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Research Paper

Preterm birth disrupts cerebellar development by affecting granule cell proliferation program and Bergmann glia

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ABSTRACT

Preterm birth is a leading cause of long-term motor and cognitive deficits. Clinical studies suggest that some of these deficits result from disruption of cerebellar development, but the mechanisms that mediate cerebellar abnormalities in preterm infants are largely unknown. Furthermore, it remains unclear whether preterm birth and precocious exposure to the *ex-utero* environment directly disrupt cerebellar development or indirectly by increasing the probability of cerebellar injury, including that resulting from clinical interventions and protocols associated with the care of preterm infants. In this study, we analyzed the cerebellum of preterm pigs delivered via c-section at 91% term and raised for 10 days, until term-equivalent age. The pigs did not receive any treatments known or suspected to affect cerebellar development and had no evidence of brain damage. Term pigs sacrificed at birth were used as controls. Immunohistochemical analysis revealed that preterm birth did not affect either size or numbers of Purkinje cells or molecular layer interneurons at term-equivalent age. The number of granule cell precursors and Bergmann glial fibers, however, were reduced in preterm pigs. Preterm pigs had reduced proliferation but not differentiation of granule cells. qRT-PCR analysis of laser capture microdissected external granule cell layer showed that preterm pigs had a reduced expression of *Ccnd1* (Cyclin D1), *Ccnb1* (Cyclin B1), granule cell master regulatory transcription factor *Atoh1*, and signaling molecule *Jag1*. In vitro rescue experiments identified *Jag1* as a central granule cell gene affected by preterm birth. Thus, preterm birth and precocious exposure to the *ex-utero* environment disrupt cerebellum by modulating expression of key cerebellar developmental genes, predominantly affecting development of granule precursors and Bergmann glia.

1. Introduction

Preterm birth occurs in ~10% of pregnancies and is associated with long-term motor and cognitive deficits (Blencowe et al., 2012; Steer, 2005). Some neurological impairments in children born preterm are considered to result from disruption of development of the cerebellum, a major center of motor-coordination that is also involved in cognition and emotion (Reeber et al., 2013; Stoodley et al., 2016; Kopecky et al., 2012).

During development, cerebellar neurons originate from two germinal zones that arise in early embryonic cerebellar anlage: the cerebellar ventricular zone, which gives rise to GABAergic neurons, such as Purkinje cells and molecular layer interneurons, and the rhombic lip, which gives rise to glutamatergic cerebellar neurons, such as granule cells (Chizhikov and Millen, 2013). Upon exiting the ventricular zone, Purkinje cells migrate radially toward the cerebellar surface forming a multilayered Purkinje cell layer, which later resolves into a Purkinje cell monolayer. Dendrites of Purkinje cells form the molecular layer, which becomes populated by molecular layer interneurons (Leto et al., 2016). Granule cells, which are the most numerous neuronal type in the cerebellum and the entire brain, exit the rhombic lip as proliferating precursors. They migrate tangentially along the outer surface of the cerebellar anlage forming a secondary germinal matrix – the external granule cell layer (EGL). Granule cell precursors proliferate in the outer EGL in response to Shh and Jag1 signaling molecules (Dahmane and Ruiz i Altaba, 1999; Solecki et al., 2001; Hatten and Roussel, 2011), translocate to the inner EGL as they exit the cell cycle and initiate differentiation, and migrate radially along Bergmann glial fibers to

Abbreviations: Nat. born term, naturally born term pigs; C-sect. term, term pigs delivered via c-section; EGL, external granule cell layer; IGL, internal granule cell layer; PCs, Purkinje cells; ML, molecular layer; BG, Bergmann glia; LCM, laser capture microdissection; pH 3, phospho-Histone 3; CDKIs, inhibitors of cyclin-dependent kinases

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form the internal granule cell layer (IGL) below the Purkinje cell layer (Basson and Wingate, 2013; Sillitoe and Joyner, 2007).

In humans, the peak of granule cell proliferation occurs during the third trimester of pregnancy, which coincides with extensive radial migration and accumulation of differentiated granule neurons in the IGL, the cerebellar folia formation, and maturation of Purkinje cells (Raaf, 1944; Rakic and Sidman, 1970; Volpe, 2009; Ten Donkelaar and Lammens, 2009). These critical events make the cerebellum potentially vulnerable to consequences of preterm birth. Indeed, human MRI studies have associated preterm birth with reduced cerebellar volume (Messerschmidt et al., 2005; Limperopoulos et al., 2005a,b; Volpe, 2009; Brossard-Racine et al., 2015; Arhan et al., 2017). It is important to note, however, that preterm infants have high rates of cerebellar hemorrhage, white matter injury because of hypoxia-ischemia or infection, and infarction, all of which independently correlate with reduced cerebellar volume (Limperopoulos et al., 2010; Yoo et al., 2014; Ranger et al., 2015; Jeong et al., 2016; Kim et al., 2016; Pierson and Al Sufiani, 2016; Matsufuji et al., 2017).

