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Interleukin-1 receptor antagonist ameliorates the pain hypersensitivity, spinal inflammation and oxidative stress induced by systemic lipopolysaccharide in neonatal rats

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Abstract

Perinatal inflammation-induced reduction in pain threshold may alter pain sensitivity to hyperalgesia or allodynia which may persist into adulthood. In this study, we investigated the anti-inflammatory protective effect of interleukin-1 receptor antagonist (IL-1ra), an anti-inflammatory cytokine, on systemic lipopolysaccharide (LPS)-induced spinal cord inflammation and oxidative stress, thermal hyperalgesia, and mechanical allodynia in neonatal rats. Intraperitoneal (i.p.) injection of LPS (2 mg/kg) or sterile saline was performed in postnatal day 5 (P5) rat pups, and IL-1ra (100 mg/kg) or saline was administered (i.p.) 5 min after LPS injection. Pain reflex behavior, spinal cord inflammation and oxidative stress were examined 24 hours after LPS administration. Systemic LPS exposure led to a reduction of tactile threshold in the von Frey filament tests (mechanical allodynia) and pain response latency in the tail-flick test (thermal hyperalgesia) of P6 neonatal rats. Spinal cord inflammation was indicated by the increased numbers of activated glial cells including microglia (Iba1+) and astrocytes (GFAP+), and elevated levels of pro-inflammatory cytokine interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2), and

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prostaglandin E2 (PGE2) 24 h after LPS treatment. LPS treatment induced spinal oxidative stress as evidenced by the increase in thiobarbituric acid reactive substances (TBARS) content in the spinal cord. LPS exposure also led to a significant increase in oligodendrocyte lineage population (Olig2+) and mature oligodendrocyte cells (APC+) in the neonatal rat spinal cord. IL-1ra treatment significantly reduced LPS-induced effects including hyperalgesia, allodynia, the increased number of activated microglia, astrocytes and oligodendrocytes, and elevated levels of IL-1β, COX-2, PGE2, and lipid peroxidation (TBARS) in the neonatal rat spinal cord. These data suggest that IL-1ra provides a protective effect against the development of pain hypersensitivity, spinal cord inflammation and oxidative stress in the neonatal rats following LPS exposure, which may be associated with the blockade of LPS-induced pro-inflammatory cytokine IL-1β.

Keywords

Interleukin-1 receptor antagonist; lipopolysaccharide; hyperalgesia; spinal cord inflammation; oxidative stress

Introduction

Reduction in pain threshold is viewed as an important behavioral component of sickness behaviors following inflammation (Wegner et al., 2014). This event in early life, especially during the perinatal period or preterm birth, may alter pain sensitivity to hyperalgesia (an increased sensitivity to painful stimuli) or allodynia (a pain response to normally non-painful stimulation), which may last into adulthood (Beggs et al., 2012; Grunau, 2013; LaPrairie and Murphy, 2010; Walker, 2014). Lipopolysaccharide (LPS), an endotoxin originating from gram-negative bacteria, is commonly used to investigate the relationship between the changes in pain sensitivity and central nervous system (CNS) inflammation (Hsieh et al., 2018; Wang et al., 2011; Wegner et al., 2014). The LPS-induced inflammation model, which is associated with pain facilitation, has been supported by evidence that LPS induces several inflammatory mediators such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) or prostaglandin E2 (PGE2), which play important roles in the pathophysiology of acute or chronic pain (Hsieh et al., 2018; Sabedra Sousa et al., 2018; Wang et al., 2011).

IL-1β, the principal species of the IL-1 family, is a key pro-inflammatory cytokine involved in development, maintenance and propagation of pain sensitivity (Ren and Torres, 2009). IL-1β can be released from neuronal cells, microglia or astrocytes in the CNS, and produces additional allogenic substances (Ren and Torres, 2009; Zhang et al., 2016). Intrathecal IL-1β is reported to induce thermal hyperalgesia and allodynia through activation of p38 mitogen-activated protein kinase phosphorylation/inducible nitric oxide synthase/nitric oxide signaling in the rat spinal cord (Reeve et al., 2000; Sung et al., 2005; Sung et al., 2017). Intrathecal injection of LPS has also been shown to produce mechanical hyperalgesia caused by the release of IL-1β through *ex vivo* activation of microglial Toll-like receptor-4 (TLR-4) in the dorsal horn (Ren and Torres, 2009). Therefore, the relationship between the IL-1β and spinal inflammation has an important impact on the mechanism of pain sensitivity.

Our recent studies have shown that neonatal exposure to LPS through intracerebral (i.c.) injection in the rat brain at P5, to mimic human intrauterine infection during late gestation,

resulted in long-lasting hyperalgesia through a significant increase of activated microglia and IL-1β expression in the brain (Fan et al., 2005a; Fan et al., 2005b; Wang et al., 2011). Our study also demonstrated that systemic neonatal LPS intraperitoneal (i.p.) injection induced thermal hyperalgesia and mechanical allodynia, which may be associated with the increased number of activated microglia and astrocytes, and the upregulation of inflammatory cytokine IL-1β, inflammatory pain mediator PGE2 and cyclooxygenase-2 (COX-2) in the rat spinal cord (Hsieh et al., 2018). Therefore, the objective of the current study is to determine whether systemic treatment with the anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1ra) provides protective effects against neonatal LPS-induced pain hypersensitivities. More specifically, this study evaluates hyperalgesia and allodynia, and potential inflammatory factors due to the blockade of the positive feedback glia-IL-1β loop following neonatal LPS-induced spinal cord inflammation and oxidative stress.

Materials and methods

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO, USA). Recombinant rat IL-1ra was purchased from Cell Sciences (Newburyport, MA, USA). Monoclonal mouse antibodies against neuron-specific nuclear protein (NeuN), adenomatous polyposis coli (clone CC1, APC-CC1), glial fibrillary acidic protein (GFAP), and polyclonal rabbit antibodies against oligodendrocyte transcription factor 2 (Olig2), and ionized calcium binding adapter molecule 1 (Iba1) were purchased from Millipore (Billerica, MA, USA) and Wako Chemicals USA (Irvine, CA, USA), respectively. Polyclonal goat antibodies against cyclooxygenase-2 (COX-2) and IL-1β were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Novus Biologicals (Littleton, CO, USA), respectively. ELISA kits for immunoassays of rat IL-1β, IL-6, TNF-α, prostaglandin E2 (PGE2), COX-2, and corticosterone (CORT) were purchased from R&D Systems (Minneapolis, MN, USA), NeoScientific (Cambridge, MA, USA), and Enzo (Farmingdale, NY, USA), respectively.

Animals

Ordered timed pregnant Sprague-Dawley rats were delivered to the animal facility on day 19 of gestation. The dams were housed in individual cages inside an animal suite within the facility that was managed on a 12-h light/dark cycle and at constant temperature (22 ± 2°C). The dams were monitored daily until day of birth, which was immediately recorded as postnatal day 0 (P0). After delivery of the pups, each litter was adjusted to ten pups per litter to minimize the effect of litter size on body weight and spinal cord size. All procedures for animal care were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center or Fu Jen Catholic University. All investigators made a concerted effort to minimize the number of animals used and their suffering.

Animal treatment

Rat pups were separated into four groups: Saline+Saline (n=16), Saline+IL-1ra (n=16), LPS +Saline (n=16), and LPS+IL-1ra (n=16). Intraperitoneal injections of LPS (2 mg/kg, from

Escherichia coli, serotype 055: B5) or sterile saline (total volume of 0.1 ml) was performed in 5-day old Sprague-Dawley rat pups of both sexes as previously described (Hsieh et al., 2018). The male and female ratio was kept equal in each group (8 males and 8 females). A total volume of 0.1 ml of a solution containing 0.1% BSA in sterile saline (saline-BSA) or 100 mg/kg of IL-1ra in saline-BSA was injected 5 minutes after the LPS injection (Lan et al., 2015). Body weights were measured and behavioral tests were recorded twenty-four hours post-injection. Eight pups from each group were sacrificed by decapitation for fresh lumbar spinal cord tissue collection. The remaining eight pups from each group were sacrificed via transcardiac perfusion with normal saline followed by 4% paraformaldehyde for spinal cord section preparation. Consecutive frozen lumbar spinal cord sections (the lumbar enlargement of the spinal cord, L4-L5) were prepared in a cryostat at a thickness of 10 μm for immunohistochemistry examination.

von Frey filament test

This test assesses mechanical nociception (cutaneous mechanical sensitivity) of rats. Each rat was placed individually inside an elevated acrylic box with a wire mesh floor (Dynamic Plantar Anesthesiometer, UGO BASILE, Italy) and was allotted 5 minutes to acclimatize to the new environment. Mechanical thresholds for flexion withdrawal reflexes in response to punctuate mechanical stimulation of the plantar surface of the rat's hind paw was tested using von Frey filaments that exert a reproducible stimulus strength in grams ranging from 0.236 to 0.384 mm in diameter with marking forces of 0.02–0.60 g. Response threshold is defined as the von Frey filament which produces reflex paw withdrawal in three out of five applications (Fairbanks et al., 2000; Hsieh et al., 2018; Vega-Avelaira et al., 2013). Filaments were applied to the plantar surface of one hind paw five times at 10 s intervals alternately, and five times at 10 s intervals alternately to the other hind paw after 3 minutes of rest.

Tail-flick test

The tail-flick test uses an infrared source to assess thermal nociceptive threshold in rats. The tail-flick test was performed as described by our previous study with modifications at 24 h after LPS injection (Brown et al., 1997; Hsieh et al., 2018; Wang et al., 2011; Wongchanapai et al., 1998). Rats were first habituated to experimenter handling and to being placed into a plastic cylindrical tube. A shallow groove in the Plexiglas plate immobilized the rat's tail during the trials of tests. Half of the rat's tail from the tip to the base of tail was placed under radiant heat (Analgesia Test Tail-Flick Type 812, Columbus Instruments, Columbus, OH, USA). Pain sensitivity was measured by tail-flick latency defined as the time from the onset of radiant heat to tail withdrawal. A mandatory cut-off time of 10 s was set to prevent thermal injury to the rats. Tail-flick latency was then calculated as the average of 3 tail-flick latencies.

