation at gut level after inflammatory stimuli. Indeed, a limitation of our study is that it is not possible to know whether the important role of TLR is in the brain, the periphery, or both. Indeed, further studies with specific TLR-4 knockout mice for each compartment/cellular type expressing this receptor are needed.

Our results suggest that TLR-4 is relevant in the activation of NF-κB proinflammatory pathways elicited by stress exposure in the brain PFC. The loss of functional TLR-4 allows the C3H/HEJ strain to be less susceptible to acute insults, such as brain ischemia (15,40) or axotomy-induced neurodegeneration (53). As commented above, more studies are necessary to demonstrate whether TLR-4 deficiency affords neuroprotection in chronic neuroinflammatory conditions or in relapse-remitting courses of disease. In this vein, an

**Figure 9.** Intestinal decontamination prevents stress-induced nuclear factor-kappa B (NF-κB) p65 activity and inducible nitric oxide synthase (iNOS) and cyclooxygenase type 2 (COX-2) upregulation. **(A)** Western blot detection of NF-κB p65 subunit and densitometric analysis of the band of interest in brain prefrontal cortex nuclear extract samples of control and stressed C3H/HeN (HeN) mice with the respective administration of vehicle or antibiotic (ATB). The densitometric data are normalized by SP1 (lower band) and are representative of three experiments. *$p < .05$ vs. CONTROL (HeN); #$p < .05$ vs. STRESS (HeN). One-way analysis of variance (ANOVA) following Newman-Keuls post hoc test. **(B)** Transcriptional activity of NF-κB p65 subunit in nuclear extracts from samples of control and stressed HeN mice with the respective administration of vehicle or antibiotic. The data represent the mean ± SEM ($n = 6$). **$p < .01$ vs. CONTROL (HeN); #$p < .05$ vs. STRESS (HeN). One-way ANOVA following Newman-Keuls post hoc test. Western blot detection of iNOS **(C)** and COX-2 **(D)** and densitometric analysis of the bands of interest in brain prefrontal cortex nuclear extract samples of control and stressed HeN mice with or without antibiotic. The densitometric data are normalized by β-actin (lower band) and are representative of three experiments. *$p < .05$ vs. CONTROL (HeN). One-way ANOVA following Newman-Keuls post hoc test. A.U., arbitrary units; OD, optical density.

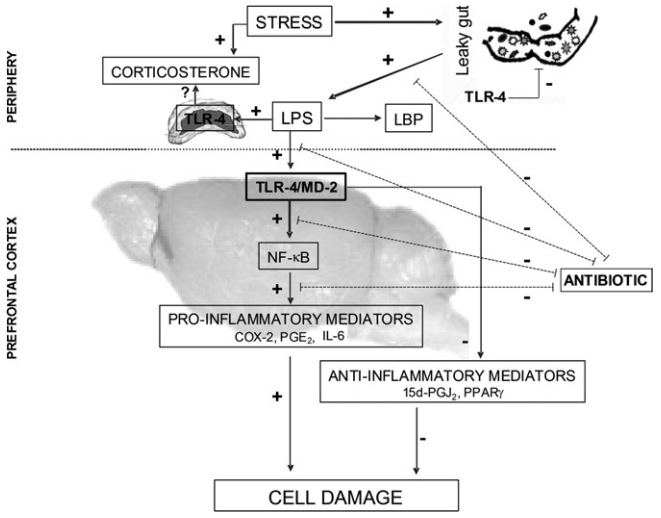


**Figure 10.** Schematic view of the role of toll like receptor-4 (TLR-4) on stress-induced neuroinflammation. COX-2, cyclooxygenase type 2; IL-6: interleukin-6; iNOS, inducible nitric oxide synthase; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; MD-2, myeloid differentiation protein-2; NF-κB, nuclear factor-kappa B; $PGE_2$, prostaglandin E2; 15d-$PGJ_2$, 15d-Prostaglandin $J_2$; PPARγ: peroxisome proliferators activated receptor γ; (−), inhibition; (+), stimulation.

interesting study has shown that TLR-4 mediates the imbalance of Th1/Th2 cytokines in chronic restraint stress-induced immune suppression (54). However, blocking TLRs may lead to inappropriate immune responses such as allergic Th2 responses or immunological tolerance (55), illustrating the need for more studies to fully understand its therapeutic potential.

