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Mild prenatal hypoxia-ischemia leads to social deficits and central and peripheral inflammation in exposed offspring

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ABSTRACT

Hypoxic-ischemic encephalopathy (HIE) resulting from intrauterine or perinatal hypoxic-ischemia (HI) is a leading cause of long-term neonatal neurodisability. While most studies of long-term outcome have focused on moderate and severe HIE in term infants, recent work has shown that those with mild HIE may have subtle neurological impairments. However, the impact of mild HI on pre-term infants is much less clear given that pre-term birth is itself a risk factor for neurodisability. Here we show that mild HI insult alters behaviour, inflammation and the corticosterone stress response in a rat model of pre-term HIE. Mild HI exposure led to social deficits in exposed offspring at postnatal day 30, without impairments in the novel object recognition test nor in the open field test. This was also accompanied by elevations in circulating adrenocorticotropic hormone and corticosterone indicating an exaggerated stress response. There were also elevations in *il-1β* and *il-6* but not *tnf-α* mRNA and protein in the brain and blood samples. In summary we find that a mild HI exposure leads to social deficits, central and peripheral inflammation, and an abnormal corticosterone response which are three core features of autism spectrum disorder. This shows that mild HI exposure may be a risk factor for an abnormal neurodevelopmental outcome in pre-term offspring.

1. Introduction

Hypoxic-ischemic encephalopathy (HIE) is a leading cause of death and long-term disability in neonates, hypoxic-ischemic encephalopathy is a consequence of a hypoxic insult arising from intrauterine or perinatal asphyxia (Locatelli et al., 2008; McIntyre et al., 2013; Harteman et al., 2013). This may be due to prolonged partial asphyxia secondary to reduced placenta blood flow, prolonged or obstructed labour, or an acute sentinel event such as uterine rupture, placental abruption or acute cord occlusion (Volpe, 2012). This leads to reduced cerebral perfusion which results in an inadequate supply of oxygen and glucose to the developing brain (Armstrong et al., 2012; Barberi et al., 1999). Reduced cerebral perfusion combined with hypoxia is known as hypoxia-ischemia (HI) (Fatemi et al., 2009). If a HI event damages the developing brain, an evolving encephalopathy is seen that is clinically graded as mild, moderate or severe (Fatemi et al., 2009; Shah and Perlman, 2009; Volpe, 2012).

The patterns of brain injury that result from HIE, and the long-term neurological outcomes depend on the grade of HIE and on the gestational age at the time the insult occurs (Logitharajah et al., 2009; Cabaj et al., 2012). The majority of studies on long-term neurodevelopmental outcome have focused on term infants with moderate and severe HIE, and have reported increased rates of a range of motor and non-motor neurological disability, (Mwaniki et al., 2012) including cognitive impairments (Lindstrom et al., 2008) and epilepsy (Kharoshankaya et al., 2016). Term infants with mild HIE have been considered to have a normal outcome, however, recent work has shown that these infants have cognitive impairments at five years (Murray et al., 2016). There is also some evidence that HI conditions increase the risk of autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) in exposed children (van Handel et al., 2007; Getahun et al., 2013; Getahun et al., 2013). Multiple studies have reported that prenatal and perinatal complications that can cause HI are associated with an increased risk of ASD. In particular, recent work in human

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populations has shown that neonatal respiratory distress and other markers of hypoxia were associated with increased risk of ASD in males in a twin study (Froehlich-Santino et al., 2014). Moreover, a recent meta-analyses also demonstrated an increased risk of ASD in children with neonatal hypoxia (Modabbernia et al., 2016).

In contrast to term infants, studying long-term neurodevelopment outcome in preterm infants (those born <37 weeks) with HIE is more difficult given that preterm birth is itself a risk factor for a poor neurodevelopment outcome and often occurs with co-existent pathologies. The result of this is that the contribution of mild HI exposure to long-term neurodevelopmental outcomes in preterm infants is unclear (Gopagondanahalli et al., 2016). Moreover, studying this is particularly difficult in very low gestational age (VLGA) infants (born <32 weeks') which occurs in 1% of singleton and 9% of twin pregnancies (Schaaf et al., 2011). Modelling HI exposure in VLGA infants has been carried out in rodents by examining the impact of HI exposure on the rat brain just before birth. This is because key neurodevelopmental processes that occur in humans from 23 to 32 weeks of gestation occur in the rat brain around birth (Semple et al., 2013). Previous work in these rat models of preterm HIE have examined the functional impact primarily on three core domains; motor deficits, social deficits and anxiety (Vazquez-Borsetti et al., 2016) and cognition and learning (Saraceno et al., 2016; Barkhuizen et al., 2017). While the functional impact of preterm exposure to moderate and severe HI has been the focus of intensive investigation (Barkhuizen et al., 2017), given the emerging clinical picture showing that term infants with mild HIE have subtle, long-term impairments without overt neurological injury (Murray et al., 2016), there is a need to better understand the contribution of mild-HI exposure to the long-term neurodevelopmental outcomes in preterm offspring.

