Intrauterine growth restriction alters term fetal baboon hypothalamic appetitive peptide balance

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Abstract

Neurons controlling appetite are located in the hypothalamic arcuate nuclei (ARH). Offspring appetite regulation has been shown to be modified by dysregulation of ARH nuclear development. Most ARH developmental studies have been in altricial rodents whose hypothalamic development is predominantly postnatal. In primates including humans, much development of hypothalamic appetite regulatory centers occurs before birth. We hypothesized that i) appetitive peptides are abundantly expressed by 90 percent gestation (0.9G), ready for postnatal function; ii) by 0.9G, intrauterine growth restriction (IUGR) increases the orexigenic:anorexigenic peptide ratio; iii) IUGR increases fetal glucocorticoid receptor (GR) expression; and iv) IUGR decreases STAT3, which signals inhibition of appetite. We developed a fetal baboon IUGR model resulting from reduced maternal nutrition. Pregnant baboons were fed *ad libitum*, controls (CTR; $n=24$), or 70% CTR diet to produce IUGR ($n=14$). C-section was performed at 0.9G. In CTR ($n=7$) and IUGR ($n=6$) fetal brains, ARH appetite regulatory peptides (neuropeptide Y (NPY) and proopiomelanocortin (POMC)) were quantified immunohistochemically. Fetal plasma cortisol was raised in IUGR fetuses. We observed that NPY and POMC were well expressed by 0.9G. IUGR increased NPY, GR, and active phosphorylated GR and decreased POMC and phosphorylated form of STAT3. We conclude that IUGR dysregulates ARH development in ways that will reset the appetitive neuropeptide balance in favor of increased appetite drive in postnatal life. We postulate that changes in peptide abundance are in part due to increased fetal cortisol and ARH GR. These changes may contribute to predisposition to obesity in IUGR offspring.

Key Words

- Fetus
- baboon
- IUGR
- appetitive peptides
- hypothalamic arcuate nucleus

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Introduction

Effective central hypothalamic neural regulation of appetitive behavior is indispensable for maintaining a healthy phenotype and good lifetime health. The hypothalamic arcuate nuclei (ARH), situated near the floor of the third ventricle, contain first-order neurons

that sense and respond to nutrient and hormone signals (Bouret & Simerly 2006, Grayson *et al.* 2006, Bouret 2010). Neurons expressing neuropeptide Y (NPY) and agouti-related protein (AgRP) provide an orexigenic drive while neurons expressing proopiomelanocortin (POMC) and

cocaine- and amphetamine-regulated transcript (CART) are anorexigenic. Neurons containing these appetitive neuropeptides are connected in a complex fashion to several other brain areas. The major ontogenic development of this complex energy and appetite control system occurs postnatally in altricial species such as rodents (Bouret & Simerly 2006, Grayson *et al.* 2006, Bouret 2010). In contrast, in the few studies that have been conducted in precocial species – sheep (Adam *et al.* 2008) and the Japanese macaque (Grayson *et al.* 2006), indications are that maturation begins in fetal life.

Dysfunctional development of these hypothalamic neuronal structures in rodents leads to an increased appetitive drive postnatally (Kirk *et al.* 2009, Steculorum & Bouret 2011b, Sarr *et al.* 2012). We have developed a nonhuman primate, baboon model of intrauterine growth restriction (IUGR) to determine the effects of this common pathophysiological state on the ARH. To our knowledge, there are no studies on fetal IUGR, expression of appetite-regulating neuropeptides, and mechanisms involved in their action in any precocial species.

Our model of IUGR is produced by moderate maternal nutrient restriction (MNR) in which nutrient-restricted mothers eat 70% of the global diet of *ad libitum*-fed controls throughout pregnancy (Nijland *et al.* 2007). This degree of maternal, and subsequent fetal, nutrient restriction produces decreased nutrient availability to the fetus (McDonald *et al.* 2012) and results in IUGR accompanied by major changes in the fetal brain frontal cortex (Antonow-Schlorke *et al.* 2011), fetal liver (Nijland *et al.* 2010), kidney (Cox *et al.* 2006b), and placenta (Schlabritz-Loutsevitch *et al.* 2007). Fetal cortisol is also elevated (Nijland *et al.* 2010) and offspring show an altered postnatal phenotype with decreased peripheral glucose disposal and elevated fasting glucose (Choi *et al.* 2011) and behavior (Rodriguez *et al.* 2012). Glucocorticoids are known to increase NPY in the hypothalamus (Jeanrenaud & Rohner-Jeanrenaud 2000) and upregulate ARH NPY gene expression (Shimizu *et al.* 2008) in keeping with their well-known drive to increase appetite. We hypothesized that i) as baboons are a precocial species, both orexigenic and anorexigenic appetitive peptides would be well expressed in the fetal ARH by 90 percent gestation (0.9G) and thus ready for postnatal function; ii) by term, IUGR increases ARH orexigenic:anorexigenic peptide balance; iii) in the setting of the increased fetal cortisol levels, we have previously described (Nijland *et al.* 2010) that IUGR increases fetal ARH glucocorticoid receptor (GR) expression; and iv) as STAT3 inhibits the orexigenic peptide NPY (Bates *et al.* 2003, Diano *et al.* 2011), IUGR

