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Abnormal Response of the Neuropeptide Y-Deficient Mouse Reproductive Axis to Food Deprivation But Not Lactation

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Neuropeptide Y (NPY) plays a key role in both food intake and GnRH secretion. Food deprivation elevates hypothalamic NPY activity and suppresses LH and gonadal steroid secretion. Similarly, lactation up-regulates NPY expression as food consumption increases and estrous cycles cease. These observations suggest that NPY coordinates reproductive suppression in response to energy deficiency; if so, the reproductive axis of NPY knockout (KO) mice should be impervious to lactation and food deprivation. We monitored food consumption, body weight, and estrous cyclicity during lactation in NPY KO mice with large and small litters. NPY KO mice with either litter size resembled wild types (WTs) in weight regulation and food consumption. Large-litter mothers had longer anestrous periods and smaller pups at weaning, but NPY KOs and WTs did not differ in either respect. We also examined the LH response of NPY KO mice to 48 h without food. Basal levels of LH in ovariectomized NPY KO animals decreased in response to fasting, but LH levels in intact and estrogen-treated ovariectomized NPY KO animals did not. In contrast, WTs consistently showed fasting-induced suppression of LH. Our findings suggest that other systems can sustain the hyperphagia of lactation and NPY alone is not responsible for suppressing cyclicity during lactation. Nevertheless, the suppression of basal LH release that accompanies food deprivation in normal female mice appears to require the steroid-dependent actions of NPY. (*Endocrinology* 144: 1780–1786, 2003)

CIRCUMSTANCES THAT TAX an animal's energy resources strongly affect the reproductive axis. Because lactation demands that the mother expend a great amount of energy on the production of milk, being pregnant during lactation endangers the mother's reproductive success (1–3). In most mammalian species, lactation is accompanied by a cessation of ovarian cycles and profound hyperphagia. Although rodents exhibit a postpartum ovulation following parturition, the implantation of any resulting embryo is delayed to ensure that peak energy demands of pregnancy and lactation do not coincide (2, 3). In addition, this ovulation is followed by an infertile period that lasts until weaning. Because blockade of milk production in the latter half of the lactational period increases LH secretion even while pups persist in suckling (4), some have theorized that the increased caloric demand of producing milk for young suppresses the reproductive axis (4, 5).

Energy reserves can also be threatened by scarce food or increased demands from thermoregulation or exercise. Inadequate food intake causes most species to divert energy away from sexual maturation, maintenance of pregnancies, and secretion of reproductive hormones. In the female laboratory mouse, a 20% loss of body weight causes ovulation to cease (6). GnRH release, as reflected by LH secretory patterns, is extremely sensitive to food deprivation; pulsatile secretion is inhibited during fasting and returns within 1–2 h after food is reintroduced (7).

Hypothalamic neuropeptide Y (NPY) influences both energy homeostasis and the release of reproductive hormones; thus, it has been suggested, may coordinate the reproductive response to energy deficiency (reviewed in 8). In the mouse, chronic central infusion of NPY markedly enhances food intake and suppresses LH, leading to obesity and hypogonadism (9). In addition, food restriction increases NPY levels in the arcuate (ARC), paraventricular, and ventromedial nuclei of the hypothalamus (10) and NPY gene expression in the ARC (11). Similarly, lactating mice show elevated ARC levels of NPY as well as a dramatic increase in NPY in the median eminence that disappears rapidly after pup removal (12, 13). Nevertheless, the role of NPY in lactation or fasting-induced infertility remains controversial.

Attempts to demonstrate an inhibitory effect of NPY on the reproductive axis of lactating animals have yielded mixed results. Chronic exogenous NPY administration to lactating rats fed *ad libitum* resulted in a profound decline in milk production and smaller pup size, accompanied by a significantly shorter period of lactational diestrus (14), possibly because of NPY suppression of prolactin (PRL) secretion (13, 15). On the other hand, NPY Y5 receptor stimulation in lactating animals lengthens the period of acylicity (16). Furthermore, lactating rats restricted to 50% of *ad libitum* food levels for the first 2 wk postpartum show a significant increase in the length of lactational diestrus associated with high levels of hypothalamic NPY (17).

