Ontogeny of neuropeptide Y expression in response to deprivation in lean Zucker rat pups

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Kowalski, Timothy J., Thomas A. Houpt, Jeongwon Jahng, Nori Okada, Streamson C. Chua, Jr., and Gerard P. Smith. Ontogeny of neuropeptide Y expression in response to deprivation in lean Zucker rat pups. *Am. J. Physiol.* 275 (*Regulatory Integrative Comp. Physiol.* 44): R466–R470, 1998.—Hypothalamic neuropeptide Y (NPY) activity is believed to play an important role in the response to food deprivation in adult rats. Little is known, however, about the role of the hypothalamic NPY system in the control of food intake in the preweanling rat. To address this issue, we examined the effect of deprivation on arcuate nucleus preproNPY expression in lean Zucker rat pups, using in situ hybridization. PreproNPY expression within the arcuate nucleus was localized to cells in the medial portion. Twenty-four hours of food, water, and maternal deprivation significantly increased the relative abundance of preproNPY mRNA in pups on postnatal *day* (P) *2*, *P9*, *P12*, and *P15* by 14–31%. This response, however, was not observed on *P5*. The absence of an effect on *P5* and the magnitude of the response at the other ages tested were not correlated with the amount of weight lost during deprivation.

in situ hybridization; arcuate nucleus; neonate; starvation

NEUROPEPTIDE Y (NPY) is an orexigenic peptide when administered centrally and is most potent when injected into the paraventricular nucleus of the hypothalamus (PVN) (25). The arcuate nucleus is the primary site of NPY synthesis in the hypothalamus (1), and an arcuo-paraventricular NPYergic projection exists (2). This arcuo-paraventricular NPYergic projection is believed to play a role in feeding behavior because its activity is correlated with deprivation state; arcuate nucleus preproNPY mRNA, PVN immunoreactive NPY, and release of NPY into the PVN all increase during periods of food deprivation and return to basal levels with refeeding (4, 12, 20). Hypothalamic NPY expression, peptide levels, and release rates are higher in many animal models of obesity (21, 30), and chronic intracerebroventricular administration of NPY mimics many of the physiological and behavioral symptoms present in obesity (33). These observations suggest that increased hypothalamic NPY activity is involved in the etiology of obesity and its associated metabolic and behavioral disturbances, such as hyperphagia, hyperinsulinemia, and hypercorticosteronemia.

Despite its apparent importance in the ingestive and metabolic response to food deprivation and in animal models of obesity, little is known about the ontogeny of the hypothalamic NPY system. Studies in neonatal rats have demonstrated that administration of NPY into the PVN can increase both milk and water intake at postnatal *day* (P) *2* and selectively increase milk intake via an intraoral catheter on *P15* (5). Although pups at *P15* are behaviorally responsive to exogenous NPY (e.g., they increase food intake), it is unknown if an increase in NPY expression in the arcuate nucleus due to nutritional deprivation is present at this young age. Information regarding the ontogeny of presynaptic responses to deprivation in the arcuo-PVN NPYergic pathway is required to achieve a better understanding of the role of NPY in normal feeding behavior and in the development of abnormal ingestive behaviors, such as hyperphagia and anorexia. To address this issue, we investigated the effect of food, water, and maternal deprivation on arcuate nucleus NPY expression in lean Zucker rat pups on *P2–P15*. The relative abundance of preproNPY mRNA in fed and deprived animals was quantified using in situ hybridization to provide anatomic resolution. The results demonstrate that a 24-h period of deprivation results in significantly higher preproNPY mRNA in the arcuate nucleus as early as *P2*, with a transient loss of responsiveness at *P5*.

