Gene expression changes in the hypothalamus provide evidence for regionally-selective changes in IL-1 and microglial markers after acute stress

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A R T I C L E   I N F O

Article history:
Received 23 January 2009
Received in revised form 17 April 2009
Accepted 29 April 2009
Available online 21 May 2009

Keywords:
Corticosterone
Stress
Norepinephrine
Microglia
Neuroinflammation
Hypothalamus
Interleukin-1

A B S T R A C T

Recent work from our laboratory and others has shown that certain stressors increase expression of the pro-inflammatory cytokine interleukin-1β (IL-1) in the hypothalamus. The first goal of the following studies was to assess the impact of acute stress on other key inflammatory factors, including both cytokines and cell surface markers for immune-derived cells resident to the CNS in adult male Sprague Dawley rats exposed to intermittent footshock (80 shocks, 90 s variable ITI, 5 s each). While scattered changes in IL-6 and GFAP were observed in the hippocampus and cortex, we found the hypothalamus to be exquisitely sensitive to the effects of footshock. At the level of the hypothalamus, mRNA for IL-1 and CD14 were significantly increased, while at the same time CD200R mRNA was significantly decreased. A subsequent experiment demonstrated that propranolol (20 mg/kg i.p.) blocked the increase in IL-1 and CD14 mRNA observed in the hypothalamus, while the decrease in CD200R was unaffected by propranolol. Interestingly, inhibition of glucocorticoid synthesis via injection of metyrapone (50 mg/kg s.c.) plus aminoglutethimide (100 mg/kg s.c.) increased basal IL-1 mRNA and augmented IL-1 and CD14 expression provoked by footshock. Injection of minocycline, a putative microglial inhibitor, blocked the IL-1 response to footshock, while CD14 and CD200R were unaffected. Together, these gene expression changes (i) provide compelling evidence that stress may provoke neuroinflammatory changes that extend well beyond isolated changes in a single cytokine; (ii) suggest opposing roles for classic stress-responsive factors (norepinephrine and corticosterone) in the modulation of stress-related neuroinflammation; (iii) indicate microglia within the hypothalamus may be key players in stress-related neuroinflammation; and (iv) provide a potential mechanism (increased CD14) by which acute stress primes reactivity to later immune challenge.

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1. Introduction

Pro-inflammatory cytokines in the central nervous system play a prevalent role in a wide variety of physiological processes such as sleep onset (Krueger and Karnovsky, 1995; Krueger and Majde, 1995), the development of sickness behaviors (Kent et al., 1996), and activation of the hypothalamic–pituitary–adrenal axis (Turnbull and Rivier, 1995). Pro-inflammatory cytokines have also gained notoriety as mediators of pathological CNS states as diverse as major depression (Dunn et al., 2005), propagation of seizure activity (Rizzi et al., 2003), neurodegeneration (Rothwell and Strijbos, 1995), autoimmune disorders such as multiple sclerosis (Elenkov and Chrousos, 2002), as well as normal aging (Frank et al., 2006). More recently, increased expression of the pro-inflammatory cytokine IL-1β (IL-1) within the brain has also been observed after exposure to certain acute stressors. For instance, increased IL-1 has been observed in the hypothalamus after exposure to immobilization (Minami et al., 1991; Shintani et al., 1995), tail-shock (Nguyen et al., 1998, 2000; O’Connor et al., 2003) and foot-shock (Blandino et al., 2006; Deak et al., 2003, 2005). It is noteworthy to mention, however, that increased IL-1 expression does not appear to be a universal response to all stressors (e.g., Deak et al., 2003; Plata-Salaman et al., 2000). Instead, there appears to be a threshold of stress that is necessary for IL-1 to be increased in the CNS (Deak et al., 2005). However, most studies have focused exclusively on IL-1 expression, failing to take into account the possibility that other inflammatory factors may be influenced by stressor exposure. Experiment 1 therefore examined a number of cytokines and cell surface markers that might be expected to change as a function of stressor exposure.

In particular, since prior work indicated that rats exposed to acute stress evinced a faster brain cytokine and corticosterone response to later LPS injection (Johnson et al., 2002a,b), we hypothesized that increased expression of CD14 and/or TLR4, two...
cognate receptors for LPS that are prevalent on microglia, might be responsible for the priming-like effects of prior stressor exposure toward later immune challenge. Considering the emergent role for the CD200–CD200 receptor interaction for maintaining microglia in a quiescent state (Frank et al., 2006; Minas and Liversidge, 2006), and convergent evidence for microglial involvement in neuroinflammatory consequences of stressor exposure (Blandino et al., 2006), we hypothesized that stressor exposure might reduce the expression of either CD200 or its receptor, thereby reflecting a transition of microglia toward a primed or activated state. We chose to use RT-PCR to examine gene expression changes in these studies so that a panel of targeted inflammatory factors could be examined in the same tissue. In doing so, we sought to determine whether changes observed in multiple factors might be regulated by common (or disparate, as the case may be) mechanisms.

Though much is already known regarding IL-1 expression during stress, very little information is available regarding expression of cell surface markers in the CNS by stress. For instance, intraperitoneal (Johnson et al., 2005), intracisternal magna (Johnson et al., 2006) and intracerebral ventricular (Maruta et al., 1997) administration of the β-adrenergic agonist isoproterenol led to increased expression of hypothalamic IL-1 protein. Moreover, when isoproterenol or desipramine were administered prior to stress, the expression of hypothalamic IL-1 was significantly increased (Blandino et al., 2006; Johnson et al., 2005). Further evidence comes from the findings that systemic injection of propranolol, a β-adrenergic receptor antagonist, blocked the IL-1 response to several stressors (Blandino et al., 2006; Johnson et al., 2005). Collectively, these studies support a role for norepinephrine in driving increased hypothalamic IL-1 through interaction with β-adrenergic receptors.