NPY mediation of the suppression of reproduction during fasting has been challenged by results from NPY-deficient animals. Erickson *et al.* (18), in their initial characterization of the NPY knockout (KO) mouse, food-restricted male wild-type (WT) and NPY KO mice and analyzed the degree to which testosterone levels were altered in the two genotypes. A 48-h fast was found to inhibit testosterone levels to the same degree in WT and KO animals, suggesting inhibition of

Abbreviations: AGRP, Agouti-related peptide; ARC, arcuate; E_2, 17β-estradiol; KO, knockout; NPY, neuropeptide Y; OVX, ovariectomy; OVXed, ovariectomized; PRL, prolactin; WT, wild-type.

LH secretion. They also examined the effect of 48 h of food deprivation on the vaginal cytology of female mice. Recovery of cyclicity following refeeding required an equal amount of time for both genotypes.

Using female NPY null-mutant mice, we tested the hypothesis that NPY mediates lactation and fasting-induced suppression of LH release. Specifically, we examined the impact of NPY deficiency on food consumption, body weight regulation, and estrous stage throughout the lactational period in mothers with small or large litters. In addition, we investigated the effect of a 48-h fast on basal LH release in the NPY KO animals. Because the influence of both fasting (19, 20) and NPY (21, 22) on LH release can be modified by steroid hormones, we went on to examine the impact of ovariectomy and steroid replacement on these animals.

Materials and Methods

Animals

All animal and surgical experimental procedures were conducted with the explicit approval of the Northwestern University's Animal Care and Use Committee. NPY KO mice were generously provided by Dr. Richard Palmiter from the University of Washington (Seattle, WA). The NPY-deficient mice were previously generated using homologous recombination techniques and maintained on a C56BL × 129SV background (23). The NPY −/− mice were bred in the Center for Experimental Animal Resources at Northwestern University under standard conditions. Mice of strain 129/SVIMJ were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as WT controls in experiments. Animals were housed singly in a humidity- and temperature-controlled room, with Purina rodent chow and water available *ad libitum*. Lighting was maintained on a 14-h light, 10-h dark cycle, with lights on at 0500 h and off at 1900 h.

Reagents

17β-Estradiol (E_2) was purchased from Sigma (St. Louis, MO). Silicon tubing (0.04-in. internal diameter, 0.085-in. outer diameter) was obtained from Helix Medical (Carpinteria, CA), and silicon type A medical adhesive was supplied by Dow Corning Corp. (Midland, MI).

Experimental design

Experiment 1. Adult female KO and WT mice less than 6 months old were used in the lactation studies. All animals were fed standard (not high-fat) rodent chow. The date of parturition was recorded as d 1. On d 3, the number of pups for each of the experimental mothers was normalized to three for the small litters and 6–11 for the large litters using age- and genotype-matched surrogate pups when necessary. Weights of male and female pups were recorded on d 22.

In a subset of each group (small litters: n = 7 WT, n = 6 KO; large litters: n = 3 WT, KO), vaginal cytology and the weight of the mother were measured starting on d 3, thus avoiding the normal postpartum ovulation that occurs on the morning of d 2 or 3. Measurements continued until weaning and the resumption of cyclicity. Because the initial weights of the mothers varied by up to 8 g, maternal weights are reported as deviation from the mean of weights recorded over the lactational period for each individual animal, thus emphasizing changes during this period. Maternal food consumption was calculated in the small-litter group by recording the mass of food given each morning and then subtracting what food remained in the food receptacle or bedding the following day. Because food consumed mirrored maternal weight change but exhibited higher variability, this measure was omitted for the large litters. Estrous cyclicity was monitored by daily inspection of vaginal cytology according to the guidelines of Bingel and Schwartz (24).

Experiment 2. The fasting experiments consisted of placing the mouse in a clean cage with no food (water was freely available) for 48 h beginning at 1000 h. In intact animals, fasting began on diestrus I. These animals had previously exhibited a consistent 5-d cycle so that they were killed on the morning of presumptive proestrus. A control group was sham fasted by placing the animal into a new cage with food and water at 1000 h, again on diestrus I for intact animals. Bilateral ovariectomy (OVX) was performed on groups of WT and control animals 6 or 20 d before the fast. Because length of OVX did not affect the results, data were later pooled. Mice were anesthetized with an 80-mg/kg ip injection of ketamine (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) and 5 mg/kg (ip) xylazine (Gemini SA, Burns Veterinary Supply, Inc., Rockville Center, NY).