METHODS

Animals. Lean Zucker rats aged 2, 5, 9, 12, and 15 days were used in the experiments. All animals were the progeny of Zucker heterozygotes (+/*fa*) derived from the Vassar College colony (Poughkeepsie, NY). Mating pairs and pregnant females were housed in Plexiglas containers with wood shavings as bedding. Animals received pelleted chow and water ad libitum and were maintained on a 12:12-h light-dark cycle (0700–1900) at 22 ± 2°C. Pregnant females were checked daily for pups, and the day pups were first seen was termed *P0*. On *P4*, animals assigned for study on *P9*, *P12*, or *P15* were ear clipped for identification and tissue collection (for genotyping, see *Determination of genotype at Lepr*). Pups studied at *P2* or *P5* were marked for identification using a dorsal subcutaneous injection of ink before the deprivation period. A total of 128 pups (+/+, $n = 39$; +/*fa*, $n = 89$) from 18 litters were used, with 3–5 litters used at each age. The pups used in this study were derived from litters of 7–12 animals, with 14 of the 18 litters having 9–12 pups. Not all littermates were used in this study (only +/+ and +/*fa* animals were used).

Food deprivation. Twenty-four hours before tissue preparation (see *Tissue preparation*), the dam was removed from the home cage. Pups were weighed and randomly assigned to one of two groups; one-half of the pups were left in the home cage, and the dam was returned, and the other pups were housed together in a humidified incubator at a temperature that maintained thermoneutrality (34 ± 1°C for pups aged 2 or 5

days and 33 ± 1°C for pups aged 9, 12, or 15 days) under constant dim light. Those housed away from the dam in the incubator for the 24-h period were deprived of both food and water but not sibling interaction.

Tissue preparation. Twenty-four hours after the start of the deprivation period (0700–0900), animals were weighed and given an overdose of pentobarbital sodium intraperitoneally. When unresponsive, animals were transcardially perfused with 0.9% NaCl, 0.5% $NaNO_2$, and 100 U/ml heparin followed by ice-cold 4% buffered paraformaldehyde (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were removed, postfixed for 24 h in 4% buffered paraformaldehyde, and cryoprotected in 30% sucrose at 4°C for 48 h before sectioning.

In situ hybridization. Forty-micrometer frontal sections were cut using a sliding microtome and placed into ice-cold 2× saline sodium citrate (SSC, 0.15 M NaCl/0.015 M sodium citrate). Six to ten sections through the midregion of the arcuate nucleus were taken. Hybridizations were carried out in glass vials, and sections from a starved and fed animal were hybridized in the same vial. The tissue was prehybridized in 1 ml of 60% formamide, 0.02 M Tris pH 7.4, 1 mM EDTA, 10% dextran sulfate, 0.8% Ficoll, 0.8% polyvinylpyrrolidone, 0.8% BSA, 2× SSC, 0.1 M dithiothreitol, and 1.6 mg/ml herring sperm DNA for 2 h at 48°C. After 2 h, radiolabeled probe was added ($\sim 1.0 \times 10^7$ counts·min^{-1}·$vial^{-1}$) and incubated for 16–20 h at 48°C. Hybridization was performed using a 511-bp [α-^{35}S]dATP random prime-labeled cDNA-encoding preproNPY (10). Sections were then sequentially rinsed in 2×, 1×, 0.5×, 0.25×, and 0.125× SSC for 15 min at 48°C and placed into 0.1 M phosphate buffer for mounting. The tissue sections were mounted onto gelatin-coated slides, air dried, and apposed to X-ray film (β-max, Amersham, Arlington Heights, IL) for 12–24 h. Each litter was processed together, and slides were exposed to the same film. Slides were then dipped into photoemulsion (Kodak, Rochester, NY) and exposed at 4°C for 1 wk for histological verification of probe labeling.

Probe hybridization in the arcuate nucleus was quantified by densitometry of autoradiograms using the MCID system (Imaging Research). The relative optical density (ROD) of hybridization signal in the arcuate nucleus, the area of hybridization in the arcuate nucleus (A), and the product of the ROD and A (ROD × A, a measure of total hybridization in the arcuate nucleus) were determined from 6–10 sections/animal. The mean ROD, A, and ROD × A values from each animal were used in the calculations.

