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Effects of Glucocorticoid on Microglia Cell Functions

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Effects of Glucocorticoid on Microglia Cell Function

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Submitted in partial fulfillment of the requirement for the degree
Of Master of Science in Biology from the Department of Biological Sciences at Seton Hall
University
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ABSTRACT

Glucocorticoids (GCs) are released from the hypothalamic-pituitary-adrenal-axis in response to physiological and psychological stressors. GCs initiate their signaling pathway by binding to Type I mineralocorticoid receptor (MR) or Type II GC receptor (GR) which are members of a large family of nuclear receptors, and have traditionally been credited with anti-inflammatory and immunosuppressive actions in the periphery making them a pharmacological tool to treat a variety of autoimmune diseases. Recent evidence has suggested that the actions for GCs may be more complicated in the central nervous system (CNS). Microglia cells, the resident macrophage in the brain, act as a primary response component of CNS inflammatory action. To gain insight into the microglia response to GCs, time- and dose-dependent effects of dexamethasone, a synthetic corticosteroid, on microglial morphology and mRNA expression of GR, MR, toll-like receptor 4 (TLR4), cluster differentiation 14 (CD14), and myeloid differentiation factor (MyD88) were examined. The type I mineralocorticoid receptor (MR) was found to be downregulated at 100 nM dose of dexamethasone after 3 days of treatment. The 10 nM and 1 μ M concentration of dexamethasone did not elicit the same repressive effects on MR as the 100 nM treatments. The type II receptor GR and inflammatory mediators TLR4, CD14, and MyD88 did not show significant changes in mRNA expression following dexamethasone treatment. Additionally, the percentage of amoeboid cells seemed to increase after exposure to 100 nM dexamethasone for two and three days. These data suggest that chronic exposure to dexamethasone may have significant effects on microglial activation and the MR mRNA expression without significantly affecting the mRNA expression of upstream mediators of LPS signaling pathway in microglial cells.

INTRODUCTION

Invertebrates and more complex organisms have a variety of mechanisms for responding to stress. In mammals, physiological changes ensue at the onslaught of the stress response activating the hypothalamic-pituitary-adrenal (HPA) axis which ultimately results in the release of glucocorticoids (GCs). GCs are a group of steroid hormones that regulate a variety of metabolic and immunologic responses decreasing accessory activities such as digestion and growth in reaction to stress. The endogenous GC corticosterone is involved in basal activities maintaining the coordination of circadian events like the sleep/wake cycle, food intake, and permissive regulation of the organisms' sensitivity to stress (De Kloet et al., 1998). In the central nervous system (CNS), the GCs at basal levels have been shown to increase synaptic plasticity, decrease neuronal cell death, and facilitate certain levels of hippocampus-dependent cognition (McEwan and Magarinos, 2001).

GCs have demonstrated anti-inflammatory action in the periphery reducing expression of pro-inflammatory cytokines including IL-1 β , IL-6, and TNF- α and enhancing anti-inflammatory molecules including IL-10 and TGF- β (Correale et al., 1998; Gayo et al., 1998). GCs' anti-inflammatory properties have been utilized pharmacologically to modulate inflammation in instances of allergies, asthma, and autoimmune disease, and as a post-operative measure to alleviate neuroinflammation (Hockey et al., 2009). While the clinical doses of GC are administered as anti-inflammatory agents, the complexity, duration, and severity of the physiological stress response present a more challenging picture of GC mediated actions in the brain. The immune response in the CNS is distinct from other tissues due to the blood brain barrier (BBB) which allows for a specialized microenvironment that is largely separate from that of the systemic organs (Risau and Wolberg, 1990). The lack of professional antigen presenting

cells, and the limited expression of major histocompatibility complex, along with the presence of the BBB create an immunologically privileged environment in the CNS (Wekerle et al., 1986).

Recent investigations of glucocorticoid response in the CNS present an array of studies that challenge and support the classical view of GCs' anti-inflammatory action. At the cellular level, dexamethasone, a synthetic corticosteroid, has been shown to reduce oxidative damage associated with inflammation (Golde et al. 2003). In contrast, rats exposed to chronic stress through restraint show an increase in microglia density and activation in multiple brain regions (Tynan et al., 2010). Low concentrations of corticosterone inhibit the expression of proinflammatory cytokines $IL-1\beta$ and $TNF-\alpha$ at the mRNA level in the rat hippocampus following an excitotoxin kainic acid stimulus (MacPherson et al., 2005), while high levels of corticosterone in rats exposed to unpredictable stress increase the levels of LPS-induced inflammatory signaling mediator $NF-\kappa B$ in the hippocampus (Munhoz et al., 2006). Evidence for a proinflammatory GC response is also indicated by the ability of endogenous GC to induce synthesis of macrophage migration inhibitory factor (MIF) in the mouse CNS. MIF is credited with a wide range of proinflammatory action including induction of $TNF-\alpha$ synthesis in macrophages, iNOS activity in microglia, and T-cell activation (Dinkel et. al. 2002; Donnelly and Bucala, 1997; Leech et. al. 1999)

GCs signaling pathway utilizes Type I mineralocorticoid receptor (MR) and Type II GC receptor (GR) which are members of a large family of nuclear receptors. These receptors consist of three major functional domains: an N-terminal domain, a central DNA binding domain, and a hinge region that links N-terminal domain and DNA binding domain to a C-terminal ligand binding domain. The N-terminal domain of MR is the longest of all the steroid receptors which are subjected to a high degree of variability with less than 15% identity within the steroid receptor family. Even so each individual receptor is highly conserved with over 50% homology observed

among various vertebrate species, which suggests an important functional role for the steroid receptor family (Viengchareun et al., 2007). When unoccupied, the receptors are attached to heat shock proteins including hsp90 and hsp70 which function in maintaining receptor conformation to facilitate ligand binding. Upon ligand binding, the receptors shed their protein chaperones, homodimerize, and translocate into the nucleus where it can exert either direct or indirect regulation of the expression of their target genes (Sorrells and Sapolsky, 2006). The receptors could directly influence the transcription of its target genes through its binding to GC response elements (GRE) located in the promoter regions. When core GR binding domains are not directly available, GR may link to promoters through binding with other transcription factors so that GR is associated by protein-protein interactions rather than direct DNA binding (Lefstin and Yamamoto, 1998). Chromatin immunoprecipitation experiments have demonstrated that occupancy of GR binding sites can be influenced by post-translational modifications (Blind and Garabedian, 2008). It has also been reported that GR is subjected to post-translational modification through phosphorylation at serine/threonine residues located in DNA binding domains, which induces conformational changes and provides a possible mechanism for GR mediated transactivation (Webster et al., 1997). Furthermore, GR stability is sensitive to its phosphorylation status which can affect the half-life and ligand-dependent stabilization suggesting a critical role of post-translational modification in receptor turnover (Hittelman et al., 1999; Liberman et al., 2007).

In the hippocampus, MR has been shown to exhibit increased binding with a ten fold higher affinity for endogenous GC than GR (De Kloet et al., 1998). While MR and GR share significant homology in their DNA binding domains they do not exclusively target the same set of genes. In the rat hippocampus, activation by the respective receptors shows less than a 30% overlap of their target genes, demonstrating the potential for a receptor specific cellular response

(Datson et al. 2001). MRs' high binding affinity for GCs ($K_d = 0.5\text{nM}$) suggests that at basal levels MR is the more highly occupied receptor with only slight saturation of GR ($K_d = 5.0\text{nM}$) (De Kloet et al., 1998). Early investigations into glucocorticoid receptor binding showed that rats sacrificed early in the morning at the nadir of the HPA axis diurnal rhythm exhibited hippocampal MRs that were 80% occupied while GR was only 10% occupied. GR binding increased only when a higher level of corticosterone was introduced as is the case during stress (Reul and De Kloet, 1985; Reul et al., 1987). Differential binding by the two receptors in brain cells may play a role in the varied effects of GCs observed in the CNS (Sapolsky et al., 2000). In vitro binding studies indicate that naturally produced steroid hormones such as corticosterone and cortisol preferentially bind to MR as compared to synthetic GCs like dexamethasone which show higher affinity for GR (Rupprecht et al 1993). These receptors have been identified in cell types that are essential to the inflammatory response including dendritic cells, macrophages, lymphocytes, and microglia cells (Fuxe et al., 1985; Sierra et al., 2008).