Immunohistochemistry

Spinal cord inflammation was measured from the immunohistochemistry results of consecutive lumbar spinal cord sections between L4 to L5 (~3 mm) at a thickness of 10 μm from rats sacrificed one day (P6) after LPS injection. Primary antibodies were used with the following dilutions: Olig2 (1:500), APC-CC1 (1:200), NeuN (1:200), Iba1 (1:500), IL-1β

(1:200), GFAP (1:500), and COX-2 (1:200). Olig2 and APC-CC1 were used to identify the total and mature oligodendrocytes, respectively. NeuN was used to detect the neuron-specific nuclear protein which is primarily localized in the nucleus of the neurons with slight staining in the cytoplasm. Microglia was detected using Iba1 immunostaining, which recognizes both resting and activated microglia. Astrocytes were detected using GFAP immunostaining. COX-2 provided selective staining of inducible cyclooxygenase. Sections were incubated with primary antibodies on a plate shaker at 4°C overnight and further incubated with secondary antibodies conjugated with fluorescent dyes (Alexa Fluor 555, 1:500 or Alexa Fluor 488, 1:200; Invitrogen, Carlsbad, CA, USA) for 1 hr in the dark on a plate shaker at room temperature. 4′, 6-Diamidino-2-phenylindole (DAPI) (100 ng/mL) was used simultaneously to stain nuclei and aid in identification during the final visualization. Negative control sections were incubated in the absence of primary antibody. The resulting sections were examined under a fluorescent microscope (Nikon Ni-E, Melville, NY, USA) at appropriate wavelengths.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IL-1β, IL-6, TNF-α, COX-2, CORT, and PGE2 in the rat spinal cord 24 h after LPS exposure were measured as markers of LPS-induced inflammatory responses by ELISA as previously described (Cai et al., 2013; Fan et al., 2013; Hsieh et al., 2018; Kaizaki et al., 2013). Serum and spinal cord tissues from each pup were collected 24 h after LPS injection, when the LPS-stimulated increase in inflammatory cytokines in the rat brain reached a peak value (Pang et al., 2003). Spinal cord tissues were homogenized by sonication in 1 ml ice-cold PBS (pH 7.2) and centrifuged at 12,000×*g* for 20 min at 4°C. The supernatant was collected, followed by the Bradford method of measuring protein concentration with a 96-well plate reader (Bio-Tek instruments, Inc., VT, USA). After the protein concentration was determined, an ELISA was subsequently performed according to the manufacturer's instructions, and the 96-well plate reader was used to acquire the data. Cytokine and PGE2 contents were expressed as picograms per ml of serum, or picograms per mg protein in the spinal cord. COX-2 content was expressed as picograms per mg protein in the spinal cord. CORT content was expressed as nanogram per ml of serum.

Measurement of lipid peroxidation

Lipid peroxidation was determined in spinal cord samples by measuring malondialdehyde (MDA) levels as thiobarbituric acid-reactive substances (TBARS) (Rodrigues et al., 2013; Rosa et al., 2015; Vasconcelos et al., 2015). Briefly, the spinal tissues were homogenized in cell extraction buffer (~5 mg/200 μl) containing 2 μl of 5% BHT (Butylated hydroxytoluene) on ice, and precipitated proteins were removed by centrifugation at 12,000g for 10 minutes. 200 μl of brain supernatant was mixed with 300 μl 20% trichloroacetic acid (TCA) and incubated for 1 minute. Then 300 μl 0.67% thiobarbituric acid (TBA) was added to the mixture. The reaction was heated to 100 ºC for 60 minutes. After cooling, the mixture was centrifuged at 12,000g for 10 minutes, and the absorbance of the supernatant was determined. The number of resulting pink-stained TBARS were determined spectrophotometrically at 532 nm in a 96-well plate reader (μQuant, Bio-Tek instruments Inc., VT, USA). A calibration curve was generated using 1,1,3,3-tetramethoxypropane (malondialdehyde, MDA, ACROS Organic) as the standard, which was subjected to the

same treatment as the samples. The results were expressed as nanomoles of TBARS (MDA equivalents) per milligram of protein (nmol MDA / mg protein).

Data analysis and statistics

Spinal cord sections at lumbar L4-L5 levels were selected for the quantification of total/mature oligodendrocytes, microglia, astrocytes, IL-1β expressing cells, and COX-2 expressing cells to determine changes caused by systemic LPS injection. Immunostaining data were quantified by counting of positively stained cells. In circumstances where separation between cellular boundaries was not clearly marked, the numbers of DAPI-stained nuclei from the superimposed images were included among the cell number counts. Three digital microscopic images were randomly captured in each of the three sections and the number of positively stained cells in the three images was counted and averaged (cells/mm^2). These averaged cell counts from the three spinal cord sections was used to represent a single spinal cord. Results were standardized as the average number of cells/mm^2 to facilitate statistical comparisons among the treatment groups.

All data obtained from body weight measurement, immunostaining, and ELISA assay were presented as the mean ± SEM and analyzed by two-way ANOVA followed by the Student-Newman-Keuls test. Data from the von Frey filament test and tail flick test were analyzed by Kruskal-Wallis one-way-ANOVA on ranks, followed by Dunn's test. Results with a $p < 0.05$ were considered statistically significant.

Results

IL-1ra reduced systemic LPS-induced body weight loss, mechanical allodynia and thermal hyperalgesia

The difference between the male and female rats within the same treatment group at P6 was not significant. Therefore, data from rats of both sexes were combined and presented here. Similar to our previous study, systemic LPS injection in P5 rats resulted in a lower body weight at P6 in the LPS group as compared with the control group ($p < 0.001$) (Figure 1A). IL-1ra treatment significantly reduced systemic LPS-induced weight reduction in the P6 rat ($p < 0.001$) (Figure 1A).

The effect of systemic LPS injection on mechanical allodynia was investigated using the von Frey filament test. Systemic LPS injection significantly decreased the pain threshold at P6 in the LPS group as compared with the control group ($p < 0.001$) (Figure 1B). IL-1ra treatment significantly reduced systemic LPS-induced mechanical allodynia in the P6 rat ($p < 0.001$) (Figure 1B).

Thermal hyperalgesia in the neonatal rats was assessed by the tail-flick test, which presents with decreased removal latency (i.e., an enhanced response to stimuli). The LPS group showed a significant decrease in mean latency time in the tail-flick test compared with the control group ($p < 0.001$) with different thermal stimulation (Figure 1C, intensity = 4; and Figure 1D, intensity = 5). Treatment with IL-1ra effectively reduced systemic LPS-induced pain hypersensitivity in neonatal rats ($p < 0.001$) (Figures 1C and 1D).

IL-1ra reduced systemic LPS-induced increases in the number of oligodendrocytes in the spinal cord

Oligodendrocytes are responsible for myelination in the CNS (Traiffort et al., 2016). We characterized the total oligodendrocytes in the spinal dorsal horn and mature oligodendrocytes in the spinal white matter by oligodendrocyte lineage transcription factor 2 (Olig2+) and adenomatous polyposis coli colon CC1 (APC-CC1+) immunofluorescence staining in the lumbar spinal cord, respectively. Olig2+ cells were widespread in the spinal cord whereas APC-CC1+ mature oligodendrocytes were mainly found in the white matter of P6 rats. The scheme used NeuN (neurons, green) and DAPI (nuclei, blue) double staining sections (Figure 2I) to show representative images of regions with the most Olig2+ (Figure 2I, yellow box, Figures 2A–2D) or APC-CC1+ (Figure 2I, green box, Figures 2E–2H).

The Olig2+ (Figure 2J) or APC-CC1+ cells (Figure 2K) were counted in randomly captured images across different spinal cord areas. LPS exposure induced the increases of both total oligodendrocytes ($p < 0.001$) (Figure 2C) and mature oligodendrocytes ($p < 0.001$) (Figure 2G) in the spinal cord of P6 rats. IL-1ra treatment attenuated LPS-induced increases in total oligodendrocytes ($p < 0.001$) (Figure 2D) and mature oligodendrocytes ($p < 0.001$) (Figure 2H).

IL-1ra reduced systemic LPS-induced increases in microglia activation and inflammatory responses in the spinal cord

The effects of systemic LPS exposure on the spinal microglia, as indicated by ionized calcium binding adapter molecule 1 (Iba1+) immunofluorescence staining, were determined in the P6 rat lumbar spinal cord sections between L4 and L5 (Figure 3). In the control spinal cord, a few Iba1+ cells were identified, mostly in a resting state with a ramified shape (white arrow in Figure 3E). The LPS treatment significantly increased microgliosis as represented by the increased numbers of activated microglia ($p < 0.001$) (Figures 3C, 3G and 3I) and their morphological activation with bright staining of enlarged cell bodies and blunt process (magnified image of the arrow indicating Iba1+ cell is shown in the insert in Figure 3G). IL-1ra treatment attenuated the LPS-induced increases in the number of activated microglia in the spinal dorsal horn of P6 rats ($p < 0.001$) (Figures 3D, 3H and 3I).

The effect of LPS exposure on the expression of IL-1β cells in the spinal dorsal horn of the P6 rats was evaluated by IL-1β antibody immunofluorescence staining (Figure 4). Systemic exposure to LPS resulted in the significant increase of IL-1β expressing cells (IL-1β+) ($p < 0.001$) (Figures 4C, 4E and 4H). IL-1ra treatment attenuated the LPS-induced increased numbers of IL-1β expressing cells (Figures 4D and 4H). Images double-labeled with Iba1 showed the vast majority of IL-1β expressing (IL-1β+) cells in the dorsal horn of the LPS group were activated microglia (Iba1+) ($p < 0.001$) (Figures 4E–4G, and 4I). IL-1ra treatment also reduced the LPS-induced increase in numbers of activated IL-1β expressing microglia (Iba1/IL-1β+) in the spinal cord of the P6 rats ($p < 0.001$) (Figure 4I).

The effects of systemic exposure to LPS on inflammatory cytokine expression in the serum and spinal cord of the P6 rats are shown in Figure 5. Twenty-four hours (P6) after LPS injection, the concentrations of IL-1β, IL-6 and TNF-α in the serum of the LPS group were

dramatically increased compared to those in the control group (Figure 5A). IL-1ra treatment attenuated an LPS-induced increase in the concentrations of IL-1β, IL-6 and TNF-α in the serum of P6 rats ($p < 0.001$). The concentration of IL-1β in the spinal cords of the LPS group was also significantly increased compared to those in the control group ($p < 0.001$). IL-1ra treatment attenuated an LPS-induced increase in the concentrations of IL-1β in the spinal cord of P6 rats ($p < 0.001$) (Figure 5B).

IL-1ra reduced systemic LPS-induced increases in astrocyte activation, COX-2 and PGE2 expression in the spinal cord, and CORT and PGE2 in the serum

The effects of systemic LPS exposure on spinal astrocyte activation were investigated using glial fibrillary acidic protein (GFAP+) immunofluorescence staining in the spinal cord of the P6 rats (Figure 6). Most astrocytes were in a resting state with fine processes extending from the main cellular processes (white arrows indicated in Figure 6E) in the Saline+Saline group. Twenty-four hours following injection, the spinal cords of the LPS group showed increased numbers of activated astrocytes (GFAP+ cells) and their cellular processes showed signs of hypertrophy (white arrows indicated in Figure 6G), which is an indication of astrogliosis ($p < 0.001$) (Figures 6C, 6G and 6I). IL-1ra treatment reduced the LPS-induced increase in the number of activated astrocytes in the P6 spinal cord ($p < 0.001$) (Figures 6D, 6H, and 6I).

