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Chronic exposure to exogenous glucocorticoids primes microglia to pro-inflammatory stimuli and induces NLRP3 mRNA in the hippocampus

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Summary Chronic stress as well as chronic treatment with glucocorticoids (GCs) primes the neuroinflammatory response to a subsequent pro-inflammatory challenge. However, it remains unclear whether chronic GCs sensitize the response of key CNS immune substrates (*i.e.* microglia) to pro-inflammatory stimuli. In the present set of studies, male Sprague-Dawley rats underwent sham surgery or were adrenalectomized and then treated with varying concentrations of corticosterone (CORT; 0, 25, 50, and 75 μg/ml) administered in their drinking water. After 10 days of CORT exposure, whole hippocampus was collected and expression of glial activation markers measured or hippocampal microglia were isolated and challenged with LPS to probe for CORT-induced sensitization of pro-inflammatory responses. Chronic CORT exposure increased the gene expression of NLRP3, Iba-1, MHCII, and NF-κBIα in a concentration dependent manner. Chronic CORT (75 μg/ml) exposure potentiated the microglial proinflammatory response (TNFα, IL-1β, IL-6 and NLRP3) to LPS compared to the microglial response of sham surgery animals treated with vehicle. The present set of results demonstrate that chronic exposure to GCs primes microglia to pro-inflammatory stimuli and add to a growing body of evidence suggesting that a permissive function of GCs is that of an endogenous danger signal or alarmin.

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1. Introduction

Chronic stress primes the neuroinflammatory response to both peripheral and central pro-inflammatory challenges (Audet et al., 2011; de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Munhoz et al., 2006; Wohleb et al., 2012) and primes the pro-inflammatory response of microglia to LPS *ex vivo* (Wohleb et al., 2011). Consistent with these stress-induced priming effects, chronic stress modulates the immunophenotype of microglia as evidenced by the up-regulation of MHCII (de Pablos et al., 2006; Espinosa-Oliva et al., 2011), TLR4 (Wohleb et al., 2011), F4/80 antigen (Nair and Bonneau, 2006) and Iba-1 expression (Hinwood et al., 2012; Tynan et al., 2010). Notably, stress-induced glucocorticoids (GCs) appear to play a pivotal role in chronic stress-induced neuroinflammatory priming (de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Munhoz et al., 2006) as well as the stress-induced modulation of microglia immunophenotype (de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Nair and Bonneau, 2006). Consistent with these stress studies, chronic administration of GCs is sufficient to prime neuroinflammatory responses to a subsequent pro-inflammatory challenge (Kelly et al., 2012; Munhoz et al., 2010). However, it is unknown whether chronic GCs sensitize the response of key CNS innate immune substrates such as microglia to pro-inflammatory stimuli.

An emerging literature suggests that GCs modulate key pro-inflammatory pathways, which may serve as the basis for how stress and GCs prime pro-inflammatory immune responses (Frank et al., 2013). Of particular relevance here, GCs induce the expression of the NLRP (nucleotide-binding domain, leucine-rich repeat, pyrin domain containing protein) 3 inflammasome, which is the only known inflammasome requiring a priming stimulus that is modulated by GCs (Busillo et al., 2011). NLRP3 inflammasome assembly and activation requires a priming stimulus, which induces NLRP3 transcription, and a secondary stimulus, which induces the formation of the NLRP3 molecular scaffold. The formation and activation of the NLRP3 inflammasome in turn leads to the formation and release of active, mature IL-1β (Hornung and Latz, 2010). Busillo et al. (2011) found that GCs induce NLRP3 at both the mRNA and protein level in THP-1 cells, bone marrow-derived macrophages, and primary human monocytes *in vitro*, thereby priming NLRP3 inflammasome formation to a subsequent stimulus such as ATP, and potentiating the pro-inflammatory cytokine response. IL-1β is critical to the inflammatory response (Basu et al., 2004) and the production and release of mature IL-1β requires inflammasome formation and activation (Lamkanfi and Kanneganti, 2010). Therefore, GC-induction of NLRP3 could serve as a mechanism of stress- and GC-induced priming of neuroinflammatory processes. However, neither the effects of GCs on NLRP3 *in vivo*, in brain, or in microglia have been examined.

In the present study, we explored whether (1) microglia serve as a neuroimmune substrate of chronic GC-induced priming and (2) chronic GC exposure modulates the NLRP3 inflammasome. Prior studies have shown that stress primes neuroinflammatory processes in several brain regions including the frontal cortex, hypothalamus, and hippocampus (Johnson et al., 2002). In the present study, the hippocampus was chosen for study because of the deleterious effects of neuroinflammatory processes on hippocampus dependent cognitive function (Barrientos et al., 2012).

2. Methods

2.1. Animals

Male Sprague-Dawley rats (60—90 days old; Harlan Sprague-Dawley, Inc., Indianapolis, IN, USA) were pair-housed with food and water available *ad libitum*. The colony was maintained at 25 °C on a 12-h light/dark cycle (lights on at 0700 h). All experimental procedures were conducted in accord with the University of Colorado Institutional Animal Care and Use Committee.