Microglia cells play a critical role in the innate immune response in the brain. The innate immune system is an inherent defense system that offers an immediate response to an immune challenge. Microglia are the resident macrophages of the nervous system and function as antigen presenting cells and phagocytic scavengers that are continuously sampling the surrounding environment for even the smallest disruption, and can be activated by a variety factors such as infection, physical trauma, oxygen depletion, and neurodegeneration. The number of microglia cells present increases in response to a variety of CNS insults such as restraint induced chronic stress in mice (Tynan et al., 2010). Immunohistochemical detection indicates that the microglia cells are the first population to respond with a proliferative response to a CNS stressor such as infection or physical trauma (Postler et al., 1997; Hailer, 2008). When neuronal cell death occurs, glutamate, prostaglandins, cytokines, and other cellular contents are released into the surrounding

area resulting in activation of microglia and migration to the damaged site. The activated microglia work to restore homeostasis and are characterized by proliferation and a morphological change from the ramified resting state to the motile amoeboid morphology that is accompanied by an increase in production of pro-inflammatory cytokines such as IL-6, IL-18, and TNF- α (Sugama et al., 2007). Chemokines and co-stimulatory molecules CD40 and CD80 are also produced in the activated microglia, which are necessary for T-cell activation (Dimayuga et al, 2005).

Microglial reaction to an invading pathogen depends on toll-like receptors (TLR) that are expressed on the microglia cell surface and these TLRs act as pattern recognition receptors for pathogen-associated molecular patterns (PAMPs). LPS from gram-negative bacteria cell wall is one of such PAMPs. It is bound by TLR4 and its co-receptor CD14. The activation of TLR4 initiates recruitment of myeloid differentiation factor 88 (MyD88). MyD88 is a cytosolic adapter protein that is a key component of the signal transduction pathways for interleukin-1 and toll-like receptor. These pathways regulate extensive proinflammatory responses. The MyD88 protein consists of an N-terminal death domain and a C-terminal Toll-interleukin 1 receptor domain (Han, 2006). Following TLR4 binding, MyD88 associates with IL-1 receptor associated Kinase (IRAK) complex through its amino terminal death domain, which ultimately results in the activation of NF- κ B. NF- κ B is a crucial activator of genes encoding innate immune proteins such as cytokines, chemokines, complement proteins, and cell adhesion molecules (Beutler, 2000; Glezer et al., 2007).

Recognition of a foreign ligand, such as bacterial LPS, initiates microglia activation and the pro-inflammatory signaling cascade (Poltorak et al., 1998). The rapid up-regulation of proinflammatory products aids in defense against the immune challenge but also potentially contributes to neurological damage under conditions of chronic inflammation. For example,

activated microglia has been indicated as a contributing factor in neurodegeneration resulting from the production of oxygen and nitrogen reactive species and prolonged exposure to inflammatory cytokines (Glezer et al., 2007).

GCs are well-known to be released as a feedback mechanism to quench an inflammatory response, however, more recently they have been shown to have proinflammatory effects. Furthermore, the effects of chronic exposure to GCs in the brain have been suggested to be more complex than its acute anti-inflammatory effects in the periphery. The purpose of this study is to investigate the effects of GCs on the basal expression of innate immune mediators in microglial cells when they are not exposed to any inflammatory challenges. Microglia cells were grown *in vitro* and treated with the synthetic corticosterone analogue dexamethasone at 10 nM, 100 nM, and 1 μ M concentrations. Three days following dexamethasone exposure, cells were collected and processed to examine the relative mRNA levels of steroid receptors MR and GR, as well as the upstream components of the inflammatory response such as TLR4, CD14, and MyD88. A time course of 100 nM dexamethasone treatment was done in order to evaluate the response of microglia cell to the duration of GC exposure. RNA was isolated from the treated populations after 1, 2, and 3 days of exposure and the expression of the steroid receptors and inflammatory mediators were determined using RT-PCR analysis.

MATERIALS AND METHODS

Cell Culture

BV2 is a murine microglial cell line kindly provided by Dr. Jau-Shyong Hong from National Institute of Health. These cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % Fetal Calf Serum (FCS) and 1% pen/strep antibiotic. Cells were cultured in T-75 cm² flasks (Corning, Corning NY) at 37°C in a humidified incubator with 5% CO₂. Once microglia were grown to confluence the cells were washed with PBS and detached from the flask using trypsin. The cells were counted on a hemocytometer and plated for treatment as described below.

Dexamethasone Treatments

BV2 cells were counted using a hemocytometer and then seeded into 6 well plates. For dose response experiments, 1.0×10^5 cells per well were used for a 6 well plate. One day after seeding, cells were treated with 10 nM, 100 nM, or 1 μ M of dexamethasone (Sigma-Aldrich, St. Louis MO) for 3 days with the control cells left untreated. For time course experiments, 2.0×10^5 , 1.5×10^5 , and 1.0×10^5 cells were seeded and treated with 100 nm dexamethasone the following day for 1, 2, and 3 days respectively.

RNA Extraction

Total RNA was isolated from the cell culture plates using Trizol reagent (MRC, Cincinnati, OH). The media was aspirated from each well, then 500 μ l of Trizol was added to lyse the cells. The suspension was transferred to 1.5 ml centrifuge tubes followed by 100 μ l of chloroform. Samples were centrifuged at 4°C for 15 minutes at a speed of 12,000g. The upper

aqueous phase was transferred to a new tube and 250 μ l isopropanol was added to precipitate RNA. The sample was spun at 12,000g for 8 minutes to form a pellet. The pellet was washed in 75% ethanol and resuspended in RNase free H₂O. RNA concentrations were determined using a spectrophotometer.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reverse transcription reactions were set up using 2 μ g of total RNA, oligo (dT)₁₂₋₁₈ primer, and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, Grand Island, NY). Following cDNA synthesis, PCR amplification reactions were carried out in 0.2 ml PCR tubes with 1x PCR buffer, 0.2 mM dNTPs, 1 unit Taq DNA polymerase, 0.5 μ l of each sense and antisense primer, and 1 μ l cDNA for a total reaction volume of 20 μ l. Primers for mouse β -actin, myd88, MR, CD14, GR, TLR4 were run with an annealing temperature of 56°C for β -actin, and 57° C for the remainder of the primers. B-actin was run for 21 cycles using primers 5'-AGC-CAT-GTA-CGT-AGC-CAT-CC-3' and 5'-CTC-TCA-GCT-GTG-GTG-GTG-AA-3'. Myd88 was run for 27 cycles with primers 5'-TGT-CCC-AAA-GGA-AAC-ACA-CA-3' and 5'-ACT-GGC-CTG-AGC-AAC-TAG-GA-3'. MR was run for 30 cycles with primers 5'-GCA-AAA-TCC-CAG-ACC-GAC-TA-3' and 5'-CAG-ACC-TTG-GAG-CGT-TCT-TC-3'. CD14 was run for 25 cycles with primers 5'-CTG-ATC-TCA-GCC-CTC-TGT-CC-3' and 5'-GCT-TCA-GCC-CAG-TGA-AAG-AC-3'. GR was run for 25 cycles with primers 5'-CCA-CTG-CAG-GAG-TCT-CAC-AA-3' and 5'-AAG-GGT-CAT-TTG-GTC-ATC-CA-3'. TLR4 was run for 28 cycles with primers 5'-GGC-AGC-AGG-TGG-AAT-TGT-AT-3' and 5'-CTT-AGC-AGC-CAT-GTG-TTC-CA-3'. Following the PCR reactions, amplified DNA samples were separated on a 2.0% agarose gel. The gels were visualized and documented using the UVP GelDocIt™ imaging

system (UVP, Upland CA). The intensities of the respective bands were analyzed by digital densitometry using Vision Works[®] LS software (UVP, Upland CA).

Data Analysis

The band intensity of the RT-PCR data was normalized to β -actin. All data were presented as the mean \pm SEM for each control or treatment group. Statistical comparisons were performed using one-way ANOVA with repeated measures for the dose response experiments and a two-way ANOVA with repeated measures for time course experiments using factors of time and treatment (treated vs. untreated).

Phase Contrast Microscopy

Images of the microglia cells were obtained using an inverted phase contrast Leica DMIL microscope with a Leica DFC 300 CCD camera. Images were collected during each day of the dexamethasone treatment. Cells were quantified manually by counting the ramified, ameboid, and total cell number present on each day of treatment. Ramified cells were defined as those with oval to oblong shaped soma that have thin projections (Glenn et al., 1992). Ameboid microglial cells were identified by their enlarged cell bodies with a granular appearance and short stubby projections or lack of projections (Rock et al., 2004).

