Experimental Neurology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Preclinical chorioamnionitis dysregulates CXCL1/CXCR2 signaling throughout the placental-fetal-brain axis

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ARTICLE INFO

Keywords:
Perinatal brain injury
Chemokine
Neutrophil
Neuroinflammation
Encephalopathy of prematurity
Microglia

ABSTRACT

In the United States, perinatal brain injury (PBI) is a major cause of infant mortality and childhood disability. For a large proportion of infants with PBI, central nervous system (CNS) injury begins *in utero* with inflammation (chorioamnionitis/CHORIO) and/or hypoxia-ischemia. While studies show CHORIO contributes to preterm CNS injury and is also a common independent risk factor for brain injury in term infants, the molecular mechanisms mediating inflammation in the placental-fetal-brain axis that result in PBI remain a gap in knowledge. The chemokine (C-X-C motif) ligand 1 (CXCL1), and its cognate receptor, CXCR2, have been clinically implicated in CHORIO and in mature CNS injury, although their specific role in PBI pathophysiology is poorly defined. Given CXCL1/CXCR2 signaling is essential to neural cell development and neutrophil recruitment, a key pathological hallmark of CHORIO, we hypothesized CHORIO would upregulate CXCL1/CXCR2 expression in the placenta and fetal circulation, concomitant with increased CXCL1/CXCR2 signaling in the developing brain, immune cell activation, neutrophilia, and microstructural PBI. On embryonic day 18 (E18), a laparotomy was performed in pregnant Sprague Dawley rats to induce CHORIO. Specifically, uterine arteries were occluded for 60 min to induce placental transient systemic hypoxia-ischemia (TSHI), followed by intra-amniotic injection of lipopolysaccharide (LPS). Pups were born at E22. Placentae, serum and brain were collected along an extended time course from E19 to postnatal day (P)15 and analyzed using multiplex electrochemiluminescence (MECI), Western blot, qPCR, flow cytometry (FC) and diffusion tensor imaging (DTI). Results demonstrate that compared to sham, CHORIO increases placental CXCL1 and CXCR2 mRNA levels, concomitant with increased $CXCR2^+$ neutrophils. Interestingly, pup serum CXCL1 expression in CHORIO parallels this increase, with sustained elevation through P15. Analyses of CHORIO brains reveal similarly increased CXCL1/CXCR2 expression through P7, together with increased neutrophilia, microgliosis and peripheral macrophages. Similar to the placenta, cerebral neutrophilia was defined by increased CXCR2 surface expression and elevated myeloperoxidase expression (MPO), consistent with immune cell activation. Evaluation of microstructural brain injury at P15 with DTI reveals aberrant microstructural integrity in the callosal and capsular white matter, with reduced fractional anisotropy in superficial and deep layers of overlying cortex. In summary, using an established model of CHORIO that exhibits mature CNS deficits mimicking those of preterm survivors, we show CHORIO induces injury throughout the placental-fetal-brain axis with a CXCL1/CXCR2 inflammatory signature, neutrophilia, and microstructural abnormalities. These data are concomitant with abnormal cerebral CXCL1/CXCR2 expression, and support temporal aberrations in CXCL1/CXCR2 and neutrophil dynamics in the placental-fetal-brain axis following CHORIO. These investigations define novel targets for directed therapies for infants at high risk for PBI.

1. Introduction

Preterm birth is responsible for 75% of perinatal mortality (Goldenberg et al., 2008), with approximately 1 million deaths/year worldwide (Gravett et al., 2012). Among children born at < 28 weeks estimated gestational age (EGA), 30 to 50% will have borderline (IQ < 85) or severe (IQ < 70) cognitive delay (Anderson, 2014). Typically, deficits are cumulative, and children with cognitive and

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https://doi.org/10.1016/j.expneurol.2017.11.002
Received 16 June 2017; Received in revised form 28 October 2017; Accepted 3 November 2017
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behavioral problems often also have cerebral palsy, vision and hearing loss, epilepsy, and overall poor physical health that contributes to the prematurity-related burden of chronic disease in adulthood (Blencowe et al., 2013; Mwaniki et al., 2012). The recent recognition that prenatal care and prevention efforts are ineffective to fully reduce the burden associated with neonatal mortality and morbidity in the United States collectively emphasizes the necessity to identify novel pathophysiological mechanisms related to the perinatal brain injury (PBI) associated with preterm birth (Chen et al., 2016; Hodnett et al., 2010).