Another effect to explore in TLR-4 deficient mice submitted to chronic stressors or after chronic bacterial translocation is LPS tolerance (56), an immunosuppressed state in which proinflammatory responses attenuate over time but oxidative/nitrosative damage could remain elevated. In fact, several authors suggest that altered TLR-4 function is responsible for endotoxin tolerance (57).

Interestingly, our results showed that HeJ mice presented higher levels of the anti-inflammatory mediator PPARγ after stress. These results agree with others reporting a crosstalk between LPS/TLR-4 and PPARγ in inflammatory scenarios, such as LPS-induced acute lung injury, inflammatory bowel disease, or LPS-induced preterm delivery (58–60), and at cellular level in macrophages and other immune cells (37,61,62). Whether these results are important in the pathophysiology of chronic stress-related diseases, such as depression, remains to be elucidated. The use of more specific and potent TLR-4 inhibitors than the currently available will be needed.

Toll-like receptor-4/corticosterone interactions may be more intricate. Our results show that under basal conditions, plasma corticosterone levels tend to increase in HeJ individuals, and interestingly, stress did not increase corticosterone secretion in HeJ mice compared with HeN mice after 4 days of stress, suggesting a failure in HPA feedback regulation or a hypofunction of the axis in these animals in chronic stress conditions. Basal alterations in adrenal gland structure and corticosterone levels in absence of TLR-4 have been previously presented (14). This role of TLR-4 is suitable for exploration in neuropsychiatric diseases, such as depression, a clinical setting in which hypercortisolemia and a clear deregulation of the mechanisms that prevent a potential damaging chronic activation of the HPA axis are present (63). Thus, complementary neuroendocrine/behavioral studies on TLR-4 deficient mice submitted to chronic stress conditions, resembling a depressive-like condition, are essential.

In summary, the data presented here suggest a functional role of TLR-4 in the brain PFC after stress exposure. Furthermore, a possible protective role of antibiotic decontamination in those stress-related pathologies presenting an increased or chronic intestinal permeability (leaky gut) in their pathophysiology, such as depression, deserves further consideration, always taking into account the detrimental effects of long-term use or repeated administration of antibiotics on mucosal integrity (64). Alternatively, the use of other evidence-based treatments targeting leaky gut, such as glutamine, has been suggested (65). Similarly, a protective role for antibiotic decontamination in an experimental model of depression has been recently demonstrated (16), opening the possibility to translational studies in psychopathologies.

*This work was supported by Spanish Ministry of Science and Innovation (SAF07-63138), the Instituto de Salud Carlos III (FIS 10/0123), Centro de Investigación Biomédica en Salud Mental, and Foundation Santander-Universidad Complutense (GR 58/08). IG, JRC, and BG-B are Formación de Personal Investigador, Juan De La Cierva, and Ramón y Cajal fellows (Ministerio de Ciencia e Innovación), respectively.*

*The authors report no biomedical financial interests or potential conflicts of interest.*