RESULTS

Phase contrast imaging of microglia cells after treatment with different doses of dexamethasone

Phase contrast microscopy was used to examine morphology of microglia treatment groups. Ramified microglia cells are the resting stage of the microglia that is composed of projecting branching attached to an elongated cylindrical cell body. The amoeboid form of a microglia cell is phagolytically active exhibiting an enlarged rounded cell body with lysosomes and possible pseudopodia like processes (Becher and Antel, 1996). The microglial cells cultured *in vitro* exhibited both populations of ramified and amoeboid cells (Fig. 1). In the untreated cells after three days, 9 % of the cells appeared ramified while 4% exhibited the amoeboid phenotype. After treatment with 10 nM dose of dexamethasone for three days, 14 % of the total cell population exhibited ramified phenotype while 9 % of the total cell population had visible amoeboid morphology. After treatment with 100 nM dose of dexamethasone for three days, there were about 9% of cells exhibiting ramified phenotype and 12 % of the cell population exhibiting amoeboid morphology. After treatment with 1 μ M dexamethasone for three days, there were about 19 % of the microglia population exhibiting ramified phenotype and 12% of the cells exhibiting amoeboid characteristics (Fig 1 and Fig. 2). Even though more repeats are needed to perform statistical analysis, it seemed that dexamethasone treatment may have an effect on the morphology of microglial cells in culture.

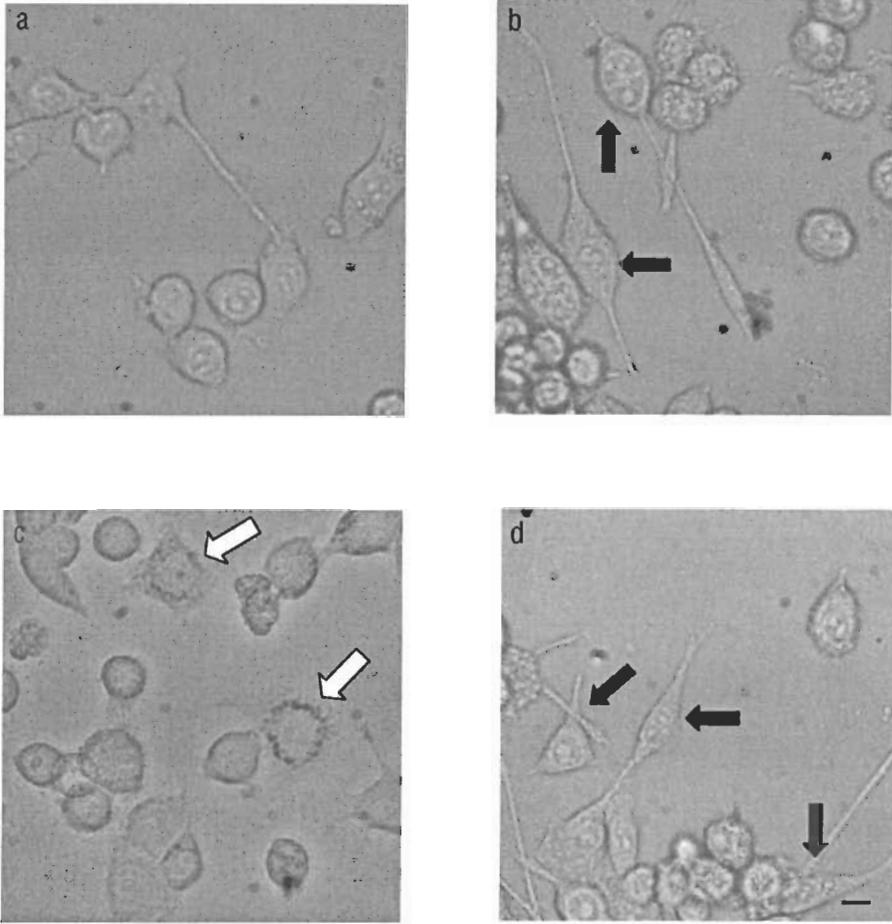


Figure 1. Representative images of microglia after three days of treatment with different concentrations of dexamethasone in culture. (a) Untreated microglia. (b) Microglia treated with 10 nM dexamethasone. Note the appearance of ramified cells (black arrow). (c) Microglia treated with 100 nM dexamethasone with amoeboid cells present (white arrow). (d) Microglia treated with 1 μ M dexamethasone with increased numbers of ramified cells observed (black arrow). Scale bar represented 50 μ m.

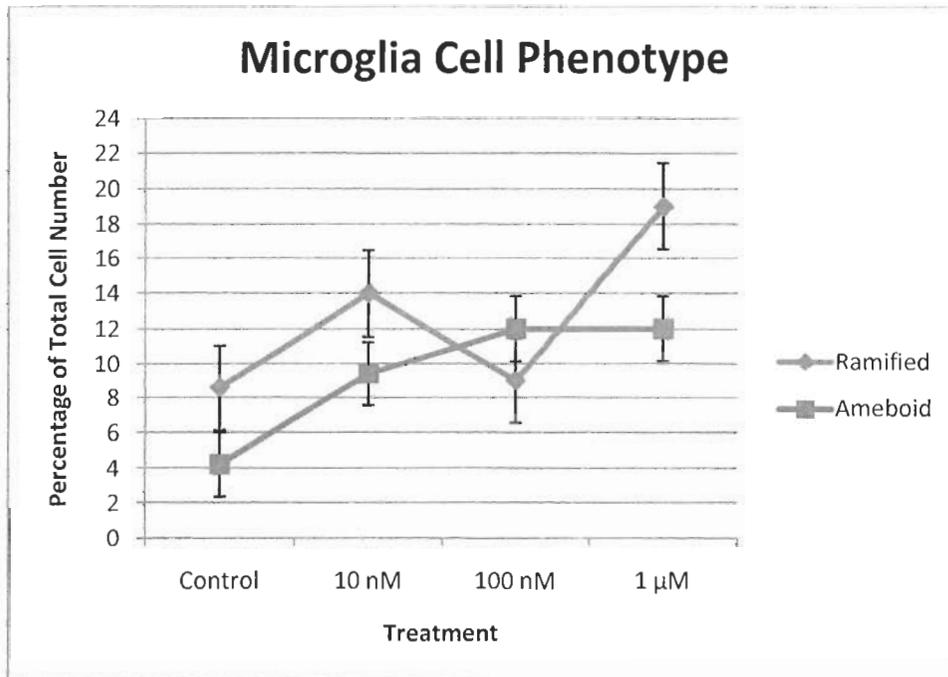


Figure 2. Percentage of ramified (blue diamond) and ameboid (red square) microglia cells after treatment with 10 nM, 100 nM, and 1 μM dexamethasone for 3 days.

Dexamethasone induced dose-dependent downregulation of Type I mineralocorticoid receptor in microglia

To examine the effects of glucocorticoid exposure on microglia expression of inflammatory mediators, microglia cells were grown *in vitro* and treated with 10 nM, 100 nM, or 1 μ M of dexamethasone. After three days of dexamethasone treatment, the cells were collected and processed for total RNA extraction. RT-PCR analysis was used to determine the expression levels of the primary type I and type II GC receptors MR and GR.

Analysis of mRNA expression shows that MR was decreased at 100 nM treatment compared to the untreated control. MR expression was unaffected at a 10 nM concentration of dexamethasone. When the concentration of the corticosteroid was increased from 100 nM to 1 μ M, the expression of MR returns to a level similar to that of the untreated control resulting in a U-shaped dose dependent expression pattern after three days of exposure to different dexamethasone concentrations (Fig. 3).

The mRNA expression of GR following exposure to different concentrations of dexamethasone was also examined. Even though the GR mRNA expression appeared to be slightly up-regulated after treatment with 10 nM, 100 nM, and 1 μ M dexamethasone as compared to control cells, but the increase was not significant (Fig. 4).

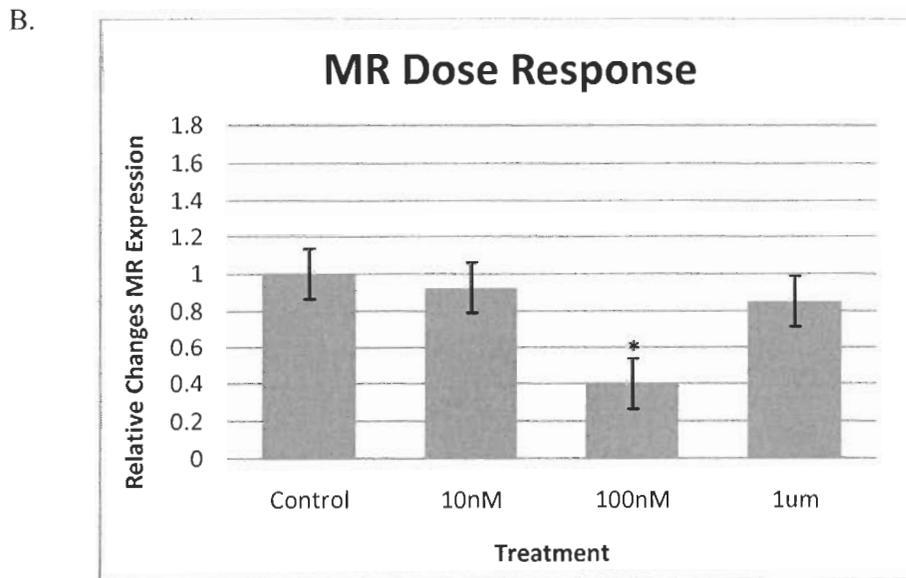
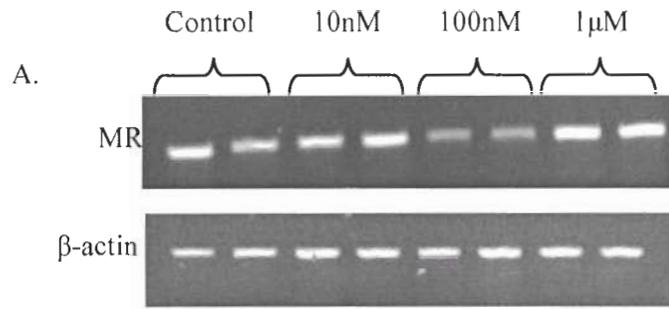


Figure 3. (A) Representative agarose gel image of MR expression in microglia cells at 3 days following treatment with 10 nM, 100 nM or 1µM of dexamethasone using semi-quantitative RT-PCR. (B) Quantitation of MR mRNA expression normalized to β-Actin. Statistical analysis was done by one way ANOVA, and values were means ±SE. (*P < 0.05).

