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MOLECULAR METABOLISM

Postnatal undernutrition delays a key step in the maturation of hypothalamic feeding circuits

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ABSTRACT

Objective: Humans and animals exposed to undernutrition (UN) during development often experience accelerated "catch-up" growth when food supplies are plentiful. Little is known about the mechanisms regulating early growth rates. We previously reported that actions of leptin and presynaptic inputs to orexigenic NPY/AgRP/GABA (NAG) neurons in the arcuate nucleus of the hypothalamus are almost exclusively excitatory during the lactation period, since neuronal and humoral inhibitory systems do not develop until after weaning. Moreover, we identified a critical step that regulates the maturation of electrophysiological responses of NAG neurons at weaning — the onset of genes encoding ATP-dependent potassium (K_{ATP}) channel subunits. We explored the possibility that UN promotes subsequent catch-up growth, in part, by delaying the maturation of negative feedback systems to neuronal circuits driving food intake.

Methods: We used the large litter (LL) size model to study the impacts of postnatal UN followed by catch-up growth. We evaluated the maturation of presynaptic and postsynaptic inhibitory systems in NAG neurons using a combination of electrophysiological and molecular criteria, in conjunction with leptin's ability to suppress fasting-induced hyperphagia.

Results: The onset of K_{ATP} channel subunit expression and function, the switch in leptin's effect on NAG neurons, the ingrowth of inhibitory inputs to NAG neurons, and the development of homeostatic feedback to feeding circuits were delayed in LL offspring relative to controls. The development of functional K_{ATP} channels and the establishment of leptin-mediated suppression of food intake in the peri-weaning period were tightly linked and were not initiated until growth and adiposity of LL offspring caught up to controls.

Conclusions: Our data support the idea that initiation of K_{ATP} channel subunit expression in NAG neurons serves as a molecular gatekeeper for the maturation of homeostatic feeding circuits.

Keywords NPY; AgRP; Leptin; Undernutrition; K_{ATP} channel; Feeding circuits

1. INTRODUCTION

Suboptimal maternal nutrition is associated with a delay in the maturation of circuits regulating diverse physiological process, including growth, reproduction and cognition [1—4]. Although "developmental delay" has negative connotations, a delay may beneficial if it provides a window during which the normal developmental program can be reinstated if adequate nutritional stores are available. Studies in humans and rodent models support the idea that early nutritional supplementation and rapid catch-up growth can mitigate impacts of maternal UN on cognitive function [5—8], but at the cost of increased risk of metabolic disease [9—12].

Little is known about mechanisms that regulate growth rates following developmental exposure to UN. Rodents exposed to moderate restriction (<50%) during lactation increase food intake in the post-weaning period such that they ultimately attain the body weight of controls; however, more severe restriction usually leads to persistent decreases in food intake and body weight [13—16]. In cases of moderate postnatal UN, there is evidence that the accelerated growth rate is supported, in part, by increased orexigenic drive in the post-weaning period. Neurons in the arcuate nucleus of the hypothalamus (ARH) that co-express neuropeptide Y (NPY), agouti-related peptide (AgRP) and gamma-aminobutyric acid (GABA) exert a powerful orexigenic influence on central circuits regulating food intake

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Abbreviations: AgRP, agouti-related peptide; ARH, arcuate nucleus of the hypothalamus; EPSC, excitatory postsynaptic current; GABA, gamma-aminobutyric acid; IPSC, inhibitory postsynaptic current; K_{ATP}, ATP-sensitive potassium channel; Kir, potassium inward rectifiying channel subunit; *Lepr*, leptin receptor; LL, large litter; NAG, NPY, AgRP, GABA, NPY, neuropeptide Y; P, postnatal day; *Pomc*, pro-opiomelanocortin; PVH, paraventricular nucleus of the hypothalamus; pSTAT3, phosphorylated signal transducer and activator of transcription 3; SUR, sulfonylurea receptor; UN, undernutrition

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[17,18]. NAG neurons are an important node of homeostatic regulation of feeding, as they are activated by signals of negative energy balance (i.e. ghrelin) and are inhibited by circulating signals of an energy replete state (i.e. leptin) [19,20]. Projections from NAG neurons that regulate food intake are formed during the suckling period [21,22], and postnatal UN due to lactation in a large litter (LL) leads to increases in the number of projections to downstream targets at 3–4 weeks of age [23–25].

Leptin is well-positioned to serve as a conduit for impacts of UN on developing feeding circuits. There is a surge in leptin levels in the first two weeks of lactation [26] that precedes its ability to modulate food intake [27]. During this period, leptin promotes axonal outgrowth from NAG neurons [24,28,29]. This neurotrophic effect is observed in immature NAG neurons that are activated by leptin [30], while leptin's actions in mature NAG neurons are inhibitory [19,20,30]. As UN can alter the timing and/or levels of leptin during the surge [24,31,32], we hypothesized that the effects of postnatal UN on orexigenic drive are mediated through impacts on leptin signaling in NAG neurons.

We previously used a combination of genetic, immunohistochemical and electrophysiological criteria to characterize the onset of leptin-mediated signaling in NAG neurons across the postnatal period. In contrast to the well-characterized effect of leptin to hyperpolarize adult NAG neurons via K_{ATP} channels [33,34], we found that all of the *Npy*-GFP + neurons [35] that responded to leptin on postnatal days 13–15 (P13–15) were depolarized [30]. Starting at P21, there was a gradual increase in the number of hyperpolarized neurons at the expense of depolarized neurons, such by P30, all of the leptin-responsive *Npy*-GFP + neurons were hyperpolarized. Moreover, we discovered that the onset of leptin-mediated hyperpolarization at weaning coincided with the onset of K_{ATP} channel subunit expression [30]. The maturation of the electrophysiological properties of NAG neurons coincides with the onset of homeostatic regulation of food intake [21,27].

