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Lung inflammation induces IL-1 β expression in hypoglossal neurons in rat brainstem

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Abstract

Perinatal inflammation is associated with respiratory morbidity. Immune modulation of brainstem respiratory control centers may provide a link for this pathobiology. We exposed 11-day old rats to intratracheal lipopolysaccharide (LPS, 0.5 μ g/g) to test the hypothesis that intrapulmonary inflammation increases expression of the proinflammatory cytokine IL-1 β within respiratory-related brainstem regions. Intratracheal LPS resulted in a 32% increase in IL-1 β protein expression in the medulla oblongata. *In situ* hybridization showed increased intensity of IL-1 β mRNA but no change in neuronal numbers. Co-localization experiments showed that hypoglossal neurons express IL-1 β mRNA and immunostaining showed a 43% increase in IL-1 β protein-expressing cells after LPS exposure. LPS treatment also significantly increased microglial cell numbers though they did not express IL-1 β mRNA. LPS-induced brainstem expression of neuronal IL-1 β mRNA and protein may have implications for our understanding of the vulnerability of neonatal respiratory control in response to a peripheral pro-inflammatory stimulus.

Keywords

Brainstem; neuro-inflammation; *in situ* hybridization; lipopolysaccharide; respiratory control

1. Introduction

Vulnerability of neonatal respiratory control is an almost universal feature of preterm birth. It may manifest as tachypnea or an irregular respiratory pattern, and frequently culminates in apnea (Abu-Shaweesh and Martin, 2008). Neonatal sepsis frequently presents with various combinations of tachypnea and apnea, and the airway is a frequent portal of entry for pathogens, both antenatally and postnatally (McKenney, 2001). We have recently developed a neonatal rat model in which endotoxin (lipopolysaccharide, LPS) is injected intratracheally into anesthetized animals who are then allowed to recover. This resulted in an increase in brainstem message for IL-1 β and an accompanying attenuation of the ventilatory response to hypoxia (Balan et al., 2011, 2012). Intratracheal LPS was also associated with a qualitative

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increase in IL-1 β immunoreactivity in the *nucleus tractus solitarius* (nTS) the first order site of termination in the brainstem for vagal afferents including oxygen sensing peripheral chemoreceptors in the carotid body (Balan et al., 2011, 2012). It remains unclear whether neurons at this and other respiratory-related sites in the brainstem actually demonstrate a quantitative increase in IL-1 β expression in response to this intrapulmonary inflammatory stimulus

Interleukin-1 is a cytokine whose biologic activity modulates the inflammatory response through a cell signaling cascade and has multiple potential effects in the central nervous system. It has been recognized for two decades that neurons in the fore- and midbrain of mature rats may express message for IL-1 receptors (Yabuuchi et al., 1994). It was assumed from these studies that IL-1 synthesized in glial cells, like microglia, might then act on IL-1 receptor expressing neurons. It is now known that sensory neurons, e.g., in dorsal root ganglia of mature rats, express IL-1 β mRNA under basal conditions (Coprav et al., 2001). In response to systemic inflammation, peripheral cytokines may elicit a central neural response by stimulating neural pathways and second messengers, or entering brain areas that lack a blood brain barrier (Licinio et al., 1997). However, the ability of the brain to elicit its own cytokine response to a systemic inflammatory stimulus is not well understood. Data from mature rats indicate that microglial cells either in or crossing the *area postrema*, one of the circumventricular organs that makes up the blood brain barrier, are likely sources for cytokine production in response to a bacterial stimulus (Wuchert et al., 2009).

In this study we initially sought to determine whether our previously observed increase in message for IL-1 β in the brainstem in response to an intrapulmonary inflammatory stimulus in early life would elicit a quantifiable increase in cytokine protein in the brainstem. We then sought to test the hypothesis that neurons in respiratory-related brainstem regions would respond to intrapulmonary LPS with a quantifiable increase in IL-1 β message expression. Finally, we focused on the hypoglossal motor nucleus, which provides neural drive to the tongue and upper airway muscles, to characterize the role of neurons versus glia in the enhancement of IL-1 β expression after LPS exposure. Demonstration of a key role for neurons in regulating cytokine expression in the brainstem might provide novel insight into mechanisms underlying the homeostasis and vulnerability of neonatal respiratory control.