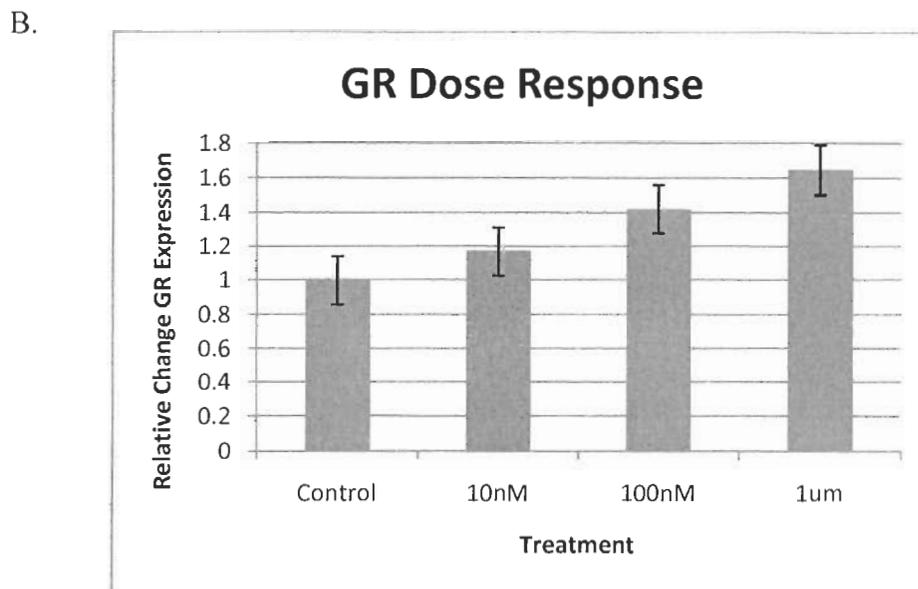
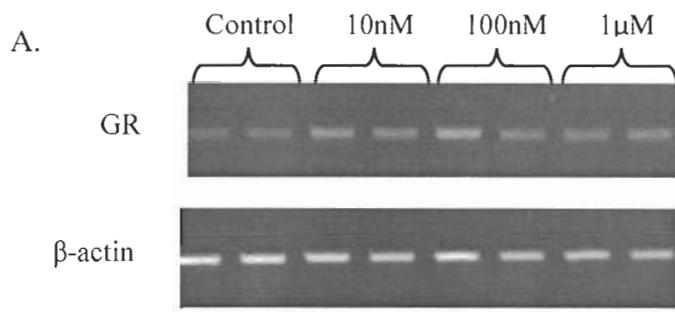


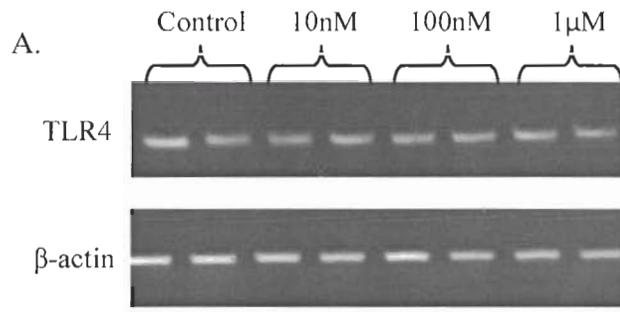
Figure 4. (A) Representative agarose gel image of GR expression in microglia cells at 3 days following treatment with 10 nM, 100 nM or 1 μ M of dexamethasone using semi-quantitative RT-PCR. (B) Quantitation of GR mRNA expression normalized to β -Actin. Statistical analysis was done by one way ANOVA, and values were means \pm SE.

Dexamethasone had no dose dependent effects on the mRNA expression of upstream mediators of LPS signaling pathway

To examine the effects of glucocorticoid exposure on microglia expression of inflammatory mediators, microglia cells were grown *in vitro* and treated with 10 nM, 100 nM, or 1 μ M of dexamethasone. Microglia cells were exposed to the dexamethasone treatment for three days and then harvested for total RNA extraction. The relative mRNA expression levels of upstream mediators of LPS signaling pathway such as TLR4, CD14, and MyD88 were determined using RT-PCR analysis.

The TLR4 mRNA level in cells treated with 10 nM and 1 μ M dexamethasone was similar to that in control cells (Fig. 5). TLR4 mRNA in cells treated with 100 nM dexamethasone appeared to be lower than that in control cells, but the difference was not statistically significant (Fig. 5).

The mRNA expression of CD14 following treatment with 10 nM and 100nM dexamethasone did not vary significantly from that in untreated microglia cells. The co-receptor CD14 appeared to be upregulated at the 1 μ M dose of dexamethasone but the change was not statistically significant (Fig. 6). The mRNA expression of Myd88 did not appear to be significantly affected by treatment with 10 nM, 100 nM, or 1 μ M of dexamethasone for three days (Fig. 7).



B.

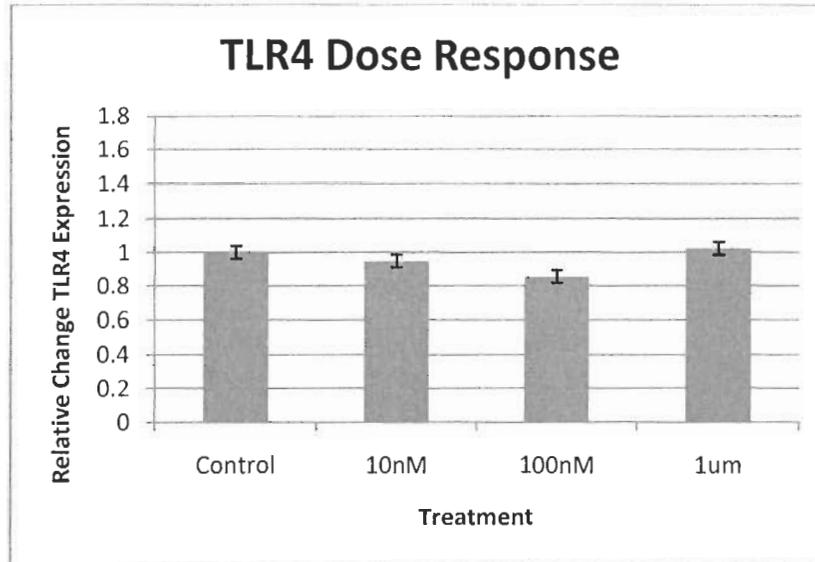


Figure 5. (A) Representative gel image of TLR4 expression in microglia cells at 3 days following treatment with 10 nM, 100 nM or 1µM of dexamethasone using semi-quantitative RT-PCR. (B) Quantitation of TLR4 mRNA expression normalized to β -Actin. Statistical analysis was done by one way ANOVA, and values were means \pm SE.