would decrease ARH p-STAT3. We measured ARH immunoreactivity of NPY as an index of orexigenic drive and POMC as an index of anorexigenic drive as well as GR, the active form of GR, phosphorylated GR (p-GR), and the active phosphorylated form of STAT3 (p-STAT3).

Materials and methods

Animals, feeding, and breeding

Thirty-eight female baboons (*Papio hamadryas*) from the Southwest National Primate Research Center (San Antonio, TX, USA), were recruited for this study and maintained in group housing. All procedures were approved by the Texas Biomedical Research Institute Institutional Animal Care and Use Committee and conducted in AAALAC-approved facilities. The caging system allows control and monitoring of food intake while still maintaining female baboons in group housing, thereby permitting normal social and physical activity (Schlabritz-Loutsevitch *et al.* 2004). Briefly, groups of 16 females carefully selected to produce a homogeneous group were assembled and socialized in the presence of a vasectomized male while eating Purina Monkey Diet 5038 (Purina, St Louis, MO, USA) *ad libitum*. After acclimation, the vasectomized male was replaced by a proven breeder male. Females were observed for turgescence (sex skin swelling) and signs of vaginal bleeding to enable timing of pregnancy (Hendrickx & Peterson 1997). Pregnancy was confirmed by ultrasound at 0.16G after which they were randomly assigned to a control group of females that continued to receive *ad libitum* feed ($n=24$) while 14 females underwent MNR and received 70% of the feed eaten by controls on a weight-adjusted basis. Once a day, baboons were passed over a scale (GSE 665; GSE Scale Systems, Livonia, MI, USA) into individual cages for feeding (Schlabritz-Loutsevitch *et al.* 2004). Food consumption, weights, and health status were recorded daily. Drinking water was continuously available.

Fetal brain preparation and histology

Cesarean sections were performed between 0800 and 1000 h at 165 days of gestation (term 184 days) under general anesthesia using techniques previously reported in detail (Schlabritz-Loutsevitch *et al.* 2007). Food was withdrawn for 16 h before surgery. Postoperative analgesia was provided with buprenorphine hydrochloride 0.015 mg/kg per day (Hospira, Inc., Lake Forest, IL, USA) for three postoperative days. Brain collection and

processing for immunohistochemistry have been described in detail (Antonow-Schlorke *et al.* 2011). Briefly, fetal brains were immediately dissected longitudinally. The right side was immersion fixed in 4% paraformaldehyde and the left side dissected and flash frozen in liquid nitrogen. Immunohistochemistry was performed in a randomly chosen subset of controls ($n=7$; males=3, females=4) and MNR pregnancies ($n=6$; males=3, females=3).

Quantitative image analysis

Fetal ARH NPY, POMC, GR, and phosphorylated active form of p-GR and the activated, p-STAT3 immunoreactive peptide were quantified by immunohistochemistry and image analysis for fraction (area immunostained/area of the field $\times$ 100%) and density (arbitrary density units (DU)). Details of antibodies used, final dilutions, and manufacturers are summarized in Table 1. Where the antigen to which the antibody was raised was available (NPY and POMC), the negative control was conducted following antibody reabsorption. Where the antigen was not available (GR, p-GR, and p-STAT3), the negative control was conducted with normal rabbit serum. In each case, the same concentration was used for the primary antibody as with the regular staining.

Images were obtained with a SPOT RT3 cooled color digital camera (2650 $\times$ 1920 pixels, Diagnostic Instruments, McHenry, IL, USA) mounted on a Nikon E600 microscope (Nikon, Inc., Melville, NY, USA). Sections (5 μ m) were cut throughout the hypothalamus and immunostained at 250 μ m intervals. Images from six slides per animal with six pictures per slide and quantified with the freely available ImageJ Software (NIH, Bethesda, MD, USA). All images were analyzed with exactly the same intensity window for each antigen for fraction (area immunostained/area of the field $\times$ 100%) and density (mean grayness of pixels detected in the field with white=0 and black=255). The threshold used was optimized after a preliminary analysis of all slides and

that threshold was then used for all sections. Analysis of the data produced was conducted by a separate, blinded investigator not associated with the microscopy.