2. Materials and Methods

2.1. Animal preparation

Studies were performed on 11 day-old Sprague-Dawley rat pups and all procedures were approved by the Case Western Reserve University Institutional Animal Care and Use Committee (IACUC). The rat pups were lightly anesthetized with a cocktail of ketamine (3 mg/100g) and xylazine (0.4 mg/100g) and 10 μ l of either saline or LPS (0.5 μ g/g) (*Escherichia coli* 0128:B12 Sigma-Aldrich, St. Louis, MO, USA) was instilled into the trachea using a Hamilton syringe as previously described (3). Four hours post LPS/saline instillation the rat pups were deeply re-anesthetized with an excess of ketamine/xylazine solution and perfused transcardially with 1x phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The brains were removed and post-fixed in the same fixative for 4 hours at 4° C. The brainstems were then removed and cryoprotected in 15% sucrose overnight. The brainstem was blocked, retaining the regions of interest, and cut coronally into 20 or 40 μ m thick free-floating sections using a cryostat (EM 1850), Leica). The sections were stored in 0.1% sodium azide/0.1M PBS solution at 4° C. For Western blot experiments, the animals were deeply anesthetized (as above) and the medulla oblongata was rapidly dissected while out frozen in liquid nitrogen.

2.2. Western blot quantification of IL-1 β protein

The medulla oblongata was homogenized in RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, from Sigma-Aldrich Inc.) containing protease inhibitors. The protein concentration of supernatant homogenate was determined using a Bio-Rad kit. The 20 μ g of denatured protein/lane was electrophoresed by SDS-PAGE. The proteins were blotted on a nitrocellulose membrane, blocked with 5% milk and incubated with anti-IL-1 β antibody (1:500, Abcam) overnight (1 hour with anti-rabbit secondary antibody at 1:5,000; Amersham). Blots were stripped and reprobed with β -actin antibody (1:3,000; Sigma) and proteins were visualized by chemi-luminescence and quantified by *ImageJ* software (<http://rsb.info.nih.gov/ij/>).

2.3. *In situ* hybridization and quantification of mRNA of IL-1 β

To determine whether the cells in respiratory related regions express IL-1 β mRNA, *in situ* hybridization was performed using digoxigenin-labeled cRNA probes. In brief, the rat IL-1 β plasmid (Pineau et al., 2006) was linearized by Eco RI and Hind III to generate the corresponding sense and antisense transcripts with SP6 and T7 promoters, respectively. The cDNA templates were digested with proteinase K and extracted with phenol-chloroform. The digoxigenin-labeled sense and antisense cRNA probes were synthesized and hybridized with IL-1 β mRNA in tissue sections using the *in situ* hybridization protocol as described previously (Zaidi et al., 2006). The intensity of IL-1 β mRNA in cells was measured using *ImageJ* (bright field images, pixel counts in arbitrary units). Pixel densities greater than 3x background were considered positive.

2.4. Colocalization of IL-1 β mRNA with neuronal marker

To determine whether neurons in respiratory-related regions of the brainstem—including nucleus tractus solitarius (nTS), rostral-ventrolateral medulla (RVLM), and hypoglossal motor nucleus—express IL-1 β mRNA, *in situ* hybridization for IL-1 β mRNA was co-localized in neurons by immunostaining for NeuN, a selective marker of neurons. First, *in situ* hybridization was performed on free-floating sections, then they were immunostained with anti-NeuN rabbit primary antibodies (1:250, Millipore) and FITC-labeled goat anti-rabbit secondary antibodies (1:300, A488, Invitrogen). For negative controls the anti-NeuN antibodies (primary antibodies) were omitted. Epifluorescence and bright-field images were merged to evaluate co-localization.

