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Research Report

Hypothalamic neurosphere progenitor cells in low birth-weight rat newborns: Neurotrophic effects of leptin and insulin

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ABSTRACT

A low birth-weight (LBW) offspring exhibits reduced hypothalamic neural satiety pathways and dysregulated signaling leading to programmed hyperphagia and adult obesity. Hypothalamic appetite circuits develop during early life, under the influence of neurotrophic hormones (leptin and insulin). Notably, LBW newborns have reduced plasma leptin and insulin levels. As neurons and glia arise from neuronal progenitor cells (NPC), we postulated that a programmed impairment of NPCs may contribute to reduced hypothalamic neural pathway development in a LBW offspring. Control dams received ad libitum food, whereas study dams were 50% food-restricted from pregnancy day 10 to 21 (LBW). At day 1 of age, hypothalamic NPCs were cultured as neurospheres (NS) and treated with leptin/insulin. We analyzed *in vitro* NPC proliferation and differentiation into neurons/astrocytes, expression of signal molecules promoting proliferation (activated Notch1 and its downstream target, Hes1) and *in vivo* NPC proliferation and migration. LBW offspring had impaired *in vivo* evidence of NPC division and migration, and reduced *in vitro* evidence of proliferation and differentiation to neurons and astrocytes, under basal and stimulated conditions. The reduced Notch1 and Hes1 expression in LBW neurosphere, under both basal and stimulated conditions, suggests a reduced progenitor cell population or reduced cell density within the neurosphere.

1. Introduction

There is convincing evidence that the *in utero* environment impacts on fetal development and alters a diversity of adult regulatory mechanisms. Consistent with the finding that low birth weight infants (LBW) have an increased risk for developing obesity in later life (Desai and Hales, 1997), poor maternal nutrition is associated with increased rates of obesity in adult male offspring (Ravelli et al., 1999). Most notably, among men born during the Dutch Hunger Winter of 1944–1945, those exposed to famine during the first half of gestation had a markedly increased risk of adult obesity (Ravelli et al., 1976).

Animal studies confirm that maternal undernutrition may lead to offspring metabolic syndrome (Desai et al., 2007a). LBW newborns often exhibit rapid catch-up growth, culminating in adult obesity. Recent evidence indicates that altered development of orexigenic and anorexigenic central pathways contribute to offspring hyperphagia and obesity. In addition, LBW human or animal offspring exhibit reduced neuronal develop-

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ment of the hippocampus, visual cortex and cerebellar cortex (Lodygensky et al., 2008; Martinussen et al., 2005; Stanley et al., 1991; Thordstein et al., 2004). The impaired appetite pathway development, and perhaps other neuronal dysfunction, has been appropriately attributed, in part, to reduced expression of neuronal growth factors in LBW or growth restricted fetuses.

In regards to appetite development, several studies point to a critical role of leptin, both *in utero* and during the newborn period. Leptin, a primary adult satiety factor, is the obesity (Ob) gene product, a 16 kDa protein synthesized by adipocytes. Leptin is transported into the brain and acts principally in the hypothalamus, activating the JAK–STAT pathway via its long form receptor (ObRb) to suppress food intake (Campfield et al., 1995).

Although leptin serves as a hypothalamic modulator of appetite/satiety in the adult, it has critical neurotrophic properties during fetal development (Bouret and Simerly, 2004). In mice, ObRb has been identified in embryonic brain as early as e10.5 by RT-PCR and at e11.5 by in situ hybridization (Udagawa et al., 2000) whereas in rodents, protein expression was demonstrated as early as e14 (Matsuda et al., 1999). Leptin's neurotrophic effect is also mediated through the extracellular signal regulated kinase (ERK1/2) pathway (Cui et al., 2006).

Leptin-deficient (*ob/ob*) mice have reduced brain weight and lower brain protein and DNA content than wild type mice (Bereiter and Jeanrenaud, 1979). The *ob/ob* mice have permanently disrupted neuronal appetite-regulatory pathways from the arcuate (ARC) to the paraventricular nucleus (PVN) which can be prevented or reversed by leptin treatment during the neonatal period, though not in adulthood (Bouret et al., 2004). In a parallel to *ob/ob* mice, growth restricted fetuses demonstrate decreased placental leptin mRNA and protein, and reduced cord blood leptin levels (Geary et al., 1999; Hoggard et al., 2001) while LBW human, rat, or calf newborns have reduced plasma leptin levels (Blum et al., 2005; Kotani et al., 2004; McMillen et al., 2004).