Determination of genotype at Lepr. Genotypes (+/+, +/*fa*) were determined according to a previously described method (6). Briefly, the A to C mutation at nt 880 of *Lepr^{fa}* introduces an *Msp* 1 restriction site, which can be used to detect the number of copies of the *Lepr^{fa}* allele. Tissue was digested with proteinase K, and genomic DNA was extracted using the Quiagen QIAamp Tissue Kit. Primers (5′-TGAAGCCCGATCCACCGCTGG-3′ and 5′-CTCTCTTACGATTGTAGAATTCTC-3′) were used to generate a 143-bp PCR fragment. PCR was performed on a Perkin-Elmer DNA thermal cycler using the following conditions: 92°C (2 min), 1 cycle; 92°C (30 s), 55°C (30 s), 72°C (1 min), 35 cycles; and 72°C (5 min), 1 cycle. The Advantage genomic PCR kit (Clontech) was used for PCR reactions. The PCR fragments were digested with *Msp* 1 (80 mU/μl final concentration) for 1.5 h and electrophoresed on a 2% agarose-2% low-melting-point agarose gel. Digestion yields an uncut 143-bp product for the wild-type allele, whereas the mutant allele (*fa*) yields both a 106-bp and a 37-bp product.

Statistical analysis. Results are presented as means ± SE. Initially, the ROD, A, and ROD × A measures within each litter were normalized to the mean value of fed +/*fa* littermates because +/*fa* pups were the most numerous in our sample. No discernible differences were seen between +/+ and +/*fa* animals (Fig. 1). The ROD, A, and ROD × A values were therefore normalized to the mean values of fed littermates, and the normalized values of all litters tested at each age were pooled for analysis. Differences in body weights and preproNPY mRNA abundance between deprived and fed groups at each age were determined using a two-way ANOVA, with the state (fed vs. deprived) and litter as independent variables. Student's *t*-test was used to reevaluate data at ages at which no litter effects were observed. Differences in response to deprivation across ages were determined using ANOVA with age as the independent variable and the normalized ROD of starved animals as the dependent variable. A P value <0.05 was considered significant.

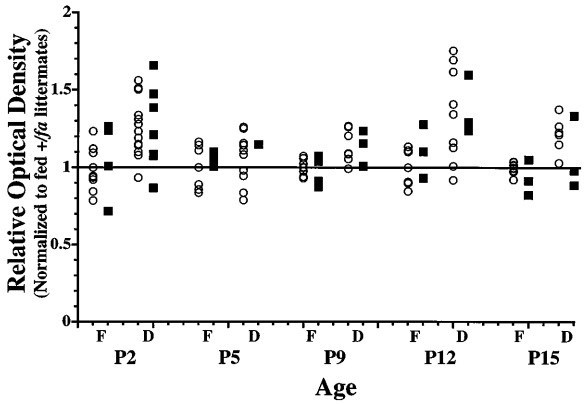

Fig. 1. Normalized relative optical density (ROD) values for individual +/+ (■) and +/*fa* (○) animals in fed (F) and deprived (D) state at each postnatal (P) age tested. ROD values of each animal are normalized to mean ROD of fed +/*fa* littermates.

RESULTS

A significantly higher ROD was seen in deprived pups at *P2*, *P9*, *P12*, and *P15* but not at *P5* (Fig. 2). Litter effects on ROD were observed only at *P15*. No difference in A was observed with deprivation at *P5*, *P9*, *P12*, or *P15*, but a significantly larger A was seen at *P2* (+16.7 ± 4.6%). The pattern of the ROD × A values with deprivation in pups aged 2–12 days was similar to that seen with the ROD values, namely, higher ROD × A values at *P2* (+41 ± 7%), *P9* (+27 ± 10%), and *P12* (+50 ± 19%) and no effect of deprivation at *P5* (+12 ±

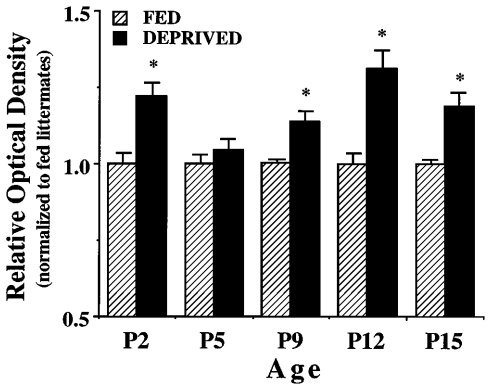

Fig. 2. Normalized ROD of fed and deprived animals at 5 ages tested (mean ± SE). *Significantly different from fed group ($P < 0.05$).