2.2. Adrenalectomy (ADX)

Bilateral ADX was aseptically performed under halothane anesthesia (Halocarbon Laboratories, River Edge, NJ, USA) as previously described (Frank et al., 2012). ADX was used to remove endogenous sources of CORT and examine whether exogenous CORT treatment is sufficient to induce microglia sensitization. All removed tissue was examined immediately to ensure complete removal of the adrenal gland. Adrenal tissue was visually inspected to assess that the adrenal gland was intact. Sham-operated animals received the identical procedure, except that the adrenal glands were gently manipulated with forceps, but not removed.

2.3. Corticosterone (CORT) treatment

Immediately after surgery, ADX animals were administered CORT (Sigma, St. Louis, MO) in their drinking water. ADX animals received either basal CORT (25 μg/ml) replacement in their drinking water since this method has been shown to mimic the normal circadian pattern of CORT secretion (Jacobson et al., 1988) or high CORT (50 and 75 μg/ml) concentrations. CORT was dissolved in 100% ETOH. Final ETOH concentration was 0.4% for all CORT conditions. CORT water was supplemented with 0.9% saline. Sham surgery animals received vehicle water (0.4% ETOH) without CORT (0 μg/ml CORT). Throughout the text and figures 0 μg/ml CORT refers to sham surgery animals. The duration of CORT or vehicle treatment was 10 days. For verification of CORT treatment, CORT levels were measured in serum and hippocampus. The effect of CORT treatment on body weight was also measured.

2.4. Tissue and blood collection

Animals were given a lethal dose of sodium pentobarbital. Animals were fully anesthetized and cardiac blood withdrawn within 3 min of injection. Animals were trans-cardially perfused with ice-cold saline (0.9%) for 3 min to remove peripheral immune leukocytes from the CNS vasculature. Brain was rapidly extracted and placed on ice and hippocampus dissected. For *in vivo* experiments, hippocampus was flash frozen in liquid nitrogen and stored at −80 °C. For *ex vivo* experiments, hippocampal microglia were immediately isolated.

2.5. *Ex vivo* immune stimulation of hippocampal microglia with LPS

Hippocampal microglia were isolated using a Percoll density gradient as previously described (Frank et al., 2006). We have previously shown (Frank et al., 2006) that this microglia isolation procedure yields highly pure microglia (Iba-1+/MHCII+/CD163−/GFAP−). In the present experiments, immunophenotype and purity of microglia was assessed using real time RT-PCR. Microglia were suspended in DMEM + 10% FBS and microglia concentration determined by trypan blue exclusion. Microglia concentration was adjusted to a density of 5×10^3 cells/100 μl and 100 μl added to individual wells of a 96-well v-bottom plate. Lipopolysaccharide (LPS; *E. coli* serotype 0111:B4; Sigma) was utilized to challenge microglia *ex vivo* as we have previously determined the optimal *in vitro* conditions under which LPS stimulates a microglia pro-inflammatory cytokine response (Frank et al., 2006). Cells were incubated with LPS (0.1, 1, 10, and 100 ng/ml) or media alone for 2 h at 37 °C, 5% CO_2. The plate was centrifuged at 1000 × *g* for 10 min at 4 °C to pellet cells and cells washed 1× in ice cold PBS and centrifuged at 1000 × *g* for 10 min at 4 °C. Cell lysis/homogenization and cDNA synthesis was performed according to the manufacturer's protocol using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen, Carlsbad, CA).

2.6. Real time RT-PCR measurement of gene expression

A detailed description of the PCR amplification protocol has been published previously (Frank et al., 2006). cDNA sequences were obtained from Genbank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences were designed using the Operon Oligo Analysis Tool (http://www.operon.com/technical/toolkit.aspx) and tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI (Altschul et al., 1997). Primers were obtained from Invitrogen. Primer specificity was verified by melt curve analyses. All primers were designed to span exon/exon boundaries and thus exclude amplification of genomic DNA (See Table 1 for primer description and sequences).

Table 1 Primer description and sequences.

Gene	Primer sequence 5′ → 3′	Function
B-Actin	F: TTCCTTCCTGGGTATGGAAT R: GAGGAGCAATGATCTTGATC	Cytoskeletal protein (housekeeping gene)
CD163	F: GTAGTAGTCATTCAACCCTCAC R: CGGCTTACAGTTTCCTCAAG	Macrophage antigen not expressed by microglia
GFAP	F: AGATCCGAGAAACCAGCCTG R: CCTTAATGACCTCGCCATCC	Astrocyte antigen
IL-1b	F: CCTTGTGCAAGTGTCTGAAG R: GGGCTTGGAAGCAATCCTTA	Pro-inflammatory cytokine
IL-6	F: AGAAAAGAGTTGTGCAATGGCA R: GGCAAATTTCCTGGTTATATCC	Pro-inflammatory cytokine
Iba-1	F: GGCAATGGAGATATCGATAT R: AGAATCATTCTCAAGATGGC	Microglia/macrophage antigen
MHCII	F: AGCACTGGGAGTTTGAAGAG R: AAGCCATCACCTCCTGGTAT	Microglia/macrophage antigen
NFKBIA	F: CACCAACTACAACGGCCACA R: GCTCCTGAGCGTTGACATCA	Induced by NFκB to inhibit NFκB function
TNFa	F: CAAGGAGGAGAAGTTCCCA R: TTGGTGGTTTGCTACGACG	Pro-inflammatory cytokine
NLRP3	F: AGAAGCTGGGGTTGGTGAATT R: GTTGTCTAACTCCAGCATCTG	Rate limiting protein in NLRP3 inflammasome formation
Caspase-1	F: ATGCCGTGGAGAGAAACAAG R: CCAGGACACATTATCTGGTG	NLRP3 inflammasome that converts pro-IL-1β to mature IL-1β
ASC	F: ACCCCATAGACCTCACTGAT R: ACAGCTCCAGACTCTTCCAT	Component of the NLRP3 inflammasome that recruits pro-caspase-1
TLR2	F: TGGAGGTCTCCAGGTCAAATC R: ACAGAGATGCCTGGGCAGAAT	Pattern recognition receptor for Gram-positive bacterial molecular motifs
TLR4	F: TCCCTGCATAGAGGTACTTC R: CACACCTGGATAAATCCAGC	Pattern recognition receptor for Gram-negative bacterial molecular motifs
GR	F: TCTCTCCTCAGTTCCTAAGG R: GATTCTCAACCACCTCATGC	Cytosolic receptor for glucocorticoids