SILASTIC (Dow Corning Corp.) capsules containing E_2 (Sigma) were made using SILASTIC silicone type A (Dow Corning Corp.) and medical silicone tubing (0.04-in. inner diameter, 0.085-in. outer diameter, Helix). Capsules were prepared by evenly mixing a measured amount of E_2 in a fixed volume of sterile adhesive and then fitting the adhesive mixture immediately into the silicon tubing and allowing the filled capsules to cure overnight. Tubing was then cut into pieces 1 cm in length, with each containing a total of 1.25 mg E_2. The capsules were stored at 4 C. Capsules were soaked in PBS for 24 h before use. They were implanted sc in the appropriate group of mice during OVX.

For blood collection, animals were anesthetized by halothane inhalation (Halocarbon Laboratories, River Edge, NJ), and blood samples were obtained via terminal cardiac puncture. Serum samples were derived by centrifugation and stored at −70 C until used for RIA.

Hormone assays

Serum LH was measured by RIA. The LH RIA used reagents provided by the NIDDK, including the LH RP-3 reference preparation. The intraassay coefficient of variation was 3.00%, and the interassay coefficient of variation was 16.6%.

Statistical analysis

Linear regression for litter size and pup weight was performed using GraphPad Software, Inc. Prism software (GraphPad Software, Inc., San Diego, CA). A two-way ANOVA was used to compare the weight and food consumption of the mothers over the lactational period. To assess differences in hormone levels between genotypes, mean and SE values for a given hormone were calculated for each of the two genotypes and compared by means of *t* tests. Hormone responses to OVX were analyzed by one-way ANOVA followed by *post hoc* comparisons using the Newman-Keuls test. For all statistical tests, differences were considered significant with $P < 0.05$.

Results

Feeding and body weight regulation of mothers during lactation

Figure 1 depicts the inverse relationship that was observed between litter size and pup weights at weaning. This relationship was observed in both WT and KO animals. At litter sizes of 9 or 10 pups, the average pup weight (for both male and female pups) decreased by 2.10 ± 0.47 g ($P = 0.0021$) in comparison with three pup litters, likely reflecting the inability of the mother to produce sufficient milk to maintain pup weights at the higher level. One null-mutant litter is recorded as having 11 pups, despite the fact that a pup died on d 18, its small size suggestive of malnutrition. All other litter sizes remained unchanged through weaning.

Previous studies have shown that body weights of lactating mice increase up to peak lactation around d 15 postpartum (4) and subsequently decrease until weaning (generally d 19 or 20). After peak lactation, the pups start to eat solid food in addition to milk (25). The maternal weights and food intake in this study followed a similar trend, although food consumption exhibited greater variability. As depicted in Fig. 2, the minimal lactational demands of a three-pup litter

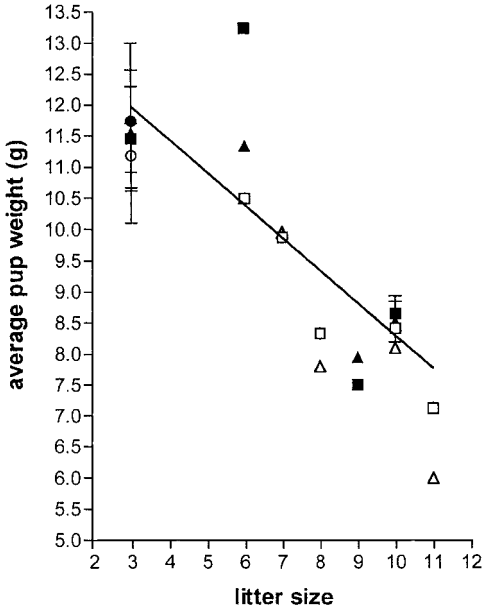

FIG. 1. Inverse correlation between litter size and pup weight. (linear regression, $r^2 = 0.6987$, $P < 0.0001$.) ■, WT male pups; ▲, WT female pups; □, NPY KO male pups; △, NPYKO female pups; ●, WT pups, gender unspecified; ○, NPY KO pups, gender unspecified.

resulted in no significant effect of day postpartum on maternal weight or food intake. Additionally, the NPY-deficient mice showed no difference in their regulation of body weight or food intake, compared with the WT mothers of small litters. In contrast, females with large litters showed a pronounced variation in weight over the course of the lactational period. Despite the considerable lactational demands, the weight regulation of KO and WT animals was similar (Fig. 3).