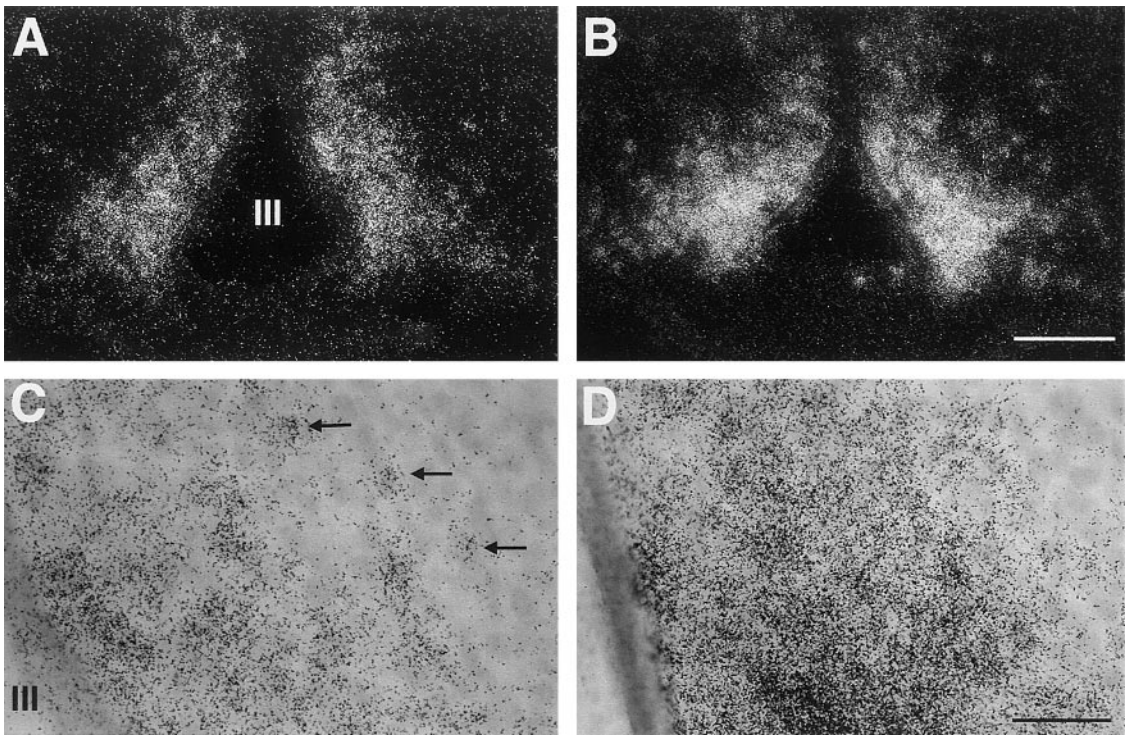

Fig. 3. Dark-field photomicrographs of prepro-neuropeptide Y (preproNPY) mRNA hybridized in arcuate nucleus of a fed (*A*) and a deprived (*B*) pup aged *P12* (×50 magnification). Bright-field photomicrographs of preproNPY mRNA hybridized in arcuate nucleus of a fed (*C*) and a deprived (*D*) pup aged *P12* (×200 magnification). PreproNPY mRNA within arcuate nucleus is localized to small cells in medial portion (arrows in *C*). III, third ventricle. Bar = 100 μm in *A* and *B* and 25 μm in *C* and *D*.

10%). Unlike the ROD value, however, the ROD × A value was not significantly higher with deprivation at *P15* (+19 ± 11%). This observation was likely due to the modest but significant increase in ROD with deprivation (+18 ± 4.7%) and large variance in A (coefficient of variation = 20% in deprived animals at this age). Litter effects on A and ROD × A were not seen at any age.