The release of endogenous corticosterone (CORT), on the other hand, is thought to constrain IL-1 expression in response to stress, while emerging evidence suggests that the relationship between CORT and inflammation in brain may not be so clear (Sorells and Sapolsky, 2007; Nair and Bonneau, 2005). Our previous work reported that bilateral adrenalectomy (ADX) potentiated the IL-1 response in brain provoked by tailshock (Nguyen et al., 1998). Though exogenous administration of CORT was later shown to normalize the IL-1 response to tailshock in ADX rats, removal of the adrenal glands does far more than simply ablate endogenous CORT. Therefore, it is not entirely clear whether the effect of surgical ADX in our previous work could be attributed unequivocally to loss of endogenous CORT. A more compelling argument for endogenous CORT constraining IL-1 expression would be the demonstration that pharmacological inhibition of glucocorticoid synthesis would augment stress-evoked IL-1 in a comparable manner as bilateral ADX. Therefore, one goal of the following series of studies was to test this hypothesis. Again, how endogenous CORT might impact the expression of cell surface markers in response to stress remains unclear.

Mechanistically, microglia appear to be well-situated to respond to stress, since microglia have been shown to express both adrenergic receptors (Maruta et al., 1997; Mori et al., 2002; Tomozawa et al., 1995) and glucocorticoid receptors (Sierra et al., 2008; Tanaka et al., 1997). Recent studies have also indicated that stress exerts a significant impact on microglial activation state, since changes in microglial proliferation (Nair and Bonneau, 2005), gene expression, (Frank et al., 2007) and morphology (Sugama et al., 2007) have all been observed following stressor exposure. Microglia are a significant and rapid cellular source of cytokines in response to CNS challenges (Streit, 2002; Streit et al., 1999), and our recent work has shown that minocycline, a putative microglial inhibitor, blocked the hypothalamic IL-1 response provoked by footshock (Blandino et al., 2006). The strategy in Experiments 2–4, therefore, was to examine whether changes in gene expression of immune cell markers observed in Experiment 1 would be subject to similar regulatory mechanisms as IL-1.

2. General materials and methods

2.1. Subjects

Adult male Sprague–Dawley rats (300–450 g) were purchased from Harlan and acclimated for 2 weeks prior to any manipulations. Colony conditions were maintained at 22 ± 1 °C with a 14:10 light–dark cycle (lights on 06:00–20:00 h). Animals were pair housed in standard transparent Plexiglas cages and had access to food and water ad libitum. In all experiments, rats were handled briefly for 3–5 min on each of 2 days prior to experimentation. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Binghamton University.

2.2. Drugs

Suppression of corticosteroid synthesis was accomplished through injection of both metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) and aminogluthemide (both purchased from Sigma Chemical Co., St. Louis, MO). Metyrapone (100 mg/kg) was dissolved in propylene glycol with gentle heating and injected subcutaneously (s.c.) at 0.5 ml/kg of body weight 2.5 h prior to experimentation. Rats that received metyrapone were also injected with aminogluthemide dissolved in propylene glycol at 100 mg/kg s.c. 1 h following the metyrapone injection. Doses, route of administration and timing of injections for inhibition of CORT synthesis were based on previously published studies (Der-Avakian et al., 2005; Plotsky and Sawchenko, 1987). Propranolol, a β-adrenergic receptor antagonist, was purchased from Sigma Chemical Co. (St. Louis, MO). Propranolol was dissolved in sterile, pyrogen free saline (0.9%) and injected intraperitoneally (i.p.) in a dose of 20 mg/kg 15 min prior to stressor exposure. Minocycline (Sigma) was dissolved in sterile filtered, deionized water with gentle heating and injected i.p. at a dose of 40 mg/kg 1 h prior to stress as described in our prior work (Blandino et al., 2006). Lipopolysaccharide (from E. coli serotype 0111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO). LPS was initially diluted in sterile, pyrogen free saline (0.9%) and aliquots were stored at −20 °C until needed. On the day of experimentation, a frozen aliquot was thawed and diluted to 100 μg/ml in pyrogen free physiological saline. LPS injections were administered intraperitoneally (i.p.), at 100 μg/kg.

2.3. Footshock paradigm

Rats were exposed to 80 inescapable footshocks (2.0 mA, 90 s variable ITI, 5 s each) over the course of approximately 2 h (Blandino et al., 2006). The footshock chambers for Experiment 1 were standard operant chambers measuring 23.5 cm × 20.5 cm × 18 cm (L × W × H; Ralph Gerbrands, Model C, Arlington, MA, USA) that were adapted to deliver shock through the grid floor (18 bars spaced 1 cm apart on center, with a diameter of 1.0 mm each). All current was delivered to the grid floor by a shocker (BRS/LVE, Model SGS-004, Beltsville, MD, USA) driven by a personal computer interface.

After completion of Experiment 1, a new footshock system was acquired and used in Experiments 2–4. These footshock chambers were operant chambers measuring 26.3 cm × 31 cm × 28.5 cm (L × W × H; Coulbourn Instruments, Habitet Chamber, Model H10-11R-TC-SF, Allentown, PA, USA) that delivered shock, via the LabLinc interface, through the grid floor (18 bars spaced 1.1 cm apart with a diameter of 4.0 mm). Though the regimen of footshocks delivered were comparable across the old and new
footshock apparatuses (80 shocks, 90 s variable ITI, 5 s each), the current was 1.0 mA in Experiments 2–4 to account for greater efficiency of current delivery with the new system (1.0 mA produced a comparable behavioral response in preliminary validation studies as a setting of 2.0 mA using the older shock system).