2.5. Quantification of IL-1 β protein expressing cell numbers

Immunostaining was performed with IL-1 β antibodies using DAB (3,3'-diaminobenzidine) a substrate for the color development in order to quantify the number of IL-1 β expressing cells in saline and LPS treated animals using bright-field microscopy. DAB was used to facilitate counting IL-1 β expressing cells using unbiased stereology (Mouton, 2002). When co-localizing with other antigens, FITC or RITC were used as fluorescent secondary antibodies for confocal microscopy. Primary antibodies were omitted for negative controls and all sections were stained using antibodies from the same lot. The specificity of antibodies was tested by using pre-absorbed antibody.

2.6. Colocalization of IL-1 β protein in neurons

To determine whether respiratory-related brainstem neurons expressed IL-1 β protein, sections were double immunostained with IL-1 β and NeuN marker and studied using confocal microscopy. Brainstem sections were selected and rinsed 2x for 10 minutes with 0.1M PBS and blocked with 5% normal donkey serum containing 1% bovine serum albumin and 0.3% Tween-20 in PBS for one hour at room temperature. The floating sections were

incubated overnight at 4° C into a fresh blocking medium containing goat anti IL-1 β 1:200 (R&D system, Inc., Minneapolis, MN, USA) and mouse anti NeuN antibodies. The sections were washed 4x for 10 minutes with PBS after every step of the experiment. The sections were incubated in RITC-donkey anti goat (A594, Invitrogen, CA) and FITC- donkey anti-mouse (A488, Invitrogen, CA) secondary antibodies, respectively. The sections were then mounted on gelatin-coated slides and coverslipped using Vectashield mounting media (Vector Laboratories, Burlingame, CA). Images of the immunostained sections were captured using a Rolera XR CCD camera (Q-Imaging, Canada) mounted on a Leica DMLB microscope (Leica Microsystems, Germany). Confocal microscopy allowed us to validate our bright-field DAB results and “bleedover” (spectrum overlap) from our fluorescent markers.

2.7 Triple labeling of IL-1 β mRNA with neuronal and microglial markers

In order to show which cells express IL-1 β mRNA a triple labeling experiment was conducted. First, the *in situ* hybridization for IL-1 β mRNA was performed on free floating sections and then these sections were immunostained with NeuN (mouse anti-NeuN antibodies, 1:250, Millipore) and the microglial marker, anti-Iba-1 (ionized calcium-binding adaptor molecule 1, 1:750, Wako Chemicals, Richmond, VA). The sections were then incubated with secondary antibodies conjugated to FITC (donkey anti mouse, A488, 1:300, Invitrogen, CA) for neuronal marker or conjugated with RITC (donkey anti rabbit, A594, 1:300, Invitrogen, CA) for microglial marker. The images of sections with three chromagens were acquired and merged to determine if there was colocalization of IL-1 β with microglia and neurons.

2.8 Stereology and densitometry

We used unbiased stereology (*StereoAnalyzer2000*, SRC, Tampa, FL) to estimate the number of cells expressing IL-1 β mRNA and protein (Mouton, 2002). For stereological counting procedures, an optical disector probe was used and semi-quantitative densitometry was performed using *ImageJ*.

2.9 Statistics and data analysis

Student's t-test was used for pairwise comparisons of Western blot band intensity (Figure 1), quantification data for IL-1 β mRNA expressing cells, intensity of mRNA in the cells, and Iba-1 protein expressing cells (Figure 3 A B and 7 respectively). All data are presented as mean $\pm$ SEM and $p < 0.05$ was considered significant.

3. Results

3.1 Intratracheal LPS stimulates cytokine protein expression in the medulla oblongata

Western blots from whole medulla oblongata of rat pups displayed a basal level of IL-1 β protein in the medulla oblongata (Fig. 1A). IL-1 β protein was increased by 32 ± 14 % (mean $\pm$ SEM) with the instillation of intratracheal 0.5 μ g/g LPS ($p < 0.05$, $n = 5$ per group, Figure 1B).

3.2 LPS treatment increases intensity of IL-1 β mRNA expression in respiratory related regions

Our experiments using *in situ* hybridization confirmed that IL-1 β mRNA was expressed by cells of the following respiratory-related regions of the medulla oblongata: nTS, rostroventral lateral medulla (RVLM) and the hypoglossal motor nuclei. Under control conditions (saline instillation) all three regions expressed a baseline level of IL-1 β mRNA (Figure 2). LPS administration did not change the numbers of IL-1 β mRNA expressing cells

in any of these regions as illustrated in Figure 2 and quantified in Figure 3A. In contrast there was a significant increase in the intensity of IL-1 β mRNA expression in cells after LPS administration (Figs. 2 & 3B).