Length of lactational anestrus of NPY KO vs. WT mice

Figure 4 shows the average length of anestrus during the lactational period. Analysis revealed a significantly longer period of anestrus in the large litters than the small litters in the NPY KO animals and a similar trend in the WTs. Nevertheless, no significant difference in length of cycle cessation was seen when comparing NPY KO and WT mothers of either litter size.

LH response to fasting in intact animals

WT and KO animals were tested for their hormonal response to a 48-h fast. Basal levels of LH measured in blood serum at 1000 h were suppressed in WT animals (Fig. 5). In contrast, NPY-deficient animals showed no reduction in LH levels following the fast. Neither WT nor NPY KO mice showed an effect of fasting on FSH or progesterone levels (data not shown).

Effects of estrogen on LH response to fasting in ovariectomized (OVXed) animals

Because removal of steroid-negative feedback by OVX causes a substantial increase in GnRH and LH release, we expected the response of NPY KO OVXed mice to resemble

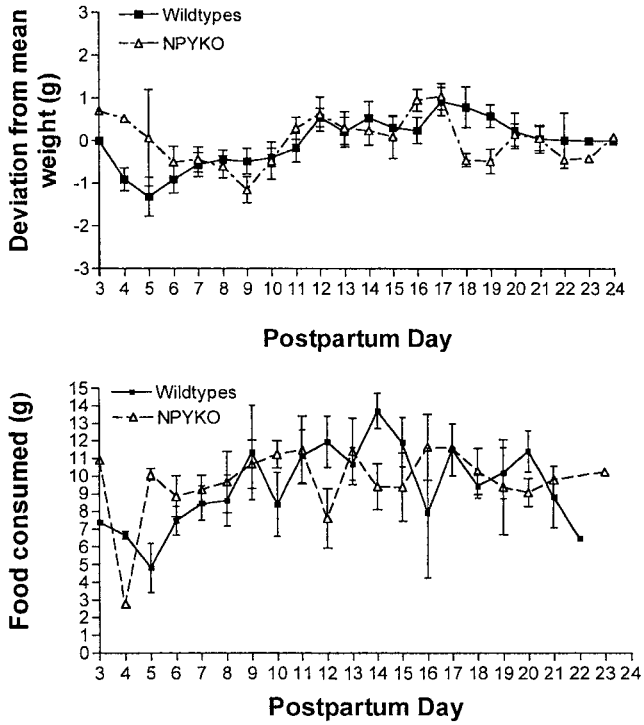

FIG. 2. a, Weights of NPY KO and WT mothers with small litters expressed as deviation from mean during lactational period. Litters consisted of three pups. Day postpartum did not affect maternal weight significantly. No differences in body weights were observed between the groups (WT, n = 7; NPYKO, n = 6). 2b, Food consumed by NPY KO and WT mothers with small litters during lactational period. Day postpartum did not influence maternal food intake significantly. No differences in food consumption were observed between the groups (WT, n = 7, NPYKO, n = 6).

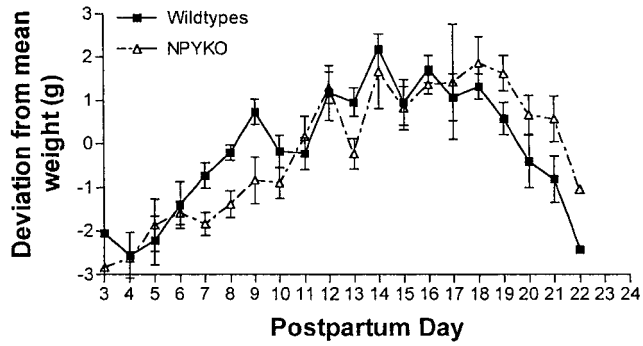

FIG. 3. Weight of NPY KO and WT mothers with large litters expressed as deviation from mean during lactational period. Litters consisted of 6–11 pups. Day postpartum had a highly significant effect on maternal weight ($P < 0.0001$). No differences in body weights were observed between the groups (WT, NPYKO, n = 3).

that of the intact animals on a larger scale. Contrary to expectations, however, although WT and KO animals did exhibit this increase in basal LH levels, both OVXed KO and WT animals showed a decrease in LH when subjected to food deprivation (Fig. 6). Despite this decrease, OVX prevented full suppression of gonadotropin levels; the fasting levels of LH in OVXed animals did not approach the levels seen in intact fasted mice.