2.4. Tissue collection and processing

Tissue was harvested following rapid decapitation (unanesthetized) immediately after footshock exposure or an equivalent time in the homecage. Trunk blood was collected into EDTA coated Vacutainers, stored on ice and plasma was immediately separated through refrigerated centrifugation. Plasma was aliquoted and stored at −20 °C until time ofRIA. For Experiment 1, brain and peripheral tissue were quickly collected and dissected on a cold plate and flash frozen and stored at −70 °C until the time of assay. For Experiments 2–4, brains were dissected on a cold plate, transferred to a 1.5-mL Eppendorf tube containing 500 µL of RNAlater (Qiagen) and stored at 4 °C for 24 h to allow penetration and then transferred to −20 °C until time of RNA extraction. Total cellular RNA was extracted using Trizol® RNA reagent and homogenized according to the manufacturer's instructions (Invitrogen). Tissue was homogenized using a motorized pestle and passed through Qiagen Qiashredder™ columns to shear residual genomic DNA and ensure thorough homogenization of samples. Equal volume of 70% ethanol was added to the supernatant and purified through RNaseasy mini columns. Columns were washed with buffer and eluted with 30 µL of RNase-free water (65 °C). Total RNA was stored at −70 °C until the time of cDNA synthesis.

Total RNA yield and purity were determined using a spectrophotometer, (BioPhotometer, Eppendorf, Hamburg, Germany), at 260/280 nm for Experiment 1 and the Experion Automated Electrophoresis System (BioRad) for Experiments 2–4. Synthesis of cDNA was performed on 5 µg of normalized total RNA from each sample using the First-strand® cDNA synthesis kit in Experiment 1 (Amersham Biosciences). Synthesis of cDNA for Experiments 2–4 was performed on 0.1–1.0 µg of normalized total RNA from each sample using the QuantiTect® Reverse Transcription Kit (Cat. No. 205313, Qiagen, Valencia, CA) which included a DNase treatment step. All cDNA was stored at −70 °C until time of assay.

2.5. Reverse-transcription polymerase chain reaction (RT-PCR)

In Experiment 1, probed cDNA was amplified using Platinum® PCR supermix reagents (Invitrogen) in the Mastercycler gradient (Eppendorf, Hamburg, Germany). The amplification program for all reactions of Experiment 1 consisted of an initial denaturation step at 94 °C for 2 min. The remaining denature, annealing and extension cycles were 45 s at 94 °C, 45 s at 60 °C and 45 s at 72 °C, respectively. Final extension was run for 7 min at 72 °C. All reactions were carried out using a PCR express thermocycler (Hybaid) using Platinum® PCR supermix reagents (Invitrogen). Number of amplification cycles was determined empirically by cycle bracketing for each primer set in each tissue compartment. Following amplification, the products were separated on 2% agarose gels, stained with SYBR GOLD (Molecular Probes), and visualized using a Kodak EDAS 290 gel system. Image gauge® software (Fuji Biomedical) was used for densitometry of PCR products. All primer sequences and accession numbers are listed in Table 1.

After completion of Experiment 1, real-time RT-PCR was acquired and subsequently used for transcriptional analysis in Experiments 2–4. Probed cDNA amplification for Experiments 2–4 was performed in a 20 µL reaction consisting of 10 µL IQ SYBR Green Supermix (BioRad), 1 µL primer (final concentration 250 nM), 1 µL cDNA template and 8 µL RNase-free water run in duplicate in a 96-well plate (BioRad) and captured in real-time using the iQ5 real-time PCR detection system (BioRad). Following a 3-min hot start (95 °C), samples underwent denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C and extension for 30 s at 72 °C for 50 cycles. An additional denaturation (95 °C, 1 min) and annealing cycle (55 °C, 1 min) were conducted to ensure proper product alignment prior to melt curve analysis. For melt curve analysis, samples underwent 0.5 °C changes every 15 s ranging from 55 °C to 95 °C. A single peak expressed as the negative first derivative of the change in fluorescence as a function of temperature indicated primer specificity to the target gene. Relative gene expression was quantified using the 2−ΔΔCT method as described previously (Livak and Schmittgen, 2001; Pfaffl, 2001) relative to a stable housekeeping gene.

2.6. Radioimmunoassay for corticosterone

Total plasma CORT levels were measured by radioimmunoassay using rabbit antiseraum (antibody B3-163, Esoterix, Tarzana, CA) as previously described (Deak et al., 2003, 2005). This antiseraum was employed due to its low cross-reactivity with other glucocorticoids and their metabolites. Assay sensitivity was 0.5 µg/dl (assay volume = 20 µL plasma). The inter- and intra-assay coefficients were 13% and 10%, respectively.

2.7. Statistical analysis

All analyses were conducted in Statview using an Analysis of Variance (ANOVA) appropriate to the study conducted (described individually below). Post hoc analyses were performed using Fisher’s Protected Least Significant Difference (PLSD) method when an overall significant main effect was observed. Criterion for rejection of the null hypothesis was always p < 0.05.

2.8. Experiment 1: molecular assessment of footshock effects in the brain

The goal of Experiment 1 was to examine regional gene expression and more broadly characterize the nature of the neuroinflammatory response provoked by acute stress. To do this, rats (n = 6–8 per group) were exposed to 80 intermittent footshocks (2.0 mA, 5 s each, 90 variable ITI) or remained in their home cages as non-stressed controls. A separate group of rats were injected with 100 µg/kg of LPS (i.p.) 2 h prior to tissue collection, to serve as a positive control for cytokine measurements using RT-PCR. Immediately after footshock or an equivalent time point for LPS and homecage controls, the hypothalamus, hippocampus, cortex and pituitary were harvested following rapid decapitation, processed for RNA and examined using gel-based RT-PCR as described above. These structures were chosen because they are all principally involved in orchestration of the stress response or have otherwise been implicated as cytokine-responsive structures.