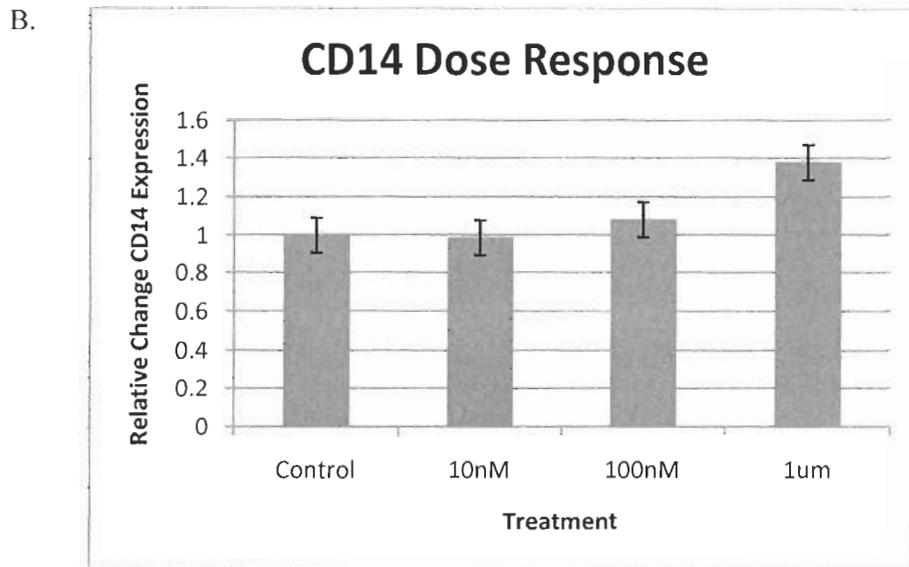
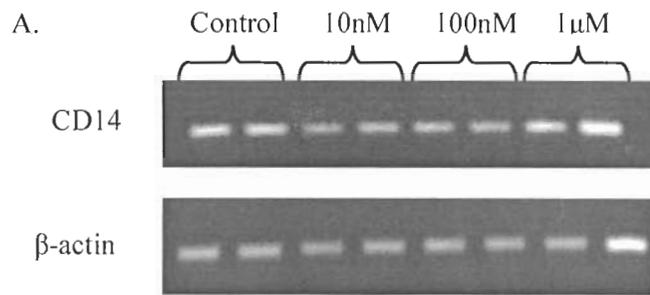


Figure 6. (A) Representative gel image of CD14 expression in microglia cells at 3 days following treatment with 10 nM, 100 nM or 1µM of dexamethasone using semi-quantitative RT-PCR. (B) Quantitation of CD14 mRNA expression normalized to β -Actin. Statistical analysis was done by one way ANOVA, and values were means \pm SE.

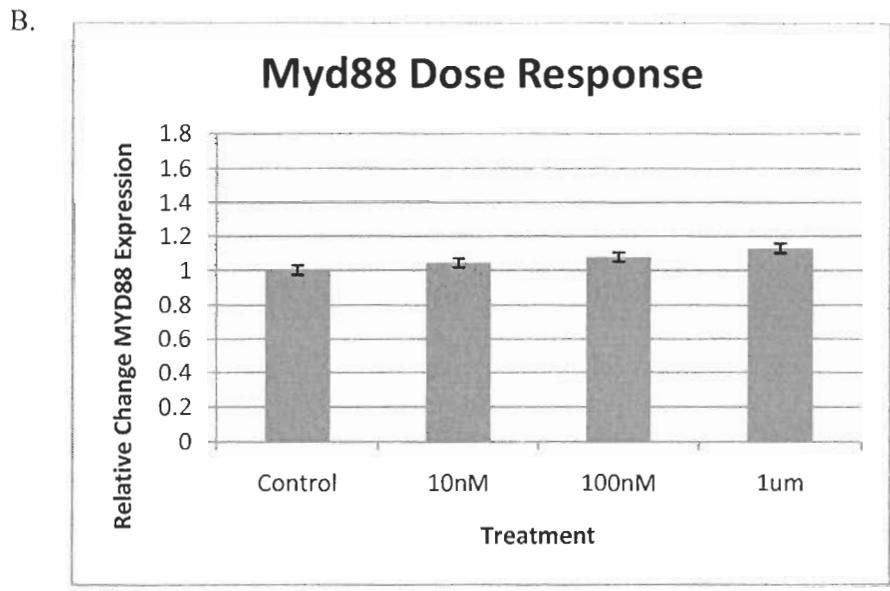
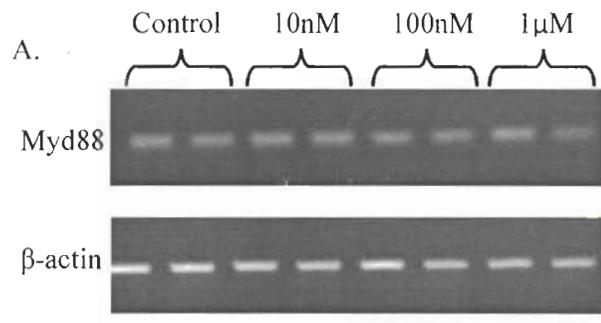


Figure 7. (A) Representative gel image of Myd88 expression in microglia cells at 3 days following treatment with 10 nM, 100 nM or 1µM of dexamethasone using semi-quantitative RT-PCR. (B) Quantitation of Myd88 mRNA expression normalized to β -Actin. Statistical analysis was done by one way ANOVA, and values were means \pm SE.

Phase contrast images of microglia cells during time course dexamethasone treatment

Phase contrast images were taken of the microglia cells after 1, 2, and 3 days of 100 nM dexamethasone treatment. The 100 nM treatment was chosen considering that at this concentration, dexamethasone significantly affected the mRNA expression of MR following three days of treatment. After the first day of time course, 3 % of the untreated cells exhibited ramified morphology and 4 % appeared amoeboid while the cells treated with 100 nM of dexamethasone contained 5 % ramified and 4 % amoeboid phenotypes in the total cell population. After second day of dexamethasone treatment, 9 % of the untreated microglia were ramified cells, while less than 1 % of the population appeared amoeboid. The amount of ramified and amoeboid cells appeared to increase on day two in treated microglia as compared to the untreated cells, with 13 % of the treated cells identified with ramified morphology and 4 % exhibiting the amoeboid phenotype. On the third day of treatment 11% of the untreated cells appeared ramified while 2 % showed amoeboid morphology. After three days of 100 nM dexamethasone treatment 10 % of the cells were ramified and 4% of the population appeared amoeboid (Fig. 8 and Fig. 9).

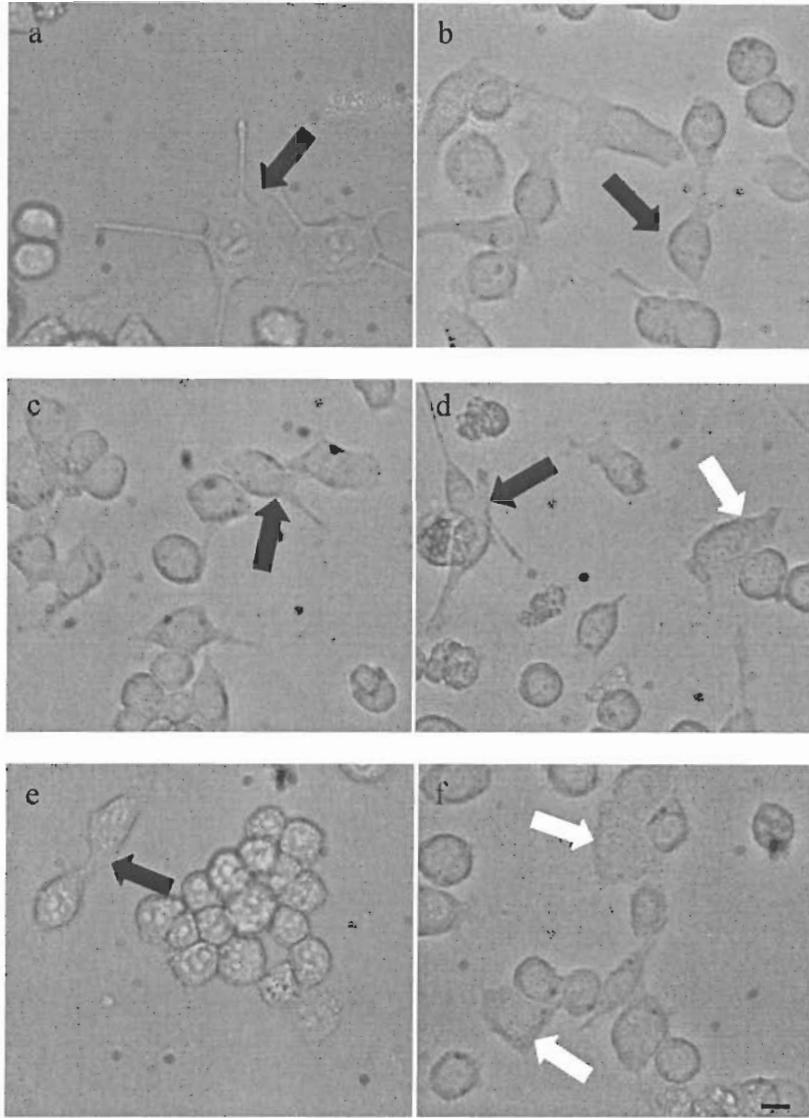
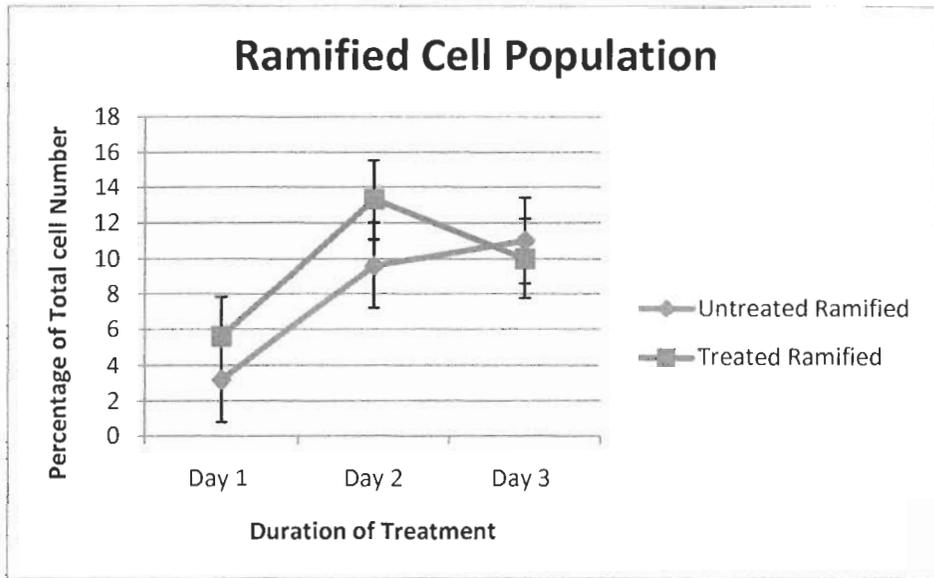