The effects of LPS exposure on COX-2 expressing cells in the P6 spinal cord was evaluated by COX-2 immunofluorescence staining (Figure 7). Twenty-four hours after injection, LPS treatment resulted in the significant increase of COX-2 expressing cells (COX-2+) ($p < 0.001$) (Figures 7C and 7H). IL-1ra attenuated the LPS-induced increased numbers of COX-2+ cells (Figures 7D and 7H). Double-labeling with GFAP showed that the majority of activated astrocytes (GFAP+) were COX-2 expressing cells (COX-2+) in the spinal cord of the LPS-injected rat ($p < 0.001$) (Figures 7E–7G, and 7I). IL-1ra treatment also reduced the LPS-induced increased numbers of activated COX-2 expressing astrocytes (GFAP+/COX-2+) in the P6 rat spinal cord ($p < 0.001$) (Figure 7I).

The effects of systemic exposure to LPS on the expression of PGE2, COX-2 and CORT in the serum and spinal cord are shown in Figure 8. Twenty-four hours after injection, the concentration of COX-2 in the spinal cord of the LPS group was significantly increased compared to those in the control group ($p < 0.001$) (Figure 8A). IL-1ra treatment attenuated LPS-induced elevated levels of COX-2 in the spinal cord of P6 rats ($p < 0.001$) (Figure 8A). The concentrations of CORT and PGE2 in the serum of the LPS group was significantly increased compared to those in the control group ($p < 0.001$) (Figures 8B and 8C). IL-1ra treatment attenuated LPS-induced elevated levels of CORT and PGE2 in the serum of P6 rats ($p < 0.001$) (Figures 8B and 8C). LPS exposure also resulted in elevated levels of PGE2 in the P6 LPS group compared to those in the control group ($p < 0.001$) (Figure 8D). IL-1ra treatment also attenuated LPS-induced increase in elevated levels of PGE2 in the spinal cord of P6 rats ($p < 0.001$) (Figure 8D).

IL-1ra reduced systemic LPS-induced increases in thiobarbituric acid-reactive substances (TBARS) content in the spinal cord

To investigate the effects of LPS and/or IL-1ra administration on oxidative stress, lipid peroxidation was assessed in spinal cord tissues by measuring thiobarbituric acid-reactive substances (TBARS), as previously established (Rodrigues et al., 2013; Rosa et al., 2015; Vasconcelos et al., 2015). Twenty-four hours following neonatal LPS injection, the TBARS content in the spinal cord of the LPS group was remarkably elevated compared with the control group ($p < 0.001$) (Figure 9). IL-1ra treatment reduced LPS-induced increases in the TBARS content in the spinal cord of P6 rats ($p < 0.001$) (Figure 9).

Discussion

Inflammation-induced perinatal pain facilitation alters pain sensitivity and results in hyperalgesia or allodynia, which may permanently affect the immature nervous system and persist throughout adulthood (Grunau, 2013; Schwaller and Fitzgerald, 2014; Walker, 2014). Illness-induced pain facilitation, such as hyperalgesia or allodynia, is one of the common aspects of inflammatory-related pain (Schwaller and Fitzgerald, 2014). Experimental data showed that LPS-induced hyperalgesia was mediated by various inflammatory cytokines such as IL-1β, IL-6 and TNF-α that largely depended on dosing and timing of LPS injection which mimic systemic inflammation or sepsis-like stimulation in clinical data (Hsieh et al., 2018; Sabedra Sousa et al., 2018; Schwaller and Fitzgerald, 2014; Vega-Avelaira et al., 2013). Exploring the most effective inflammatory mediator in the neonatal LPS-induced inflammation animal model would be helpful for discovering therapeutic agents and preventing inflammation-induced perinatal pain facilitation that may cause other pain complications later in life. Recently, various pharmacological agents including minocycline, COX-2 inhibitors, alpha-(phenylselanyl) acetophenone or green tea extract have been used to delineate the possible underlying mechanism involved in LPS-induced hyperalgesia in different nociceptive assays (Hsieh et al., 2018; Padi and Kulkarni, 2005; Sabedra Sousa et al., 2018; Yoon et al., 2012). Our previous study showed that co-administration of IL-1ra reduced i.c. LPS-induced hyperalgesia associated with an increase in IL-1β concentration in the rat brain (Wang et al., 2011). IL-1ra is a member of the IL-1 family that binds to IL-1 receptor with higher affinity than IL-1α or IL-1β but does not induce the intracellular pro-inflammatory response. (Arend et al., 1998; Rosenzweig et al., 2014). IL-1ra is also one of the blood-borne cytokines that has been shown to cross the blood-brain barrier (BBB) via saturable transport systems to enter cerebrospinal fluid and interstitial fluid spaced of the brain and spinal cord (Arend et al., 1998; Banks et al., 1995; Gutierrez et al., 1994). In the present study, we showed that systemic LPS exposure also elevated IL-1β levels in the neonatal rat spinal cord and resulted in thermal hyperalgesia and mechanical allodynia. IL-1ra treatment attenuated the LPS-induced hyperalgesia and allodynia through the blockade of LPS-induced pro-inflammatory cytokine IL-1β in the spinal cord. Based on these studies, IL-1ra may be a potential therapeutic agent in the treatment of LPS-induced hyperalgesia and allodynia resulting from brain and spinal cord inflammation.

Increased IL-1β expression and activation of microglia are commonly used as the hallmarks of spinal cord inflammation (Liu and Quan, 2018). Activation of microglia includes the

increases in cell numbers, hypertrophy in cell bodies and processes, and overexpression of specific immunofluorescence markers such as CD11b and Iba1 (Norden et al., 2016; Tsuda et al., 2005). Many studies showed that LPS exposure resulted in enriched microglia activation and the robust overexpressing of inflammatory cytokines including IL-1β and TNF-α in mRNA and protein levels in the brain, contributing to hyperalgesia and allodynia (Norden et al., 2016; Wang et al., 2011). Spinal microglia activation also resulted in tactile allodynia through the activation of p38 MAPK and purinergic P2 receptors, especially subtype P2X4 or P2X7 (Clark et al., 2010; Tsuda et al., 2004; Tsuda et al., 2003). In P2X7 knock-out mice with LPS exposure, the enhanced nociceptive transmission was strongly associated with the release of IL-1β by activated microglia in the dorsal horn of the spinal cord following activation of TLR-4 through the activation of P2X7 receptors of the spinal microglia (Clark et al., 2010). In our present study, double labeling showed that systemic LPS exposure resulted in an increase in activated microglia that also highly expressed IL-1β (IL-1β+/Iba+). IL-1ra treatment reduced systemic LPS-induced spinal cord inflammation through ameliorating increased numbers of activated microglia that were expressing IL-1β (Figure 4). IL-6 plays a crucial role in ongoing immune and inflammatory responses that are initially induced by IL-1β. Cross-reaction between IL-1β and IL-6 has been reported. IL-1β is a potent inducer of IL-6 in peripheral blood monocytes, and the production of IL-6 may be mediated by the pathway of PI3K-dependent AKT/IκB kinase targeting AP-1 (Cahill and Rogers, 2008; Tosato and Jones, 1990). Therefore, the reduced systemic level of IL-6 appeared to result from the blocking of macrophage activation in the blood stream since both minocycline (Hsieh et al., 2018) and IL-1ra (Figure 5) showed similar levels of attenuation. The modest effect of IL-1ra on IL-1β attenuation seems to be affected by the IL-1 positive feedback loop.

Apart from activated microglia, the other major glial cells, both astrocytes and oligodendrocytes, play active roles in propagating, regulating or maintaining neuroinflammation (Ji et al., 2013; Liu and Quan, 2018; Traiffort et al., 2016; Zaghloul and Ahmed, 2017). Neuron-glial and glial-glial interaction are emerging as key mechanisms involved with pain facilitation (Ji et al., 2013). Systemic LPS exposure stimulated spinal glial activation that are identified by immune-activities of various specific markers such as Iba1, ED1, P2X4 receptor, endothelial monocyte activating polypeptide II and GFAP (Guo and Schluesener, 2006; Hsieh et al., 2018; Yoon et al., 2012). IL-1 receptor type 1, a functional IL-1 receptor, is expressed in the astrocytes of the dorsal horn of carrageenan-injected rats (Choi et al., 2015). Spinal astrogliosis may not only be induced by LPS through the TLR-4 pathway like microgliosis, but also can be affected by IL-1β released from activated microglia (Choi et al., 2015; Liu et al., 2016; Norden et al., 2016). Further, other pro-inflammatory mediators such as PGE2 released from activated astrocytes through up-regulation of the COX-2 enzyme propagate pain facilitation (Font-Nieves et al., 2012). Our study showed that systemic LPS exposure resulted in increased numbers of activated microglia (Iba1+) and astrocytes (GFAP+) that also highly expressed IL-1β and COX-2, respectively. The concentration levels of IL-1β, COX-2, and PGE2 were significant elevated in the spinal cord. These above-mentioned inflammatory reactions induced by LPS exposure can be effectively reduced by IL-1ra (Figures 5 and 8).

The oligodendrocyte supports neuron functions and generates myelin that allows fast nerve conduction in the CNS (Traiffort et al., 2016). It seems that the disruption of oligodendrocyte development in the brain at early postnatal ages is closely related to LPS-induced pathology. In our previous studies, early postnatal LPS exposure at P3 led to excessive oligodendrocyte formation and neurogenesis in P6 and P12 rat brains (Fan and Pang, 2017; Pang et al., 2016). In contrast, LPS exposure at P5 resulted in a reduction of oligodendrocyte formation in P6 and P8 rat brains (Fan et al., 2013; Fan et al., 2008; Fan et al., 2005a). Other studies also showed that LPS-induced white matter injury contributed to the loss of oligodendrocyte progenitor cells in the brain (Gritsch et al., 2014; Ji et al., 2013; Zaghloul and Ahmed, 2017). These data suggest that systemic inflammation disrupts oligodendrogenesis in the early postnatal brain leading to either increased or decreased numbers of oligodendrocytes largely depending on which postnatal days LPS is injected. However, the relationship between spinal oligodendrocytes and LPS-induced hyperalgesia and allodynia in early postnatal days has not been addressed in detail.

The current study used Olig2+ to label both premature and mature oligodendrocyte lineage cells and APC-CC1+ to label only mature oligodendrocytes in the P6 rat spinal cord (Figure 2). Interestingly, the most Olig2+ cells were found in the dorsal horn whereas the most APC-CC1+ cells were found in the white matter in all examined groups. Systemic LPS exposure increased the number of both total (Olig2+) and mature oligodendrocytes (APC-CC1+) across the entire spinal cord areas (Figures 2J–K) suggesting that the disrupted oligodendrocyte biology is closely associated with early postnatal pain development in the brain (Fan and Pang, 2017; Pang et al., 2016). This might be related to the potential sprouting of Aδ fibers and their synaptogenesis in the dorsal horn which may require additional oligodendrocytes and their myelination on Aδ fibers. Nociceptive A fibers are known to form synaptic connections earlier and generate stronger excitation than C fibers during the first week after birth (Fitzgerald, 2005). Nociceptive A fibers that express tyrosine kinase receptor (TrK) B and C respond to nerve growth factor (NGF) during the development and undergo axon growth in inflammatory, hypertrophic conditions (Fitzgerald, 2005). Indeed, oligodendrocyte lineage cells produce NGF (Byravan et al., 1994) and LPS treatments stimulate microglia and astrocytes to express NGF (Cheng et al., 2019; Heese et al., 1998). Collectively, the LPS- induced upregulation of IL-1β and gliosis might trigger an excessive expression of nerve growth factor (NGF) that binds tyrosine kinase receptor (TrK) B and C and activates the sprouting of A fibers which will, in turn, strengthen synaptic outcomes in nociceptive reflex pathways resulting in hyperreflexia. The fact that IL-1ra treatment reduced the LPS-induced increase in numbers of microglia, astrocytes, and oligodendrocytes provides indirect evidence that IL-1 is a key mediator for nociceptive afferent sprouting and/or myelination in the dorsal horn. The effect of LPS treatment on spinal oligodendrocyte and specific nociceptive afferent types needs to be further studied.