2. Materials and methods

2.1. Study 1: Generation of a model of a mild pre-term HI insult

2.1.1. Animals and study design

All work was carried out under licence with ethical approval from the institutional ethics committee. Animals were maintained in a controlled environment on a 12 h light/dark cycle (lights on at 7:30 am) with *ad libitum* access to food and water. All experiments were performed in a blinded manner. Pregnant Sprague-Dawley (SD) rats were randomised into one of the following groups: (1) caesarean delivery, with no hypoxia exposure (sham, GD 22); (2) caesarean delivery following a 3, 5 or 7 min HI insult where indicated (Fig. 1). For the induction of HI, pregnant dams were induced and maintained under isoflurane (2%) anaesthesia and 21% oxygen (O_2). Following laparotomy, the infra-renal abdominal aorta and uterine arteries were ligated. The inhaled O_2 concentration was reduced to 10% for 3, 5 or 7 min to induce hypoxia. Durations longer than 7 min lead to a significant increase in offspring mortality and so were discontinued. After the HI exposure the O_2 concentration was restored from 10% to 21% to restore blood flow and O_2 saturation to induce a reperfusion injury. The pups were then immediately delivered by caesarean section (C-section) on GD 22, and manually stimulated to initiate breathing in an incubator at 37 °C for 1 h in room air. Two pups (males only) from each dam were cross fostered to a foster mother post-delivery. These foster mothers were first time mothers with foster pups only, litter sizes (n = 9–12). Maternal bonding behaviour was observed initially for rejection which was defined as any aggressive behaviour towards a pup, avoidance to nest or rejection to allow to feed. Pup weight was used as a measure to determine if feeding rejection occurred.

2.1.2. Western blotting

To assess caspase-3 activation, brains were collected on dry ice at postnatal day five (P5). Postnatal day five was chosen to assess death to encompass the combination of acute injury (24–48 h) and delayed injury response (3–5 days). Proteins homogenates of the whole brain from one pup, from three individual litters (n = 3 per group) were prepared using RIPA buffer containing a protease inhibitor cocktail (Santa Cruz Biotech., USA). Proteins were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane, that was blocked in 5% BSA in PBST (10 mM PBS + 0.1% Tween20), and incubated with primary antibodies against cleaved caspase-3 (1:500); pro-caspase-3 (1:1000); or β-actin (1:1000) (all Sigma) in 1% BSA in PBS-T for 16 h at 4 °C. Following washes, membranes were incubated with HRP-conjugated secondary antibodies (1:2000, Sigma) and were detected with the ECL-Plus system (Amersham). Images were analyzed by densitometry using Image J (NIH Image J 1.47v). Protein expression was normalized to that of β-actin.

2.1.3. TUNEL staining

To assess cell death in the tertiary phase of injury, we carried out terminal deoxynucleotidyl transferase dUPT nick-end labelling (TUNEL) staining in an additional cohort of offspring at P20. We used 2 male pups per litter from 5 L (n = 10 per group). To do this the offspring were anaesthetised at P20, and fixed by transcardial perfusion of 4% paraformaldehyde. The offspring were then culled by rapid decapitation, their brains were extracted and then post-fixed for 24 h at 4 °C in 4% paraformaldehyde. Following cryoprotection in a 30% sucrose in 10 mM PBS for 24 h, samples were snap frozen and sectioned at 20 μm intervals. TUNEL staining was performed according to the manufacturer's instructions (ApopTag; Millipore, USA). Five fields were randomly chosen in the CA1, CA2 and CA3 regions of the hippocampus and the numbers of TUNEL-positive were counted. Data are presented as the mean number of TUNEL-positive cells per field of view.

2.1.4. 28-Point neuroscore testing

Sensorimotor function was assessed using a modified version of the 28-point neuroscore (Encarnacion et al., 2011) in two additional cohorts; one of which was assessed at P20-P25, and one at P30-35. We used 2 male pups per litter from 5 L (n = 10 per group). The neuroscore test consisted of 11 tests with a maximum score of 28 (Table 1). Scoring was determined on a scale from 0 (impairment) to 28 (no impairment). Each animal was observed during each task at either P20-25 or P30-35. The 28-point neuroscore was completed for 5 days, and the mean cumulative score calculated per time period was calculated.

2.2. Study 2: Behavioural and molecular outcomes in the mild model of pre-term HI

2.2.1. Animals and study design

The rest of the study consisted of new cohorts of offspring that were randomised into three experimental groups: (1) offspring born by a normal vaginal delivery on GD22/23 (vaginal); (2) those born by C-Section without hypoxia on GD22 (C-section); and (3) those born by C-section following exposure to 3 min HI *in utero* (C-section + 3 min HI). (Fig. 1) Two pups (males only) from each dam were cross fostered to a foster mother post-delivery and monitored as outlined in Section 2.1.1. Pup weights were recorded at P1, P7, P14, P21 and P30 prior to behavioural analysis and tissue sampling at P30. For all the behavioural testing we used 1–2 male pups per litter from 5 to 6 L (n = 10 per group).

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