The removal of ovarian factors by OVX clearly altered the

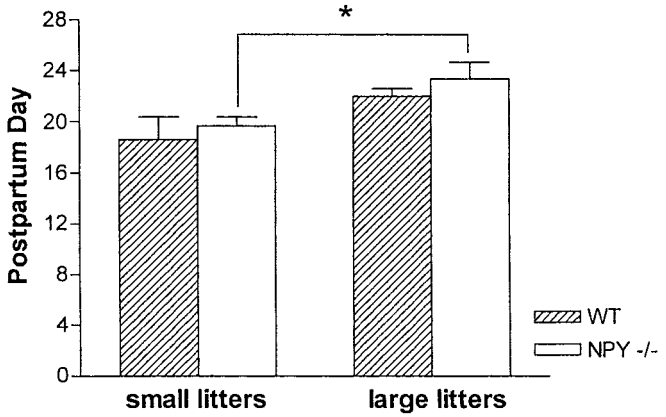

FIG. 4. Day of first postlactational estrus. Small litters consisted of three pups, n = 6; large litters consisted of 6–10 pups, n = 3. No significant difference in day of estrus cycle resumption existed between WT and NPYKO animals. NPYKO large litter day of first estrus was significantly later than NPYKO small litter first estrus day ($P = 0.036$). *, $P < 0.05$.

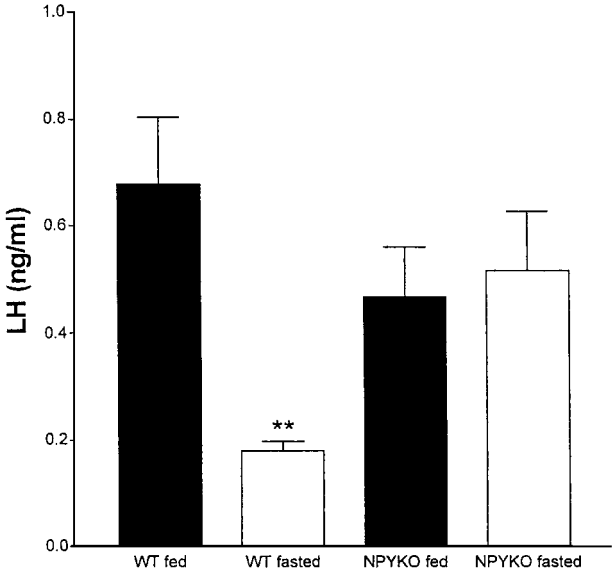

FIG. 5. LH levels of intact NPYKO and WT mice after a 48-h fast. Fast or sham fast began on diestrus I at 1000 h. WT fasted levels were significantly less than levels in animals fed *ad libitum* ($P < 0.01$, n = 7–8, all groups). **, $P < 0.01$.

responsiveness of the reproductive axis to fasting, so we investigated the effect of E_2 replacement. When OVXed animals were treated with E_2 capsules, LH levels in animals of both genotypes fed *ad libitum* fell to a range similar to that of intact animals, although levels in fed NPY-deficient mice were significantly lower than their WT counterparts. Just as seen in intact animals, fasting caused a significant suppression of LH levels in the WT animals, but LH secretion in NPY KO proved resistant to the effects of food deprivation (Fig. 7).