Identification of critical pathways underlying the developmental program shared by both the placenta and developing brain is required to minimize the consequences of PBI (Redline, 2013). Disruptions in placental function directly affect organ maturation, and the central nervous system (CNS) is particularly vulnerable due to its protracted development throughout pregnancy (Morrison et al., 2007). For a large proportion of infants with CNS injury associated with prematurity, the injury begins *in utero* with inflammation of the placenta and/or hypoxia-ischemia (HI) from placental insufficiency with resultant dysfunction in the placental-fetal-brain axis (Anblagan et al., 2016; Gopagondanahalli et al., 2016). Chorioamnionitis (CHORIO), or inflammation/infection of the placenta and surrounding membranes, has recently been recognized in a significant number of preterm and term infants with PBI (De Felice et al., 2001; Galinsky et al., 2013; Lee et al., 2013; Shevell et al., 2014; Soraisham et al., 2013; YW et al., 2003). CHORIO affects placental permeability and blood flow, disseminates HI and transmits inflammation to fetuses of all gestational ages (Dueck et al., 2009). Notably, CHORIO is the most common abnormality found in preterm deliveries, and is associated with a significant increase in systemic fetal and neonatal inflammation (O'Shea et al., 2012; O'Shea et al., 2014).

The placenta and CNS share essential underlying developmental programs and both are linked by a developing and dynamic immune system. Chemokines and cytokine ligands and receptors are major mediators of immune system communication, and require exquisite balance during development and in response to injury. The chemokine (C-X-C motif) ligand 1 (CXCL1) and its receptor, CXCR2, are essential for neural cell development and placental function (Filipovic et al., 2003; Gomez-Lopez et al., 2013; Nasu et al., 2001; Robinson and Franic, 2001; Robinson et al., 1998). CXCL1 is normally present in the uterus in humans and rats (Gomez-Lopez et al., 2013; Nasu et al., 2001), where it provides a chemotactic gradient for neutrophil trafficking to the maternal-fetal interface, creating a tissue specific immunological environment. CXCL1 signaling through CXCR2 is essential for neurodevelopment (Robinson and Franic, 2001; Robinson et al., 1998; Tsai et al., 2002). In the brain, both CXCL1 and CXCR2 are preferentially expressed on oligodendrocyte progenitor cells (OPCs), astrocytes, microglia, neurons, and monocytes as early as 19–22 weeks gestation (Filipovic et al., 2003; Robinson and Franic, 2001; Robinson et al., 1998; Lindner et al., 2008; Valles et al., 2006; Veenstra and Ransohoff, 2012), and CXCR2 activation by CXCL1 on OPCs regulates their proliferation and migration (Robinson and Franic, 2001; Robinson et al., 1998; Tsai et al., 2002). Notwithstanding these beneficial roles in development, CXCL1/CXCR2 signaling is upregulated in inflammatory placental and CNS pathology. CXCL1 is extensively elevated with intrauterine inflammation (Saini et al., 2011), and the severity of pathologic placental inflammation correlates positively with CXCL1 levels in newborns with CHORIO and funusitis (Bry et al., 2015). Similarly, CXCL1 is the dominant ligand of CXCR2 that is expressed in the inflamed CNS (Roy et al., 2012), and CXCL1 levels are directly proportional to CXCR2 signaling (Veenstra and Ransohoff, 2012; Roy et al., 2012; Kielian et al., 2001; Carlson et al., 2008; Kerstetter et al., 2009; Liu et al., 2010).