3.3 Identification of IL-1 β mRNA in hypoglossal neurons

We next sought to identify whether neurons in the hypoglossal region of the medulla express IL-1 β mRNA. Colocalization experiments employed a combination of *in situ* hybridization and immunostaining for neuronal marker. As shown in Figure 4 large motor neurons in the hypoglossal nuclei demonstrate higher intensity of IL-1 β mRNA after LPS vs. saline administration.

3.4 LPS increases IL-1 β protein expressing cells in hypoglossal motor nucleus

We sought to determine whether increased IL-1 β message in the hypoglossal region translated into increased expression of IL-1 β protein. Qualitative evaluation by fluorescence microscopy showed IL-1 β protein in neurons after both saline and LPS administration. There was a qualitative increase in protein content of fibers at this site after LPS administration (Fig 5). Unbiased stereological quantification of DAB-labeled cells ($n = 5$ per group) showed that the number of IL-1 β protein expressing cells in hypoglossal nucleus was increased by 43% [9407 ± 826 (saline) vs. 13462 ± 325 (LPS), mean $\pm$ SEM] after LPS administration ($p < 0.001$, Fig 6).

3.5. LPS administration increases the number of microglial cells in the hypoglossal motor nucleus

We sought to characterize the response of microglial cells in the hypoglossal region to intratracheal LPS. There was a 59% increase (62 ± 6.5 (saline) vs. 97 ± 3.6 (LPS), mean $\pm$ SEM, $p < 0.004$) in the number of glial cells after LPS administration (unbiased stereology) as can be seen in Figure 7.

3.6. Triple labeling using *in situ* hybridization and immunostaining show that glia in XII do not express IL-1 β mRNA

As shown in Fig. 8 IL-1 β mRNA expressing cells were colocalized with neuronal marker. LPS administration qualitatively increased the number of glial cells as compared to saline. However, IL-1 β mRNA was not colocalized with glial cell marker showing that glial cells do not express IL-1 β mRNA.

4. Discussion

To our knowledge, this is the first clear demonstration that neurons in the medulla oblongata express both message and protein for a proinflammatory cytokine in early postnatal life. Our focus is on neurons in the brainstem because respiratory control in the neonatal and post-neonatal periods is highly vulnerable to inflammatory stimuli (Abu-Shaweesh and Martin 2008). In addition, the nTS within the brainstem is an entry point for afferents from peripheral sites, including the lung (Andresen and Kunze, 1994). We have previously documented that vagal afferents may transmit an intrapulmonary signal to the brainstem (Balan et al., 2011) as early as two hours after LPS injection. The nTS is also in close proximity to the area postrema where permeability of the blood brain barrier to a systemic proinflammatory response is greatest. Interestingly, in this study we have also demonstrated IL-1 β mRNA and protein expression in neurons of the hypoglossal motor nucleus—which innervates the tongue and respiratory muscles of the upper airway. Here we focus upon the early changes in IL-1 β expression that are localized to neurons rather than glia. These findings, therefore, have potential implications for future studies that might characterize the

neuronal and glial cytokine response to a systemic inflammatory response at CNS sites within and beyond the brainstem.