Figure 8. Representative images of microglia in culture following 100 nM treatment dexamethasone for different days. (a) Untreated microglia after 1 day. (b) Microglia treated with 100 nm for 1 day. (c) Untreated microglia after 2 days. (d) Microglia treated with 100 nM for 2 days. (e) Untreated microglia after 3 days. (f) Microglia treated with 100 nM dexamethasone for 3 days. Scale bar represents 50 μ M. Ramified cells indicated by black arrows, amoeboid cells seen by white arrows.

A.



B.

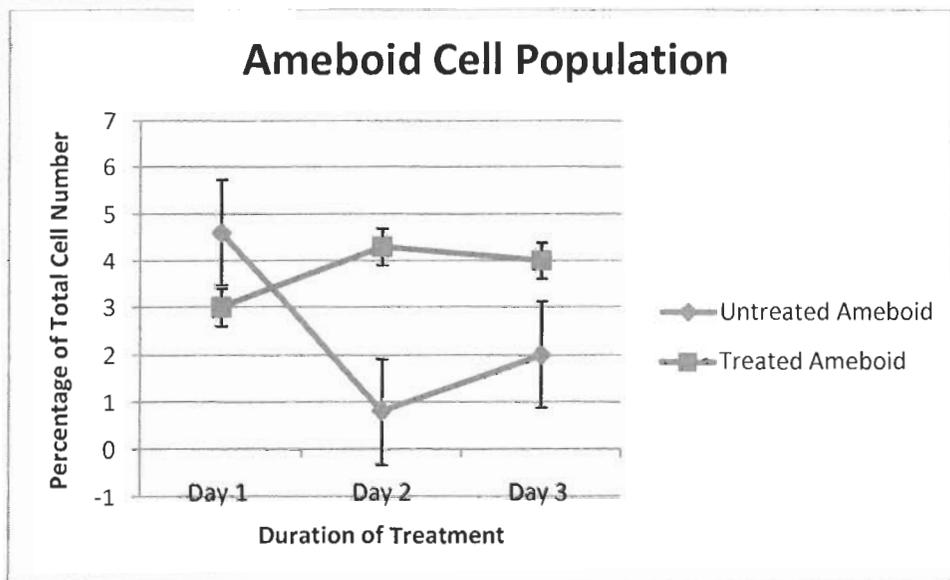


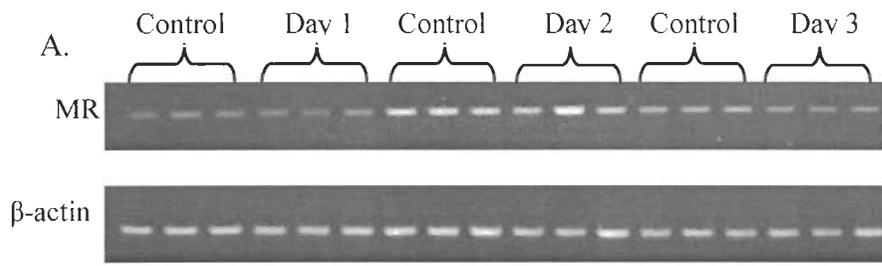
Figure 9. (A) The percentage of observed ramified cells in the untreated (blue diamond) and cells treated with 100 nM dexamethasone (red square) for 1, 2, and 3 days. (B) The percentage of ameboid microglia cells in the untreated (blue diamond) and treated (red square) cell populations after 1, 2, and 3 days.

Time dependent effects of dexamethasone on the expression of MR in microglial cells

Microglia cells were treated with 100nM dexamethasone for 1, 2, and 3 days to assess the effect of different durations of treatment on mRNA levels of the glucocorticoid receptors.

MR was expressed at low levels in both the control and the treatment cell populations on day 1 of dexamethasone exposure. The expression level of MR slightly increased in both the control and treated sets of cells by day 2 of treatment. After three days of treatment with dexamethasone, the mRNA expression of MR significantly decreased in the treated cell population as previously observed in the dose response (Fig. 10).

The GR receptor expression was consistent throughout the three days of treatment. The 100 nM concentration of dexamethasone used and the duration of treatment from day 1 to day 3 appear to have little effect of the expression of GR in the microglia cell (Fig. 11).



B.

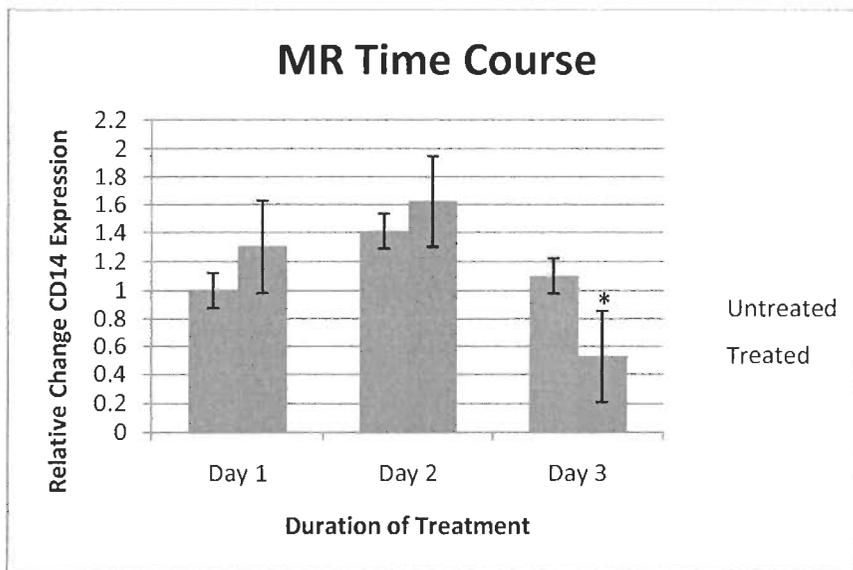


Figure 10. Representative gel image of MR RT-PCR following treatment with 100nM dexamethasone for 1, 2, or 3 days. (B) Quantitation of MR expression normalized to β -Actin. Statistical analysis was done by two way ANOVA, and values were means \pm SE. (*P < 0.05)