The systemic effect of LPS treatment on spinal cord inflammation and hypersensitivity is shown in our study similar with other reports (Hsieh et al., 2018; Sabedra Sousa et al., 2018; Vega-Avelaira et al., 2013; Yoon et al., 2012). Peripheral LPS injection can directly activate cells within the CNS by crossing the disruptive BBB (Banks et al., 2015; Varatharaj and Galea, 2017). However, only 0.025% of an intravenously injected dose of LPS was discovered to cross the murine BBB (Banks and Robinson, 2010). Thus, the effect of LPS on

the BBB is not universal based on variable experimental techniques. LPS-induced weight loss in neonatal rats is attributed to reduced intake, diarrhea, the increased substrate utilization or muscle proteolysis resulting from TNF-α induced activation of the ubiquitin-proteosome system (Dehoux et al., 2003; Fernandez-Celemin et al., 2002; Premer et al., 2002). In our present study, LPS-induced weight loss was discovered in P6 neonatal rats and the increased serum concentration of CORT levels indicated LPS-induced stress, which may be influenced by activation of the hypothalamic-pituitary-adrenal axis (Webster and Sternberg, 2004). In addition, many IL-1β, IL-6, and TNF-α receptors are expressed in the cerebral endothelial cells. Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α induced by systemic LPS injection activate the endothelial cells to change the permeability of BBB and trigger further PGE2 expression through the COX-2 enzyme (Varatharaj and Galea, 2017). Previous adult rat studies showed that LPS treatment significantly increased expressions of IL-1β, IL-6 and TNF-α in the rat serum and spinal cord (Yoon et al., 2012). In our neonatal rat study, systemic LPS exposure also dramatically induced the increased expressions of IL-1β, IL-6, TNF-α and PGE2 in the serum. However, only the increased expressions of IL-1β, COX-2 and PGE2 were discovered in the dorsal horn of the P6 rat spinal cord. The immune system and the integrity of BBB involving the systemic LPS-induced spinal cord inflammation in our neonatal rat model should be further studied.

It has been reported that redox imbalance and reactive oxygen species (ROS) play critical roles in the pathogenesis of chronic neuropathic pain (Lv et al., 2016; Zhao et al., 2016). Further damage from ROS molecules to dorsal lamina neurons leads to membrane excitability, which physiologically correlates with chronic pain (Hassler et al., 2014). Our present data show that LPS exposure increased TBARS along with pain sensitivity and IL-1ra treatment reduced LPS-induced TBARS in the spinal cord along with pain sensitivity (Figure 9). Previous studies have shown that cytokines alter the redox equilibrium by affecting GSH/GSSG shuttling and recycling (Fan et al., 2009; Fan et al., 2008; Rodrigues et al., 2013; Rosales-Corral et al., 2010). Additionally, neonatal LPS treatment induces alterations in glutathione homeostasis in the rat brain (Fan et al., 2008). Protection of anti-oxidant PBN was linked with attenuated oxidative stress induced by LPS or IL-1β, as indicated by decreased elevation of 8-isoprostane contents and by the reduced number of 4-hydroxynonenal or malondialdehyde or nitrotyrosine-positive cells following LPS or IL-1β exposure in neonatal rats (Fan et al., 2009; Fan et al., 2008). Overall, the data suggest that inflammatory events and oxidative stress occur in a synchronized manner by changing the redox environment, and that compounds with anti-inflammatory and anti-oxidant properties could be used as adjuvant therapy for some neurodegenerative disorders (Fan et al., 2009; Fan et al., 2008; Rodrigues et al., 2013).

Inflammation-induced activated glial cells may cause central plasticity on nociceptive afferents giving rise to central sensitization, ectopic firing, axon sprouting and synaptogenesis (Woolf and Salter, 2000), and second neurons to become overactive and excessively stimulated in the spinal cord dorsal horn, which results in the enhancement of pain hypersensitivity (Hsieh et al., 2018). Our present study demonstrated that systemic LPS treatment induced thermal hyperalgesia and mechanical allodynia through the increased numbers of inflammatory cytokines and activated glial cells in the dorsal horn of the rat spinal cord. Collectively, systemic treatment with IL-1ra immediately after inflammatory

stimulation successfully blocked positive feedback in the glia-IL-1β loop, significantly reduced expressions of inflammatory molecules and oxidative stress molecules, and ameliorated the development of pain hypersensitivity. Indeed, two drugs, minocycline (Hsieh et al., 2018) and IL-1ra, demonstrated very similar anti-inflammatory, anti-nociceptive effects on the inflamed neonatal spinal cord. However, their actions are attributed to different mechanisms: blocking activation (i.e. M1 polarization) of microglia by minocycline (Kobayashi et al., 2013) and modulating of the pro-inflammatory IL-1β pathway by IL-1ra (Arend et al., 1998). There was a tendency that IL-1ra treatments showed more profound attenuation of IL-1β and COX-2 in the spinal cord than minocycline. The former may be a compensatory overexpression of IL-1β in microglia due to the canceling of the IL-1 positive feedback loop as illustrated in our working model (Figure 11). The latter appears to be a result of blocking IL-1 receptor in astrocytes which has been shown to be mediated by the PKC pathway (Molina-Holgado et al., 2000). Moreover, albeit the modest attenuation of IL-1β, IL-1ra treatments in the current studies have almost completely reversed cellular (oligodendrocyte, microglia), biochemical (IL-6, COX-2, PGE2, CORT, TBARS), and behavioral (body weight, thermal hyperalgesia) changes in response to LPS-induced inflammation. This suggests that IL-1ra at the dose of 100 mg/kg may have completely or, at least, very efficiently blocked the pro-inflammatory IL-1 pathway of which activation is necessary for those inflammatory outcomes. Due to the lack of statistical comparison between two different sets of experiments and the missing analysis of oligodendrocyte and lipid peroxidation in the previous publication, however, we would not able to conclusively comment on which drug is better or worse. Considering that minocycline is a tetracycline-derived antibiotic and IL-1ra is a peptide analog, they may have to be tested for the safe and efficacious clinical use in newborn babies.

Conclusion

In summary, our data demonstrated that systemic LPS exposure resulted in extensive inflammatory responses in the early postnatal spinal cord and pain hypersensitivity including thermal hyperalgesia and mechanical allodynia. The inflammatory responses involved the activated gliosis of microglia and astrocytes through the expressions of IL-1β, COX-2 and PGE2, which induced a positive feedback loop. LPS exposure also induced ROS and related downstream molecules to further produce lipid peroxidation on cell membranes. In addition, LPS exposure resulted in upregulation of oligodendrocyte lineage cells, which may contribute to growth factor-induced sprouting. IL-1ra treatments significantly reduced LPS-induced molecular/cellular inflammatory responses and, as a result, ameliorated the development of thermal hyperalgesia and mechanical allodynia (Figure 10). These findings suggest that IL-1β is an effective mediator of spinal cord inflammation and oxidative stress during early postnatal pain facilitation, and blocking IL-1β with IL-1ra could be a potential therapy for pain hypersensitivity development during early life or later in life.

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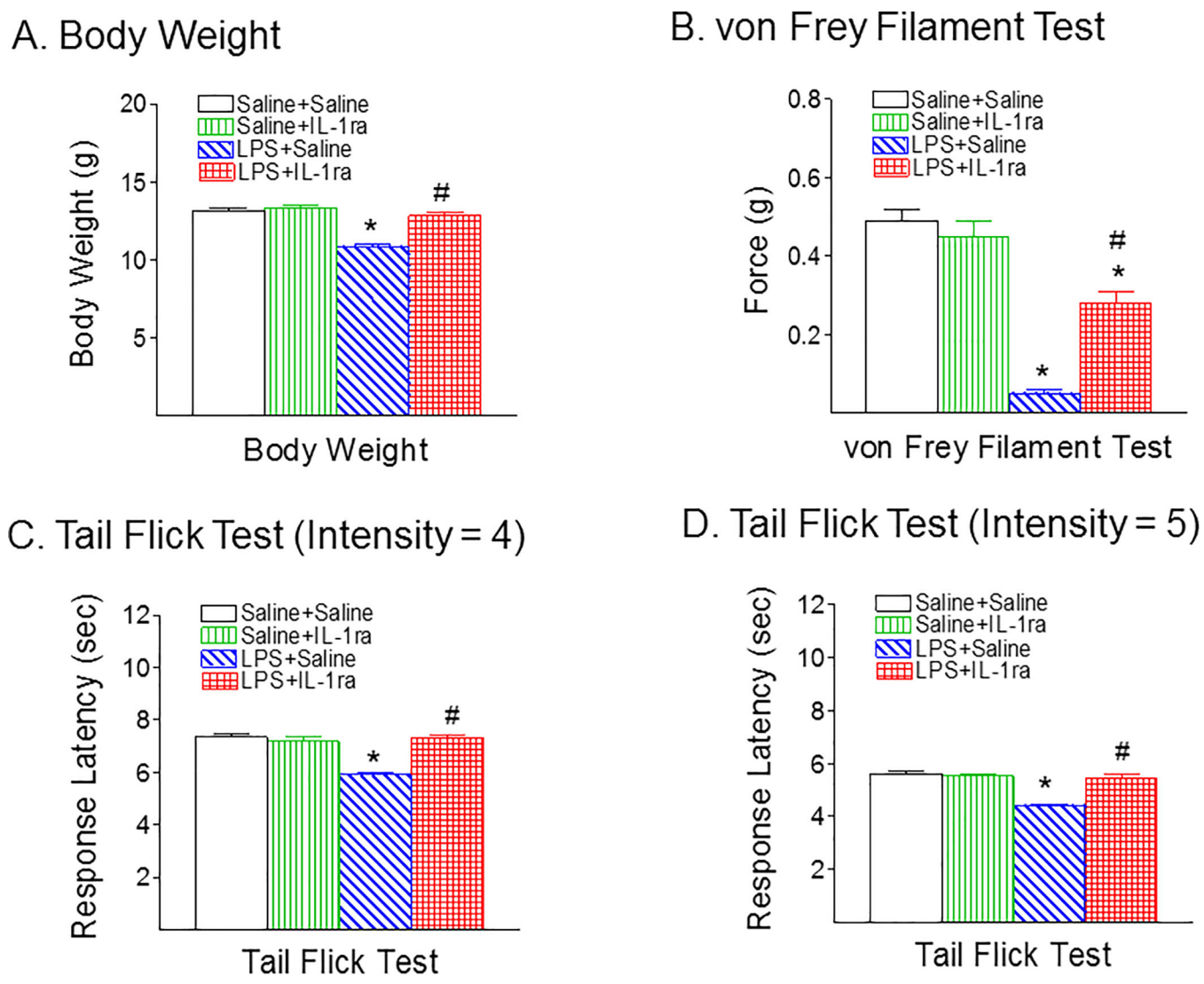

Figure 1.