Abbreviations: GFAP, glial fibrillary acidic protein; IL, interleukin; Iba-1, ionized calcium-binding adaptor molecule-1; MHCII, major histocompatibility complex II; NF-κBIα, nuclear factor kappa light chain enhancer of activated B cells inhibitor alpha; TNFα, tumor necrosis factor-α; NLRP3, nucleotide-binding domain, leucine-rich repeat, pyrin domain containing protein 3; ASC, apoptosis-associated speck-like protein containing a CARD; TLR, toll-like receptor; GR, glucocorticoid receptor.

PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA). Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA). Relative gene expression was determined by taking the expression ratio of the gene of interest to β-actin.

2.7. Serum and hippocampus CORT assay

Cardiac blood was centrifuged (10 min, 14,000 × *g*, 4 °C) and serum collected. Hippocampus was sonicated using a tissue extraction reagent (Invitrogen) supplemented with a protease inhibitor cocktail (Sigma). Homogenate was centrifuged (10 min, 14,000 × *g*, 4 °C) and supernatant collected and stored at −20 °C. Total protein was quantified using a Bradford assay. CORT was measured using a competitive immunoassay (Assay Designs, Inc., Ann Arbor, MI) as described in the manufacturer's protocol.

2.8. Hippocampal IL-1β ELISA

IL-1β protein was measured using a commercially available ELISA (R&D Systems, Minneapolis, MN). Concentrations of IL-1β protein were normalized to total protein and expressed as pg/mg total protein.

2.9. Water consumption

Animals underwent sham surgery or ADX. Immediately after surgery, sham animals received vehicle (0.4% ETOH) and ADX animals received CORT (75 μg/ml) in drinking water for 10 days. Water consumption was measured 1 d prior to surgery and on days 2, 4, 6, 8 and 10 days post-surgery.

2.10. Statistical analysis and data presentation

All data are presented as mean ± SEM. Statistical analyses consisted of ANOVA followed by *post hoc* tests (Tukey B) using Prism 5 (Graphpad Software, Inc., La Jolla, CA). Repeated measures ANOVA was used to analyze body weight data and water consumption. All data met the assumptions of ANOVA including normality of data and homogeneity of variance. Area under the LPS concentration curve (AUC) was computed using the trapezoid rule and significant mean differences in AUC determined by two-tailed *t*-test. Omnibus *F*-values are reported for each ANOVA and serve as a criterion for performing *post hoc* analyses. Threshold for statistical significance was set at $\alpha = .05$. 6 Animals per experimental group were used in each experiment.

3. Results

3.1. Serum and hippocampal CORT levels

Chronic CORT treatment induced a significant change in both serum ($F_{3,\ 18} = 27.77$, $p < 0.0001$) and hippocampal ($F_{3,\ 18} = 32.03$, $p < 0.0001$) CORT (Fig. 1). In serum (Fig. 1A), CORT treatment for 10 days resulted in a concentration dependent increase in CORT levels. CORT levels in vehicle treated intact animals did not significantly differ from levels