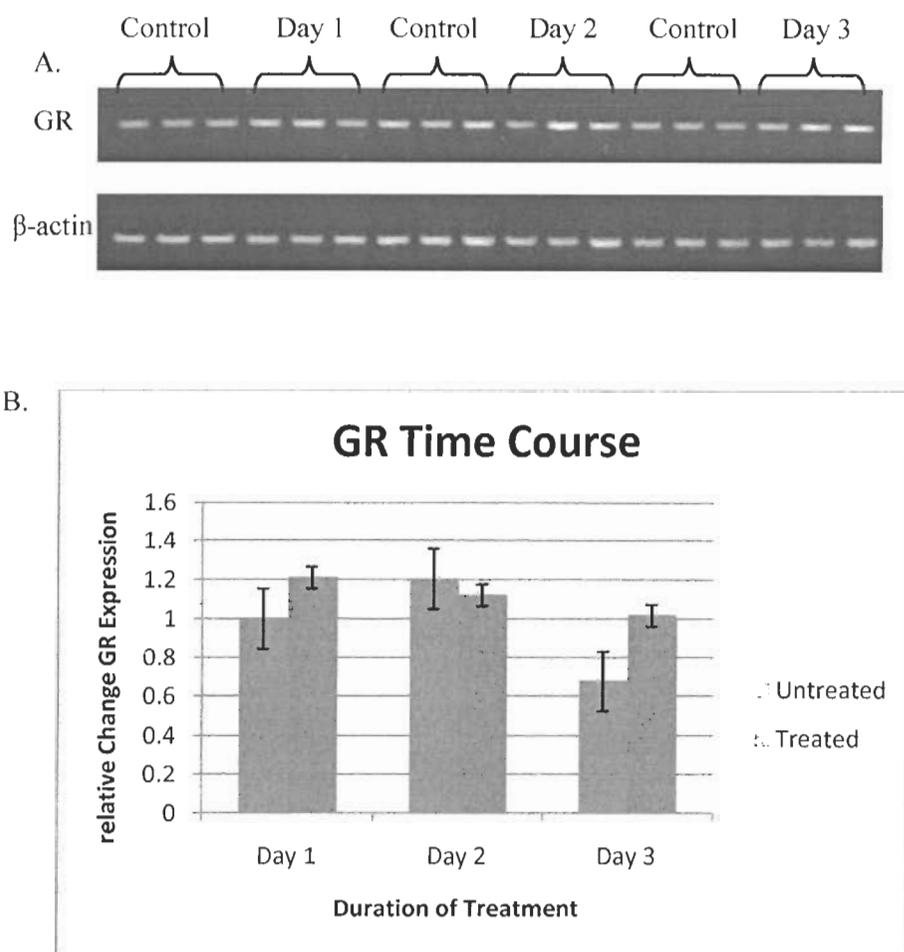


Figure 11. Representative gel image of GR RT-PCR following treatment with 100nM dexamethasone for 1, 2, or 3 days. (B) Quantitation of GR expression normalized to β -Actin. Statistical analysis was done by two way ANOVA, and values were means \pm SE.

Dexamethasone had no effects on the expression of upstream LPS signaling mediators in microglial cells

The mRNA expression of TLR4 appeared to be slightly increased after one day of dexamethasone treatment and decreased thereafter as the duration of treatment increased to two and three days, yet these changes were not statistically significant. Therefore, TLR4 mRNA expression did not appear to be significantly affected by the 100 nm treatment (Fig. 12).

No changes in the mRNA expression of CD14 co-receptor and the signal mediator Myd88 were observed after the first day of the treatment with dexamethasone. The expression of CD14 and MyD88 increased slightly on the second day of treatment, and then returned to basal level of expression thereafter, but these changes were not statistically significant. Therefore, the mRNA expression of inflammatory mediators CD14 and myD88 appeared to be largely unaffected by the exposure to 100 nM dexamethasone (Fig. 13 and Fig. 14).

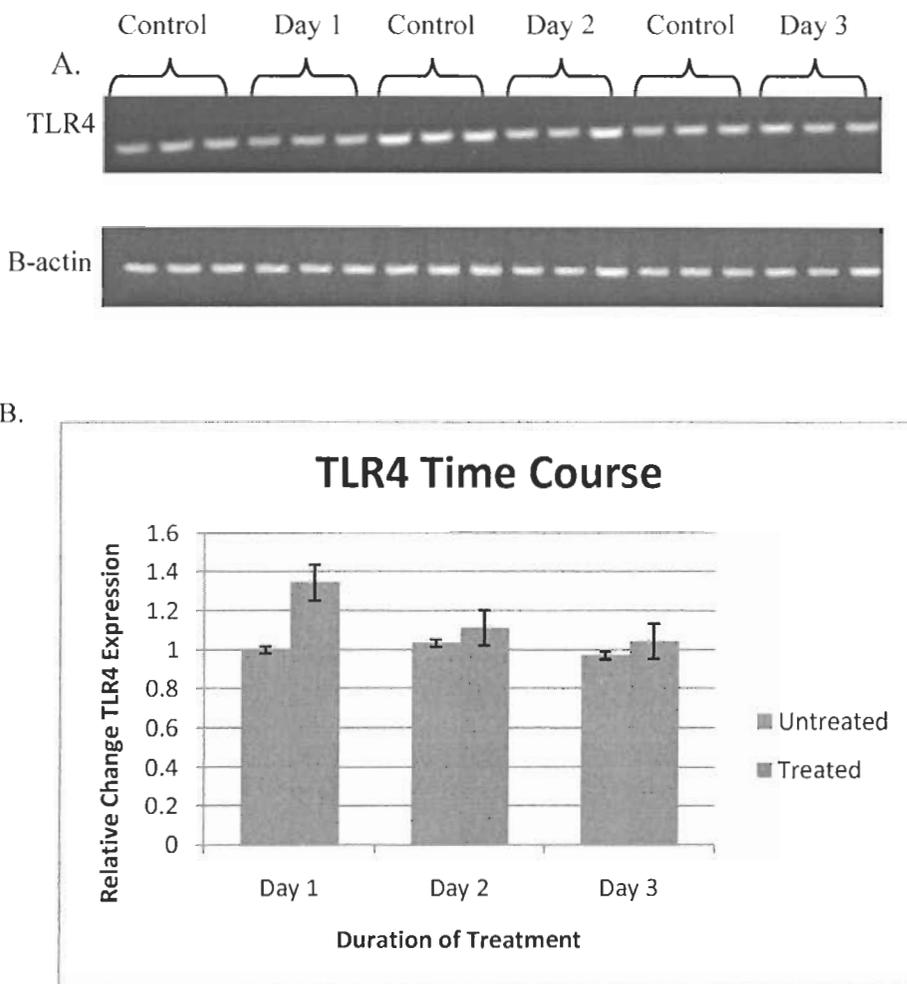


Figure 12. Representative gel image of TLR4 RT-PCR following treatment with 100nM dexamethasone for 1, 2, or 3 days. (B) Quantitation of TLR4 expression normalized to β -Actin. Statistical analysis was done by two way ANOVA, and values were means \pm SE.

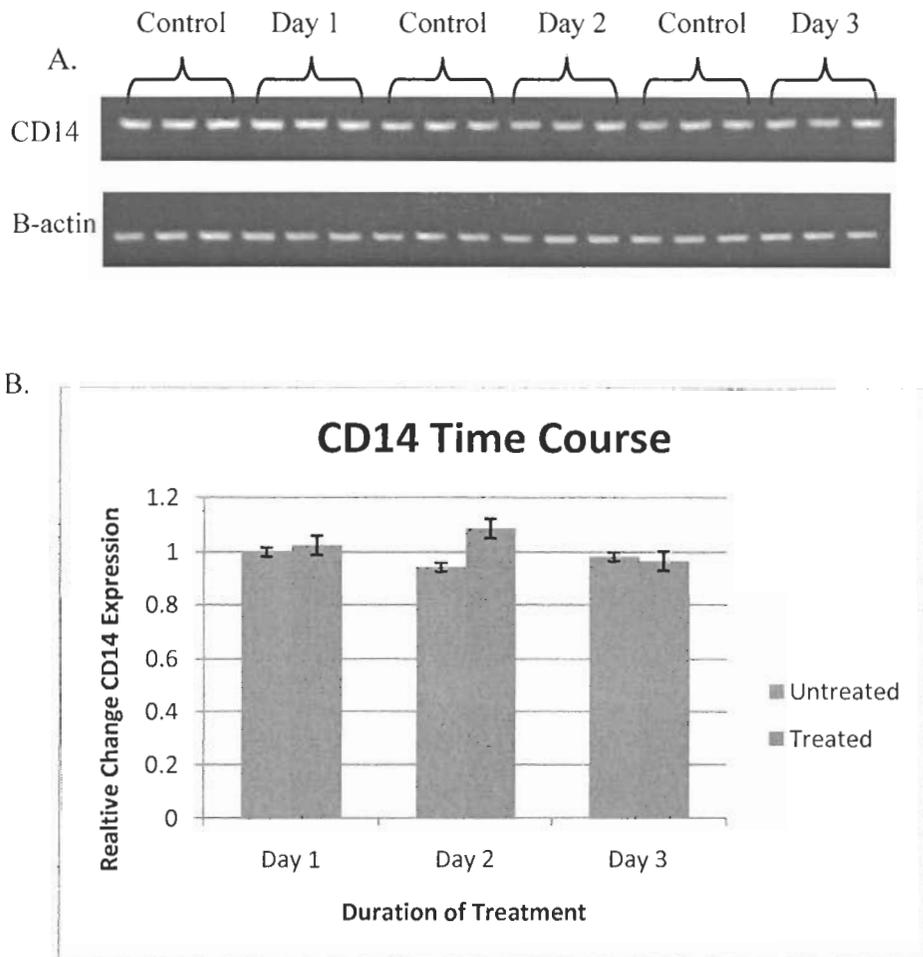


Figure 13. Representative gel image of CD14 RT-PCR following treatment with 100nM dexamethasone for 1, 2, or 3 days. (B) Quantitation of CD14 expression normalized to β -Actin. Statistical analysis was done by two way ANOVA, and values were means \pm SE.