IL-1ra attenuated systemic LPS-induced body weight loss (A), allodynia in the von Frey filament test (B), and hyperalgesia in the tail flick test (C, intensity = 4; D, intensity = 5) in neonatal rats (P6). A, Systemic LPS treatment (P5) resulted in body weight loss in the P6 rat. Treatment with IL-1ra significantly reduced LPS-induced body weight loss in the P6 rats. B, Systemic LPS treatment (P5) resulted in the reduction of withdrawal threshold (g force) for hind paw removal from a mechanical stimulation in the P6 rat. Treatment with IL-1ra significantly reduced LPS-induced pain hypersensitivity in the neonatal rats. C & D, Systemic LPS treatment (P5) resulted in reduction of mean latency times (sec) in tail removal from a thermal stimulation (C, intensity = 4; D, intensity = 5) in the P6 rat. Treatment with IL-1ra significantly reduced LPS-induced pain hypersensitivity in the neonatal rats. The results are expressed as the mean ± SEM of sixteen animals in each group. The results from body weight were analyzed by two-way ANOVA, followed by the Student-Newman-Keuls test. The results from the von Frey filament test and tail flick test were analyzed by Kruskal-Wallis one-way-ANOVA on ranks, followed by the Dunn's test.

*P < 0.05 represents significant difference for the LPS+Saline group, or LPS+IL-1ra group compared with the Saline+Saline group. #P < 0.05 represents significant difference for the LPS+IL-1ra group compared with the LPS+Saline group. (N =16)

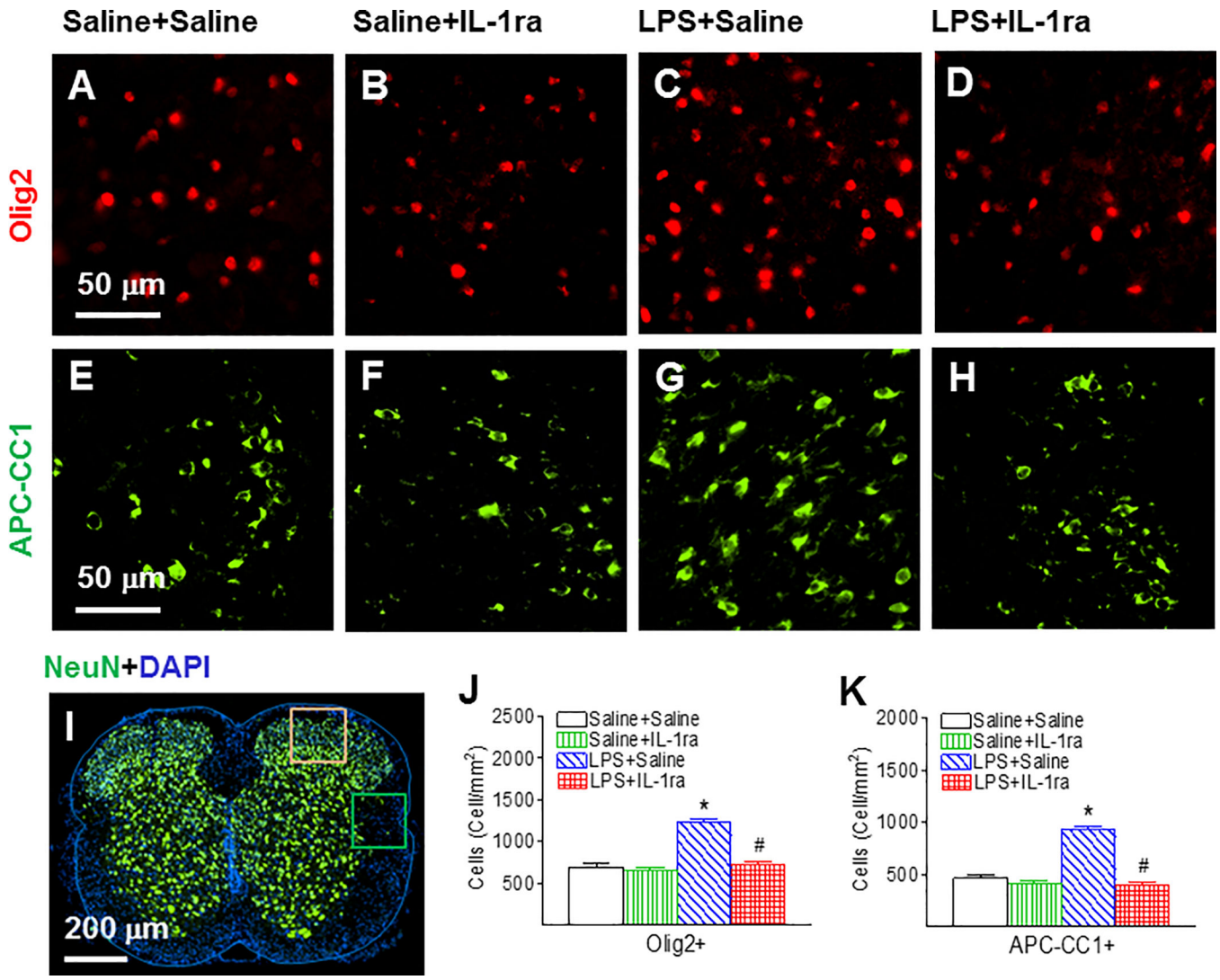

Figure 2.

IL-1ra reduced neonatal LPS-induced increases in number of oligodendrocytes, as assessed by Olig2+ staining (total oligodendrocytes) and APC-CC1+ staining (mature oligodendrocytes) in the spinal cord of the P6 rat. The scheme used NeuN (neurons, green) and DAPI (nuclei, blue) double staining section (I) presenting an overview of the detected region: yellow box for Olig2, A-D; green box for APC-CC1, E-H. Representative photomicrographs of Olig2 immunostaining (A-D, red) and APC-CC1 (E-H, green) in the rat spinal cord 24 hours (P6) after LPS injection. Oligodendrocytes were determined cross the spinal cord of all groups (A-H). LPS exposure induced the increase of numerous total oligodendrocytes (C) and mature oligodendrocytes (G). IL-1ra attenuated LPS-induced increase in total oligodendrocytes (D) and mature oligodendrocytes (H). Total oligodendrocytes (J) and the mature oligodendrocytes (K) were quantified by counting the number of Olig2+ or APC-CC1+ cells throughout the spinal cord, respectively. The results are expressed as the mean ± SEM of eight animals in each group and analyzed by two-way ANOVA, followed by the Student-Newman-Keuls test. *P < 0.05 represents significant

difference for the LPS+Saline group compared with the Saline+Saline group. $^{\#}P < 0.05$ represents significant difference for the LPS+IL-1ra group compared with the LPS+Saline group. (N=8)

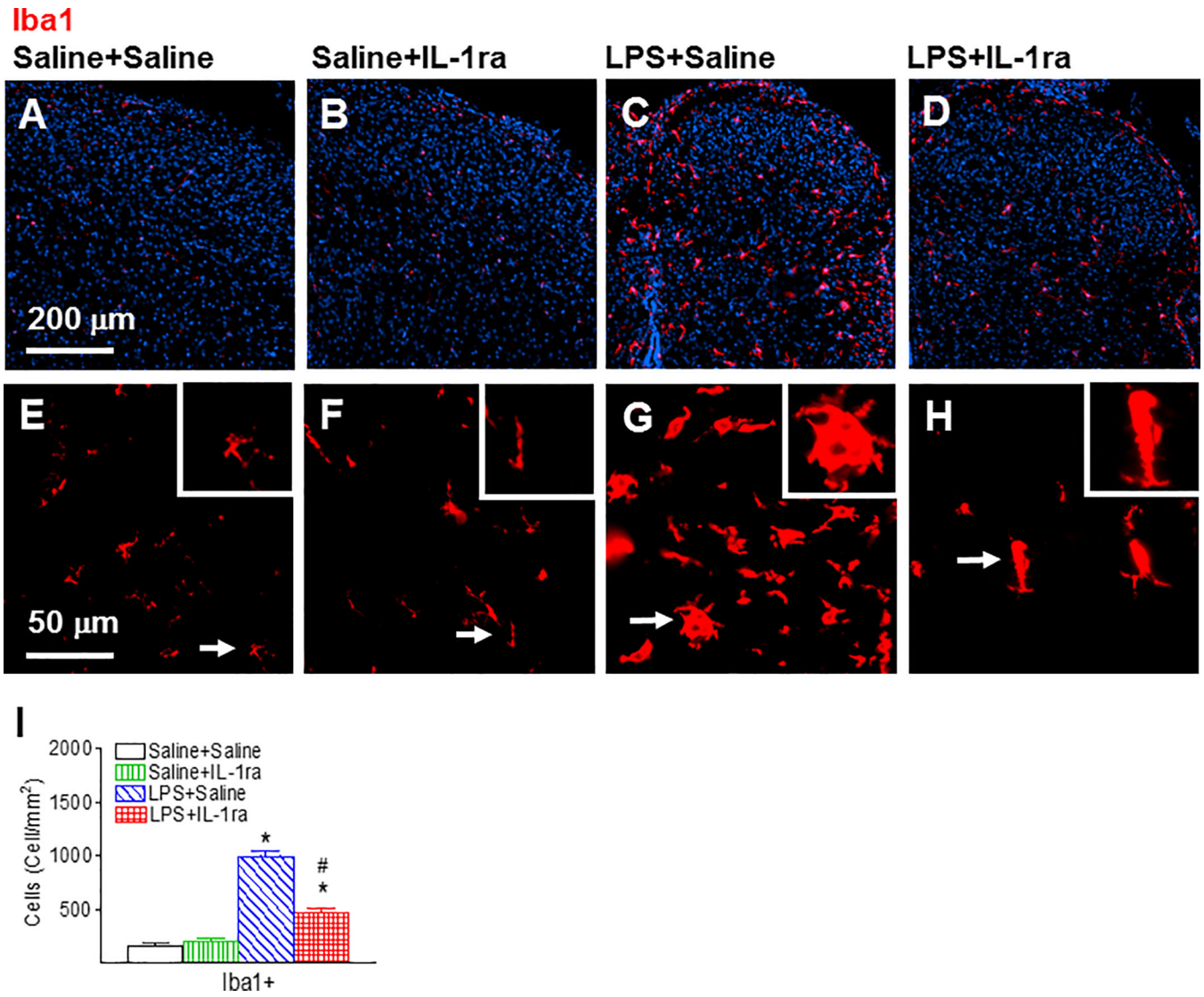

Figure 3.