*Supplementary material cited in this article is available online.*

1. Herbert TB, Cohen S (1993): Depression and immunity: A meta-analytic review. *Psychol Bull* 113:472–486.
2. Herbert TB, Cohen S (1993): Stress and immunity in humans: A meta-analytic review. *Psychosom Med* 55:364–379.
3. Sheridan JF, Dobbs C, Brown D, Zwilling B (1994): Psychoneuroimmunology: Stress effects on pathogenesis and immunity during infection. *Clin Microbiol Rev* 7:200–212.
4. Raison CL, Capuron L, Miller AH (2006): Cytokines sing the blues: Inflammation and the pathogenesis of depression. *Trends Immunol* 27:24–31.
5. García-Bueno B, Caso JR, Leza JC (2008): Stress as a neuroinflammatory condition in brain: Damaging and protective mechanisms. *Neurosci Biobehav Rev* 32:1136–1151.
6. Madrigal JL, Moro MA, Lizasoain I, Lorenzo P, Leza JC (2002): Stress-induced increase in extracellular sucrose space in rats is mediated by nitric oxide. *Brain Res* 938:87–91.
7. Esposito P, Chandler N, Kandere K, Basu S, Jacobson S, Connolly R, *et al.* (2002): Corticotropin-releasing hormone and brain mast cells regulate blood-brain-barrier permeability induced by acute stress. *J Pharmacol Exp Ther* 303:1061–1066.
8. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008): From inflammation to sickness and depression: When the immune system subjugates the brain. *Nat Rev Neurosci* 9:46–56.
9. Crack PJ, Bray PJ (2007): Toll-like receptors in the brain and their potential roles in neuropathology. *Immunol Cell Biol* 85:476–480.
10. Hanke ML, Kielian T (2011): Toll-like receptors in health and disease in the brain: Mechanisms and therapeutic potential. *Clin Sci (Lond)* 121: 367–387.
11. Akira S, Takeda K (2004): Toll-like receptor signalling. *Nat Rev Immunol* 4:499–511.
12. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr (1997): A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388:394–397.
13. Akira S, Uematsu S, Takeuchi O (2006): Pathogen recognition and innate immunity. *Cell* 124:783–801.
14. Zacharowski K, Zacharowski PA, Koch A, Baban A, Tran N, Berkels R, *et al.* (2006): Toll-like receptor 4 plays a crucial role in the immune-adrenal response to systemic inflammatory response syndrome. *Proc Natl Acad Sci U S A* 103:6392–6397.
15. Caso JR, Pradillo JM, Hurtado O, Leza JC, Moro MA, Lizasoain I (2008): Toll-like receptor 4 is involved in subacute stress-induced neuroinflammation and in the worsening of experimental stroke. *Stroke* 39: 1314–1320.