Cortisol measurements

Fetal plasma cortisol was measured via chemiluminescent immunoassay (Immulite 1000, Siemens Healthcare Diagnostics, Los Angeles, CA, USA). Within-assay coefficient of variation (CV) was 4.9% and between-assay CV was 7.9% (Nijland *et al.* 2010).

Statistical analyses

Data are presented as mean $\pm$ S.E.M. An initial comparison was made to evaluate sex differences using Student's non-paired *t*-test. As no differences were observed for any of the peptides by sex, data were pooled for each peptide. Statistical comparisons between IUGR and control groups were performed with Student's unpaired *t*-test with the Bonferroni correction. Correlations were performed by the Pearson's Product Moment Correlation. α was set at 0.05.

Results

Maternal and fetal morphometrics

At the time of recruitment to the study, non-pregnant females randomly assigned to the control group weighed 16.7 ± 0.43 kg ($n=24$) and those assigned to the MNR group weighed 16.3 ± 0.77 kg ($n=14$; $P>0.05$). Within the complete groups of animals studied, MNR decreased fetal weight: CTR ($n=24$), 795 ± 23.5 g vs MNR fetuses ($n=14$), 715 ± 21.0 g ($P<0.03$). When divided by sex, male control fetuses ($n=11$), 840 ± 33.6 g were heavier than female control fetuses ($n=13$), 757 ± 30.0 g ($P<0.05$). Male MNR fetuses ($n=8$), 749 ± 26.5 g were lighter than male CTR fetuses ($P<0.05$) and female MNR fetuses ($n=6$), 671 ± 26.0 g were lighter than female CTR fetuses ($P<0.05$). These data show that this moderate MNR challenge produced a similar degree of IUGR in males (11.0%) and females (11.4%). Animals in the subset in which immunohistochemistry was performed fell within the fetal weight values for the larger group: CTR male ($n=3$), 784 ± 46.2 ; CTR female ($n=4$), 714 ± 41.6 ; MNR male ($n=3$), 711 ± 55.2 ; and MNR female ($n=3$), 626 ± 27.1 .

Table 1 Sources of antibodies

Antigen	Final dilution	Manufacturer	Catalog no.
NPY	1:100 000	Sigma-Aldrich	M9528
POMC	1:1000	PP, Inc., Burlingame, CA, USA	H-029-30
GR	1:500	Gift from Dr M Garabedian	218
p-GR	1:500	Gift from Dr M Garabedian	S211-353
p-STAT3	1:100	SCB, Santa Cruz	SC-135649

Fetal plasma cortisol

In CTR fetuses ($n=22$), plasma cortisol was 210 ± 13.0 ng/ml and IUGR fetuses ($n=11$), 275 ± 29.5 ng/ml ($P=0.03$), an increase of more than 30%. There were no differences according to sex of fetus. Fetal plasma cortisol concentrations in the subset in which immunohistochemistry was performed were very similar to the larger group means: CTR ($n=7$), 202 ± 32.4 ng/ml and IUGR ($n=6$), 276 ± 26.6 ng/ml.

Fetal plasma metabolites

There were no differences in fetal plasma glucose (data not shown) between the control, and IUGR fetuses. Circulating concentrations of five plasma amino acids were reduced in IUGR compared with CTR fetuses (CTR values first; $\mu\text{m/l}$) asparagine, 10.1 ± 1.88 vs 3.7 ± 0.24 ; taurine, 206.3 ± 17.50 vs 140.6 ± 17.5 ; methionine, 45.1 ± 1.80 vs 38.4 ± 2.44 ; leucine, 108.3 ± 8.2 vs 73.6 ± 2.19 ; and ornithine, 54.9 ± 5.95 vs 27.8 ± 2.99 .

Distribution of appetitive peptides

NPY and POMC immunogenicity was present in abundance in both perikarya and fibers (Fig. 1A, B, D and E).

Effect of IUGR on abundance of appetitive peptides

The major changes in immunostaining for both appetitive peptides produced by IUGR were in the fraction of the area

stained, representing increased or decreased numbers of immunoreactive cells rather than density of product within each active cell (Figs 1 and 2). The fraction for NPY, which includes distribution in both neurons and fibers, hence representing the amount of immunoreactive peptide, was greatly increased in IUGR fetuses compared with CTR (Fig. 1A, B and G) while the density of staining was unchanged (data not shown). The fraction stained for POMC was decreased (Fig. 1D, E and G) while immunostaining density was unchanged (data not shown).