IL-1ra reduced neonatal LPS-induced microglia activation, as assessed by Iba1+ staining in the spinal dorsal horn of the P6 rat. Representative photomicrographs of Iba1 immunostaining (A-H, red) in the rat spinal cord 24 hours (P6) after LPS injection. DAPI (blue) was used simultaneously to identify nuclei in the final visualization (A-D). Most microglia were in a resting state with a ramified shape (white arrow indicated in E) in the rat spinal dorsal horn of the Saline+Saline group (A&E). LPS exposure induced the increase of numerous activated microglia with enlarged cell bodies and blunt processes (C&G, a magnified image of the arrow indicated Iba1+ cell is shown in the insert in G). IL-1ra attenuated LPS-induced increase in activated microglia (D&H). Microglia activation was quantified by counting the number of Iba1+ cells in the spinal dorsal horn (I). The results are expressed as the mean ± SEM of eight animals in each group and analyzed by two-way ANOVA, followed by the Student-Newman-Keuls test. *P < 0.05 represents significant difference for the LPS+Saline group, or LPS+IL-1ra group compared with the Saline +

Saline group. #P < 0.05 represents significant difference for the LPS+IL-1ra group compared with the LPS+Saline group. (N=8)

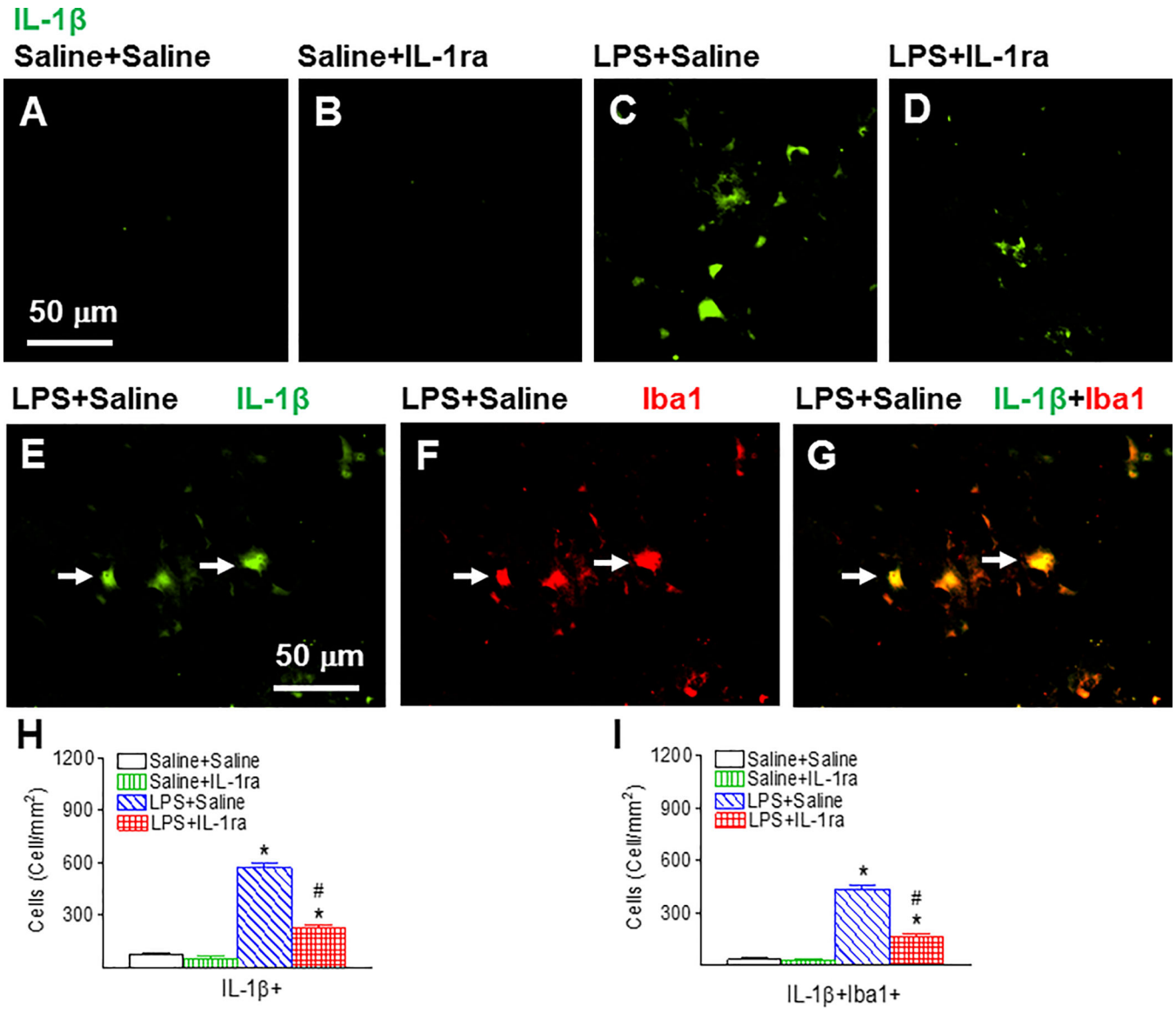

Figure 4.

IL-1ra reduced neonatal LPS-induced IL-1β expressing cells in the spinal dorsal horn of the P6 rat. LPS exposure induced the increase of IL-1β expressing cells (C). IL-1ra attenuated LPS-induced increase in IL-1β expressing cells (D). IL-1β expression was quantified by counting the number of IL-1β+ cells in the spinal dorsal horn (H). Double-labeling showed that many Iba1+ activated microglia in the spinal dorsal horn (F, red) of the LPS-injected rat spinal cord were IL-1β expressing cells (E, green). G (yellow) is a merged image of E and F. IL-1β activation was quantified by counting the number of IL-1β+ cells (H), and Iba1+ microglia with IL-1β expression was quantified by counting the number of Iba1/IL-1β+ cells in the spinal dorsal horn (I). The results are expressed as the mean ± SEM of eight animals in each group and analyzed by two-way ANOVA, followed by the Student-Newman-Keuls test. *P < 0.05 represents significant difference for the LPS+Saline group, or LPS+IL-1ra

group compared with the Saline+Saline group. $^{\#}P < 0.05$ represents significant difference for the LPS+IL-1ra group compared with the LPS+Saline group. (N=8)

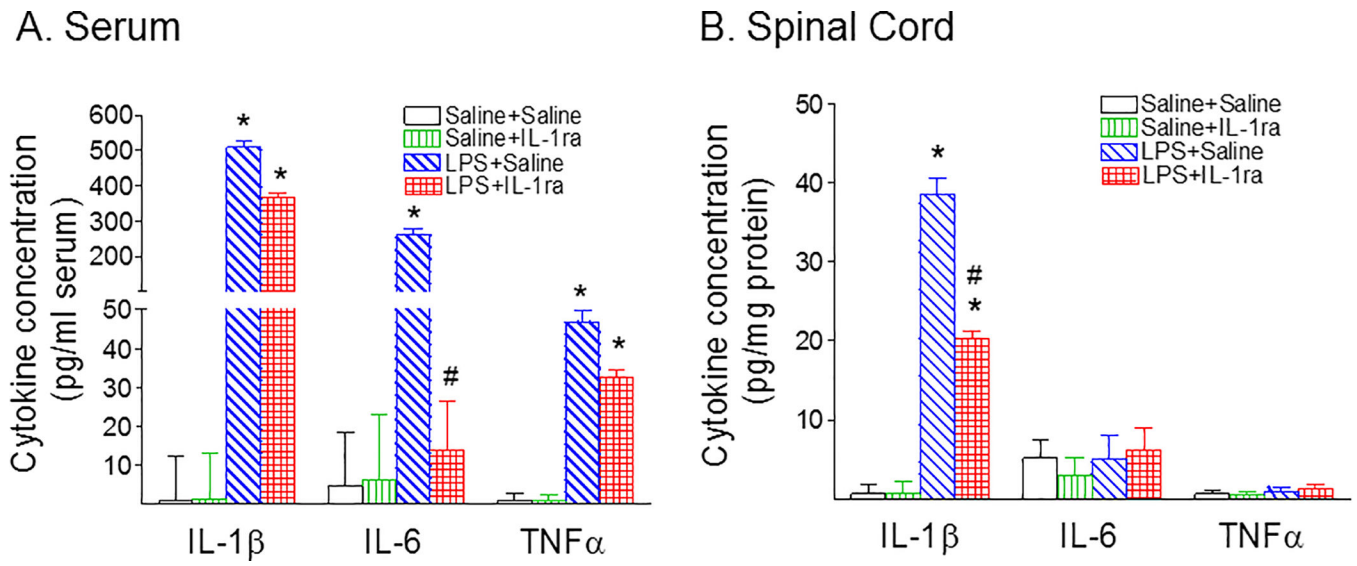

Figure 5.

IL-1ra attenuated systemic LPS exposure-induced increases in inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the rat serum (A) and spinal cord (B) 24 hours (P6) after LPS injection. A, The concentration levels of IL-1β, IL-6 and TNF-α 24 hours (P6) following LPS injection in the serum were elevated compared with the control group. IL-1ra attenuated LPS-induced increases in the concentration levels of IL-6 in the serum of P6 rats. B, Following LPS injection, the concentration of IL-1β in the spinal cord was elevated compared with the control group in the P6 rat. IL-1ra attenuated LPS-induced increase in the concentration levels of IL-1β in the spinal cord of P6 rats. The results are expressed as the mean ± SEM of eight animals in each group and analyzed by two-way ANOVA, followed by the Student-Newman-Keuls test. *$P < 0.05$ represents significant difference for the LPS +Saline group, or LPS+IL-1ra group compared with the Saline+Saline group. $^{\#}P < 0.05$ represents significant difference for the LPS+IL-1ra group compared with the LPS+Saline group. (N=8)

