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Research Paper

Neonatal erythropoietin mitigates impaired gait, social interaction and diffusion tensor imaging abnormalities in a rat model of prenatal brain injury

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ABSTRACT

Children who are born preterm are at risk for encephalopathy of prematurity, a leading cause of cerebral palsy, cognitive delay and behavioral disorders. Current interventions are limited and none have been shown to reverse cognitive and behavioral impairments, a primary determinant of poor quality of life for these children. Moreover, the mechanisms of perinatal brain injury that result in functional deficits and imaging abnormalities in the mature brain are poorly defined, limiting the potential to target interventions to those who may benefit most. To determine whether impairments are reversible after a prenatal insult, we investigated a spectrum of functional deficits and diffusion tensor imaging (DTI) abnormalities in young adult animals. We hypothesized that prenatal transient systemic hypoxia-ischemia (TSHI) would induce multiple functional deficits concomitant with reduced microstructural white and gray matter integrity, and tested whether these abnormalities could be ameliorated using postnatal erythropoietin (EPO), an emerging neurorestorative intervention. On embryonic day 18 uterine arteries were transiently occluded for 60 min *via* laparotomy. Shams underwent anesthesia and laparotomy for 60 min. Pups were born and TSHI pups were randomized to receive EPO or vehicle *via* intraperitoneal injection on postnatal days 1 to 5. Gait, social interaction, olfaction and open field testing was performed from postnatal day 25–35 before brains underwent *ex vivo* DTI to measure fractional anisotropy, axial diffusivity and radial diffusivity. Prenatal TSHI injury causes hyperactivity, impaired gait and poor social interaction in young adult rats that mimic the spectrum of deficits observed in children born preterm. Collectively, these data show for the first time in a model of encephalopathy of prematurity that postnatal EPO treatment mitigates impairments in social interaction, in addition to gait deficits. EPO also normalizes TSHI-induced microstructural abnormalities in fractional anisotropy and radial diffusivity in multiple regions, consistent with improved structural integrity and recovery of myelination. Taken together, these results show behavioral and memory deficits from perinatal brain injury are reversible. Furthermore, resolution of DTI abnormalities may predict responsiveness to emerging interventions, and serve as a biomarker of CNS injury and recovery.

1. Introduction

Globally, almost 15 million infants are born preterm each year (Blencowe et al., 2012), making preterm birth a leading cause of neurological and neuropsychiatric disability. Children born extremely preterm (< 28 weeks) are particularly at risk, and prone to a spectrum of deficits including cerebral palsy, epilepsy, hyperactivity and inattention, and impaired social interaction, memory and executive function (Anderson, 2014). Affecting up to 50% of survivors (Nosarti et al., 2012; Anderson, 2014), cognitive and behavioral deficits are the most common, often the most debilitating, and present the greatest hurdle to adult independence. As survival from preterm birth into

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adulthood improves, the need to optimize neuropsychiatric outcomes for preterm survivors is increasingly apparent (Nosarti et al., 2012). Further, a lack of resource-efficient preclinical models that adequately replicate neuropsychiatric impairments in children born preterm is a major barrier to translation of potential therapies.

The neurological deficits from preterm birth arise from a complex amalgam of developing central nervous system (CNS) abnormalities that involve white matter (WM), subplate and gray matter (GM) injuries (Robinson, 2005, Kostovic and Judas, 2006, Kinney, 2009, Volpe, 2009a, 2009b, Kostovic et al., 2014a, 2014b). Despite the shift observed from cystic periventricular leukomalacia to diffuse WM gliosis on imaging in preterm survivors at major academic medical centers (Hamrick et al., 2004; Rutherford et al., 2010; de Vries and Volpe, 2013), a concomitant improvement in neuropsychiatric outcomes has not occurred. Collectively, these data emphasize that cognitive and behavioral difficulties likely reflect complex, multiregional injury extending beyond white matter alone (Kostovic and Judas, 2006; Robinson et al., 2006; Ligam et al., 2009; Volpe, 2009a, 2009b; Kostovic et al., 2014a, 2014b; Ceschin et al., 2015).

For many preterm infants, CNS injury begins *in utero* with maternal-placental-fetal axis dysfunction (Leviton et al., 2010; Dammann and Leviton, 2014; Shevell et al., 2014; Johnson and Marlow, 2016). To facilitate translation to clinical scenarios, we developed a model of CNS injury from prematurity that: 1) replicates the intrauterine insult and capitalizes on the hemichorial, discoid placental unit in rats that is similar to humans (Jantzie and Robinson, 2015); 2) recapitulates the neuronal, oligodendroglial and subplate developmental injury (Robinson et al., 2005, Mazur et al., 2010, Jantzie et al., 2013, Jantzie et al., 2015a, 2015b, Jantzie et al., 2016); and as shown here produces a sustained spectrum of functional deficits in mature animals. We hypothesized that this model would be an effective platform to study and support testing of neuro-repair strategies for CNS injury from preterm birth.