16. Garate I, Garcia-Bueno B, Madrigal JL, Bravo L, Berrocoso E, Caso JR, *et al.* (2011): Origin and consequences of brain Toll-like receptor 4 pathway stimulation in an experimental model of depression. *J Neuroinflammation* 8:151.
17. Takeuchi O, Akira S (2001): Toll-like receptors; their physiological role and signal transduction system. *Int Immunopharmacol* 1:625–635.
18. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M (1999): MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 189:1777–1782.
19. Means TK, Golenbock DT, Fenton MJ (2000): The biology of Toll-like receptors. *Cytokine Growth Factor Rev* 11:219–232.
20. Madrigal JL, Moro MA, Lizasoain I, Lorenzo P, Castrillo A, Boscá L, Leza JC (2001): Inducible nitric oxide synthase expression in brain cortex after acute restraint stress is regulated by nuclear factor kappaB-mediated mechanisms. *J Neurochem* 76:532–538.
21. Koedel U, Merbt UM, Schmidt C, Angele B, Popp B, Wagner H, *et al.* (2007): Acute brain injury triggers MyD88-dependent, TLR2/4-independent inflammatory responses. *Am J Pathol* 171:200–213.
22. Collins SM (2001): Stress and the gastrointestinal tract IV. Modulation of intestinal inflammation by stress: Basic mechanisms and clinical relevance. *Am J Physiol Gastrointest Liver Physiol* 280:G315–G318.
23. Soderholm JD, Perdue MH (2001): Stress and gastrointestinal tract. II. Stress and intestinal barrier function. *Am J Physiol Gastrointest Liver Physiol* 280:G7–G13.
24. Ponferrada A, Caso JR, Alou L, Colon A, Sevillano D, Moro MA, *et al.* (2007): The role of PPARgamma on restoration of colonic homeostasis after experimental stress-induced inflammation and dysfunction. *Gastroenterology* 132:1791–1803.
25. Maes M, Kubera M, Leunis JC (2008): The gut-brain barrier in major depression: Intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. *Neuro Endocrinol Lett* 29:117–124.
26. Leonard B, Maes M (2012): Mechanistic explanations how cell-mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression. *Neurosci Biobehav Rev* 36:764–785.
27. Maes M, Coucke F, Leunis JC (2007): Normalization of the increased translocation of endotoxin from gram negative enterobacteria (leaky gut) is accompanied by a remission of chronic fatigue syndrome. *Neuro Endocrinol Lett* 28:739–744.
28. Poltorak A, He X, Smirnova I, Liu MY, Van HC, Du X, *et al.* (1998): Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science* 282:2085–2088.
29. Velders GA, van OR, Hagoort H, Verzaal P, Guiot HF, Lindley IJ, *et al.* (2004): Reduced stem cell mobilization in mice receiving antibiotic modulation of the intestinal flora: Involvement of endotoxins as cofactors in mobilization. *Blood* 103:340–346.
30. Kiank C, Holtfreter B, Starke A, Mundt A, Wilke C, Schutt C (2006): Stress susceptibility predicts the severity of immune depression and the failure to combat bacterial infections in chronically stressed mice. *Brain Behav Immun* 20:359–368.
31. Depke M, Fusch G, Domanska G, Geffers R, Volker U, Schuett C, Kiank C (2008): Hypermetabolic syndrome as a consequence of repeated psychological stress in mice. *Endocrinology* 149:2714–2723.
32. Olfe J, Domanska G, Schuett C, Kiank C (2010): Different stress-related phenotypes of BALB/c mice from in-house or vendor: Alterations of the sympathetic and HPA axis responsiveness. *BMC Physiol* 10:2.
33. Schreiber E, Matthias P, Muller MM, Schaffner W (1989): Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 17:6419.
34. Das NP, Ratty AK (1987): Studies on the effects of the narcotic alkaloids, cocaine, morphine, and codeine on nonenzymatic lipid peroxidation in rat brain mitochondria. *Biochem Med Metab Biol* 37:258–264.
35. Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
36. García-Bueno B, Madrigal JLM, Lizasoain I, Moro MA, Lorenzo P, Leza JC (2005): Peroxisome proliferator-activated receptor gamma activation decreases neuroinflammation in brain after stress in rats. *Biol Psychiatry* 57:885–894.
37. Necela BM, Su W, Thompson EA (2008): Toll-like receptor 4 mediates cross-talk between peroxisome proliferator-activated receptor gamma and nuclear factor-kappaB in macrophages. *Immunology* 125:344–358.
38. Prinz M, Garbe F, Schmidt H, Mildner A, Gutcher I, Wolter K, *et al.* (2006): Innate immunity mediated by TLR9 modulates pathogenicity in an animal model of multiple sclerosis. *J Clin Invest* 116:456–464.
39. Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K (2006): Role of toll-like receptor signalling in Abeta uptake and clearance. *Brain* 129: 3006–3019.
40. Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I (2007): Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. *Circulation* 115:1599–1608.
41. Griffiths MR, Gasque P, Neal JW (2010): The regulation of the CNS innate immune response is vital for the restoration of tissue homeostasis (repair) after acute brain injury: A brief review. *Int J Inflam* 2010:151097.
42. Kigerl KA, Lai W, Rivest S, Hart RP, Satoskar AR, Popovich PG (2007): Toll-like receptor (TLR)-2 and TLR-4 regulate inflammation, gliosis, and myelin sparing after spinal cord injury. *J Neurochem* 102:37–50.
43. Romanovsky AA, Steiner AA, Matsumura K (2006): Cells that trigger fever. *Cell Cycle* 5:2195–2197.
44. Fukata M, Michelsen KS, Eri R, Thomas LS, Hu B, Lukasek K, *et al.* (2005): Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am J Physiol Gastrointest Liver Physiol* 288:G1055–G1065.
45. Shang L, Fukata M, Thirunarayanan N, Martin AP, Arnaboldi P, Maussang D, *et al.* (2008): Toll-like receptor signaling in small intestinal epithelium promotes B-cell recruitment and IgA production in lamina propria. *Gastroenterology* 135:529–538.
46. Bailey MT, Engler H, Powell ND, Padgett DA, Sheridan JF (2007): Repeated social defeat increases the bactericidal activity of splenic macrophages through a Toll-like receptor-dependent pathway. *Am J Physiol Regul Integr Comp Physiol* 293:R1180–R1190.
47. Tsan MF, Gao B (2004): Endogenous ligands of Toll-like receptors. *J Leukoc Biol* 76:514–519.
48. Glezer I, Zekki H, Scavone C, Rivest S (2003): Modulation of the innate immune response by NMDA receptors has neuropathological consequences. *J Neurosci* 23:11094–11103.
49. Kizaki T, Izawa T, Sakurai T, Haga S, Taniguchi N, Tajiri H, *et al.* (2008): Beta2-adrenergic receptor regulates Toll-like receptor-4-induced nuclear factor-kappaB activation through beta-arrestin 2. *Immunology* 124:348–356.
50. Du Q, Min S, Chen LY, Ma YD, Guo XL, Wang Z (2012): Major stress hormones suppress the response of macrophages through down-regulation of TLR2 and TLR4. *J Surg Res* 173:354–361.
51. Tang SC, Lathia JD, Selvaraj PK, Jo DG, Mughal MR, Cheng A, *et al.* (2008): Toll-like receptor-4 mediates neuronal apoptosis induced by amyloid beta-peptide and the membrane lipid peroxidation product 4-hydroxynonenal. *Exp Neurol* 213:114–121.
52. Olson JK, Miller SD (2004): Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *J Immunol* 173:3916–3924.
53. Kilic U, Kilic E, Matter CM, Bassetti CL, Hermann DM (2008): TLR-4 deficiency protects against focal cerebral ischemia and axotomy-induced neurodegeneration. *Neurobiol Dis* 31:33–40.
54. Zhang Y, Liang L, Wu W, Gao Y, Chen ZB, Liang ZY, Liang TB (2008): Resuscitation with hydroxyethyl starch solution prevents CD4+ T-lymphocyte apoptosis and modulates the balance of T helper type 1 and T helper type 2 responses in the rat with traumatic virgule/shill hemorrhagic shock. *Shock* 30:692–698.
55. Ishii KJ, Uematsu S, Akira S (2006): 'Toll' gates for future immunotherapy. *Curr Pharm Des* 12:4135–4142.
56. Biswas SK, Lopez-Collazo E (2009): Endotoxin tolerance: New mechanisms, molecules and clinical significance. *Trends Immunol* 30:475–487.
57. Qureshi ST, Larivière L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D (1999): Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med* 189:615–625.
58. Inoue K, Takano H, Yanagisawa R, Ichinose T, Sadakane K, Yoshino S, *et al.* (2005): Effects of 15-deoxy-Delta12,14-prostaglandin J2 on the expression of Toll-like receptor 4 and 2 in the murine lung in the presence of lipopolysaccharide. *Clin Exp Pharmacol Physiol* 32: 230–232.