Given the placental-fetal interface plays a critical role in relaying the effects of gestational insults to the developing brain, we propose CXCL1/CXCR2 signaling contributes to the pathophysiology of PBI in our preclinical rat model of CHORIO. Consistent with the concept that CHORIO leads to sustained aberrant immune function in adult offspring (Lin et al., 2010), with perturbations in chemokines essential to inflammatory pathophysiology, we hypothesized that upregulation of CXCL1/CXCR2 commencing *in utero* would negatively affect the fetal microenvironment and trajectory of CNS development. These investigations are among the first studies to connect CXCL1/CXCR2 dysregulation in the placental-fetal-brain axis to impaired neurodevelopment and chronic brain injury, and elucidate novel therapeutic targets for preterm infants with PBI.

2. Materials & methods

The Institutional Care and Use Committee (IACUC) at the University of New Mexico Health Sciences Center and Johns Hopkins University approved all experimental procedures.

2.1. Induction of chorioamnionitis (CHORIO/TSHI ± LPS)

A unique challenge to neurodevelopmental research is accurately recapitulating the intact maternal-placental-fetal unit, in which the compartment-specific responses to inflammation and HI can be studied in unison. As placental structure and function is of significant clinical importance to neurologic sequelae in preterm survivors (Redline, 2013; Badawi et al., 1998; Bastek et al., 2011), we use a prenatal model of *in utero* transient systemic HI (TSHI) and intra-amniotic lipopolysaccharide (LPS) administration in pregnant rats (Jantzie et al., 2014a; Jantzie et al., 2015a; Maxwell et al., 2015). This approach capitalizes on an intact maternal-placental-fetal unit and hemichorial discoid placenta, and allows investigation of inflammation transduction throughout the placental-fetal-brain axis. Injury is induced on embryonic day 18 (E18) in Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) as previously described (Jantzie et al., 2014a; Jantzie et al., 2015a; Maxwell et al., 2015). We and others have shown that injury at E18 occurs before subplate axons infiltrate the cortical plate on E21 (Jantzie et al., 2015b; Kanold and Luhmann, 2010; Kostovic et al., 2014; Kostovic and Judas, 2010), and near the beginning of pre-oligodendrocyte appearance (Jantzie et al., 2013; Mazur et al., 2010; Robinson et al., 2005). Thus, this models both the gray and white matter neural cell injury in preterm infants born at 24–28 weeks gestation (Jantzie et al., 2015b; Mazur et al., 2010; Robinson et al., 2005). Briefly, a laparotomy is performed and uterine arteries are clamped for 60 min, followed by intra-amniotic injections of LPS at 4 μg/sac (0111:B4, Sigma, St. Louis, MO). Sham controls undergo anesthesia and laparotomy for 60 min without arterial clamping or LPS injections. Pups are born at E22, approximately equal to 30–32 weeks in human gestation. We have previously reported the effects of TSHI and LPS alone, and in concert, on CNS pathological hallmarks, functional motor outcomes, histologic placental injury, and expression of common pro-inflammatory cytokines (Jantzie et al., 2015a; Maxwell et al., 2015). For each experiment described, equal numbers of male and female pups were used in each assay, and data represents true n (individual pups) from at least 4 different dams per condition.

2.2. Placenta, serum and brain collection

Placentae were harvested at E19 (24 h following induction of CHORIO). We chose this acute time point to evaluate placental inflammation for two reasons. First, the temporal proximity to induction of the *in utero* insult; and second, to eliminate any potential confounding factors of later gestational ages due to initiation of labor (rat term labor commences on E22) (Jantzie et al., 2014a; Jantzie et al., 2015a; Maxwell et al., 2015). Serum was collected from rat pups from postnatal day (P) 2 to P15, and brains were collected from E19 to P15. Specifically, tissues were rapidly dissected and flash frozen and stored at − 80 °C until final analyses. Serum was prepared from whole blood collected from individual pups and centrifuged at 6000 RCF at 4 °C for

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