Analysis of the emulsion-coated cresyl violet-stained sections showed that the preproNPY mRNA in the arcuate nucleus is localized to small cells within the medial region (Fig. 3), as previously demonstrated (4). The deprivation-induced increase in preproNPY mRNA was specific to the arcuate nucleus at *P12*. This was evidenced by no differences in preproNPY mRNA in the cortex or the thalamic reticular nucleus of fed and deprived animals (cortex, 1.00 ± 0.03 vs. 0.95 ± 0.02; reticular nucleus, 1.00 ± 0.04 vs. 0.99 ± 0.05; normalized ROD in fed vs. deprived animals, respectively).

A significant effect of age ($F = 2.71$, $P < 0.05$) on the response to deprivation (expressed as normalized ROD of deprived animals) was seen. Post hoc analysis revealed that the ROD on *P5* was significantly smaller than the ROD on *P2* and *P12*, but not on *P9* or *P15*, and that the ROD on *P12* was significantly larger than *P9*. A significant effect of age on the relative change in body weight was observed in fed ($F = 2.6$, $P < 0.05$) and starved ($F = 5.9$, $P < 0.001$) animals (Table 1). Animals on *P9* gained significantly more weight than those on *P2*, *P5*, and *P15*. Weight loss in animals aged 2 days was significantly larger than weight loss at other ages; weight loss in animals aged 9 days was significantly smaller than those aged 15 days.

These data demonstrate that the failure to respond to deprivation on *P5* was not due to inadequate weight loss of the starved animals or weight gain of those left with the dam. Furthermore, the pattern of weight loss and gain across the other ages does not appear to

Table 1. *Body weights of fed and 24-h food- and water-deprived lean Zucker rat pups*

Age	*n*	Initial Body Weight, g	Final Body Weight, g	%Change
P2				
F	12	5.7 ± 0.13	6.4 ± 0.17	11.8 ± 1.5[a]
D	19	5.6 ± 0.07	5.0 ± 0.08	−10.9 ± 0.5[a]
P5				
F	11	7.7 ± 0.36	8.5 ± 0.43	10.7 ± 2.9[a]
D	9	7.4 ± 0.35	6.8 ± 0.34	−8.7 ± 0.7[b]
P9				
F	12	14.9 ± 0.51	17.9 ± 0.72	18.2 ± 1.7[b]
D	10	15.7 ± 0.42	14.6 ± 0.39	−7.0 ± 0.5[b,c]
P12				
F	10	21.9 ± 1.23	25.3 ± 1.49	15.6 ± 2.3[a]
D	12	21.7 ± 1.22	19.8 ± 1.08	−8.7 ± 0.6[b]
P15				
F	12	25.5 ± 0.97	28.7 ± 1.08	12.8 ± 0.5[a]
D	9	25.1 ± 0.99	22.8 ± 0.79	−9.1 ± 0.8[b,d]

Values are means ± SE. F, fed; D, deprived at each postnatal (P) age. Values with different superscripts are significantly different ($P < 0.05$) from animals at other ages within same nutritional state.

account for the differences in the magnitude of the preproNPY expression with deprivation.

DISCUSSION

This study demonstrates that a 24-h period of food, water, and maternal deprivation increases the relative abundance of preproNPY mRNA in the arcuate nucleus of lean Zucker rat pups as early as *P2*. The relative increase in preproNPY mRNA abundance (expressed as normalized ROD) seen with deprivation at the ages tested was between 15 and 30%. This range is smaller than that seen in adult rats, in which 24- to 96-h food deprivation increases arcuate nucleus preproNPY mRNA 50–100% (4, 11, 21, 29). The reason for the difference in the magnitude of response between neonates and adults is not known, but it could be due to the differences in the nature of the deprivation paradigm used as well as the immaturity of brain structures or humoral signals involved in mediating this response.

The mechanism by which food deprivation increases arcuate nucleus preproNPY expression in adult rats is not clear and may involve one or several hormones and neurotransmitters such as insulin, glucocorticoids, serotonin, dopamine, corticotropin-releasing hormone, and leptin (3, 7, 14, 22, 23, 27). Adult arcuate nucleus cells have receptors for insulin (15), glucocorticoids (9), and leptin (17), and studies in vivo have demonstrated that administration of insulin or leptin decreases and glucocorticoids increase preproNPY expression and release (22, 23, 31). Similar evidence exists for serotonin and dopamine, with serotonergic agents decreasing and dopaminergic antagonists increasing NPY expression or peptide levels (7, 14). The ontogeny of these putative arcuate nucleus NPY regulatory systems and their involvement in deprivation-induced changes in hypothalamic NPY, however, have not been investigated.