We chose to stimulate cytokine production by instillation of LPS intratracheally to the lung. Direct pulmonary exposure was readily accomplished in 11 day-old rat pups as previously described (Balan et al., 2011). Available comparative data suggest that, at the age used in this study, brain growth approximates that of a full term human infant (Davis et al., 1974). However, we acknowledge that the maturity of the immune system at this stage in rats may be inherently different than seen in humans. This model attempts to simulate the introduction of endotoxin into the respiratory system either antenatally in the presence of infected amniotic fluid, or postnatally in infants with pneumonia. We recognize that our model comprising intrapulmonary LPS exposure may initiate inflammation that is compartmentalized to the lung, initiate a blood-borne proinflammatory response, or both. Prior studies have employed injection of *E. coli*, endotoxin, LPS, or recombinant IL-1 β via the intraperitoneal route to simulate neonatal sepsis in rat pups (Ladino et al., 2007, Olsson et al., 2003). As IL-1 β does not readily cross the blood brain barrier, a likely mechanism for the resultant apnea was thought to be IL-1 β -induced activation of the cyclooxygenase-2 pathway and release of prostaglandin E2 which can cross the blood brain barrier with resultant respiratory depression (Hofstetter et al., 2007). Indeed, based upon data published from our group (Gresham et al., 2011) we have shown that both *in vivo* and *in vitro* rat models show changes in frequency of breathing rhythm in response to LPS or IL-1 β . Our current data suggest that intrapulmonary or systemic infection actually stimulates IL-1 β production from neurons within respiratory related regions of the brainstem. The role of this newly synthesized neuronal IL-1 β in altering the neurotransmitter balance regulating respiratory control needs to be a subject for future study. Glia are also known to produce and release cytokines constitutively (Beurel et al. 2009) thus the absence of IL-1 β in microglia may be due to transcriptional regulation though this does not explain the lack of *in situ* staining in the microglia that were present. The lower LPS dose, shorter timing and intrapulmonary route of administration may be few reasons for this observation.

While LPS exposure significantly increased the intensity of IL-1 β mRNA, the number of IL-1 β mRNA expressing cells did not change. The majority of neurons at all three sites appeared to express IL-1 β mRNA under control conditions of intratracheal saline instillation. It is now widely recognized that cytokines serve an important trophic role and mediate a diverse array of functions in neural tissues of the central nervous system (Deverman et al., 2009). However, the normal roles that cytokines have during CNS development may be disturbed, based on animal models of maternal infection, and potentially contribute to a variety of brain disorder via neuroimmune mechanisms (Ellman et al., 2009). The proinflammatory cytokine IL-1 β may exert antiproliferative, antineurogenic and progliogenic adverse effects under such conditions by binding to interleukin receptors as demonstrated in embryonic rat hippocampal neural precursor cells (Green et al., 2012). Experiments in cultured embryonic hippocampal neurons have demonstrated the ability of IL-1 β to induce interleukin receptor expression in such neurons (Friedman, 2001). Study of such adverse effects during development has focused largely on synthesis of cytokines by microglial tissues in the CNS and on cortical structures rather than the brainstem (Hagberg and Mallard, 2005).

Our study shows that microglia in the hypoglossal motor nucleus do not express IL-1 β but they surround or make connections with the IL-1 β expressing neurons and their number increases with the LPS induced inflammation in lungs. The neurons may produce and take up IL-1 β from adjacent glia releasing cytokines. Other investigators have shown that peripheral and central injection of LPS can increase microglia in the CNS but the changes reported occur at 24 hours or later, in contrast to our experiments (Campbell et al., 2012,

Hernandez-Romero et al., 2012). Obviously, we find it curious that we do not see IL-1 β in microglia but we believe this may be due to the relatively short time window (post-LPS) that we are looking at. We have previously reported changes in IL-1 β message as early as 2 hours after LPS injection and we believe that we are looking at the earliest change in expression by focusing on 2 to 4 hours. Additionally, (Jeong et al., 2010) showed that systemic administration of LPS increased the expression and number of glia cells in *substantia nigra* within 4–8 h. This may be due to either migration or increased expression of Iba-1 protein in the existing glia. However, the glia we see do not seem to exhibit an “activated” phenotype (Stence et al., 2001, and Kaizaki et al., 2013). (Buchanan et al., 2010) also have shown the increase of glia after 4h of intraperitoneal LPS but it seems unlikely that we are seeing the proliferation of glia in such a short time. The moderate increase in the number of glia we observed may indicate an early microglial response to LPS. Additionally, it is possible that low expression of IL-1 β may have been undetectable using our methods. To address this issue, in future experiments different LPS doses, timing, and route of administration should be studied. Longitudinal studies over longer time periods will need to be performed to determine which cells express IL-1 β , other cytokines, and their corresponding receptors to better understand the consequences of their activation on cardiorespiratory control.

