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A critical postnatal period of heightened vulnerability to lipopolysaccharide

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ABSTRACT

Evidence of respiratory abnormalities and vulnerability to infection during a critical period of development have been implicated in Sudden Infant Death Syndrome (SIDS). Here we investigated whether the acute hypoxic ventilatory response (HVR) exhibits a heightened vulnerability to the endotoxin lipopolysaccharide (LPS) during a critical period of development. The acute HVR was measured 2 h after an i.p. injection of saline or LPS (0.1 mg/kg) at various postnatal (P) ages (P5, P10, or P20 days). LPS attenuated the early (1–2 min) and late (4–6 min) phase of the acute HVR in P10 but not P5 or P20 rats. The P10 age group exhibited the largest increase in brainstem TNFα and iNOS mRNA expression following LPS. LPS also caused a higher mortality rate in P10 rats (48%) compared to P5 (12%) and P20 (0%) age groups. After stratifying LPS treated P10 rats into survivors vs non-survivors, only the latter exhibited an attenuated HVR (specifically the early phase). Thus, the heightened vulnerability to endotoxin exposure during this critical period of development is characterized by a depression of the ventilatory response to acute hypoxia in association with an increased incidence of mortality. These data share similarities with some of the circumstances surrounding a SIDS scenario, including evidence of infection, increased brainstem cytokine expression, a disturbance in respiratory control, and a peak incidence of mortality during a critical period of development.

1. Introduction

The second postnatal week of life in the rat, particularly around postnatal (P) age 12 days (P12), represents a critical window of development characterized by unique neurochemical changes within specific brainstem cardio-respiratory control regions (Liu and Wong-Riley, 2002, 2003, 2010b; Liu et al., 2002). We showed previously that sustained hypoxia exposure (SH, 11% O_2 for 5 days) between P11-15 (which encompasses the P12 time point) eliminated the ventilatory response to acute hypoxia (HVR) (Mayer et al., 2014). These respiratory disturbances were also associated with a uniquely high incidence of mortality which occurred not during SH *per se*, but several days after (~3 days) the rats were returned to room air. However, younger (P1) or older (P21) rats that received the same exposure were relatively resistant to SH. These data indicate that the critical window may be a period during which mechanisms mediating at least the ventilatory response to acute hypoxia exhibit a heightened vulnerability to prolonged (days) hypoxia exposure. The cause of the vulnerability is unknown, although it was associated with increased microglia and reduced serotonin (5-HT) expression within the nucleus of the solitary tract (nTS) where afferent inputs from the carotid body (CB) are integrated (MacFarlane et al., 2015). Minocycline (an inhibitor of "activated" microglia) prevented the changes in microglia and 5-HT expression, protected against the attenuated HVR, and improved survival rates. These data suggest the heightened vulnerability of the HVR during the critical window of development may involve a microglia/inflammatory-mediated disturbance in brainstem neurochemistry (MacFarlane et al., 2015).

Although there are well-documented changes in brainstem neurochemistry during the second postnatal week of life in the rat, there are also unique changes in the postnatal profile of constitutive microglia and cytokine expression. The number of microglia in the mouse brain increases during the immediate postnatal period, peaking at ~P14 before a rapid decline thereafter toward adult levels (Nikodemova et al., 2015). The P10-15 age window is also a time when the brain exhibits a high level of CD11b-positive cells (surface protein marker of microglia) that are in a proliferative rather than apoptotic state (Nikodemova et al., 2015). Further, constitutive expression of microglial iNOS mRNA increases, whereas

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IL-16 decreases between P3 and P21 (Crain et al., 2013). Microglia are the resident immune cells of the CNS and play an important role in healthy CNS development which includes synaptic pruning, remodeling, and neurogenesis (Paolicelli et al., 2011; Wake et al., 2011). However, cytokines have a potent effect on respiratory neural control. Infusion of prostaglandin to neonatal sheep and piglets disrupted central respiratory rhythm and led to apnea (Guerra et al., 1988; Long, 1988; Tai and Adamson, 2000). Application of TNFα directly to the carotid body attenuated its sensitivity to hypoxia *in vitro* (Fernandez et al., 2008). Further, neonatal rats exposed to the endotoxin lipopolysaccharide (LPS) also exhibited an attenuated carotid body sensory response to acute hypoxia and increased IL-1β and IL-6 gene expression (Gauda et al., 2013). LPS also decreased the acute hypoxic ventilatory response in piglets (McDeigan et al., 2003), whereas in 7-day old rats it also caused respiratory depression, increased mortality after anoxia, and impaired autoresuscitation (Olsson et al., 2003). Interestingly, constitutive mRNA expression of the toll-like receptor 4 (TLR4, a receptor activated by LPS) in brain microglia also increases between P3-P21 (Crain et al., 2013).

Collectively, these data suggest there may be a critical window of development during which certain characteristics of the respiratory neural control system may be vulnerable to pro-inflammatory challenges. Specifically, we tested the hypotheses that there is a critical period of development (e.g. at P10) during which the respiratory control system exhibits a heightened vulnerability to LPS, characterized by: 1) an attenuated HVR; 2) increased mRNA expression for various inflammatory markers (TNFα, TLR4 and iNOS); and 3) whether they are associated with a higher incidence of mortality (vs younger (P5) and older (at P20) age groups).