The higher arcuate nucleus preproNPY mRNA level after deprivation was not observed at *P5*. This was not due to less change in body weight gain or loss in these animals relative to other ages (Table 1). This is interesting because food- and water-deprived animals will independently ingest more milk than fed animals of the same age at *P6* (8) and *P5* (T. Kowalski, unpublished observations). It is not clear if the absence of a significant response to deprivation was due to the inability to detect a small increase or if it represents nonresponsiveness of NPY-expressing arcuate nucleus neurons at this age. One possible explanation for the lack of a significant increase in arcuate nucleus NPY mRNA at *P5* with deprivation may be the transient decline in circulating corticosterone after birth and low abundance of brain type II glucocorticoid receptor at this age (16). Several studies in adult rats indicate that glucocorticoids are required for increased arcuate nucleus NPY expression (13, 28) and that arcuate nucleus NPY-expressing neurons have been shown to contain type II glucocorticoid-like immunoreactivity (9).

Our results indicate that at ages when NPY injection into the PVN increases intraoral milk and water intake (*P2*) or selectively increases milk intake (*P15*) (5), deprivation increases the expression of NPY (Fig. 2). These data, as well as studies demonstrating that pups ingesting food away from the dam increase their intake with deprivation (8), indicate that the NPY system may be operating from birth. It is not known, however, when the arcuate nucleus NPYergic projections to the PVN are established, although NPY-like immunoreactivity is seen in both the arcuate nucleus and PVN as early as embryonic *day 18* (32).

Because our animals were deprived of food and water, osmotic effects of water deprivation on NPY expression may have modified the response. Studies in adult rats have demonstrated that water deprivation also increases arcuate nucleus NPY mRNA, but to a lesser extent than food deprivation, whereas both food and water deprivation yields results similar to food deprivation alone (18). The effect of food deprivation on NPY expression in hydrated, normovolemic pups is required to determine if dehydration changes the response to food deprivation alone.

It is possible that maternal deprivation also contributed to our results. Several studies have revealed that the absence of maternal interaction modifies the development of the hypothalamic-pituitary-adrenal axis (19). Evaluation of the role of glucocorticoids in the regulation of hypothalamic NPY expression at these ages is needed to address this issue. The influence of maintaining the deprived animals under constant light is unknown. Animals at *P2* deprived under the same light cycle as the colony room, however, showed a similar response [1.00 ± 0.01 vs. 1.20 ± 0.02, normalized ROD of fed ($n = 3$) and deprived ($n = 3$) pups, respectively]. The effect of lighting conditions at the other ages tested requires further investigation.

In summary, deprivation of food, water, and maternal interaction significantly increases the relative amount of NPY mRNA within the arcuate nucleus of lean Zucker rats aged 2, 9, 12, and 15 days but not 5 days. Although effects of heterozygosity in Zucker rat pups have been shown for fat mass and fat-free dry mass (24, 26), preproNPY expression appeared to be no different between the +/+ and +/*fa* genotypes in both the fed and deprived states. The mechanisms responsible for the deprivation-induced increase in arcuate nucleus NPY mRNA, as well as the changes in hypothalamic NPY synthesis, release, and responsiveness in preweanling animals remain to be determined.

Perspectives

These data demonstrate that arcuate nucleus NPYergic neurons are responsive to nutritional and maternal deprivation as early as *P2*. It is interesting that hypothalamic NPY expression is responsive to deprivation at ages when rat pups demonstrate a behavioral response to both deprivation [ingesting more milk when tested in independent ingestion paradigms after deprivation (8)] or central administration of NPY (5). Unfortunately, the causal relationships among these observations have not been demonstrated. This will require developmental studies that correlate NPY expression, peptide level, and release with intake in independent ingestive tests as a function of maternal or nutritional

deprivation. When these results are available, it will be possible to compare the controls of NPY that operate in preweanling rats with those that have been described in adult rats.

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