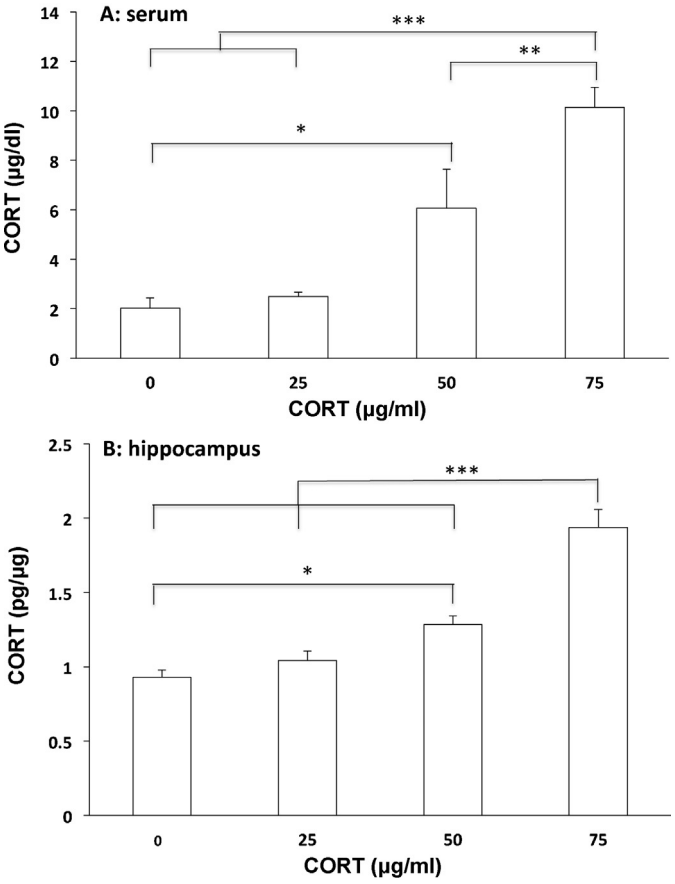

Figure 1 Effect of chronic CORT exposure on serum and hippocampal CORT levels. Animals were ADX or subject to sham surgery. Sham surgery animals (0 μg/ml) were administered vehicle (0.4% ETOH) and ADX animals were administered CORT (25, 50, and 75 μg/ml) supplemented with 0.9% saline in their drinking water for 10 days. On day 10 of treatment, serum (Panel A) and hippocampal (Panel B) CORT levels were measured. Data are presented as the mean + SEM. Significant group differences between different CORT treatment groups are designated as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

observed in ADX animals treated with 25 μg/ml CORT, indicating that a concentration of 25 μg/ml CORT restores CORT levels to basal levels. In hippocampus (Fig. 1B), CORT treatment resulted in a pattern of CORT levels similar to CORT levels observed in serum. The correlation between serum and hippocampal CORT levels was $r = 0.89$ ($p < 0.0001$).

3.2. Change in body weight post-surgery

Change in body weight post-surgery was significantly modulated by CORT treatment (Fig. 2, CORT × time interaction, $F_{9,\ 60} = 4.12$, $p < 0.001$). At 2 days post-surgery, CORT treatment (50 and 75 μg/ml) induced a significant reduction in body weight compared to vehicle treatment and 25 μg/ml CORT. Similar effects of CORT treatment on body weight were observed at 4 and 7 days post-surgery. At 10 days post-surgery, CORT treatment induced a significant graded reduction in body weight (75 μg/ml CORT < 50 μg/ml < 25 μg/ml CORT and vehicle). At each time point post-surgery, body

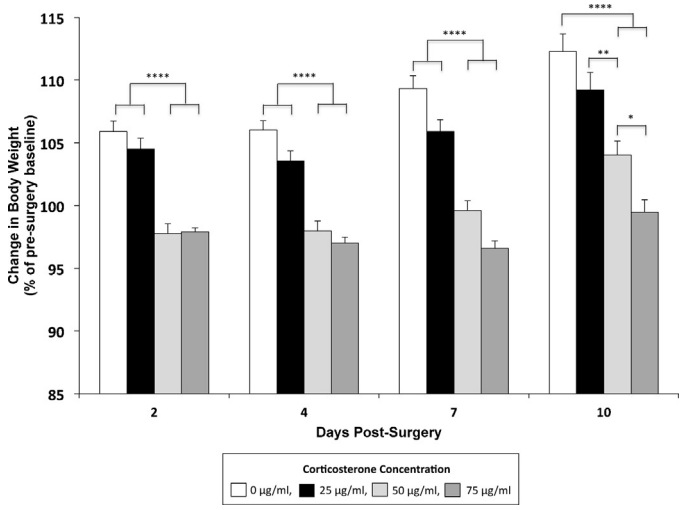

Figure 2 Effect of chronic CORT exposure on body weight. Animals were ADX or subject to sham surgery. Sham surgery animals (0 μg/ml CORT) were administered vehicle (0.4% ETOH) and ADX animals were administered CORT supplemented with 0.9% saline in their drinking water for 10 days. Percent change in body weight was measured 2, 4, 7 and 10 days post-surgery in animals treated with 0 μg/ml, 25 μg/ml, 50 μg/ml, and 75 μg/ml CORT. Data are presented as the mean + SEM. Significant group differences between different CORT treatment groups are designated as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.0001$.

weight change did not significantly differ between vehicle treated and 25 μg/ml CORT treated animals.

3.3. Macrophage/microglia activation markers and inflammasome components

In hippocampus, CORT treatment induced a significant change in the steady state mRNA levels for Iba-1 ($F_{3,\ 20} = 7.1$, $p < 0.01$), MHCII ($F_{3,\ 20} = 4.59$, $p < 0.05$), NLRP3 ($F_{3,\ 20} = 8.79$, $p < 0.001$), and NF-κBIα ($F_{3,\ 20} = 12.0$, $p < 0.0001$). *Post hoc* analyses (Fig. 3) showed that CORT induced a concentration dependent increase in Iba-1, MHCII, NLRP3, and NF-κBIα. CORT failed to significantly alter the steady state mRNA levels of ASC, caspase-1, IL-1β, TLR2, TLR4 and the glucocorticoid receptor (GR) (Supplementary Fig. 1). Hippocampal CORT levels were modestly correlated with gene expression levels of Iba-1 ($r = 0.58$, $p < 0.01$), and MHCII ($r = 0.55$, $p < 0.01$), while hippocampal CORT levels were highly correlated with NLRP3 ($r = 0.70$, $p < 0.0001$) and NF-κBIα ($r = 0.77$, $p < 0.0001$) expression levels.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2013.11.006.