2. Experimental procedures

All procedures were carried out in accordance with the National Institute of Health (NIH) guidelines for care and use of laboratory animals and were approved by the Animal Care and Use Committee at Case Western Reserve University. Time-pregnant Sprague-Dawley rats were purchased from a commercial vendor (Charles River, colony P09) and were later observed to give birth in the animal facility of the institution; experiments were performed on 5, 10, and 20 day old male rats.

2.1. LPS injections

On the day of the experiment, male rats from individual litters were removed from the mother, weighed, and divided into treatment groups that received either intraperitoneal (i.p.) injection of saline or LPS (0.1 mg/kg). LPS was dissolved in sterile saline (0.9% NaCl) and briefly sonicated before injection. Typically, the pups in a given litter were evenly divided into two groups so that half received saline and the other half received LPS. Treatment groups, therefore consisted of saline and litter mates injected with LPS for each of the three age groups (P5, 10 and 20). After the pups were injected they were returned to the dam for 1.5 h before again being removed and placed inside a whole body plethysmograph for measurements of ventilation. Saline injected rats served as control groups for each age-matched comparison. In this study, the P10 age group was uniquely sensitive to LPS compared to P5 and P20 rats (see results). Therefore, we investigated whether LPS at an earlier time point (P5) would elicit a pre-conditioning effect following a second injection at P10 (pre-$T_{(5)}$, n = 12). At the end of plethysmography, rats were returned to the dam for normal rearing. Additional rats were sacrificed to collect brainstem tissue for mRNA analysis at 2 h post-injection.

2.2. Plethysmography and measurement of ventilation

Ventilatory responses to acute hypoxia (10% O_2) and hypercapnia (5% CO_2) using whole-body plethysmography have been described in detail previously (Mayer et al., 2014). Briefly, 1.5 h after injection of saline or LPS, rats were placed in a plethysmograph for the measurement of ventilation (VE) using the barometric method. A mass flow controller (Aalborg, 0–5L/min; NY, USA) was used to regulate continuous flow through the chamber. After a 30 min rest period (i.e. precisely 2 h post-injection), a baseline measurement of VE was made. The airflow through the chamber was then increased to deliver acute hypoxia (10% O_2, 6 min), returned to room air (21% O_2, 5 min), and then acute hypercapnia (5% CO_2, 6 min). However, since animals at each age were a different size (Table 1), we used two different sized custom-made perspex plethysmography chambers. Baseline flow rates for the small and large chambers were 300 and 600 ml/min, respectively. The small chamber (90 ml volume) was used to perform measurements on P5 and P10 rats, whereas the larger chamber (420 ml) was used for P20 pups. To ensure equal washout times were associated with the different dead space volumes of each chamber time constants were determined using a 5 min N_2 perturbation at various flow rates. Based on these equiv-

Table 1
Body weight (BW), body temperature (Tb) and baseline ventilatory variables in P5, P10, and P20 rats ~2 h after i.p. injection of saline or LPS (0.1mg/kg). Note, given the higher mortality rate in P10 age group, data are provided after the rats were stratified into survivors (LPS(s)) and non-survivors (LPS(NS)) of LPS.

Age	Treatment Group	Variable					
		Mortality	BW (g)	Tb (°C)	VE (ml/g/min)	VT (ml/g)	fR (br/min)
P5	Saline	0% (0/11 rats)	11.7 ± 0.2	35.2 ± 0.1	1.22 ± 0.13	0.011 ± 0.001	114.2 ± 9.9
	LPS	18% (2/9 rats)	11.7 ± 0.3	35.0 ± 0.3	0.97 ± 0.06	0.014 ± 0.001*	70.3 ± 6.1*
P10	Saline	0% (0/13 rats)	21.9 ± 0.7	36.2 ± 0.2	1.34 ± 0.08	0.0102 ± 0.0009	142.0 ± 7.4
	$LPS_{(S)}$	N/A	20.3 ± 1.1	35.8 ± 0.4	1.16 ± 0.08	0.0096 ± 0.0007	129.4 ± 13.2
	$LPS_{(NS)}$	48% (10/21 rats)	21.5 ± 1.0	34.7 ± 0.4*#	0.92 ± 0.05*#	0.0106 ± 0.0005	88.4 ± 6.6*#
P20	Saline	0% (0/10 rats)	52.2 ± 3.2	36.9 ± 0.2	1.09 ± 0.07	0.0082 ± 0.0007	136.4 ± 5.4
	LPS	0% (0/10 rats)	46.6 ± 2.1	37.4 ± 0.2*	1.20 ± 0.07	0.0082 ± 0.0004	147.0 ± 4.9

Values are mean ± 1S.E.M. *Significantly different from saline treated rats ($p < 0.05$) within an age group. #significant difference between LPS(NS) and LPS(S) in the P10 age group. Values in parenthesis indicate the proportion of rats that died. N/A, not applicable since this group represents survivors of LPS. Abbreviations are indicated in the text.