2.9. Experiment 2: pharmacological blockade of β-adrenergic receptors

Since propranolol has been shown to block the increase in hypothalamic IL-1 protein provoked by footshock (Blandino et al., 2006), we predicted pretreatment with propranolol would block the footshock-induced increase in hypothalamic IL-1 mRNA as well. Additionally, like IL-1, increased CD14 expression has been shown to be a cAMP dependent process (Liu et al., 2000). Since increased cAMP has been shown following β-adrenergic receptor activation (Stone et al., 1990), we predicted that pretreatment with propranolol would also block the footshock-induced increase in
hypothalamic CD14 mRNA. To test this, rats \( (n = 7–8 \text{ per group}) \) were pretreated with 20 mg/kg propranolol i.p. or vehicle and exposed to 80 intermittent footshocks (1.0 mA, 5 s each, 90 variable ITI) or remained in their home cages as non-stressed controls. Immediately after footshock or an equivalent time for controls, tissue was harvested via rapid decapitation and stored in RNA later as described above.

2.10. Experiment 3: pharmacological suppression of corticosterone

The findings of Experiment 1 demonstrated that footshock increased CD14 mRNA within the hypothalamus. Since CORT is a primary effector of the HPA response and has potent anti-inflammatory properties, we hypothesized that inhibition of the footshock-induced increase in CORT would augment the IL-1 and CD14 mRNA responses to stress. To test this, rats \( (n = 7–8 \text{ per group}) \) were either pretreated alpha-gluthemide + metyrapone (1.5 and 2.5 h prior to footshock, respectively) or vehicle and later exposed to either 80 intermittent footshocks (1.0 mA, 5 s each, 90 variable ITI) or remained in the home cage for an equivalent amount of time as controls. Rats were killed by rapid decapitation and tissue was collected as previously described for later analysis via real-time RT-PCR.

2.11. Experiment 4: pharmacological inhibition of microglia via the putative microglial inhibitor, minocycline

Our previous work showed that minocycline blocked the increase in IL-1 protein produced by footshock (Blandino et al., 2006). The goal of this experiment was to determine whether minocycline would significantly reduce changes in IL-1, CD14 and CD200R gene expression provoked by footshock. To do this, rats \( (n = 8 \text{ per group}) \) were injected with 0 or 40 mg/kg of the putative microglial inhibitor, minocycline (i.p.) and returned to their home cages. Sixty minutes later, rats were exposed to footshock (80 shocks, 1.0 mA, 5 s each, 90 s variable ITI) or remained in their home cages as non-stressed controls. Immediately after footshock or an equivalent time for controls. Tissue was harvested via rapid decapitation and stored in RNA later as described above.

3. Results

3.1. Experiment 1: molecular assessment of footshock effects in the brain

Data from this experiment was analyzed using a single factor ANOVA with three levels. Because the overarching goal was to determine the difference between footshock control and shocked rats rather than comparisons between footshock and LPS, both effects were annotated in Table 2. For clarity, we present the results from Experiment 1 by region.

3.1.1. Hypothalamus

In the hypothalamus, we observed a significant increase \( [F(2,19) = 8.83, p < 0.05] \) in IL-1 mRNA in footshock and LPS treated rats relative to non-stressed controls (data summarized in Table 2). Analysis of additional cytokines in this structure revealed effects on hypothalamic IL-6 mRNA \( [F(2,19) = 4.18, p < 0.05] \) and IL-10 mRNA \( [F(2,19) = 10.79, p < 0.05] \). Post hoc analysis revealed that hypothalamic IL-10 mRNA was significantly increased in both footshock \( (p < 0.05) \) and LPS \( (p < 0.05) \) groups in comparison to non-manipulated controls. IL-10 mRNA in LPS treated rats was also significantly increased in comparison to rats exposed to footshock \( (p < 0.05) \). The increase in IL-6 mRNA was solely driven by LPS \( (p < 0.05) \) in comparison to non-stressed controls. An overall significant main effect of hypothalamic CD14 mRNA was observed \( [F(2,19) = 6.41, p < 0.05] \), with rats exposed to either LPS or footshock demonstrating a significant decrease in comparison to non-manipulated controls \( (p < 0.05) \) for both. No significant difference was observed between LPS and footshock \( (p > 0.05) \). Hypothalamic CD200R mRNA was significantly decreased \( [F(2,18) = 15.15, p < 0.05] \), with rats exposed to either LPS or footshock demonstrating a significant decrease in comparison to non-manipulated controls \( (p < 0.05) \) for both. No difference in hypothalamic CD200R mRNA was observed between footshock and LPS groups \( (p < 0.05) \). No significant main effects were observed on GAPDH mRNA \( [F(2,19) = 0.96, p > 0.05], CD200 mRNA [F(2,19) = 0.67, p > 0.05], MHCI II mRNA [F(2,19) = 0.06, p > 0.05], TLR 4 mRNA [F(2,19) = 2.11, p > 0.05] or GFAP mRNA [F(2,19) = 0.82, p > 0.05].