59. Rousseaux C, Desreumaux P (2006): [The peroxisome-proliferator-activated gamma receptor and chronic inflammatory bowel disease (PPAR-gamma and IBD)]. *J Soc Biol* 200:121–131.
60. Pirianov G, Waddington SN, Lindstrom TM, Terzidou V, Mehmet H, Bennett PR (2009): The cyclopentenone 15-deoxy-delta 12,14-prostaglandin J(2) delays lipopolysaccharide-induced preterm delivery and reduces mortality in the newborn mouse. *Endocrinology* 150:699–706.
61. Inoue H, Tanabe T, Umesono K (2000): Feedback control of cyclooxygenase-2 expression through PPARgamma. *J Biol Chem* 275:28028–28032.
62. Dasu MR, Park S, Devaraj S, Jialal I (2009): Pioglitazone inhibits Toll-like receptor expression and activity in human monocytes and db/db mice. *Endocrinology* 150:3457–3464.
63. Gillespie CF, Nemeroff CB (2005): Hypercortisolemia and depression. *Psychosom Med* 67(suppl 1):S26–S28.
64. Levy J (2000): The effects of antibiotic use on gastrointestinal function. *Am J Gastroenterol* 95(suppl 1):S8–S10.
65. Maes M, Leunis JC (2008): Normalization of leaky gut in chronic fatigue syndrome (CFS) is accompanied by a clinical improvement: Effects of age, duration of illness and the translocation of LPS from gram-negative bacteria. *Neuro Endocrinol Lett* 29:902–910.