Distribution of GR, p-GR, and p-STAT3 and effects of IUGR

GR, p-GR, and p-STAT3 were distributed in neuronal nuclei throughout the ARH. GR (Fig. 2A, B and J) and p-GR (Fig. 2D, E and J) increased with IUGR while p-STAT3 (Fig. 2G, H and J) was decreased in IUGR fetuses compared with CTR while immune product density was unchanged (data not shown).

Correlation analysis

ARH NPY in individual fetuses correlated positively with fetal plasma cortisol ($P=0.02$; $r=0.65$) while the correlations with the other peptides (POMC, GR, p-GR, and p-STAT3) were not significant.

Figure 1

NPY (A – CTR; B – IUGR; negative control C) and POMC (D – CTR; E – IUGR; negative control F) immunoreactive peptide expression in the hypothalamic arcuate nucleus (ARH) of fetuses from mothers fed as *ad libitum* controls (CTR, $n=7$) or from mothers fed 70% of CTR diet (IUGR, $n=6$) from

0.15 to 0.9 gestation (G, term ~ 184 days). Micron bar applies to all panels. (G) Summary of group data expressed as % area immunostained in the ARH of CTR group fetuses (closed columns) vs IUGR fetuses (open columns). Data are mean $\pm$ S.E.M. * $P<0.05$ compared with own control.

Figure 2

(A) Glucocorticoid receptor (GR; A – CTR; B – IUGR; negative control C), phosphorylated GR (p-GR; D – CTR; E – IUGR; negative control F) and phosphorylated form of STAT3 (p-STAT3; G – CTR; H – IUGR; negative control I) peptide expression via immunohistochemistry in the hypothalamic arcuate nucleus (ARH) of fetuses from mothers fed as *ad libitum*

controls (CTR, $n=7$) or from mothers fed 70% of CTR diet (IUGR, $n=6$) from 0.15 to 0.9 gestation (G, term ~184 days). Micron bar applies to all panels. (J) Summary of group data expressed as % area immunostained in the ARH of CTR group fetuses (closed columns) vs IUGR fetuses (open columns). Data are mean $\pm$ S.E.M. * $P < 0.05$ compared with own control.

Discussion

Poor fetal nutrition and IUGR are associated with later-life increased appetite and obesity in altricial rodent models (Tarry-Adkins & Ozanne 2011, Sarr *et al.* 2012) but no data exist to determine the pathophysiological effects on appetite regulatory systems in primates. As most studies on abnormal development of the ARH have been conducted in altricial rodents, we sought to develop a nonhuman primate model in which we could observe changes in the structures responsible for production of appetitive neuropeptides in the setting of IUGR. Studies in nonhuman primates are required to aid translation to human fetal development. We have previously shown several marked prenatal (Cox *et al.* 2006a, Nijland *et al.* 2007, 2010, Antonow-Schlorke *et al.* 2011, Kamat *et al.* 2011, Rodriguez *et al.* 2012, Li *et al.* 2013) and postnatal (Choi *et al.* 2011, Rodriguez *et al.* 2012) phenotypic changes in this IUGR model.

No studies exist in fetal rodents on normative ontogeny of ARH appetite peptide expression or in the pathophysiological state of IUGR. This is understandable given both the practical difficulties involved in studying the fetal rodent brain and the likelihood that the majority of perinatal development in this hypothalamic system in rodents is postnatal and therefore fetal studies may not be

informative. In contrast to rodents, the development of appetite regulatory neural circuits in precocial species occurs prenatally. Even in these species, however, development of these systems in response to pathological situations such as IUGR has received little attention (Sorensen *et al.* 2002, Grayson *et al.* 2006).

Our observations show a well-developed regulatory system with appetitive peptides expressed abundantly by the end of gestation, indicating a greater degree of development in the nonhuman primate fetus than rodents at this stage of development.

The nutritional challenge imposed produced a 10% reduction in late gestation weight. In the Dutch Hunger Winter study, the decrease in birth weight of babies who later showed increased predisposition to a variety of chronic diseases was ~200 g – just under 10% of birth weight of controls (Smith 1947). Thus, our IUGR model has relevance to the human epidemiologic data (Barker 1998, Nathanielsz 1999). In addressing our second hypothesis that there would be a change in the balance of orexigenic and anorexigenic peptides in the setting of IUGR, it was clear that the changes observed were in the fraction of the brain area stained rather than the overall density, indicating that IUGR is associated with more (NPY) or fewer (POMC) cells becoming active in producing their secretory product. There is now clear evidence that