3.1.2. Hippocampus

In the hippocampus, a main effect on IL-6 was observed \( [F(2,19) = 261.82, p < 0.05] \) with rats exposed to footshock demonstrating a decrease \( (p < 0.05) \) and LPS an increase \( (p < 0.05) \), in comparison to non-manipulated controls. IL-6 was also significantly decreased in footshocked rats in comparison to LPS treated rats \( (p < 0.05) \). Main effects of treatments were also observed on IL-1 \( [F(2,19) = 14.68, p < 0.05] \) and IL-2 \( [F(2,19) = 2.57, p > 0.05] \) with
both increased within the hippocampus, but these effects were driven by the LPS treated rats (p < 0.05), as footshock did not significantly differ from either non-manipulated controls or LPS treated rats (p > 0.05). No effect was observed on CD200 mRNA \( F(2, 19) = 0.50, p > 0.05 \), CD200R mRNA \( F(2, 19) = 0.03, p > 0.05 \), MHC II mRNA \( F(2, 19) = 0.14, p > 0.05 \), CD14 mRNA \( F(2, 19) = 0.03, p > 0.05 \), TLR4 mRNA \( F(2, 19) = 2.5, p > 0.05 \), GFAP mRNA \( F(2, 18) = 1.73, p > 0.05 \), or GAPDH mRNA \( F(2, 19) = 1.09, p > 0.05 \).

### 3.1.3. Cortex

In the cortex, an overall significant main effect in GFAP mRNA was observed \( F[2, 19] = 3.49, p < 0.05 \) and interestingly, this effect was observed after footshock but not LPS, such that GFAP mRNA in rats exposed to footshock was significantly decreased in comparison to both LPS and non-manipulated controls (p < 0.05). Several other main effects of treatment were observed on factors within the cortex, all of which reflect increased mRNA expression in response to LPS and not footshock IL-1: \( F[2, 19] = 39.11, p < 0.05 \); IL-6: \( F[2, 19] = 526.74, p < 0.05 \); IL-10: \( F[2, 19] = 13.38, p < 0.05 \); CD14: \( F[2, 19] = 4.85, p < 0.05 \). No effects were observed on the remaining transcripts GAPDH \( F[2, 19] = 0.77, p > 0.05 \), CD200 \( F[2, 19] = 1.32, p > 0.05 \), CD200R \( F[2, 19] = 1.67, p > 0.05 \), MHC II \( F[2, 19] = 1.64, p > 0.05 \), and TLR4 \( F[2, 19] = 0.50, p > 0.05 \).

### 3.1.4. Pituitary gland

In the pituitary gland, an overall significant increase in IL-6 mRNA was observed \( F[2, 19] = 64.24, p < 0.05 \) in rats exposed to footshock. Post hoc analysis demonstrated a significant increase in IL-6 mRNA following both footshock and LPS exposure in comparison to non-manipulated controls (p < 0.05). Additionally, IL-6 mRNA was significantly elevated in LPS exposed rats in comparison to rats exposed to footshock (p < 0.05). Additional main effects of treatment were observed on several factors in the pituitary, including IL-1 \( F[2, 19] = 159.53, p < 0.05 \), IL-10 \( F[2, 19] = 56.78, p < 0.05 \), CD14 \( F[2, 19] = 8.77, p < 0.05 \), TLR4 \( F[2, 18] = 7.59, p < 0.05 \) and CD200R \( F[2, 19] = 4.93, p < 0.05 \). As in the cortex, these effects all reflect altered mRNA expression in response to LPS but not footshock. No other significant effects of footshock were seen in the pituitary on GAPDH mRNA \( F[2, 19] = 1.50, p > 0.05 \), CD200 mRNA \( F[2, 19] = 1.39, p > 0.05 \) and MHC II mRNA \( F[2, 19] = 1.15, p > 0.05 \).

### 3.2. Experiment 2: pharmacological blockade of β-adrenergic receptors

Data from Experiment 2 were analyzed using a 2 × 2 ANOVA. While there was no main effect of footshock on hypothalamic IL-1 \( F[1, 30] = 0.12, p < 0.05 \), there was a significant main effect of propranolol injection \( F[1, 30] = 20.42, p < 0.05 \) resulting in a significant interaction between stress condition and injection \( F[1, 30] = 13.74, p < 0.05 \) (see Fig. 1). Post hoc analysis revealed that in vehicle injected rats, footshock significantly increased IL-1 expression relative to non-stressed controls. Not only did propranolol block the acute rise in IL-1 produced by footshock, it reduced IL-1 gene expression significantly below control levels. A main effect of footshock on hypothalamic CD14 mRNA also failed to reach significance \( F[1, 30] = 3.19, p = 0.08 \), while a main effect of propranolol injection was observed \( F[1, 30] = 8.71, p < 0.01 \). A significant interaction was observed between stressor condition and injection \( F[1, 30] = 4.32, p = 0.05 \). Post hoc analysis revealed that hypothalamic CD14 mRNA was significantly increased in vehicle-
footshock rats compared to vehicle-injected controls, an effect that was completely blocked by pretreatment with propranolol. A main effect of footshock was also observed on hypothalamic CD200R mRNA, such that footshock treated rats compared to non-stressed controls exhibited lower CD200R expression \( [F(1, 30) = 6.27, p < 0.05] \), and no interaction between stress condition and injection was observed \( [F(1, 30) = 0.001, p > 0.05] \). As expected, plasma concentrations of CORT were significantly increased by footshock \( [F(1, 30) = 244.16, p < 0.05] \), while propranolol was without influence on plasma CORT \( [F(1, 30) = 3.12, p > 0.05] \). Additionally, no significant interaction was observed \( [F(1, 30) = 2.34, p > 0.05] \).