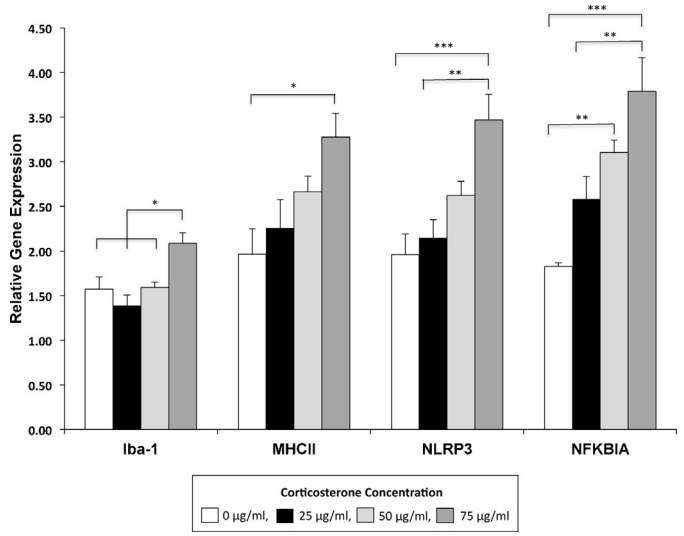

Figure 3 Effect of chronic CORT exposure on hippocampal macrophage/microglia activation markers and NLRP3 inflammasome components. Animals were ADX or subject to sham surgery. Sham surgery animals (0 μg/ml CORT) were administered vehicle (0.4% ETOH) and ADX animals were administered CORT supplemented with 0.9% saline in their drinking water for 10 days. Animals were treated with 0 μg/ml, 25 μg/ml, 50 μg/ml, and 75 μg/ml CORT. Ten days post-treatment, relative gene expression was measured in hippocampus. Data are presented as the mean + SEM. For each gene, significant group differences between different CORT treatment groups are designated as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

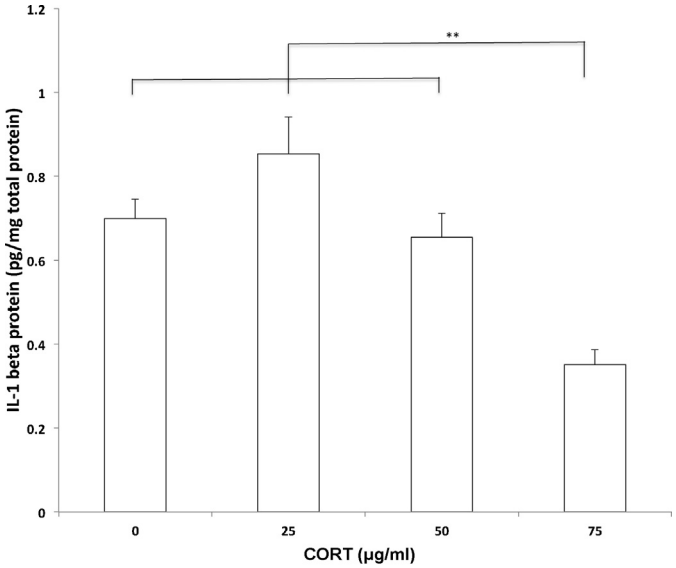

Figure 4 Effect of chronic CORT exposure on hippocampal IL-1β protein. Animals were ADX or subject to sham surgery. Sham surgery animals (0 μg/ml CORT) were administered vehicle (0.4% ETOH) and ADX animals were administered CORT supplemented with 0.9% saline in their drinking water for 10 days. Animals were treated with 0 μg/ml, 25 μg/ml, 50 μg/ml, and 75 μg/ml CORT. Ten days post-treatment, IL-1β protein levels were measured in hippocampus. Data are presented as the mean + SEM. Significant group differences between different CORT treatment groups are designated as $^{**}p < 0.01$.

3.4. CORT effects on hippocampal IL-1β protein

CORT treatment had a significant effect on IL-1β protein levels ($F_{3,\ 20} = 12.33$, $p < 0.0001$). *Post hoc* analyses (Fig. 4) showed that a CORT concentration of 75 μg/ml resulted in a significant reduction in IL-1β protein levels compared to the 0, 25, and 50 μg/ml CORT conditions.

3.5. CORT effects on water consumption

To assess the effects of CORT treatment on sickness behavior, the effect of CORT treatment (75 μg/ml) on water consumption was measured. CORT treatment significantly modulated water consumption behavior (CORT × time interaction, $F_{4,\ 48} = 6.83$, $p = 0.0002$). *Post hoc* analyses (Fig. 5) showed that CORT treatment significantly increased water consumption at 6 and 8 days post-surgery.