development of the appetitive centers is affected by impairment of nutrient availability during early growth and development. In one study, newborn rats were reared either in litters of ten pups or food restricted in litters of 20. At 25 and 380 days of postnatal life, the ARH was evaluated for gene expression of *Npy*, *Agrp*, *Pomc*, and *Cart* (Remmers *et al.* 2008). Poor early postnatal nutrition reduced body size. When they reached adulthood rats food restricted as pups showed reduced *Pomc* and *Cart* mRNA. In restricted offspring, the ratio of ARH NPY and AgRP to the anorexigenic peptides POMC and CART was increased at 25 days, but not at 380 days. Thus, decreased nutrient availability in early postnatal days produces changes in the ARH that favor food ingestion (Remmers *et al.* 2008). These findings are similar to our observations, the major difference being that the period of nutritional deprivation was postnatal in the rodent study while in the fetal baboon in this study, the challenge and study of these changes are prenatal.

The responsiveness of the fetal ARH to nutritional inputs in sheep, a precocial species, has been investigated in one study that showed that infusion of glucose at 0.86–0.93G increased *Pomc* mRNA while *NPY* and *AgRP* were unchanged. This important study shows that the regulatory systems are beginning to function in late gestation in precocial species and that the time scale of maturation of responsiveness by the various transmitters may differ (Muhlhausler *et al.* 2004).

Based on studies primarily in sheep, it is now recognized that glucocorticoids play a central role in maturation of several fetal physiological systems as term approaches. These include the kidney, lung, gut, as well as brain structures (Thomas *et al.* 1978, Fowden *et al.* 2006). As IUGR increased fetal plasma cortisol in our model, we hypothesized that increase in activity of the glucocorticoid system would play a role in the observed changes in appetitive neuropeptides. We first determined whether GR and p-GR are present in the fetal baboon ARH; both were detected in abundance. Glucocorticoids are known to increase NPY in the hypothalamus (Jeanrenaud & Rohner-Jeanrenaud 2000) and upregulate ARH *Npy* gene expression (Shimizu *et al.* 2008) in keeping with their well-known drive to increase appetite. Both total and active-GR were increased in IUGR. In their review, McMullen *et al.* (2012) introduced the concept of glucocorticoids as a general 'Gatekeeper' mechanism responsible for a variety of developmental programming outcomes. Our hypothesis for a role of glucocorticoids in the development of the neuropeptide systems is supported by the observations in rodent species described earlier in which

the period of greatest development of the appetitive peptides is between postnatal days 6 and 21, the same time when the neonatal rodent adrenal increases activity in a fashion that resembles the increase seen during late fetal life in precocial species (Daniels *et al.* 1973). Further studies will be needed to elucidate these mechanisms.

Finally, we sought to determine whether these IUGR-associated changes are accompanied by altered cell signaling through p-STAT3. The signaling factor p-STAT3 that inhibits the orexigenic peptide NPY (Bates *et al.* 2003, Diano *et al.* 2011) was abundantly expressed throughout the ARH and was decreased by IUGR.

In addition to the regulation of the ARH-feeding centers by dietary metabolites and glucocorticoids. The ARH integrates several peripheral signals to regulate appetitive drive. Among these are leptin, insulin, and ghrelin. Rat pups delivered by obese mothers show a blunted postnatal leptin peak and develop leptin resistance (Kirk *et al.* 2009). The gastric peptide hormone ghrelin plays a key role in appetite regulation and has a stimulatory effect on perinatal growth (Steculorum & Bouret 2011a). Ghrelin stimulates NPY production and insulin inhibits this effect (Maejima *et al.* 2011). The extent to which these and other mediators regulate hypothalamic appetitive peptides in the late gestation primate fetus remains to be elucidated.

Conclusions

Related to our four hypotheses, we have demonstrated that by 0.9G, the fetal baboon ARH abundantly expresses *NPY* and *POMC*, two of the key peptides that regulate appetitive behavior and that IUGR changes the balance of these two regulators in favor of orexigenic NPY. IUGR produces increased circulating fetal cortisol as well as both GR and p-GR. Finally, IUGR decreases p-STAT3. Thus, IUGR results in an orchestrated set of changes all of which would support increased appetite. If these differences persist postnatally, they could play a role in the increased orexigenic drive shown in rodent offspring of undernourished mothers. To our knowledge, this is the first demonstration of the presence and distribution of protein expression for key appetitive peptides in the ARH of a developing nonhuman primate combined with demonstration that IUGR alters their abundance in a way that would increase appetitive drive.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C L, T J M, M J N, and P W N designed the study. M J N and P W N were responsible for the animal management. C L and T J M conducted the immunohistochemistry. C L and P W N wrote the paper.

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