3.3. Experiment 3: pharmacological suppression of corticosterone

Data from Experiment 3 were analyzed using a \( 2 \times 2 \) ANOVA. As expected, a main effect of stress was observed on plasma CORT \( [F(1, 27) = 59.65, p < 0.05] \) and metyrapone injection \( [F(1, 27) = 25.61, p < 0.05] \) as well as a significant stress by injection interaction \( [F(1, 27) = 54.76, p < 0.05] \). Post hoc analysis revealed that footshock significantly increased circulating plasma CORT compared to non-stressed controls and the footshock-induced increase in CORT was blocked by pretreatment with metyrapone (see Fig. 2). These data provide important verification for the efficacy of the metyrapone plus amino-glutethimide cocktail to inhibit stress-increased plasma CORT to approximately 7 µg/dl (Dinkel et al., 2003; Stein and Sapolsky, 1988) and are requisite to the evaluation of inflammatory factors.

A main effect of footshock exposure on hypothalamic IL-1 mRNA \( [F(1, 27) = 18.83, p < 0.05] \) replicated the findings in Experiments 1 and 2 and a significant main effect of metyrapone on hypothalamic IL-1 was also observed \( [F(1, 27) = 21.18, p < 0.05] \). Importantly, a significant interaction between footshock and metyrapone exposure was observed on hypothalamic IL-1 mRNA \( [F(1, 27) = 9.56, p < 0.05] \). Post hoc analyses revealed that despite an approximate 2-fold increase in hypothalamic IL-1 expression for both vehicle-footshock and metyrapone home cage treated rats, these groups failed to reach significance relative to vehicle home cage controls. Importantly, IL-1 was significantly increased (a 9-fold increase) in the metyrapone-footshock group when compared to the vehicle-non-stressed group.

As in prior experiments, footshock significantly increased hypothalamic CD14 mRNA \( [F(1, 27) = 10.09, p < 0.05] \). A main effect of metyrapone was also observed on hypothalamic CD14 mRNA, where metyrapone-treated rats demonstrated increased hypothalamic CD14 mRNA compared to vehicle-injected controls \( [F(1, 27) = 10.60, p < 0.05] \), while no interaction between stress condition and injection on hypothalamic CD14 mRNA was observed \( [F(1, 27) = 0.15, p > 0.05] \). Lastly, a main effect of footshock on CD200R mRNA \( [F(1, 26) = 7.92, p < 0.05] \) also observed, but no main effect of drug condition \( [F(1, 26) = 2.09, p > 0.05] \) or interaction between footshock and metyrapone treatments \( [F(1, 26) = 0.79, p > 0.05] \) were observed. Together, these findings support a role for the stress-evoked rise in CORT in constraining the increase in IL-1 and CD14 produced by stress. However, CORT does not appear to influence the reduction in CD200R.

3.4. Experiment 4: pharmacological inhibition of microglia via the putative microglial inhibitor minocycline

Data from Experiment 4 were analyzed using a \( 2 \times 2 \) ANOVA. As expected, a main effect of stress \( [F(1, 27) = 42.65, p < 0.05] \) and minocycline \( [F(1, 27) = 6.27, p < 0.05] \) were observed on plasma CORT and CD14 mRNA, such that footshock and metyrapone home cage treated rats, these groups failed to reach significance relative to vehicle home cage controls. Importantly, IL-1 was significantly increased (a 9-fold increase) in the metyrapone-footshock group when compared to the vehicle-non-stressed group.

As in prior experiments, footshock significantly increased hypothalamic CD14 mRNA \( [F(1, 27) = 10.09, p < 0.05] \). A main effect of metyrapone was also observed on hypothalamic CD14 mRNA, where metyrapone-treated rats demonstrated increased hypothalamic CD14 mRNA compared to vehicle-injected controls \( [F(1, 27) = 10.60, p < 0.05] \), while no interaction between stress condition and injection on hypothalamic CD14 mRNA was observed \( [F(1, 27) = 0.15, p > 0.05] \). Lastly, a main effect of footshock on CD200R mRNA \( [F(1, 26) = 7.92, p < 0.05] \) also observed, but no main effect of drug condition \( [F(1, 26) = 2.09, p > 0.05] \) or interaction between footshock and metyrapone treatments \( [F(1, 26) = 0.79, p > 0.05] \) were observed. Together, these findings support a role for the stress-evoked rise in CORT in constraining the increase in IL-1 and CD14 produced by stress. However, CORT does not appear to influence the reduction in CD200R.

![Fig. 1](image-url)

Rats were injected i.p. with 20 mg/kg of propranolol or equivolume vehicle. Fifteen minutes later, rats were either given 80 inescapable footshocks (1.0 mA, 90 s variable ITI, 5 s each) over the course of approximately 2 h (black bars) or remained in home cages as non-stressed controls (white bars). As expected, footshock significantly increased CORT and this effect was unaffected by pretreatment with propranolol. (A) Footshock significantly increased hypothalamic IL-1 (B) and CD14 (C) mRNAs and both were blocked by pretreatment of propranolol. Hypothalamic CD200R mRNA, on the other hand, was significantly reduced by footshock (D), regardless of propranolol exposure. All data are expressed as \( 2^{-\Delta \Delta Ct} \) ± SEM. *Significant difference between groups connected by bars (\( p < 0.05 \)).
CORT as well a significant stress by minocycline interaction \( F(1, 27) = 9.83, p < 0.05 \) (see Fig. 3). Post hoc analysis revealed that footshock vehicle treated rats had significantly higher circulating plasma CORT compared to home cage vehicle controls. Additionally, minocycline alone significantly increased circulating plasma CORT compared to home cage controls \( p < 0.05 \), although this increase was significantly less in comparison to footshock vehicle treated rats \( p < 0.05 \). Importantly, there was no significant difference between footshock rats that were pretreated with either vehicle or minocycline, indicating minocycline did not alter the HPA response to stress as measured by circulating plasma CORT \( p < 0.05 \). Stress once again increased hypothalamic IL-1 mRNA, indicated by a main effect \( F(1, 27) = 41.41, p < 0.05 \). A main effect of minocycline was also observed on hypothalamic IL-1 mRNA \( F(1, 27) = 4.89, p < 0.05 \) as well as a significant stress by minocycline interaction \( F(1, 27) = 4.34, p < 0.05 \). Post hoc analysis revealed that footshock vehicle rats had a significant increase in hypothalamic IL-1 expression compared to home cage control \( p < 0.05 \). While minocycline had no effect on basal hypothalamic IL-1 expression, minocycline significantly attenuated the footshock-induced increase in hypothalamic IL-1 mRNA. Despite this attenuation, hypothalamic IL-1 was still significantly elevated relative to non-stressed controls in rats that received stress plus minocycline. No main effects of stressor exposure \( F(1, 27) = 2.20, p > 0.05 \) or minocycline injection \( F(1, 27) = 3.04, p > 0.05 \) were observed on hypothalamic CD14 mRNA, but a significant stress by minocycline interaction was observed \( F(1, 27) = 4.71, p < 0.05 \). Post hoc analysis revealed that minocycline increased CD14 mRNA expression in both home cage and footshock treated rats in comparison to home cage vehicle treated rats \( p < 0.05 \).