Several limitations need to be considered. We chose to administer only one dose of LPS, 0.5 μ g/g as we considered this to be a modest dose. In our previous studies we employed 0.1 μ g/g of intratracheal LPS which did up regulate cytokine expression in brainstem but neuronal labeling of IL-1 β in the nTS was not apparent (Balan et al., 2012). It is also possible that saline infusion in the control group may have stimulated afferent pulmonary pathways and contributed to baseline brainstem cytokine expression in our control group, although an effect of LPS was clearly observed. Finally, only one time point of sacrifice at four hours post instillation was used for both *in situ* hybridization and immunostaining studies. It is possible that a greater qualitative effect on protein expression in both cell bodies and fibers might have been observed if a series of multiple time points had been selected.

In summary we believe we have demonstrated, for the first time, that intrapulmonary inflammation in a rat pup model increases neuronal expression of IL-1 β mRNA in the brainstem. Given the instability of cardiorespiratory control in early postnatal life, these findings should stimulate further study of the physiologic role of proinflammatory cytokines on respiratory related neurons under both *in vitro* and *in vivo* conditions. Our findings also should stimulate parallel investigation of the potential vulnerability of cortical structures to proinflammatory conditions, and the role of neuronal cytokines on resultant pathobiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Abu-Shaweesh JM, Martin RJ. Neonatal apnea. What's new? *Pediatr Pulmonol.* 2008; 43:937–944. [PubMed: 18780339]

2. Andresen MC, Kunze DL. Nucleus tractus solitarius--gateway to neural circulatory control. *Annu Rev Physiol.* 1994; 56:93–116. [PubMed: 7912060]
3. Balan KV, Kc P, Hoxha Z, Mayer CA, Wilson CG, Martin RJ. Vagal afferents modulate cytokine-mediated respiratory control at the neonatal medulla oblongata. *Respir Physiol Neurobiol.* 2011; 178:458–464. [PubMed: 21397055]
4. Balan KV, Kc P, Mayer CA, Wilson CG, Belkadi A, Martin RJ. Intrapulmonary lipopolysaccharide exposure upregulates cytokine expression in the neonatal brainstem. *Acta Paediatr.* 2012; 101:466–471. [PubMed: 22176020]
5. Beurel E, Jope RS. Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain. *Journal of Neuroinflammation.* 2009; 6:9. [PubMed: 19284588]
6. Buchanan JB, Sparkman NL, Johnson RW. A neurotoxic regimen of methamphetamine exacerbates the febrile and neuroinflammatory response to a subsequent peripheral immune stimulus. *J Neuroinflammation.* 2010; 7:82. [PubMed: 21092194]
7. Campbell LR, Pang Y, Ojeda NB, Zheng B, Rhodes PG, Alexander BT. Intracerebral lipopolysaccharide induces neuroinflammatory change and augmented brain injury in growth-restricted neonatal rats. *Pediatr Res.* 2012; 71(6):645–652. [PubMed: 22337231]
8. Copray JC, Mantingh I, Brouwer N, et al. Expression of interleukin-1 beta in rat dorsal root ganglia. *J Neuroimmunol.* 2001; 118:203–211. [PubMed: 11498255]
9. Davis, JA.; Dobbing, J. *Scientific Foundations of Paediatrics.* Philadelphia: Saunders; 1974. p. 565-577.
10. Deverman BE, Patterson PH. Cytokines and CNS development. *Neuron.* 2009; 64:61–78. [PubMed: 19840550]
11. Ellman LM, Susser ES. The promise of epidemiologic studies: neuroimmune mechanisms in the etiologies of brain disorders. *Neuron.* 2009; 64:25–27. [PubMed: 19840544]
12. Friedman W. Cytokines regulate expression of the type 1 interleukin-1 receptor in rat hippocampal neurons and glia. *Exp Neurol.* 2001; 168:23–31. [PubMed: 11170718]
13. Green HF, Treacy E, Keohane AK, Sullivan AM, O'Keeffe GW, Nolan YM. A role for interleukin-1beta in determining the lineage fate of embryonic rat hippocampal neural precursor cells. *Mol Cell Neurosci.* 2012; 49:311–321. [PubMed: 22270046]
14. Gresham K, Boyer B, Mayer C, Foglyano R, Martin R, Wilson CG. Airway inflammation and central respiratory control: results from in vivo and in vitro neonatal rat. *Respir Physiol Neurobiol.* 2011; 178:414–421. [PubMed: 21609789]
15. Hagberg H, Mallard C. Effect of inflammation on central nervous system development and vulnerability. *Curr Opin Neurol.* 2005; 18:117–123.
16. Hernández-Romero MC, Delgado-Cortés MJ, Sarmiento M, de Pablos RM, Espinosa-Oliva AM, Argüelles S, Bández MJ, Villarán RF, Mauriño R, Santiago M, Venero JL, Herrera AJ, Cano J, Machado A. Peripheral inflammation increases the deleterious effect of CNS inflammation on the nigrostriatal dopaminergic system. *Neurotoxicol.* 2012; 33(3):347–360.
17. Hofstetter AO, Saha S, Siljehav V, Jakobsson PJ, Herlenius E. The induced prostaglandin E2 pathway is a key regulator of the respiratory response to infection and hypoxia in neonates. *Proc Natl Acad. Sci. USA.* 2007; 104:9894–9899. [PubMed: 17535900]
18. Jeong H-K, Jou I, Joe EH. Systemic LPS administration induces brain inflammation but not dopaminergic neuronal death in the substantia nigra. *Exp Mol Med.* 2010; 42(12):823–832. [PubMed: 20962566]
19. Kaizaki A, Tien LT, Pang Y, Cai Z, Tanaka S, Numazawa S, Bhatt AJ, Fan LW. Celecoxib reduces brain dopaminergic neuronal dysfunction, and improves sensorimotor behavioral performance in neonatal rats exposed to systemic lipopolysaccharide. *J Neuroinflammation.* 2013; 5(10(1)):45. [PubMed: 23561827]
20. Ladino J, Bancalari E, Suguihara C. Ventilatory response to hypoxia during endotoxemia in young rats: role of nitric oxide. *Pediatr Res.* 2007; 62:134–138. [PubMed: 17597656]
21. Licinio J, Wong ML. Pathways and mechanisms for cytokine signaling of the central nervous system. *J Clin Invest.* 1997; 100:2941–2947. [PubMed: 9399938]