3.6. Priming of hippocampal microglia

RT-PCR analysis of microglia immunophenotype showed that Iba-1 and MHCII exhibited high gene expression levels (*i.e.* similar to β-actin levels), whereas CD163 and GFAP failed to amplify through 35 cycles of PCR suggesting that the isolation

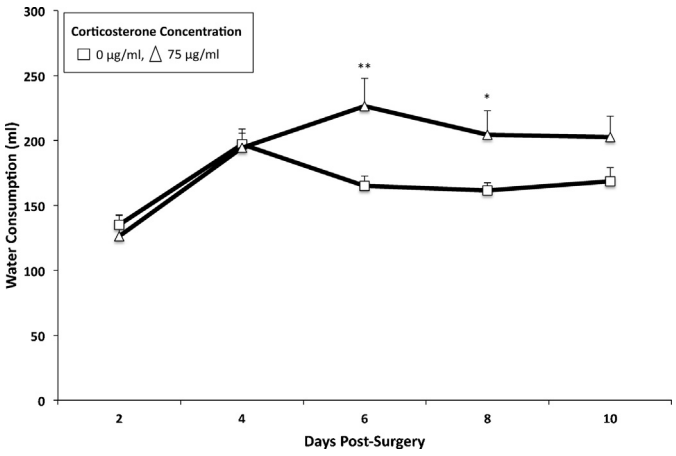

Figure 5 Effect of chronic CORT exposure on water consumption behavior. Animals were ADX or subject to sham surgery. Sham surgery animals (0 μg/ml CORT) were administered vehicle (0.4% ETOH) and ADX animals were administered CORT (75 μg/ml) supplemented with 0.9% saline in their drinking water for 10 days. Water consumption was measured at 2, 4, 6, 8 and 10 days post-surgery. Data are presented as the mean + SEM. At each time-point post-surgery, significant group differences between vehicle and CORT treatment are designated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

procedure yielded highly pure microglia (data not shown). CORT treatment significantly modulated the microglia pro-inflammatory response to LPS *ex vivo* (Fig. 4) for IL-1β (CORT × LPS interaction, $F_{4,\ 50} = 2.56$, $p < 0.05$), IL-6 (CORT × LPS interaction, $F_{4,\ 50} = 5.53$, $p < 0.001$), TNFα (CORT × LPS interaction, $F_{4,\ 50} = 4.82$, $p < 0.01$) and NLRP3 (CORT × LPS interaction, $F_{4,\ 50} = 3.82$, $p < 0.01$). CORT failed to significantly modulate LPS-induced NF-κBIα expression, though the main effect of LPS on NF-κBIα expression was significant ($F_{4,\ 50} = 16.6$, $p < 0.0001$) (data not shown). *Post hoc* analysis showed that compared to vehicle treatment, CORT treatment potentiated the pro-inflammatory response of IL-1β (100 ng/ml LPS, $p < 0.05$), IL-6 (1, 10 and 100 ng/ml LPS, $p < 0.05$), TNFα (0.1 and 1 ng/ml LPS, $p < 0.01$; 10 and 100 ng/ml LPS, $p < 0.05$) and NLRP3 (100 ng/ml LPS, $p < 0.05$). To capture the cumulative pro-inflammatory response of microglia to LPS *ex vivo*, area under the LPS concentration curve was determined for vehicle and CORT treated animals. Compared to vehicle treatment, CORT treatment resulted in a significant increase in the area under the curve for IL-1β ($p = 0.03$), IL-6 ($p = 0.01$), TNFα ($p = 0.01$) and NLRP3 ($p = 0.02$) (Fig. 6).

4. Discussion

An emerging literature has demonstrated that chronic stress and GCs modulate the immunophenotype of CNS macrophages and microglia (de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Hinwood et al., 2012; Munhoz et al., 2010; Nair and Bonneau, 2006; Wohleb et al., 2011). Likewise, the present results show that chronic exposure to exogenous GCs up-regulates the expression of the macrophage/microglia activation antigens MHCII and Iba-1, replicating the results of prior studies (de Pablos et al., 2006; Hinwood et al., 2012; Tynan et al., 2010) that stress/GCs alter the immunophenotype of these myeloid cells. Here, whole hippocampus expression of MHCII and Iba-1 was measured. Unfortunately, MHCII and Iba-1 antigens are expressed by both CNS macrophages (perivascular macrophages) and microglia, thereby precluding conclusions as to the cell type influenced by GCs.

The effects of chronic stress and GCs on macrophage/microglia immunophenotype would suggest a GC-induced shift in the activation state of these myeloid cells. Indeed, stress and GCs prime the neuroinflammatory response to a subsequent pro-inflammatory challenge (de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Kelly et al., 2012; Munhoz et al., 2006, 2010) suggesting that GC-induced immunophenotypic changes reflect a fundamental change in the functional state of CNS innate immune effectors. In prior studies of chronic GC exposure on neuroinflammatory priming (Kelly et al., 2012; Munhoz et al., 2010), the pro-inflammatory challenge was administered *in vivo*, thereby precluding determination of the CNS substrate(s) primed by GCs. That is, because pro-inflammatory stimuli (*e.g.* LPS) signal through multiple innate immune cell types, determination of GC-induced priming effects was confounded. To address this issue here, hippocampal microglia were isolated after 10 days of *in vivo* GC treatment and exposed to LPS directly, thereby probing the activation state (primed) of this particular CNS innate immune population of cells. Importantly, macrophage (CD163) and astrocyte (GFAP) antigen expression was undetectable in the isolated microglia preparations, indicating that the cell isolation procedure yielded highly pure microglia (Iba-1+/MHCII+ cells), a finding consistent with our prior work (Frank et al., 2006). Hippocampal microglia isolated from GC-exposed animals showed a potentiated response to LPS, which demonstrates that chronic GC exposure primes microglia to pro-inflammatory stimuli, and thus microglia could serve as a CNS substrate of chronic GC effects. The effects of chronic GCs on microglia priming are consistent with our prior findings showing that acute GC exposure (a single bolus injection) also primes microglia to a subsequent pro-inflammatory challenge (Frank et al., 2010). It is important to note that the present results do not exclude the possibility that other CNS macrophage populations (*e.g.* perivascular macrophages) may be sensitive to the priming effects of GCs. A growing literature shows that multiple macrophage populations are directly primed by GCs (Busillo and Cidlowski, 2013) suggesting that macrophages, regardless of their micro-environmental milieu, may be primed by GCs.

