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Placental CpG methylation of HPA-axis genes is associated with cognitive impairment at age 10 among children born extremely preterm

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1. Introduction

The neuroendocrine system serves as an interface between the brain and many of the peripheral endocrine systems and is integral for maintaining homeostasis throughout the body (Tsigos and Chrousos, 2002). Exposure to exogenous chemicals such as endocrine disrupting compounds (EDCs) in the environment may alter the neuroendocrine system resulting in detrimental health effects, including reproductive disorders and cancer (Toni, 2004; Uzumcu et al., 2012). One of the major components of the neuroendocrine system is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis comprises interactions among the hypothalamus, the pituitary gland and the adrenal glands and plays a critical role in regulating physiological and biological responses to stressors (Kinlein et al., 2015). Importantly, it has been shown that components of the HPA system are sensitive to disruption by various environmental toxicants and stressors (Kitraki et al., 2015; Lee and Sawa, 2014).

Several genes are key to functioning of the HPA axis system (Lee and Sawa, 2014). Among these are Nuclear Receptor Subfamily Group 3C Member 1 (*NR3C1*), FK506 Binding Protein 5 (*FKPB5*), and Brain-Derived Neurotrophic Factor (*BDNF*). *NR3C1* encodes for the glucocorticoid receptor, which regulates mechanistic negative feedback actions that inhibit HPA axis activity (Keller-Wood and Dallman, 1984). *FKPB5* is involved in glucocorticoid signaling (Wochnik et al., 2005). *BDNF* promotes synaptic plasticity and regulation of Corticotropin-Releasing Hormone (CRH) in the hypothalamus, and is involved in regulating and maintaining homeostasis in the HPA axis during times of stress (Cowansage et al., 2010; Jeanneteau et al., 2012; Naert et al., 2015). Importantly, all of these genes are critical to the brain's defenses against stress-inducing exposures (Lee and Sawa, 2014).

In addition to their role in neuronal processes, an interesting feature is that many HPA axis-associated genes also control fetal readiness for birth, survival after birth and timing of birth (Wood and Keller-Wood, 2016). The mechanistic basis for this is that HPA axis-associated genes control multiple biological functions in the placenta such as cellular proliferation, nutrient transport, and trophoblast growth (Gao et al., 2012; Kawamura et al., 2009; Padmini et al., 2012) (Fig. 1). In the context of environmental exposures, the placenta regulates gene expression and hormone production in response to exposures and thus functions as an environmentally-responsive biosensor during fetal development (Gheorghe et al., 2010). There is evidence that disruption of normal placenta physiology is associated with later life health effects. Specifically, placental physiological measures such as weight and vascularity have been linked to later life health such as cardiovascular disease and hypertension (Barker et al., 1990). Furthermore, recent studies have shown that epigenetic marks (*i.e.* DNA methylation) in the

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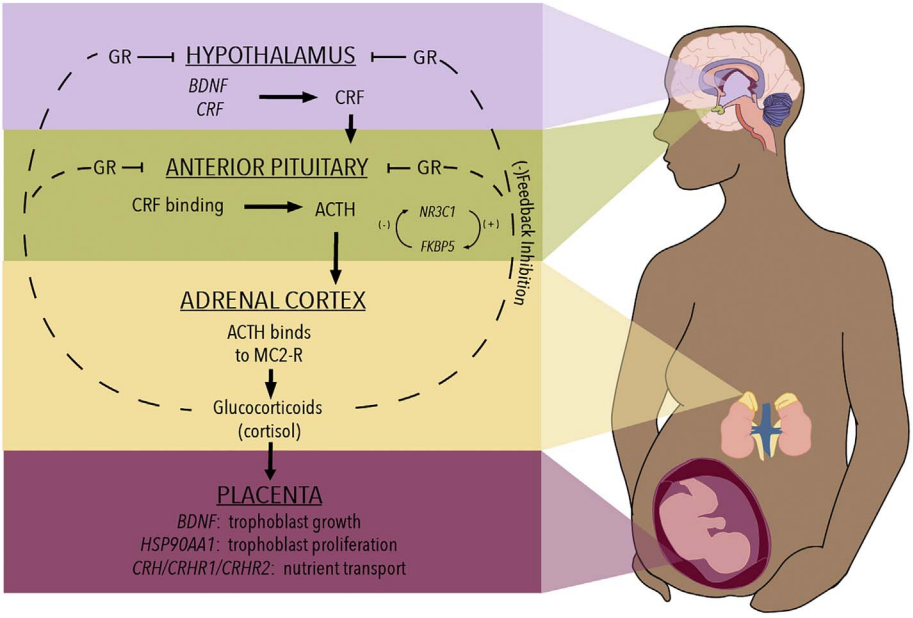