In these studies, we explored whether effects of moderate postnatal UN to increase post-weaning growth rates are associated with impairments in the maturation of systems that provide negative feedback to the orexigenic actions of NAG neurons. We found that UN delays the development of homeostatic regulation of feeding, which is tightly correlated with the onset of K_{ATP} channel expression and the maturation of electrophysiological properties of NAG neurons.

2. MATERIALS AND METHODS

2.1. Animals

All mouse protocols were overseen and approved by the Columbia University Medical Center or the Oregon Health and Science University Institutional Animal Care and Use Committees. Mice were maintained in a temperature (22+/− 1 °C) and light controlled (12 h light: 12 h dark) barrier facility. Mice were weaned at P21 and had *ad libitum* access to chow (13.2% calories from fat, 5053; PicoLab Rodent Diet 20) and autoclaved drinking water. *Npy-hrGFP* mice (B6.FVB-Tg(Npy-hrGFP)1Lowl/J, Stock No: 006417) were purchased from The Jackson Laboratory. The number of pups per litter was adjusted at P3 to n = 8 (control litter) or n = 12 (LL litter) in a randomized way. Main exclusion criteria for any experiment were based on: a) any sign of sickness and b) body weight outliers: mice with a deviation of 2 times the standard deviation from the mean value of the group. Data from males and females were combined, as we did not detect any gender differences in the endpoints examined. Mice were allocated to saline vs. leptin treatment groups on the basis of body weight, ensuring that the distribution of body weights was similar. Experimenters were not blinded to the experimental groups of animals. Mice were weighed 3 times per week. Body composition was assessed on a biweekly basis using nuclear magnetic resonance imaging (Minispec, Bruker). Naso-anal length was measured bi-weekly on anesthetized animals (4% isoflurane).

2.2. Immunohistochemistry

Mice were injected with 0.9% saline or leptin (4 mg/kg, i.p., National Hormone Peptide Program) and after 45 min were transcardially perfused with saline followed by 4% paraformaldehyde in 0.1M PB under avertin (2.5 mg/10 g, Sigma) anesthesia. Pups under P21 were injected and anesthetized in their breeding cage. Mice older than P21 were fasted for 3 h before injection, to minimize fasting-induced NAG neuronal activation. After perfusion, brains were removed, postfixed overnight, washed with cold PBS and submerged in 30% sucrose before embedding in O.C.T medium. Brains were kept frozen at −80 °C until sectioning. 10 μm-thick coronal sections were acquired from the paraventricular nucleus of the hypothalamus (PVH) to the caudal ARH (Allen Brain Atlas coordinates: −0.08 to −2.0 mm from bregma). For ARH immunohistochemistry, sections from the medial ARH area were incubated with rabbit anti-c-Fos (1:500, Calbiochem, #PC38 or 1:500, Cell Signaling # 2250) or rabbit anti-phospho-STAT3 (pSTAT3) (1:500, Cell Signaling Technology, #9131) [30], followed by a goat anti-rabbit-Cy3 secondary antibody (1:500, Jackson ImmunoResearch Laboratories, #115-165-205), and nuclear staining with DAPI (1:500). An additional antigen retrieval step was performed before pSTAT3 staining; slides were incubated in 1% NaOH, 1% H_2O_2 for 20 min before primary antibody incubation. Digital images were captured using a Nikon Eclipse 80i equipped with a Retiga EXi camera and X-Cite 120 fluorescent illumination system. Cell counts were performed using Adobe Photoshop CS5, and experimenters were blinded to the experimental groups. *Npy*-GFP$^+$, c-Fos$^+$ and pSTAT3$^+$ cells were only counted if they colocalized with a DAPI signal from at least 12–18 hemisections per mouse.

2.3. Quantification of fibers in the PVH

Sections were incubated with α-AgRP [36] (1:500, Phoenix Pharmaceuticals #H0035-57) or α-melanocyte stimulating hormone (α-MSH) (1:1000 rabbit A 2-7-83, kindly provided by Sharon Wardlaw). Fluorescent images were captured from 8 to 12 sections per mouse, and the Fiji version of the ImageJ program was used for quantitative analyses of immunoreactive AgRP or α-MSH fibers in the PVH. Briefly, all images were taking using the same settings and adjusted with same threshold to eliminate background. Images were binarized and the percent of the total area that contained fluorescent signals >2 pixels^2 was calculated.

2.4. Leptin-induced suppression of food intake

Mice were isolated at P25 and daily food intake and body weight were measured until P29 (Baseline). At P29, food was removed at lights out, and, the next morning, mice were injected with 0.9% saline or leptin (4 mg/kg, i.p.) and provided with *ad libitum* access to food. Food intake and body weight changes were measured after 24 h.

2.5. Quantitative expression analysis

Brains from 4 to 6 animals per group and age were extracted and maintained in cold artificial cerebrospinal fluid (aCSF). Brains were sectioned in 300 μm coronal slices in a vibratome (Leica, VT1200). Sections containing *Npy*-GFP$^+$ fluorescence in the ARH were carefully microdissected under a fluorescent scope, using a scalpel to cut a triangular area encompassing the GFP$^+$ neurons from both sides of the ARH, while the median eminence was discarded. Following mRNA