While the phenomenon of stress and GC-induced neuroinflammatory priming has been well characterized, the neurobiological mechanism of GC priming has yet to be clarified. Of relevance here are a set of studies that have assessed the direct effects of GCs *in vitro* on innate immune signaling pathways as well as priming of pro-inflammatory responses. Several studies have shown that GCs upregulate the pattern recognition receptors, TLR2 and TLR4 on multiple peripheral cell types *in vitro* (Galon et al., 2002; Hermoso et al., 2004; Rozkova et al., 2006; Sakai et al., 2004; Shibata et al., 2009). Interestingly, the present results failed to show an effect of chronic GCs on TLR2 and TLR4 expression, which may be due to experimental differences in the type of GC used and of perhaps greater relevance, the use of *in vitro* systems *versus in vivo* effects. TLR4 recognizes the LPS motif that is present in the cell membrane of all Gram-negative bacteria, while TLR2 recognizes lipoteichoic acid that characterizes Gram-positive bacteria (Kawai and Akira, 2007). Interestingly, TLR2 and TLR4 signaling has been co-opted by endogenous danger signals or danger associated molecular patterns (DAMPs) such as HMGB1, which are thought to alert microglia to a variety of internal conditions such as cellular stress, damage or death (Kawai and Akira, 2010). Because DAMPs can activate TLR signaling and produce inflammatory responses, TLRs are thought to discriminate "danger" from "non-danger" (Bianchi, 2007). Several studies have shown that GCs can potentiate the pro-inflammatory effects of DAMPs *in vitro* (Busillo et al., 2011; Ding et al., 2010), amplify the pro-inflammatory effects of the pro-inflammatory cytokines TNFα (Lannan et al., 2012; Smyth et al., 2004), IL-6 (Dittrich et al., 2012; Smyth et al., 2004) and LIF (Langlais et al., 2008), induce NF-κB (Smyth et al., 2004) and amplify the pro-inflammatory effects of the transcription factors NF-κB, AP-1 and STAT3 (Busillo and Cidlowski, 2013).

Of particular importance to the present study, Busillo et al. (2011) found that GCs directly upregulate the expression of NLRP3 and prime the pro-inflammatory response to ATP, a well characterized DAMP. NLRP3 forms a multi-protein complex with ASC and pro-caspase-1. This complex is termed the NLRP3 inflammasome because it activates caspase-1, which is the rate-limiting enzyme in the maturation of the