Akin to leptin, insulin gains access to the hypothalamus by means of a saturable receptor-mediated process and diffusion from the median eminence (Schwartz et al., 1992). Insulin regulates hypothalamic anorexigenic responses via a membrane-bound tyrosine kinase, which in turn, activates PI3K signaling cascade (Plum et al., 2006). Although insulin has been considered a peripheral hormone, a series of studies (Schechter and Abboud, 2001; Schechter et al., 1999) have demonstrated the presence of insulin synthesis machinery in the fetal brain. Insulin receptors are widely expressed throughout the brain, suggesting a role in neuronal growth (Chiu et al., 2008; Chiu and Cline, 2010). Exogenous insulin promotes cell growth and serves as a trophic factor in fetal neuron cell culture (Aizenman and de, 1987). Rat fetal brain cell culture at e16 expresses preproinsulin mRNA and insulin immunoreaction, and stimulates axonal growth in insulin medium (Schechter et al., 1999). Insulin further stimulates ERK1/2 phosphorylation, suggesting that neurite growth may be mediated via this signaling pathway (Schechter et al., 1998).

Our studies have shown that maternal food restriction during rat pregnancy results in LBW newborns with decreased plasma leptin and insulin levels (Desai et al., 2005, 2007b). Importantly, LBW offspring exhibit dysregulated hypothalamic leptin and insulin signaling with subsequent impaired anorexigenic responses, increased food intake and obesity (Desai et al., 2007c). Thus, maternal food restriction that causes LBW programs offspring hyperphagia by impairing hypothalamic neuronal development and altering neuronal signaling pathways in the developing circuits that regulate appetite. As neurons and glia arise from neural stem progenitor cells (NPC), we postulated that a programmed impairment of NPCs may contribute to reduced hypothalamic neural pathway development. The present studies utilized a model in which NPC grown in culture form neurosphere colonies, which can be proliferated into further NPCs or differentiated into neurons and astrocytes, dependent upon the culture medium.

2. Results

2.1. *In vivo* NPC proliferation and migration

Fig. 1A demonstrates the presence of neurons (NeuN) throughout the hypothalamic region. Consistent with the neural stem cell/NPC localization, nestin-positive staining is observed bordering the third ventricular region. Astrocytes labeled with GFAP are detected along the hypothalamic epithelium layer, and this is notably less in LBW newborns.

Following BrdU injection, control offspring brains demonstrated incorporation in the peri-third ventricular region, indicating NPC proliferation, and migration of BrdU positive cells away from the midline. LBW brains visually and quantitatively exhibited decreased BrdU staining around the

Fig. 1 – A: Evidence of hypothalamic NPCs and GFAP in 1 day newborn. Brain from 1 day newborn was fixed, sectioned (20 μm) and stained for markers of NPC (nestin), neuronal (NeuN), astrocyte (GFAP) and nuclei (DAPI). Upper images show evidence of hypothalamic NPCs around third ventricular (V) region and lower images show hypothalamic surface (HS) GFAP (astrocyte). Images shown are at ×40 magnification. B: *In vivo* NPC proliferation and migration: hypothalamic immunostaining of BrdU incorporation. Food-restricted (n=3) and control (n=3) pregnant dams were injected with BrdU (50 mg/kg/day, i.p.) from e17–e19. After birth, brains were collected from 1 day old Control (■) and LBW (■) newborn males. The images (×20) show hypothalamic BrdU (cell proliferation) and DAPI (nuclear marker) immunostaining around the third ventricular (V) region. C: *In vivo* NPC proliferation and migration. Food-restricted (n=3) and control (n=3) pregnant dams were injected with BrdU (50 mg/kg/day, i.p.) from e17–e19. After birth, brains were collected from 1 day old Control (■) and LBW (■) newborn males. Three brains per litter were frozen, and three sections per brain were immunostained. Cell proliferation was determined by counting BrdU positive cells in third ventricle and midline. Migration rate was determined by counting BrdU labeled cells in the area between 30 μm to 100 μm from midline. The average of BrdU-labeled cell numbers of three sections represented one brain and the average of three brain cell numbers represented one litter. Values are mean ± SE; *P < 0.05 vs. Control.