Survival after early preterm birth requires intensive care. Some of the protocols that are used for survival of preterm infants have the potential to compromise cerebellar development. For example, the glucocorticoids administered to many preterm infants (often up to 85–100%) to enhance lung maturation or because of low blood pressure (Volpe, 2009; Baud and Gressens, 2011; Tam et al., 2011) inhibit cerebellar growth by repressing Shh signaling and disrupting proliferation of granule cell precursors (Heine et al., 2011; Tam et al., 2011). The cerebellum is sensitive to blood oxygen saturation, and the mechanical ventilation and hyperoxia required for some preterm infants because of extreme lung immaturity or respiratory distress are known to affect cerebellar development (Scheuer et al., 2018; Biran et al., 2011; Rees et al., 2009). Thus, it remains unknown (1) whether preterm birth and subsequent precocious exposure to the *ex-utero* environment directly compromise cerebellar developmental program or indirectly by increasing the probability of cerebellar injury, including that resulting from clinical interventions required for treatment of extracerebellar problems resulting from preterm birth, and (2) what cerebellar populations and molecular mechanisms are primarily affected by preterm birth.

Recently, Haldipur et al. (2011) compared cerebellar tissue samples from infants that were born preterm and died shortly after birth with those from stillborn infants (fetuses that died in utero, without any exposure to the *ex-utero* environment) and found that the first group had a reduced proliferation of granule cell precursors because of downregulated Shh signaling, and decreased density of Bergmann glia. Preterm infants born after 34 weeks also had a reduced number of Purkinje cells. Although this study suggests granule cells, Bergmann glia and Purkinje cells as potential targets of preterm birth, it is important to note that many of the preterm infants had sepsis/inflammation and/or were affected by other confounding factors. Using stillborn infants as study controls further complicated interpretation of the results, highlighting the need for analyzing a healthy animal model.

Although laboratory rodents are commonly used to study brain development, unlike infants, the mouse cerebellum is underdeveloped at birth, with the peak of granule cell proliferation, the development of cerebellar foliation, IGL formation and maturation of Purkinje cells occurring after birth (Raaf, 1944; Rakic and Sidman, 1970; Leto et al., 2016). In contrast, preterm pigs share similarities with preterm infants in organ anatomy, physiology, and patterns of cerebellar development, including the development of granule cells and Purkinje cells (Larsell, 1954; Sangild et al., 2013; Eiby et al., 2013; Radlowski et al., 2014; Choudhri et al., 2014). Moreover, the compatibility of preterm pigs with neonatal intensive care unit protocols and technologies provides an opportunity to study the consequences of prematurity and postnatal care on brain development.

A recent study found very limited differences in expression of several developmentally important genes in the cerebellum of preterm pigs relative to those born at term, which was interpreted as evidence of limited effect of preterm birth on cerebellar development (Bergström et al., 2016). In the aforementioned study, however, gene expression was analyzed using total cerebellar RNA. Since development of different cerebellar populations is controlled by different genetic programs (Chizhikov and Millen, 2013; Basson and Wingate, 2013; Sillitoe and Joyner, 2007; Goldowitz and Hamre, 1998), it is likely that preterm birth modulates expression of genes in a cell-type specific manner. Thus, gene expression differences in preterm subjects may be hard to detect by analyzing total cerebellar RNA, highlighting the need to analyze individual cerebellar cell types.

In the current study, by analyzing healthy pigs with cell-type specific markers, we demonstrated that preterm birth and precocious exposure to the *ex-utero* environment compromise cerebellar development. Furthermore, we identified specific cerebellar cellular populations and molecular mechanisms that are affected by preterm birth.

2. Materials and methods

2.1. Pigs and tissue collection

All aspects of the study that involved live pigs were performed according to an established protocol (Choudhri et al., 2014; Caminita et al., 2015) that was approved by the Institutional Animal Care and Use Committee of the University of Memphis. All pigs used in this study shared a consistent genetic lineage, were born to sows that had been artificially inseminated using a consistent source of semen, and were pathogen-free. Preterm pigs were delivered on gestational day 105 (91% of average 115-day term) (Choudhri et al., 2014) via c-section and maintained for 10 days, until term-equivalent age, when they were euthanized for brain collection. After delivery, the pigs had an umbilical artery catheter and feeding tube placed and were individually housed in incubators. Each pig received a single dose of maternal serum (5 ml per kg via the umbilical catheter) to provide passive immunity and compensate for the lack of colostrum. The pigs received parenteral nutrition for the first 24 h (8 ml/kg × h) and were then converted to enteral milk replacer (24 ml/kg every 3 h for a total of 192 ml/kg × day). The compositions of the parenteral nutrition solution and milk replacer were previously described (Choudhri et al., 2014) and were formulated to meet the energy and nutrient requirements of newborn pigs.

As controls, we used naturally born term pigs that were euthanized within 8 to 12 h after birth. Another set of controls were pigs that were delivered at term via c-section to assess possible influence of birth mode. Since normal gestation length of the pig is 113–116 days (Tilley et al., 2007), we performed c-section at gestational day 113 to avoid delivery by natural birth; the pigs were euthanized immediately after the c-section.

None of the pigs had any evidence of compromised health or required medical interventions prior to necropsy. Upon necropsy, the entire brain was immediately removed; the cerebellum was isolated and cut into ~1 cm thick pieces and processed for immunohistochemistry, laser capture microdissection or cerebellar slice cultures. None of the pigs had evidence of brain hemorrhage or any other type of brain injury observed either during initial macroscopic brain examination or subsequent microscopic examination of cerebellar sections (> 40 sections per cerebellum, including those from medial vermis, lateral vermis, and hemispheres). Animals of both sexes were analyzed.

2.2. Immunohistochemistry and detection of apoptotic cells

Cerebellar tissue for immunohistochemistry was fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS) for three days at 4 °C, rinsed in cold PBS, cryoprotected with 30% sucrose, embedded in OCT and stored at −80 °C until sagittally sectioned on a