An example of such an intervention is erythropoietin (EPO) (McPherson and Juul, 2010, Wu et al., 2012, Jantzie et al., 2013, Jantzie et al., 2014a, 2014b, Leuchter et al., 2014, Ohls et al., 2014, Fauchere et al., 2015, Jantzie et al., 2015a, 2015b, O'Gorman et al., 2015, Jantzie et al., 2016). Without ligand present, unbound EPO receptors trigger cell death (Knabe et al., 2004; Knabe et al., 2005; Mazur et al., 2010; Ott et al., 2015). After perinatal brain injury (PBI), neural cell EPOR expression increases without concomitant EPO ligand expression, and exogenous EPO restores balanced EPOR signaling (Spandou et al., 2004; Mazur et al., 2010; Ott et al., 2015). Exogenous EPO crosses the blood-brain barrier to enhance recovery after CNS injury (Brines et al., 2000; Xenocostas et al., 2005; Mazur et al., 2010; Gonzalez et al., 2013; Jantzie et al., 2013). Thus, to improve the evaluation of emerging interventions for deficits from preterm birth, we used prenatal injury and neonatal EPO to test whether changes in mature, sophisticated outcomes, including social interaction could be detected in a rat model. Indeed, we show using translatable diffusion tensor imaging (DTI) metrics and behavioral assessment that postnatal EPO treatment restores impaired complex gait deficits and social interaction, and normalizes DTI abnormalities and aberrant microstructure in white matter and deep gray matter that are associated with CNS injury from preterm birth.

2. Methods

All procedures were performed in accordance with NIH Guide for Care and Use of Laboratory Animals with approval by Institutional Animal Care and Use Committees at University of New Mexico Health Sciences Center and Boston Children's Hospital. Rats were housed in standard colony rooms with lighting on from 7 am to 7 pm, and food and water available *ad libitum*.

2.1. Transient systemic hypoxia-ischemia

Embryonic day (E) 18 pregnant Sprague-Dawley dams underwent isoflurane anesthesia and laparotomy, as previously described (Robinson, 2005; Mazur et al., 2010). Like humans, rodent oligodendrogenesis begins prenatally, and differentiation and myelination continue postnatally. In rats, pioneering subplate neurons exit the thalamus by embryonic day 16 (E18) and arrive in the cortex by postnatal day 2 (P2) (Kanold and Luhmann, 2010). For prenatal transient systemic hypoxia-ischemia (TSHI), uterine arteries were transiently occluded for 60 min, while shams underwent laparotomy only for 60 min. This approach accurately recapitulates TSHI injury through intact maternal-placental-fetal units, and capitalizes on changes individual fetal microenvironments. Pups were born at term (E22) and reared with dams until postnatal day 21 (P21), when they were weaned and housed in single sex groups (2–3 per cage). Prenatal TSHI produces a reproducible insult with consistent fetal mortality and postnatal growth and neuropathological, biochemical and functional outcomes (Robinson et al., 2005, Mazur et al., 2010, Jantzie et al., 2013, Jantzie et al., 2014a, Jantzie et al., 2014b, Jantzie et al., 2015a, 2015b, Jantzie et al., 2016). For all experiments, both sexes, and rats generated from at least two separate litters were used. The average litter size for all experiments were similar, with sham litters an average of 10.4 ± 0.5 pups and TSHI litters at 11.6 ± 0.6 pups ($p > 0.05$). The average pup size also did not differ between groups with sham weighing 7.3 ± 0.05 g on P1, TSHI pups weighing 7.1 ± 0.1 g and TSHI + EPO pups weighing 7.2 ± 0.1 g at P1, consistent with our previously published reports of no significant differences between Sham and TSHI litters or pups, and the cumulative effects of *in utero* TSHI on litter size, postnatal survival and offspring body weight (Jantzie et al., 2014a, 2014b).

2.2. Postnatal EPO treatment

Using an established, clinically-relevant dosing regimen (Mazur et al., 2010; Jantzie et al., 2014b, 2015a, 2016), P1 pups from all litters were individually randomized to receive either EPO (2000 U/kg, R&D Systems, Minneapolis, MN) or vehicle (sterile saline) intraperitoneally once daily from P1 to P5, a dosing regimen comparable to those used in human neonatal neuro-reparative trials. Rodents are born at a time equivalent to the human third trimester, with P9 approximately equivalent to term in human gestation (Jantzie and Robinson, 2015). Thus, EPO or vehicle administration at P1 to P5 is approximately equivalent to 30 to 35 weeks gestation in humans. Previous studies showed this dosing regimen improved stride length and seizure threshold, and demonstrated no unexpected findings with EPO-treated sham animals (Mazur et al., 2010).

2.3. Behavioral testing

Three rat cohorts (each cohort consisting of a sham and a prenatal TSHI litter) were sequentially tested for gait, and open field (OF) testing at P25–30. Specifically, Gait was tested at P25-P26, and then Open field was tested at P28–30.Two separate cohorts (4 litters) underwent social interaction testing at P30-P32.

2.4. Gait analysis

Computerized gait analysis was performed on P25-P26 (Jantzie et al., 2014a, 2015a). Briefly, digital video of each rat running on a backlit transparent treadmill set at 30 cm/s was acquired with a high-speed camera and analyzed using Digigait software (Mouse Specifics, Framingham, MA). Digigait software analyses identifies individual paw prints and allows calculation of multiple gait metrics and kinematic measurements based on the position, area and timing of each paw step. Similarly, posture, cadence, stance duration, well as braking, swing and propulsion phases of each step are measured. Propulsion was defined as