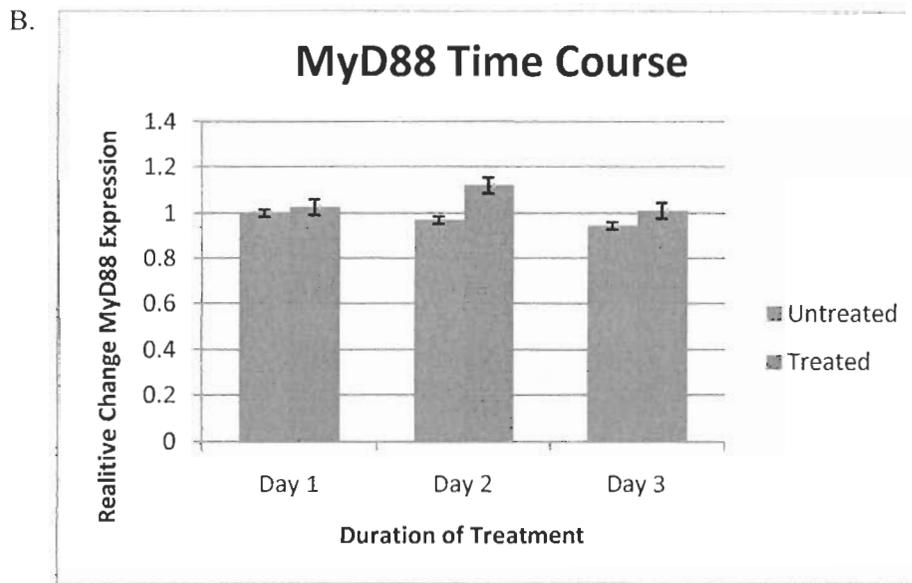
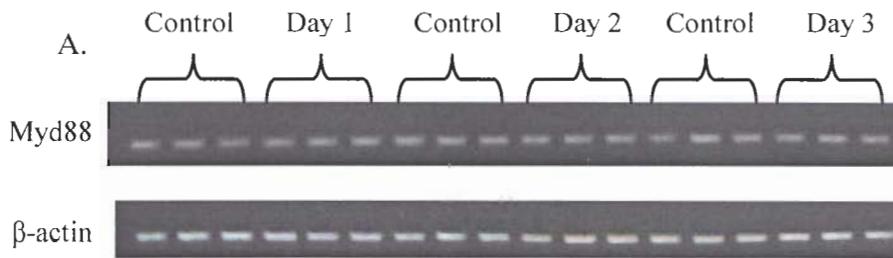


Figure 14. Representative gel image of Myd88 RT-PCR following treatment with 100nM dexamethasone for 1, 2, or 3 days. (B) Quantitation of Myd88 expression normalized to β -Actin. Statistical analysis was done by two way ANOVA, and values were means \pm SE.

DISCUSSION

The current study investigated the effects of dexamethasone treatment on microglial function using BV2 microglia cell line as an *in vitro* model. GCs have traditionally been credited with anti-inflammatory action which is why they are so commonly used in clinical settings. Recently growing evidence has demonstrated possible pro-inflammatory effects for GCs in the CNS. In this study the treatment of microglia cells with 100 μ M dexamethasone appeared to increase the percentage of amoeboid cell population after two and three days of treatment as compared to controls. While components of the innate immune response, namely TLR4, myd88, and CD14, showed little variation in mRNA expression after treatment with 10 nM, 100 nM and 1 μ M dexamethasone, the mRNA expression of MR significantly decreased in microglial cells after treatment with 100 nM of dexamethasone for three days.

In the dose response experiment, the number of observed amoeboid cells after treatment with 100 nM and 1 μ M dexamethasone for three days appeared to be higher than that after treatment with 10 nM dexamethasone and controls, suggesting that the 100 nM and 1 μ M dexamethasone treatment may increase activation of the microglia cells even though these experiments remained to be repeated. Furthermore, it would be advantageous to identify microglia activation markers such as ionized calcium-binding adaptor molecule-1 (Iba-1) and major histocompatibility complex II (MHC-II) in the treated cell population to molecularly differentiate the resting cell from the activated cells in populations exposed to GCs (Tynan et al. 2010).

Dexamethasone exposure for three days at 100 nM showed a significant decrease in the expression of the type I mineralocorticoid receptor (MR). Treatment with increasing concentrations of dexamethasone produced a U-shaped curve of effects on MR expression. Dexamethasone did not have effects on MR expression at 10nM, significantly inhibited the gene

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expression at 100nM, while 1 μ M dexamethasone did not inhibit the expression of MR. This dose dependent relationship between MR mRNA expression and dexamethasone exposure is similar to observations reported in hippocampal cultures where mid-range doses (0.2 μ M – 0.6 μ M) of corticosterone is able to inhibit excitotoxin induced expression of cytokines IL-1 β and TNF- α (MacPherson et al., 2005).

In the current study the 100 nM dose of Dexamethasone responsible for MR downregulation is significantly higher than the Kd of MR (0.5-1 nM) (Luttge et al. 1989; Rupprecht et al. 1993), making it less likely that MR is involved in the U-shaped expression response. The treatment dose was almost 100 times higher than the Kd of MR, therefore presumably receptor saturation and the maximal point of MR-mediated action would have been reached at significantly lower concentrations of dexamethasone, including the 10 nM treatment investigated in this study. This suggests that the differential effects of MR expression after corticosteroid exposure are not in fact mediated by the MR receptor, but instead rely on the GR receptor. If GR is mediating MR regulation at GC doses that above that of MR saturation, the type II receptor would be responsible for the divergent effects seen in this study at the 100 nM and 1 μ M treatment, specifically the MR downregulation and the return to control level MR expression at the 1 μ M dose of dexamethasone since GR would be the primary receptor binding the glucocorticoid present. The observed relationship between MR and GR has previously only been suggested in an investigation of glucocorticoid receptors and cortisol regulation in the gills of Atlantic salmon where GR was targeted using the antagonist RU486 (Kilerich et al 2007). Future studies are needed to investigate the relationship between GR and MR expression.

The current study demonstrated the suppressive effects of 100 nM dexamethasone, after three days, on MR expression in microglia cells. The effects of dexamethasone on the expression of MR followed a U-shaped pattern where levels of MR were maintained at the lowest and

highest concentrations (10 nM and 1 μ M) of dexamethasone and only suppressed under the midrange concentration (100 nM). These data suggest that expression of the MR receptor at the mRNA level in microglia cells is concentration dependent, which may have consequences on MR regulated functions in microglia under varying conditions of the stress response. While MR has been found to regulate salt homeostasis in periventricular tissue, and stabilization of excitability and facilitation of memory storage in the hippocampus (De Kloet et al., 2000), the specific basal function of MR in microglia cells has yet to be determined. Further studies are needed to identify the downstream signaling effects of altered MR repression following treatment with dexamethasone. Investigations into the mechanisms by which glucocorticoids regulate the expression of MR at the mRNA level would contribute to the understanding of how the MR mRNA expression is affected by dexamethasone in a concentration dependent manner.

In this study, the mRNA expression of inflammatory mediators TLR4, CD14, and Myd88 did not appear to be affected by the dexamethasone treatment, which suggests that glucocorticoid exposure for extended period of time does not have sustained effects on the innate immune machinery.

In summary, after exposure to dexamethasone at 100 nM and 1 μ M doses for three days, there appeared to be a higher percentage of amoeboid cells as compared to the ones treated 10 nM dexamethasone and controls. Following three days of dexamethasone treatment at 100 nM, the expression of MR was significantly downregulated while the 10 nM and 1 μ M dose did not exert the same repressive effects on MR expression. MR downregulation by 100 nM of dexamethasone was not observed until day three of the time course with no apparent change in regulation detected on the first or second day of the treatment. The type II receptor GR was not significantly affected by dexamethasone at different concentrations for the duration of exposure examined here. Inflammatory mediators TLR4, CD14, and MyD88, which are integral to the LPS mediated

immune response, were not significantly affected by the dose or duration of dexamethasone treatment examined. These findings add to the body of investigations recognizing glucocorticoid induced actions in the CNS. MR is an important component of the microglia cell response to the glucocorticoids released as a result of stress. MR down-regulation at a midrange dose of dexamethasone may significantly affect the microglia inflammatory cell response. While the mechanisms underlying the repression of MR remains to be elucidated further investigation into how MR expression was repressed after exposure to certain level of glucocorticoids may provide relevance to the role of microglia during the stress response in the CNS.

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