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Do prenatal immune activation and maternal iron deficiency interact to affect neurodevelopment and early behavior in rat offspring?

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ABSTRACT

Infection and iron deficiency are common during pregnancy and studies have described altered brain development in the offspring as a result of these individual maternal exposures. Both exposures have been identified as risk factors for schizophrenia yet they have never been modeled simultaneously. We developed a rat model of prenatal immune activation on a background of maternal iron deficiency to determine whether these factors interact to affect neurodevelopment and early behavior in offspring. Pregnant rats were placed on iron sufficient (IS) or iron deficient (ID) diets from E2 to P7, and administered LPS or saline on E15/16. Iron was reduced in liver, spleen, serum and placenta from ID dams by E15. LPS administration on E15 caused greater induction of serum interleukin-6 and tumor necrosis factor-α in ID dams compared to IS dams. Offspring (P0, P7) from ID dams had reduced iron in spleen, liver and brain compared to IS, which normalized by P21. Pups from ID dams showed differences in forelimb grasp and acoustic startle, whilst pups from LPS dams displayed differences in grip ability, geotaxis reflex, cliff avoidance and acoustic startle. Offspring from LPS dams displayed reduced locomotor activity at P7 and P60; offspring from ID dams showed no change. Our findings show effects of prenatal LPS and maternal iron deficiency were additive, such that offspring exposed to both insults displayed more neurodevelopmental abnormalities than offspring exposed to one alone. Yet surprisingly there was no interaction between factors, suggesting independent mechanisms of action.

1. Introduction

Epidemiological studies indicate that prenatal immune activation, as a result of an infection during pregnancy, is highly prevalent in developed and developing countries (Velu et al., 2011). Maternal iron deficiency during prenatal and early postnatal life is also extremely common (Milman, 2011), with an estimated global incidence of 42% (McLean et al., 2009). This is due, in part, to increased iron requirements during pregnancy compared to the non-pregnant state (Bothwell, 2000). Maternal iron status may also play an important role in modulating immune responses during pregnancy as evidenced, for example, by the observation that subclinical iron deficiency is strongly and independently associated with bacterial vaginosis in early pregnancy (Verstraelen et al., 2005). Hence, it is likely that prenatal infection and maternal iron deficiency will occur simultaneously in many pregnancies. Epidemiological studies also indicate that these two early insults are associated with negative neurobehavioral outcomes for offspring. For example, strong evidence describes an association between maternal infection with either viral or bacterial pathogens during pregnancy and later development of schizophrenia or autism in offspring (Atladottir et al., 2010; Brown and Derkits, 2010). Recent evidence also suggests an association between maternal iron deficiency and schizophrenia in the offspring (Insel et al., 2008; Sorensen et al., 2010). Given that we understand schizophrenia and other psychiatric disorders to be neurodevelopmental in origin, considering the early life phenotype that results from these prenatal manipulations is an important component of understanding disease pathophysiology.

Multiple rodent models of prenatal immune activation using systemic administration of bacterial endotoxins or viral mimics, or peripheral injection of more localized immunogens, have described structural and neurochemical alterations in the brains of offspring, as well as significant behavioral deficits during development and at adulthood (for review see Boksa, 2010). The inflammatory cytokine, interleukin-6 (IL-6), is thought to be one of the key mediators of the central and peripheral immune response that is responsible for the downstream effects of prenatal immune activation (Luheshi et al., 1997; Smith et al., 2007). As with maternal immune activation, maternal iron deficiency has also been shown to have negative neurobehavioral consequences for the offspring in animal models. Offspring from iron deficient rat dams display reduced birth weight, altered lipid and monoamine metabolism, disrupted brain development, as well as cognitive dysfunction and hypertension during adulthood (Beard

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et al., 2006; Brunette et al., 2010; Gambling et al., 2002; Gambling et al., 2003; Morath and Mayer-Proschel, 2002).

While prenatal immune activation and maternal iron deficiency have individually been shown to affect neurodevelopmental outcomes, the relationship between the two insults may be crucial for determining the short and long-term health of offspring. Yet no study has investigated the interaction between these factors in a pregnant animal. Interestingly, Pagani et al. (2011) recently demonstrated that, in non-pregnant mice, iron deficiency results in enhanced induction of IL-6 in response to administration of the bacterial endotoxin, lipopolysaccharide (LPS). If iron deficiency also enhances LPS-induced cytokine responses in pregnant animals, this might result in augmented abnormalities in offspring exposed to the two prenatal insults compared to prenatal LPS alone. Furthermore, exposure to LPS is known to cause a decrease in serum iron (Deschemin and Vaulont, 2013; Kemna et al., 2005), an effect which is mediated by IL-6 and hepcidin. Hence, there appears to be a circular relationship between iron status and inflammatory events. Iron deficiency may predispose an organism to an enhanced pro-inflammatory response, while the inflammatory cascade it induces may further decrease circulating and stored iron.