Footshock vehicle rats had increased hypothalamic CD14 expression in comparison to home cage control vehicle treated rats. No main effects of stress \( F(1, 27) = 0.27, p > 0.05 \), minocycline \( F(1, 27) = 1.11, p > 0.05 \) or a stress by minocycline interaction \( F(1, 27) = 0.06, p > 0.05 \), were observed on hypothalamic CD200R expression.

**4. Discussion**

The current series of experiments replicated the increase in hypothalamic IL-1 protein and mRNA that we have reported elsewhere after footshock (Blandino et al., 2006; Deak et al., 2003, 2005). The present data significantly expand what is known about neuroinflammatory consequences of stress by showing that footshock not only increased CD14 mRNA and decreased expression of CD200R mRNA, but that these changes were taking place predominantly in the hypothalamus. These results indicate dynamic changes in neuroinflammatory processes that extend well beyond increased IL-1. Though some other changes were observed elsewhere after footshock (decreased IL-6 and GFAP mRNA expression in the hippocampus and increased IL-6 expression in the pituitary), the prevalence of neuroinflammatory effects observed within the hypothalamus was particularly striking. The reactivity of the hypothalamus to footshock exposure probably reflects a more general role of the hypothalamus as a major site of integration for the stress response (Li et al., 2006; Sawchenko and Swanson, 1981; Swanson and Sawchenko, 1983). Since cytokine responses have
been observed in quiescent microglia (Van Dam et al., 1995) and are deemed an early marker of microglial activation (Mori et al., 2002), these findings may indicate an early transitional phase of microglia to a primed or activated state as a result of acute stressor exposure (see Fig. 4 for schematic).

We have previously shown that pretreatment with propranolol blocked the increase in hypothalamic IL-1 protein after footshock (Blandino et al., 2006). We extend these findings here by demonstrating pretreatment with propranolol also blocked the expression of hypothalamic IL-1 mRNA, indicating a role for norepinephrine in regulating IL-1 gene expression at the level of the hypothalamus in response to footshock. Furthermore, we demonstrate that inhibition of CORT synthesis during stressor exposure led to approximately a 9.5-fold increase in stress-provoked IL-1 expression within the hypothalamus. Importantly, this finding demonstrates the suppressive effects of CORT on IL-1 expression during stressor exposure, without exposing an animal to any prior surgical procedures and without the loss of circulating epinephrine (and other adrenal-derived factors) that accompanies full ADX. Together, these data suggest activation of β-adrenergic receptors is necessary for footshock induction of IL-1 within the hypothalamus, while stress-induced increases in CORT would constrain the IL-1 response during stress (Fig. 4).

Since β-adrenergic receptors have been shown to be coupled to cAMP induction (Mori et al., 2002; Stone et al., 1990) and a cAMP-protein kinase A dependent pathway has also been shown to regulate CD14 expression (Iwahashi et al., 2000), we tested if blockade of β-adrenergic receptors would also block the stress provoked increase in hypothalamic CD14 mRNA. We show for the first time that footshock-increased hypothalamic CD14 expression and this stress-provoked effect is modulated through β-adrenergic receptors. It should be noted that CD14 expression has also been shown to increase as a result of IL-1 stimulation. Thus, the blockade of CD14 expression may be due to either blockade of either cAMP production by propranolol or the downstream production of IL-1 protein (Blandino et al., 2006; Johnson et al., 2005). Future experiments will be necessary to determine whether CD14 is increased in tandem with, or as a result of, increased IL-1 expression. From the present data we can simply conclude that the two effects of footshock appear to be uniformly regulated.

Evidence suggests the stress-induced increase in hypothalamic CD14 mRNA may not only be indicative of early microglial activation in response to stress, but may also relate to sensitized corticosterone and cytokine responses to later antigen challenge (Johnson et al., 2002a,b). It has been postulated that microglial activation is not simply characterized as a morphological change, but rather a
Minocycline has also been shown to block LPS-induced up-regulation of numerous receptors and secretory products (Nakamura, 2002; Rock et al., 2004). Since quiescent microglia do not express CD14 (Rock et al., 2004), our data suggest that increased CD14 mRNA in combination with the increase in IL-1 expression, may be indicative of early microglial activation within the hypothalamus. A shift in activation state of microglia may indeed be the case since animals exposed to tailshock 24 h prior to LPS challenge demonstrate a faster increase in cytokine responses across several brain structures including the hypothalamus both in vivo (Johnson et al., 2002b) and ex vivo (Frank et al., 2007). Therefore, the footshock-induced increase in CD14 mRNA may indicate an early shift in differentiation and later sensitization of microglia.