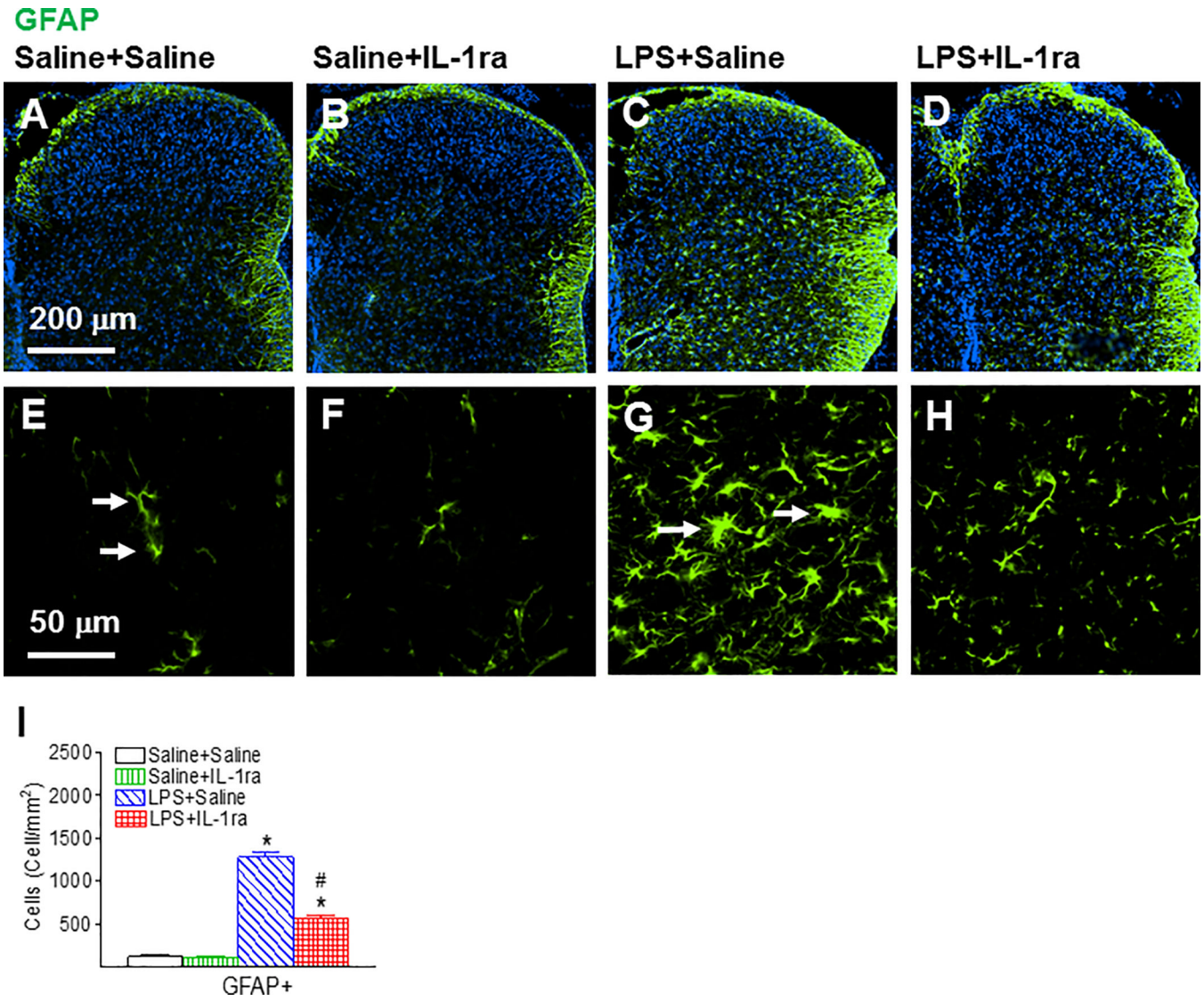

Figure 6.

IL-1ra reduced neonatal LPS-induced astrocyte activation, as assessed by GFAP+ staining in the spinal dorsal horn of the P6 rat. Representative photomicrographs of GFAP immunostaining (A-H, green) in the rat spinal cord 24 hours (P6) after LPS injection. DAPI (blue) was used simultaneously to identify nuclei in the final visualization (A-D). Most astrocytes were in a resting state with fine processes extending from the main cellular processes (arrows indicated in E) in the rat spinal dorsal horn of the Saline+Saline group (A&E). LPS exposure induced the increase of numerous activated astrocytes with hypertrophy of cellular processes (C&G, arrows indicated in G). IL-1ra attenuated LPS-induced increase in activated astrocytes (D&H). Astrocyte activation was quantified by counting the number of GFAP+ cells in the spinal dorsal horn (I). The results are expressed as the mean ± SEM of eight animals in each group and analyzed by two-way ANOVA, followed by the Student-Newman-Keuls test. *P < 0.05 represents significant difference for the LPS+Saline group, or LPS+IL-1ra group compared with the Saline+Saline group. $^{\#}$P <

0.05 represents significant difference for the LPS+IL-1ra group compared with the LPS +Saline group. (N=8)

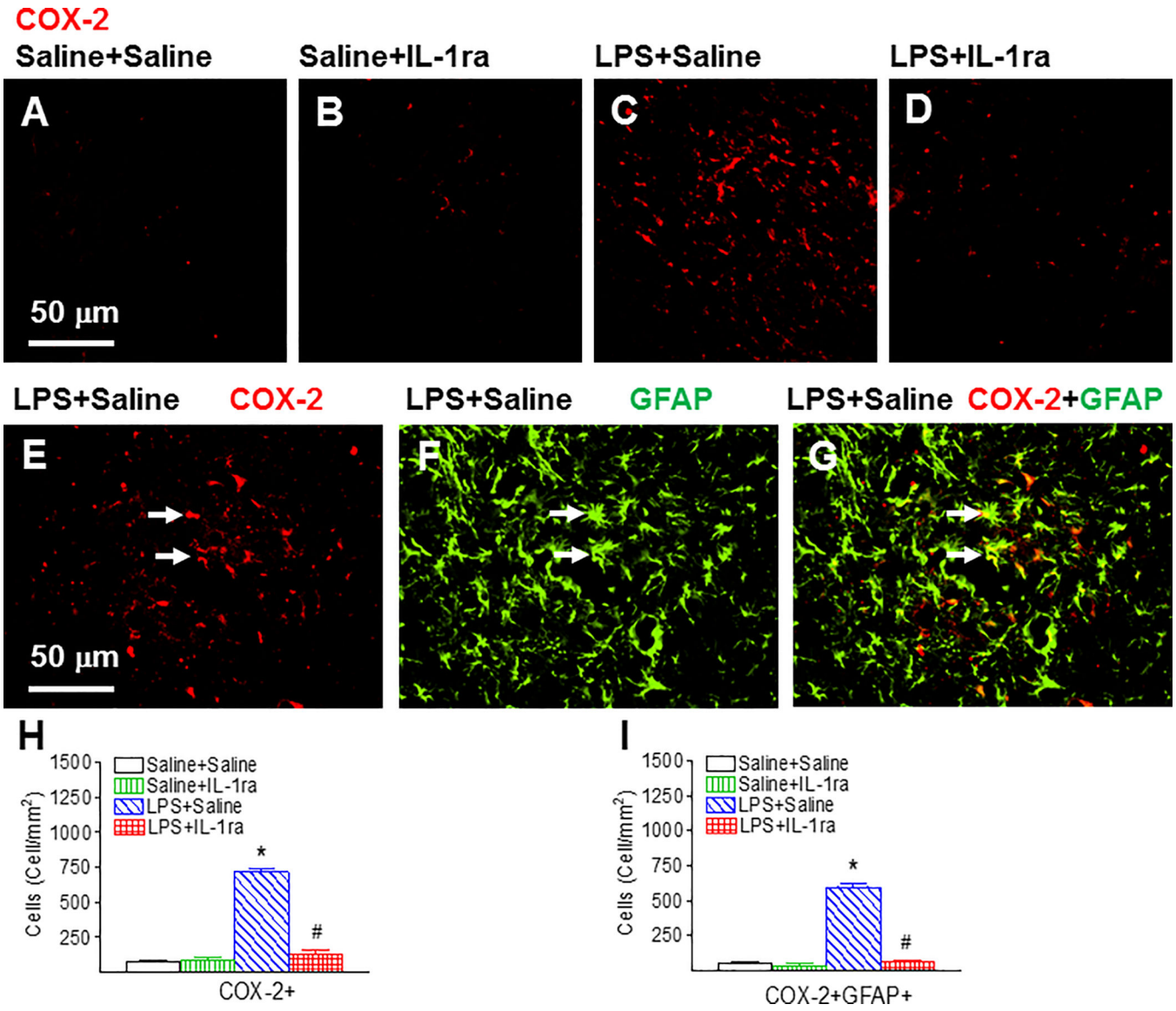

Figure 7.

IL-1ra reduced neonatal LPS-induced COX-2 expressing cells in the spinal dorsal horn of the P6 rat. LPS exposure induced the increase of COX-2 expressing cells (C). IL-1ra attenuated LPS-induced increase in COX-2 expressing cells (D). Double-labeling showed that many GFAP+ activated astrocytes in the spinal dorsal horn (F, green) of the LPS-injected rat were COX-2 expressing cells (E, red). G (yellow) is a merged image of E and F. COX-2 activation was quantified by counting the number of COX-2+ cells (H), and GFAP+ astrocytes with COX-2 expression was quantified by counting the number of GFAP/COX-2+ cells in the spinal dorsal horn (I). The results are expressed as the mean ± SEM of eight animals in each group and analyzed by two-way ANOVA, followed by the Student-Newman-Keuls test. *$P < 0.05$ represents significant difference for the LPS+Saline group, or LPS+IL-1ra group compared with the Saline+Saline group. $^{\#}P < 0.05$ represents significant difference for the LPS+IL-1ra group compared with the LPS+Saline group. (N=8)