Therefore, the objective of this study was to characterize the interaction between iron deficiency and immune system activation in pregnant animals, and to consider the ramifications of these early life events on the offspring's neurodevelopment. To accomplish this we combined two well described models of maternal iron deficiency and prenatal immune activation in the rat. We chose a moderate model of iron deficiency in which the pregnant dam is placed on an iron deficient diet from early pregnancy until postnatal day 7 (P7) (Brunette et al., 2010; Jorgenson et al., 2005; Rao et al., 2011). This and similar models have been shown to produce developmental delays in the offspring (Beard et al., 2006), persistent deficits in sensorimotor and hippocampal-dependent behaviors (Felt et al., 2006) and alterations in hippocampal size and neurochemistry (Rao et al., 2011). To induce prenatal immune activation, we chose a model of LPS administration at embryonic day 15 and 16 (E15, 16). In rats, exposure to LPS during late gestation has been shown to result in subtle but significant alterations in early neuromotor functions and neonatal behaviors (Baharnoori et al., 2010) as well as behavioral changes in the adult offspring (Fortier et al., 2004, 2007).

The first specific aim of this study was to provide an in-depth characterization of this novel combined model of prenatal LPS exposure on a background of maternal iron deficiency during pregnancy. We measured pro-inflammatory cytokines (IL-6 and TNF-α) that are produced in response to LPS administration as well as the changes in iron levels in dam tissue and placenta as a result of an iron deficient diet. We assessed the impact of maternal diet and prenatal LPS treatment on iron status and body weight in the offspring. The second aim was to test whether prenatal LPS exposure and maternal iron deficiency interact to affect the early neurodevelopmental profile of resulting offspring. We characterize the offspring's early neurodevelopment using a battery of tests modified from the SHIRPA protocol (Rogers et al., 1997), and describe locomotor activity in post-weaning life. We hypothesize that prenatal LPS administration, on a background of maternal iron deficiency, will result in potentiation of the subsequent pro-inflammatory responses in the dam, resulting in a more severe postnatal phenotype in the offspring.

2. Methods

2.1. Animals

Timed pregnant Sprague Dawley rats were obtained from Charles River at embryonic day 2 (E2). Dams were housed individually, on a 12 h light/dark cycle (lights on 0800 h) at a constant temperature of 21 ± 2 °C with food and water available ad libitum. All animals were treated in accordance with guidelines from the Canadian Council on Animal Care (www.ccac.ca) and protocols approved by the McGill University Animal Care Committee.

2.1.1. Dietary manipulation of iron intake

Upon arrival at E2, pregnant dams were placed on either an iron deficient (background iron 3–8 ppm, ID) or iron sufficient (200 mg/kg, IS) modified AIN-93G diet (Harlan Teklad Diets, Madison WI, USA). Diet was powdered, to allow quantification of food intake. Dams continued on their respective diets throughout pregnancy, until postnatal day 7 (P7), at which time all dams were placed on Global 18% Protein Diet (iron content, 200 mg/kg, Harlan Teklad Diets, Madison WI, USA).

2.1.2. Administration of LPS to pregnant rats

On E15 and E16, dams on iron sufficient and iron deficient diets received an injection of either LPS (50 μg/kg, i.p, *Escherichia* coli serotype 0111:B4, Lot 42k4120, Sigma–Aldrich, Canada) or 0.9% saline (4 ml/kg, i.p.). Injections were administered between 10:00 a.m. and noon. One cohort of dams was sacrificed two hours post-injection in order to determine inflammatory cytokine response and dam iron status. Otherwise, pregnancies continued to birth as normal.

2.1.3. Offspring

Offspring were weighed and sexed within 24 h of birth. Cohorts were tested for neonatal behaviors at P6–P18 or sacrificed at P0, P7 and P21 (weaning) for tissue iron measurement. At weaning offspring were separated by sex and were group housed 2–3 per cage and fed standard rat chow (Global 18% Protein Diet, Harlan Teklad Diets, Madison WI, USA). Offspring were weighed weekly and were either used for locomotor activity testing (P25, P60) or sacrificed for iron analysis (P60).

2.2. Quantification of non-heme iron in tissue and serum

Non-heme iron content in tissue and serum was determined using a ferrozine assay (Gkouvatsos et al., 2011). Briefly, livers, spleen, brains and placenta were removed (either whole, or portions, depending on the age of the animal) and snap frozen. Whole blood was collected, left at room temperature for 30 min, then centrifuged (2000*g*, 15 min at 4 °C) and serum was collected. Tissues and serum were stored at −80 °C. Samples were dehydrated overnight at 106 °C then weighed and incubated in an acid mixture (3 M HCl in 10% TCA; 20–30 mg tissue/ml of acid mixture) for 48 h at 65 °C. Samples were centrifuged (10,000*g*, 5 min, room temperature) and supernatant collected and stored at 4 °C. Using a 96 well plate, 50 μL of sample were incubated for 30 min at room temperature with 200 μL ferrozine reagent (including 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid, Sigma–Aldrich, USA). A standard curve was prepared according to the manufacturer's instructions (QuantiChrom Iron Assay kit, Gentaur, UK). Samples and standards were read at 562 nm (Spectra Max 190, Molecular Devices, USA). Non-heme iron content was calculated per milligram of dry tissue, or per organ in the case of P0 and P7 spleens.

2.3. Complete blood cell count analysis

EDTA anti-coagulated blood samples were used to obtain a complete blood count (CBC) using a Vet ABC Analyser. A white blood cell differential was determined from microscopic analysis of whole blood smears, obtained using Camco Quik Stain (Fisher Scientific, USA).