Our data suggest the adrenergic receptor mediated increase in CD14 mRNA during stress is tempered by the concomitant rise in CORT. A similar effect of CORT on CD14 following LPS exposure has also been observed (Nadeau and Rivest, 2002). For instance, rats given LPS directly into the striatum demonstrated significantly increased CD14 mRNA expression in the brain. Interestingly, this effect was blocked when rats were injected with CORT 6 h prior to intrastratial LPS. Furthermore, a reversal of the CORT suppression of LPS-induced CD14 expression was obtained when rats were pretreated with the glucocorticoid receptor antagonist RU486. Collectively, these findings indicate that CD14 mRNA within the brain is modulated by endogenous CORT under both basal and stressful conditions.

The finding that CD14 was increased despite pretreatment with the putative microglial inhibitor minocycline also requires discussion. While the mechanism of action of the antibiotic effects of minocycline are known (Jonas and Cuhna, 1982), the anti-inflammatory effects through inhibition of microglia are still under investigation. For instance, minocycline been shown to significantly reduce interleukin-1β converting enzyme (ICE) and inducible nitric oxide synthase (iNOS) mRNAs by 70% and 30%, respectively, following ischemic challenge. These findings indicate that minocycline does not exert anti-inflammatory effects equivalently across all factors. Minocycline has also been shown to block LPS-induced increases in p38, ERK 1/2 and JNK1/2 MAP kinases, but this drug was unable to block these same responses during oxidative stress (Nikodemova et al., 2006), suggesting the efficacy of minocycline may be dependent upon the precipitating stimulus. Thus, while minocycline significantly reduced stress-dependent changes in IL-1 protein (Blandino et al., 2006) and mRNA (present data), minocycline may not do so equivalently across all factors in response to footshock exposure.

CD200R, a membrane bound receptor found on cells of myeloid lineage, directly interacts with the CD200 ligand expressed by neurons (Fig. 4), endothelial and other cells and serves to inhibit myeloid cell activation and differentiation (Barclay et al., 2002; Zhang et al., 2004). Therefore, the decrease in hypothalamic CD200R mRNA following both footshock and LPS exposure may indicate a potential shift in differentiation of myeloid type cells. Interestingly, the decreased CD200R expression after footshock was not impacted by pretreatment with propranolol, metyrapone or minocycline, suggesting that changes in CD200R are regulated independently from IL-1 and CD14. One question that remains to be answered regarding the decreased expression of CD200R is whether these changes reflect active repression of the CD200R gene, or a more passive gene transcriptional ‘cost’ that occurs as a response to changing gene expression patterns. Indeed, this may explain why the reduction in CD200R was overall rather small (~25% change from controls in Experiments 1–3). Though it is not presently clear why the effect of footshock on CD200R failed to replicate in Experiment 4, it can be noted that there was a trend for CD200R to be increased after minocycline injection that may have masked the influence of footshock.

One limitation of the present studies is that only a single time point after acute stress was examined, since many of these factors would be expected to show time-dependent expression patterns. A related issue is that only a single exposure to acute stress was imposed on subjects, which may significantly impact the nature of the neuroinflammatory changes produced by stress. For instance, Kwon et al. (2008) reported increased IL-1 in both the PVN and hippocampus after 2 h of immobilization (restraint in a Plexiglas tube) repeated over 4 days. Though this effect appeared to be localized to...
neurons, it should be noted that the controls employed in these studies were not particularly convincing. Nair and Bonneau (2005), on the other hand, observed microglial proliferation after 15 h of restraint exposure over 4 days, an effect that was blocked by pretreatment with MK-801, implicating a role for glutamate. In light of these findings, it will be very important to examine the cell-type specificity for the main effects observed here with real-time RT-PCR using in situ hybridization. Such studies are already under way in our laboratory. From a functional standpoint, it will also be informative to examine protein levels for CD14 and CD200 receptor, since gene expression changes are not always coupled to changes in functional protein. However, the greater specificity provided by the RT-PCR approach (relative to use of antibodies) combined with the ability to examine all factors in the same samples offers unique advantages. Nevertheless, the next logical step will be to evaluate the timecourse and anatomical specificity of these changes at the protein level. Such studies are already under way in our laboratory.

Taken together, the findings reported here extend what was previously thought to be an isolated change in IL-1 to changes in IL-1, CD14 and CD200R at the transcriptional level. These findings were highly reproducible across not only multiple experiments but also across two different RT-PCR platforms. The observation that norepinephrine changes in IL-1 and CD14 and CORT constrained these effects, while CD200 receptor alterations were independently regulated, suggests that stress provokes changes in neuroinflammatory processes beyond simple cytokine expression. Importantly, Garcia-Bueno et al. (2008) recently demonstrated similar mechanisms in the regulation of cortical prostaglandins, indicating conservation of these processes across inflammatory pathways. Ultimately, this neuroinflammatory tone (Fig. 4) may regulate the expression of sickness-like behaviors, recuperative responses, sensitivity to later LPS challenge and accelerated aging of the CNS produced by stress (see Deak, 2007; Hennessy et al., 2001, for reviews).

Acknowledgments

This work was supported by grants from the National Science Foundation (NSF Grant Nos. 0549987 and 0822129), Hope for Depression Research Foundation (HDRF Grant No. 6-008), and the Center for Development and Behavioral Neuroscience (CDBN) at Binghamton University. We also thank Sheri Zola and Joanna Brice for their excellent technical assistance. Any opinions, findings, conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the above stated funding agencies.

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