Discussion

The blockade of ovulation in lactating mothers arises from suppression of the basal pulsatile LH secretion necessary for follicular development (26, 27). However, controversy exists

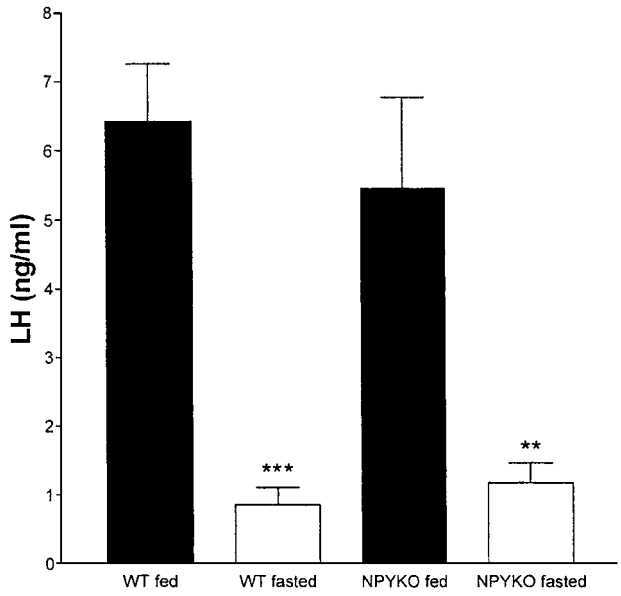

FIG. 6. LH levels of OVX NPYKO and WT mice after a 48-h fast. Fasting significantly suppressed LH levels of WT and NPYKO animals ($P < 0.001$ and $P < 0.001$, respectively; n = 10–12, all groups). **, $P < 0.01$; ***, $P < 0.001$.

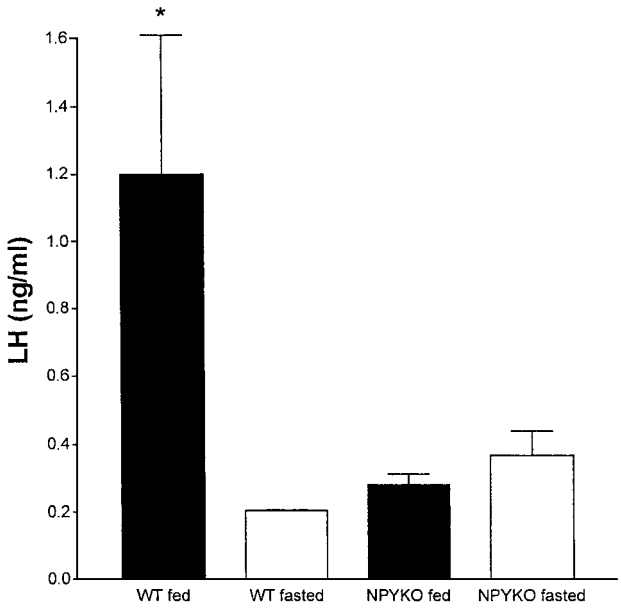

FIG. 7. LH levels of OVX and estradiol-treated NPYKO and WT mice after a 48-h fast. LH levels in WT mice fed *ad libitum* were significantly greater than all other groups. LH levels were not different between fasted *vs.* fed ovariectomized, estrogen-treated NPYKO mice ($P < 0.05$, n = 5, all groups). *, $P < 0.05$.

concerning the means by which lactation exerts this suppression. In rhesus monkeys, projections to the hypothalamus carrying input from the suckling stimulus alone can often maintain lactational anestrus, yet in some monkeys, elevated levels of PRL are required to suppress cyclicity (28). In fact, PRL has been shown to directly suppress neuronal GnRH release (29–31). Others have suggested a third mechanism, namely that the caloric expenditure of lactation suppresses estrous cycles through mechanisms similar to those

engaged during food deprivation (4, 5). The relative importance of these mechanisms in the rodent may depend on the stage of lactation, with PRL levels and/or metabolic factors becoming more important than the suckling stimulus in late lactation (32–34).

Given its involvement in the hypothalamic control of energy intake and GnRH release, NPY may convey information about the metabolic status of an animal to the reproductive axis. To investigate the role of NPY in postpartum anestrus, we measured food consumption, body weight regulation, and timing of the resumption of cyclicity in the NPY KO mouse. Our data suggest that the suppression of estrous cycles during lactation and the reestablishment of cyclicity after weaning can occur independently of the actions of NPY. The absence of NPY in the KO mothers did not impair cessation of their estrous cycles over the course of the lactational period. These results suggest that nonmetabolic mechanisms such as direct actions by PRL or the suckling stimulus may play a dominant role in the suppression of cyclicity during lactation, at least in mice. As is the case with all null-mutant animals, alternative signaling pathways may have compensated for any role played by NPY. One might speculate that ghrelin-expressing (35), melanin-concentrating hormone-expressing (5, 36, 37), or orexin-expressing (5, 38, 39) neurons could also link energy regulation and control of gonadotropin secretion.