22. McKenney WM. Understanding the neonatal immune system: high risk for infection. Crit Care Nurse. 2001; 21(6):35–47. [PubMed: 11767763]
23. Mouton, PR. Principles and Practices of Unbiased Stereology: An Introduction for Bioscientists. Johns Hopkins University Press; 2002.
24. Olsson A, Kayhan G, Lagercrantz H, Herlenius E. IL-1 beta depresses respiration and anoxic survival via a prostaglandin-dependent pathway in neonatal rats. Pediatr Res. 2003; 54:326–331. [PubMed: 12761362]
25. Pineau I, Barrette B, Vallières N, Lacroix S. A novel method for multiple labeling combining *in situ* hybridization with immunofluorescence. J Histochem Cytochem. 2006; 54:1303–1313. [PubMed: 16899759]
26. Stence N, Waite M, Dailey ME. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. Glia. 2001; 33(3):256–266. [PubMed: 11241743]
27. Wuchert F, Ott D, Rafalzik S, Roth J, Gerstberger R. Tumor necrosis factor- α , interleukin-1 β and nitric oxide induce calcium transients in distinct populations of cells cultured from the rat area postrema. J Neuroimmunol. 2009; 206:44–51. [PubMed: 19081643]
28. Yabuuchi K, Minami M, Katsumata S, Satoh M. Localization of type I interleukin-1 receptor mRNA in the rat brain. Brain Res Mol Brain Res. 1994; 27:27–36. [PubMed: 7877451]
29. Zaidi SI, Jafri A, Martin RJ, Haxhiu MA. Adenosine A2A receptors are expressed by GABAergic neurons of medulla oblongata in developing rat. Brain Res. 2006; 1071:42–53. [PubMed: 16413509]

Highlights

- Perinatal inflammation increases proinflammatory cytokines in the brainstem.
- LPS administration increased IL-1 β expression in brainstem respiratory regions.
- LPS exposure also increased microglia numbers though not IL-1 β mRNA expression.