Fig. 1. The HPA axis involves interactions among the hypothalamus, anterior pituitary, and adrenal cortex. Genes that are critical to the HPA pathway include, Brain-derived neurotrophic factor (*BDNF*), FK506 binding protein 5 (*FKBP5*), Corticotropin-releasing hormone/factor (*CRF/CRH*), and Glucocorticoid receptor (*NR3C1*). Ultimately, the HPA axis cascade results in the production of cortisol, which has the potential to cross the placental barrier. Cortisol has been shown to regulate fetal readiness for birth and infant survival. HPA-axis associated genes are also involved in trophoblast growth and proliferation as well as nutrient transport.

placenta of HPA-axis genes are associated with neurobehavioral outcomes in infants (Appleton et al., 2015; Conradt et al., 2013; Monk et al., 2016; Paquette et al., 2014). The placenta is thus of great interest for study as it: (1) mediates fetal exposures to exogenous compounds, (2) regulates fetal nutrition, (3) controls the production of fetal and maternal cortisol, (4) produces additional hormones key for fetal development, and (5) is a key regulator of the fetal environment.

Because HPA axis-associated genes are known to regulate placental growth and function and these are known to influence overall fetal development, we hypothesized that placental DNA (CpG) methylation of targeted genes is predictive of later life cognitive function. To address this, we utilized placental samples from the Extremely Low Gestational Age Newborns (ELGAN) cohort to investigate the relationship between placental CpG methylation changes in HPA axis-associated genes and cognitive functioning at age 10. Our study is among the first to investigate the use of placental CpG methylation to predict later life cognitive function in mid-childhood. The data provide novel insights into potential mechanistic relationship of CpG methylation as a driver of fetal development and later life cognition in mid-childhood.

2. Methods

2.1. ELGANs study subject recruitment and sample collection

Details regarding the recruitment of ELGAN participants have been discussed elsewhere (O'Shea et al., 2009). Briefly, infants born before 28 weeks of gestation at one of the 14 ELGAN sites between 2002 and 2004 were eligible for enrollment in the study. Participating mothers provided informed consent following admission to the hospital, before birth, or immediately following birth. Study procedures were approved by the Institutional Review Board at each of the 14 participating ELGAN sites (O'Shea et al., 2009). After recruitment, a total of 1506 infants and 1249 mothers enrolled in the ELGAN study (ELGAN1). Of 1200 ELGAN survivors, 1102 (92%) underwent clinical evaluations at age 2 years. For the second clinical evaluation at age 10, 889 returned for follow up (ELGAN2). Although placental specimens were collected from the large majority of ELGAN participants, specimens of sufficient size for epigenetic analysis include 438 children. For the current study, a total of 228 mother-infant pairs were selected from the 889 ELGAN2 cohort based on the availability of placental samples with data on CpG methylation and cognitive function. Thus, the data presented here represents a subset of placenta from children who display deficits in cognitive function and controls.

Participating women gave permission for collection of a sample of their placenta for the ELGAN study. Upon delivery, placentas were placed into a sterile exam basin and taken to the sampling room, at which point, biopsies of the placentas were collected. To expose the chorion, the amnion was pulled back using sterile technique at the midpoint of the longest distance between the cord insertion and the edge of the placental disk. A tissue sample was collected by applying traction to the chorion and the underlying trophoblast tissue and cutting a sample out at the base of this tissue structure. The tissue sample was subsequently placed into a cryo-vial that was immediately submerged in liquid nitrogen. Placental samples were shipped to the University of North Carolina at Chapel Hill for processing and were stored at −80 °C prior to shipment (Onderdonk et al., 2008).

2.2. DNA extraction and assessment of DNA methylation

A small subsample of placental tissue (~0.2 g) was cut from the frozen biopsy sample and rinsed with sterile 1 X PBS to wash away any residual blood. Samples were then homogenized in Buffer RLT with β-mercaptoethanol (Qiagen, Valencia CA). An AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Valencia CA) was utilized to extract DNA and RNA sequences that were > 18 nucleotides in length, according to the manufacturer's instructions. To analyze placental CpG methylation, extracted DNA sequences were bisulfate-converted using the EZ DNA methylation kit (Zymo Research, Irvine, CA) and then subsequently hybridized on the Illumina HumanMethylation 450 BeadChip© array (n = 132) and the Illumina HumanMethylation850 Bead Chip array (n = 96) (Illumina, Inc., San Diego, CA), which assesses the DNA methylation levels of 486,428 and 853,307 individual probes at single nucleotide resolution, respectively. Data were integrated as it has been proposed that high variability methylation sites are well conserved between the two platforms (Logue et al., 2017). Methylation levels were calculated and expressed as β values (β = intensity of the methylated allele (M) / (intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100). Data were normalized for both arrays using the *minfi* package in R (Aryee et al., 2014). Specifically, image files were used to produce background-corrected and quantile normalized β-values. Subsequently, β-values with a $p > 0.01$ were removed from analysis, leaving a total of 237 HPA-axis associated probes available for analysis. To demonstrate that the sites included in the analysis were suitable for integration, a regression analysis was