Our findings also revealed no evidence of a contribution of NPY to maintenance of metabolic balance during lactation, even with high-energy expenditure caused by large litter sizes. These data recall previous studies showing no effect of the NPY KO genotype on fasting weight loss and refeeding (23, 40). Redundant pathways may well take over the metabolic functions of NPY in the KO animals. The high degree of colocalization of agouti-related peptide (AGRP) and NPY suggest that they could be coordinately regulated during lactation. Indeed, AGRP gene expression is significantly elevated in a subset of the AGRP neurons in the ARC during lactation (41). Proopiomelanocortin neurons may also help maintain weight regulation during lactation because proopiomelanocortin gene expression changes across pregnancy and lactation (42).

NPY activity has been proposed to mediate the neuroendocrine response to starvation, including the suppression of the reproductive axis. In contrast to previous studies using NPY-deficient male mice, we have shown that a 48-h fast causes a near-complete suppression of basal LH levels in the WT females but producing no effect on LH in the NPY KO animals. Thus, NPY appears play a key role in the fasting-induced suppression of reproductive hormone secretions in females but possibly playing less of a requisite role in the male. This type of selective alteration in the reproductive axis of NPY KO mice has been seen before, *e.g.* in the size of the preovulatory LH surge (43). Our results also suggest that NPY plays a more integral role in the reproductive responsiveness to fasting than to lactation.

Both the effects of E_2 replacement on fasted females and the apparent contrast with NPY KO males (18) point to an important role for sex steroids in the actions of NPY during fasting. Studies by Cagampang *et al.* (19, 20) found that rats possess both a steroid-dependent and steroid-independent component to fasting-induced suppression of LH release. Administration of an opioid antagonist reversed the steroid-independent suppression seen in OVXed animals (44). Our WT mice exhibited a similar dual response to fasting in that LH levels fell lower in the intact and estrogen-treated conditions than in the OVXed animals. The KO animals may retain a steroid-independent mechanism for LH suppression but lacking a steroid-dependent mechanism that involves NPY. In addition, a sexually dimorphic mechanism may be at work. The female reproductive axis is more sensitive to food restriction during pubertal development than that of the male (45), and food restriction decreases estrogen receptor immunoreactivity in hypothalamic areas of female but not male mice (46). Our results demonstrate that neither ovarian hormones nor the actions of NPY are required for restraint of LH release; nevertheless, in the presence of estrogen, NPY may serve to suppress the hypothalamic-pituitary-gonadal axis fully during fasting. Additional studies are needed to elucidate the precise actions of steroid hormones in the mouse fasting response.

These studies show that NPY KO animals retain their ability to respond to the negative feedback effects of estrogen. When the steroid is removed, LH levels increase as they do in WT animals, and E_2 replacement returns LH levels to the normal range. In fact, the NPY KO animals may be more sensitive to this negative feedback effect of estrogen because OVXed, E_2-replaced KO mice display lower levels of basal LH than their WT counterparts. This result is unexpected because NPY administration in the presence of low E_2 levels generally reduces LH secretion (47, 48). It should be noted, however, that a general trend toward lower LH levels in the KO animals was seen in the intact and OVXed animals as well. It is conceivable that the absence of NPY mildly impairs LH secretion under any circumstance.

The results of these studies suggest that non-NPY systems can sustain the hyperphagia of lactation and NPY alone does not mediate the lactational suppression of cyclicity. Our findings also illustrate that NPY is required for the suppression of basal LH release that normally accompanies food deprivation in female mice. NPY suppression of GnRH-induced basal LH release may occur through one or more NPY receptor subtypes (16, 49–54). Sainsbury *et al.* (54) found that deletion of the Y4 receptor restores fertility to 50% of female ob/ob mice. In addition, Pralong *et al.* (55) found that starvation has fewer reproductive consequences in male mice lacking Y1 or Y5 receptors, with the Y1 null-mutant mice showing the least severe phenotype. Thus, NPY may act through Y1, Y4,or Y5 receptors to signal fasting-induced alterations in energy balance, triggering LH suppression. The specific population of receptors involved in such a role and the relative contribution of these subtypes remain to be identified and fully characterized.

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