Figure 1. Effect of intratracheal LPS on IL-1β protein expression in rat medulla oblongata

Panel A: Western blot of two representative medulla oblongata samples. The basal level of IL-1β protein in the medulla oblongata increased with the instillation of 0.5 μg/g LPS. Panel B: Quantification of IL-1β protein expression in medulla oblongata expressed as mean ± SEM of the ratios of IL-1β to β-actin proteins. The IL-1β protein expression increased 32 ± 14% with LPS treatment as compared to saline (* $p < 0.05$, $n=4$ per group).

Figure 2. Effect of LPS on intensity of IL-1 β mRNA expression in respiratory related regions
 The left column shows the respiratory related regions, nTS, RVLM and hypoglossal motor nuclei (Hy) of the medulla oblongata. The middle and right columns show *in situ* hybridization micrographs of IL-1 β mRNA. Top row shows sense control. A majority of cells in these respiratory related regions expressed baseline IL-1 β mRNA that increased after LPS administration (Bar=50 μ m, n=5 per group).

A

B

Figure 3. Quantification of number and intensity of IL-1β mRNA expressing cells in respiratory related regions after LPS treatment

Panel A: LPS administration did not change the number of IL-1β expressing cells in the nTS, RVLM and hypoglossal motor nuclei. Panel B: in contrast there was a significant increase in the intensity of IL-1β mRNA expression in cells of all these regions after LPS administration (* $p < 0.05$, for LPS versus. Saline, $n=5$ per group, values are expressed as mean $\pm$ SEM of pixel density measured in arbitrary units).

Figure 4. Identification of IL 1-β mRNA in hypoglossal motoneurons

Colocalization of IL1-β mRNA expression (*in situ* hybridization) with neuronal marker (NeuN, immunostaining). Left panel is IL-1β mRNA, middle panel is neuronal marker (NeuN) and the right panel is the merged images showing that IL-1β expressing cells are neurons. (Bar=50 μm, n=5 per group).

Figure 5. Colocalization of IL-1 β protein in hypoglossal motoneurons

Left panel is IL-1 β protein, middle panel is neuronal marker, NeuN, and the right panel is the merged images showing that neurons express IL-1 β protein. The yellow arrows indicate neurons and white arrows indicate fibers. The numbers of IL-1 β fibers and the intensity of IL-1 β expression is qualitatively increased by LPS (Bar=50 μ m, n=5 per group).

Figure 6. Quantification of IL-1β protein expressing cells in hypoglossal motor nucleus

The IL-1β protein expressing cells were immunostained using DAB as substrate. The number of IL-1β protein expressing cells significantly increased in the hypoglossal neuron (panel A) by LPS administration (panel D) compare to saline (panel B) (* $p < 0.05$, for LPS versus. Saline; $n=5$ per group; values are expressed as mean $\pm$ SEM, Bar=50 μ m).). Panel C, negative control without the IL-1β antibodies.

Figure 7. Quantification of glial cells in hypoglossal motor nucleus

The expression of glial cell protein (labeled with Iba-1) was significantly increased (panel A) after LPS instillation (panel D) as compared to saline (panel B). (* $p < 0.05$, for LPS vs. Saline, $n=3$ per group, values are expressed as mean $\pm$ SEM, Bar=50 μ m). Panel C, negative control without the Iba-1 antibodies.

Figure 8. Triple labeling using *in situ* hybridization and immunostaining show that glia in XII do not express IL-1 β mRNA

Labeling for IL-1 β mRNA (*in situ* hybridization), glial cells and neuronal marker (immunostaining) were performed in same sections. Thin white arrows represent the glial cells expressing Iba-1 protein (panel A); thick arrows represent the cells expressing IL-1 β mRNA (panel B). The thick arrows in panel C represent neurons immunostained with neuronal marker, NeuN. In panel D the merged image of panels A, B and C shows that the IL-1 β message is colocalized with neurons but not with glial cells. Although, glial cells are surrounding the neurons. (Bar=50 μ m, n=3 per group).