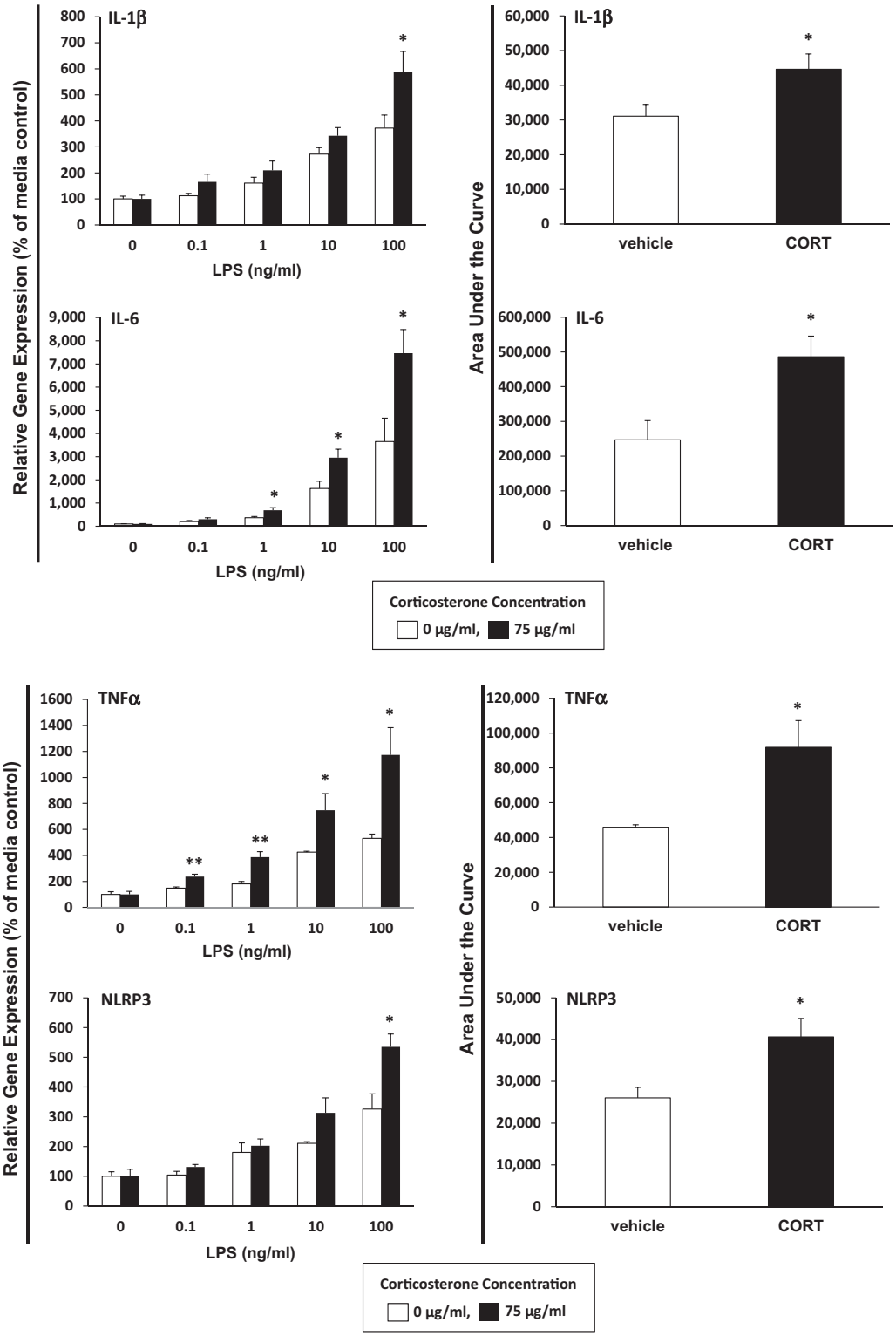

Figure 6 Effect of chronic CORT exposure on priming of hippocampal microglia. Animals were ADX or subject to sham surgery. Sham surgery animals (0 μg/ml CORT) were administered vehicle (0.4% ETOH) and ADX animals were administered CORT (75 μg/ml) supplemented with 0.9% saline in their drinking water for 10 days. Ten days post-treatment, hippocampal microglia were isolated and exposed to LPS for 2 h and relative gene expression measured. Data are presented as the mean + SEM. In the left hand column, vehicle (0 μg/ml) and CORT (75 μg/ml) treatment effects on pro-inflammatory cytokine levels were compared for each concentration of LPS. Significant mean differences are designated $^{*}p < 0.05$, $^{**}p < 0.01$. In the right hand column, the area under the LPS concentration curve is presented for each gene and means compared for vehicle and CORT treated animals. Significant mean differences are designated $^{*}p < 0.05$.

pro-inflammatory cytokine IL-1β (Lamkanfi and Kanneganti, 2010). The NLRP3 inflammasome is the only known inflammasome that is primed by GCs (Busillo and Cidlowski, 2013), which suggests that NLRP3 may play a similar role in GC-induced neuroinflammatory priming.

Consistent with these *in vitro* effects of GCs on NLRP3, the present results show that chronic GC treatment up-regulates the expression of NLRP3 *in vivo*. However, GC treatment failed to modulate the expression of the NLRP3 inflammasome components ASC and caspase-1, which is consistent with *in vitro* studies showing that NLRP3 is uniquely sensitive to the effects of GCs (Busillo et al., 2011). Here, GC treatment also did not affect steady state IL-1β expression, which is consistent with *in vitro* data that GCs prime NLRP3 inflammasome formation through selective upregulation of NLRP3 expression, but not IL-1β expression (Busillo et al., 2011). Interestingly, GC treatment suppressed IL-1β protein levels in the hippocampus and increased drinking behavior suggesting that chronic GC treatment induced anti-inflammatory phenotypes, while also modulating pathways involved in pro-inflammatory phenotypes (NLRP3 mRNA). In addition, chronic GC treatment increased the expression of NF-κBIα, which replicates the finding of a prior study (Munhoz et al., 2010). GCs directly induce the expression of NF-κBIα, which functions to inhibit NF-κB signaling by retaining NF-κB in the cytosol (Scheinman et al., 1995). However, NF-κBIα expression is also induced by NF-κB transcriptional activity and is routinely used as an index of the pro-inflammatory drive of NF-κB (Sun et al., 1993). Accordingly, it is unclear from the present data what role, if any, NF-κBIα plays in GC-induced neuroinflammatory priming. Interestingly, Munhoz et al. (2010) found that chronic GCs increased NF-κBIα expression as well as other anti-inflammatory mediators and p65 NF-κB transcriptional activity. These data suggest that GCs may prime neuroinflammatory processes through broad signaling pathways such as NF-κB and simultaneously induce both pro- and anti-inflammatory processes. In other words, GCs may set in motion an opponent process, which summates to form either an anti-inflammatory or pro-inflammatory response to a subsequent challenge depending upon the severity of the GC-inducing stressor and timing of the immunological threat in relation to the stress experience (Frank et al., 2013).

While the present results are correlative in nature, they provide a basis to investigate the role of NLRP3 in GC-induced neuroinflammatory priming. A considerable literature suggests that NLRP3 is a sensor of a diverse array of endogenous danger signals (Leemans et al., 2011). In light of the effects of GCs on NLRP3, stress-induced GCs may be conceptualized as an endogenous danger signal or alarmin, which alerts or primes the organism's innate immune system to potential immunological threats such as injury or infection. This permissive effect of GCs may prepare an organism to cope with immunological threats that are more likely to occur during a fight/flight emergency.

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Conflict of interest

None declared.

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