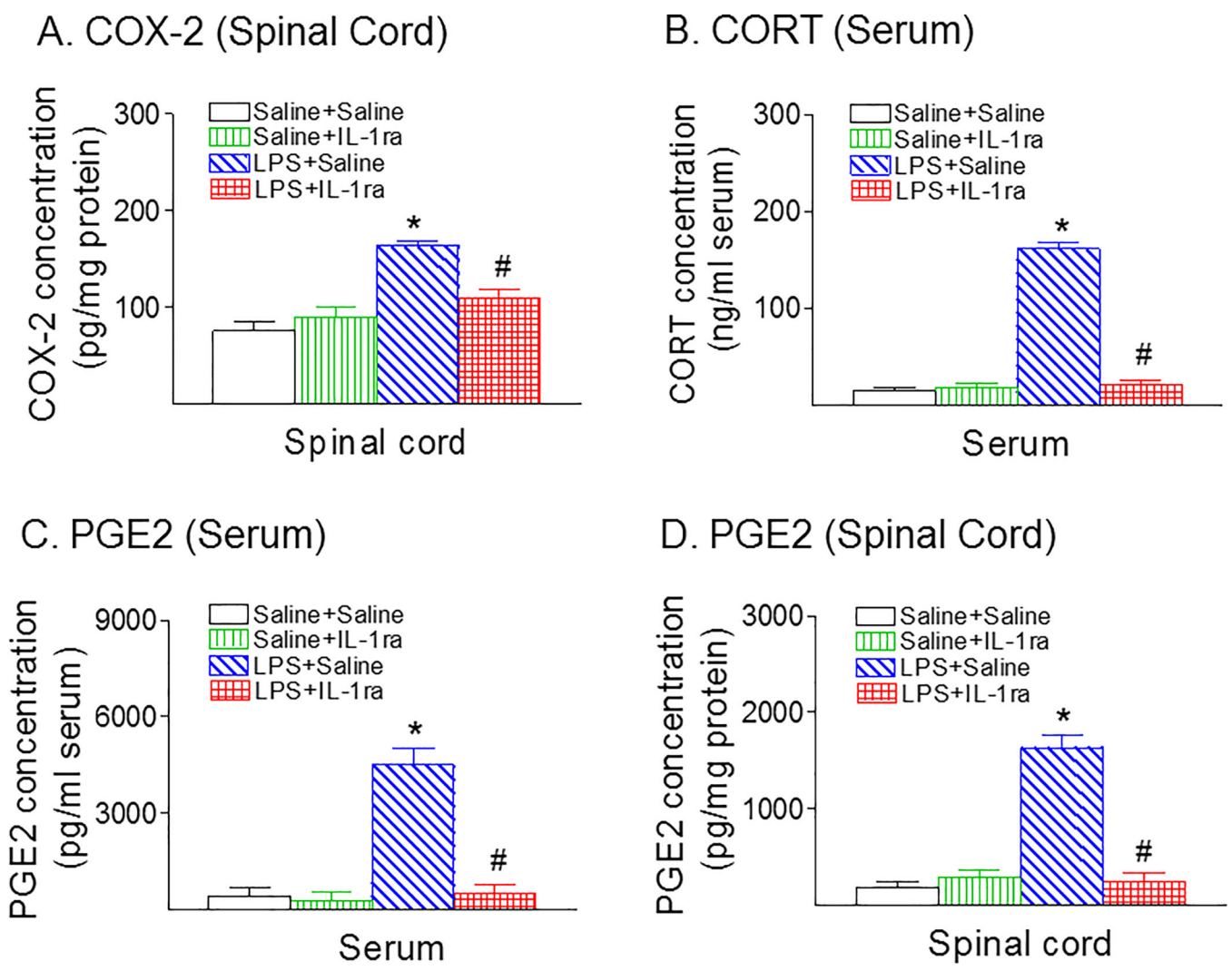

Figure 8.

IL-1ra attenuated systemic LPS exposure-induced increases in cyclooxygenase-2 (COX-2) in the rat spinal cord (A), corticosterone (CORT) in the rat serum (B), and prostaglandin E2 (PGE2) in the rat serum (B) and spinal cord (C) 24 hours (P6) after LPS injection. A, Following LPS injection, the concentration of COX-2 in the spinal cord was elevated compared with the control group in the P6 rat. IL-1ra attenuated LPS-induced increase in the concentration levels of COX-2 in the spinal cord of P6 rats. B, The concentration levels of corticosterone 24 hours (P6) following LPS injection in the serum were elevated compared with the control group. IL-1ra attenuated LPS-induced increase in the concentration levels of corticosterone in the serum of P6 rats. C&D, The concentration levels of PGE2 24 hours (P6) following LPS injection in the serum (C) and spinal cord (D) were elevated compared with the control group. IL-1ra attenuated LPS-induced increase in the concentration levels of PGE2 in the serum and spinal cord of P6 rats. The results are expressed as the mean ± SEM of eight animals in each group and analyzed by two-way ANOVA, followed by the Student-Newman-Keuls test. *P < 0.05 represents significant difference for the LPS+Saline group

compared with the Saline+Saline group. $^{\#}P < 0.05$ represents significant difference for the LPS+IL-1ra group compared with the LPS+Saline group. (N=8)

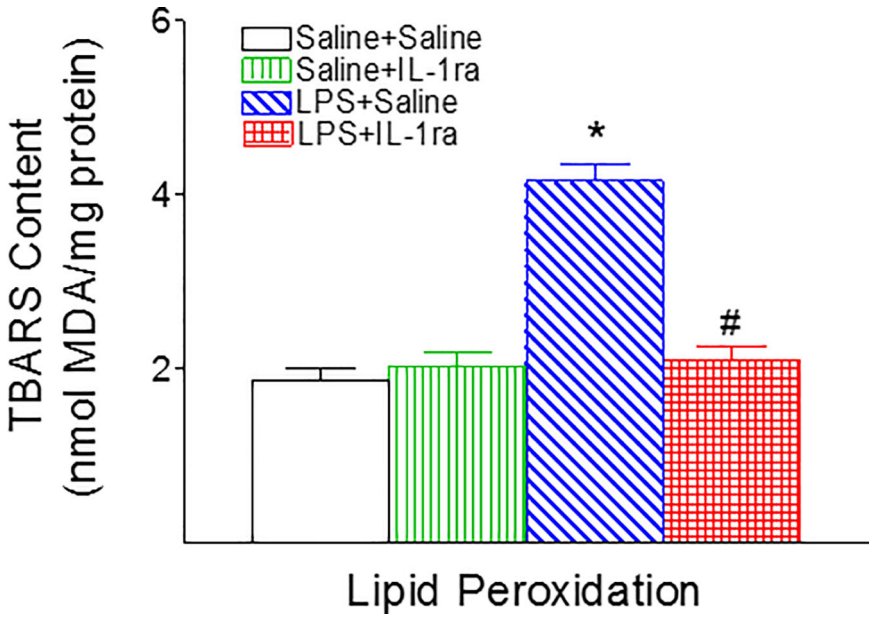

Figure 9.

IL-1ra attenuated systemic LPS exposure-induced increases in thiobarbituric acid-reactive substances (TBARS) content in the rat spinal cord 24 hours (P6) after LPS injection. Following LPS injection, the TBARS content in the spinal cord was elevated compared with the control group in the P6 rat. IL-1ra attenuated LPS-induced increase in the TBARS content in the spinal cord of P6 rats. The results are expressed as the mean ± SEM (malondialdehyde, MDA equivalents) of eight animals in each group and analyzed by two-way ANOVA, followed by the Student-Newman-Keuls test. *$P < 0.05$ represents significant difference for the LPS+Saline group compared with the Saline+Saline group. $^{\#}P < 0.05$ represents significant difference for the LPS+IL-1ra group compared with the LPS+Saline group. (N=8)

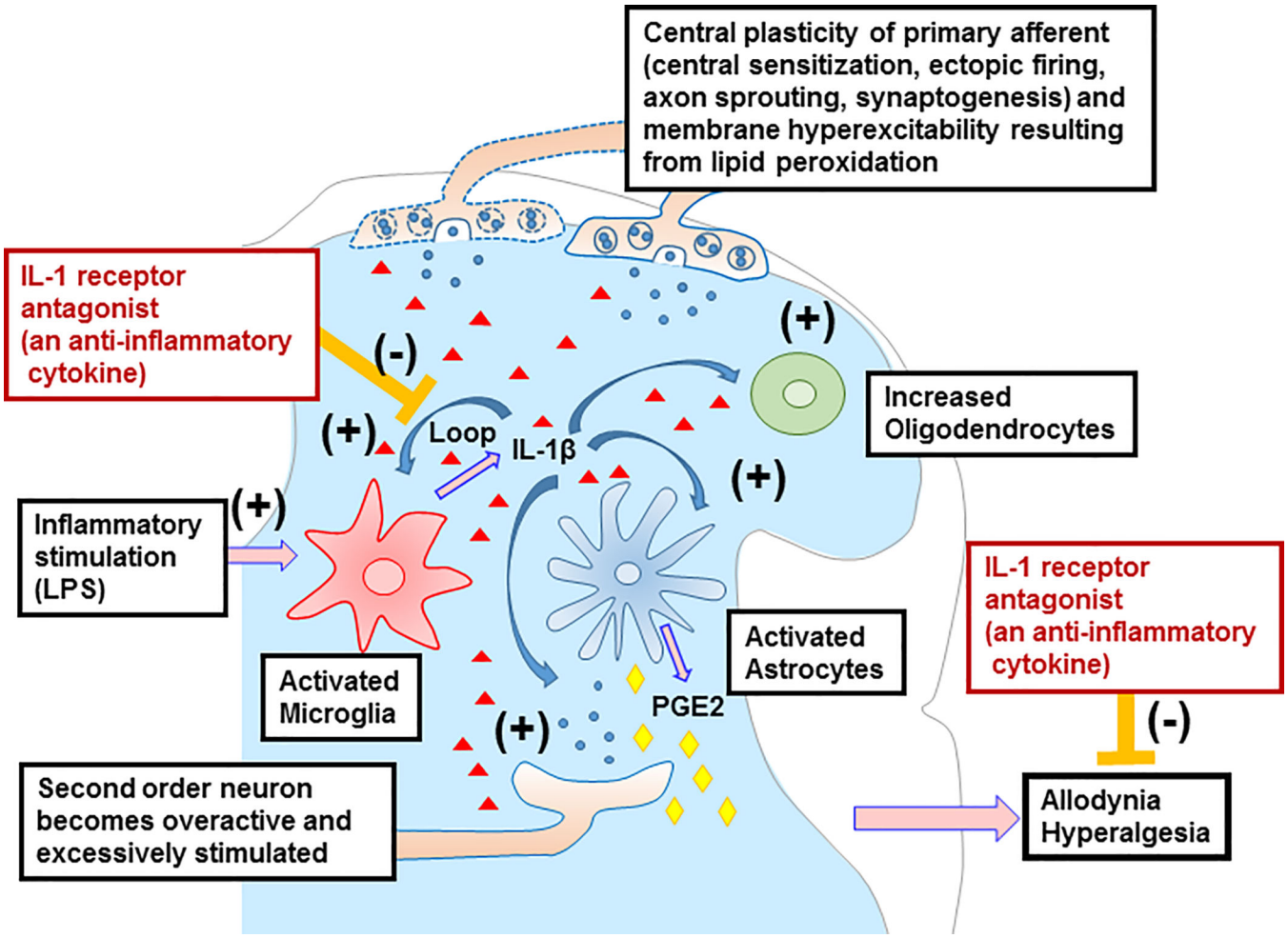

Figure 10.

Schematic illustration of glia-glia and neuron-glia interaction in the spinal cord dorsal horn in neonatal systemic inflammation-induced pain hypersensitivity. Systemic LPS exposure led to activation of microglia and astrocytes, increased oligodendrocyte lineage cells and upregulation of pain mediators such as pro-inflammatory cytokine interleukin-1β (IL-1β) and prostaglandin E2 (PGE2) which might form a positive feedback loop of inflammatory responses in the spinal cord. In addition, LPS exposure also induced reactive oxygen species (ROS) and related downstream molecules to further produce lipid peroxidation on cell membranes. These inflammatory effects may supplement neurotrophic factors including nerve growth factor (NGF) that cause nociceptive primary afferents to undergo central plasticity (central sensitization, ectopic firing, axon sprouting, synaptogenesis), second order dorsal horn neurons to become overactive and excessively stimulated in spinal nociceptive signal processing, and, as a result, the enhancement of pain hypersensitivity (allodynia and hyperalgesia) in inflamed neonatal rats. These compromises in gliosis, oxidative stress responses, and behaviors are attenuated by IL-1 receptor antagonist (IL-1ra, an anti-inflammatory cytokine), demonstrating that pro-inflammatory cytokine IL-1β plays a central role in mediating those pathological conditions.