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Attenuation of Luteinizing Hormone Surges in Neuropeptide Y Knockout Mice

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Key Words
Neuropeptide Y · Gonadotropins · Gonadotropin-releasing hormone · Gonadal steroids · Knock out mice

Abstract
To clarify the role of neuropeptide Y (NPY) in the regulation of the reproductive axis, these experiments evaluated the extent to which reproductive hormone secretions may be compromised in the absence of NPY expression. In NPY knockout (NPY-KO) and wild-type (WT) mice, hormone secretions were analyzed under conditions of basal release, following ovariectomy (OVX), in proestrus, after estrogen treatments which induce gonadotropin surges and after injection of gonadotropin-releasing hormone (GnRH). Radioimmunoassays of serum from metestrous females revealed that basal luteinizing hormone (LH), follicular-stimulating hormone (FSH), estrogen and progesterone levels, as well as hypothalamic GnRH tissue concentrations, were not different between the two genotypes. The LH and FSH levels and GnRH tissue concentrations were likewise similar in WT and NPY-KO mice 5 and 10 days following OVX. Significant differences in LH levels were observed however when animals were exposed to pheromone stimulation (male mouse urine) to induce preovulatory LH surges. In proestrous animals, mean LH levels at 18.30–19.00 h were reduced by about 66% in NPY-KO versus WT mice (4.33 ± 1.12 ng/ml in the WT mice vs. 1.47 ± 0.42 ng/ml in the NPY-KO mice, $p = 0.028$). Despite diminishment of LH surges in NPY-KO mice, corpora lutea were equally abundant in the ovaries of NPY-KO and WT mice. In an additional experiment, a surge-inducing regimen of estradiol-17-β (E_2) and estradiol benzoate (E_2B) was administered to OVX animals. The LH surges in the NPY-KO animals treated in this manner were again diminished by approximately 50% compared to corresponding values in WT animals (WT mice 7.33 ± 0.97 ng/ml, NPY-KO mice 3.58 ± 0.74 ng/ml; $p = 0.0063$). To assess the contribution of altered pituitary responsiveness to the diminishment of LH surges, LH responses to a GnRH challenge (200 ng/kg subcutaneously) were determined; NPY-KO animals exhibited LH responses that were significantly reduced compared to values in WT mice (WT mice 4.88 ± 0.56 ng/ml, NPY-KO mice 3.00 ± 0.41 ng/ml; $p = 0.013$). Taken together, these observations do not support the idea that NPY plays a major role in the regulation of basal gonadotropin secretion or in mediating negative feedback actions of gonadal hormones. They demonstrate however that preovulatory NPY release is required for normal amplification of the LH surge that occurs on proestrus. Involvement of NPY in the generation of normal LH surges is partially mediated by the ability of the peptide to prime the anterior pituitary gland to GnRH stimulation.

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Introduction

Neuropeptide Y (NPY) neurons have been implicated in the regulation of reproductive hormone secretions in a variety of physiological contexts. There is considerable evidence, for example, that a preovulatory NPY surge facilitates preovulatory release of gonadotropin-releasing hormone (GnRH) in proestrous rats [1, 2], and to potentiate GnRH-induced luteinizing hormone (LH) secretion from pituitary gonadotropes [3–7]. These effects appear to be mediated by NPY Y1 receptors expressed in hypothalamic [8, 9] and pituitary [6] cells respectively. Other studies have demonstrated that intracerebroventricular NPY applications can induce changes in basal LH secretion in a steroid dependent manner. NPY is stimulatory in the presence of gonadal steroids [8] and inhibitory in their absence, e.g. in gonadectomized animals. Inhibitory actions appear to be mediated by NPY Y5 receptors [10] and/or Y2 receptors [11]. It has also been proposed that NPY exerts inhibitory actions on the reproductive axis prior to the onset of puberty, and that these may wane as the pubertal acceleration of GnRH pulsatility proceeds [12].

Given the multiplicity of effects of exogenous NPY, the physiological importance of endogenous NPY actions have been difficult to establish. Immunoneutralization of NPY in the brain [13] or in the peripheral circulation [1] has been shown to block or attenuate gonadotropin surges in rats, supporting an obligatory role for the peptide in the generation of preovulatory GnRH and LH surges. Infusion of NPY antibodies into the hypothalamus of monkeys has been found to interrupt GnRH pulsatility [14], implicating synaptic NPY release in the facilitation of basal GnRH pulse generation. Recent studies have also made use of specific NPY receptor subtype antagonists to demonstrate that presumptive blockade of NPY actions in the hypothalamus induces precocious pubertal development [15]. Such immunoneutralization or pharmacological studies however cannot exclude partial preservation of endogenous NPY action. Moreover, they may effect hormone secretions by mechanisms unrelated to their ability to antagonize the actions of NPY. We have therefore attempted to assess the physiological relevance of NPY activity by examining the reproductive axis of animals carrying a deletion of the NPY gene. Gene targeting techniques were recently used by Erickson et al. [16] to generate lines of NPY-deficient mice, and the phenotype of these animals has thus far been characterized by an increased susceptibility to seizures and, surprisingly, lack of an overt feeding or reproductive impairment [16–18]. The present experiments were designed to examine more closely whether reproductive hormone deficits accompany the targeted ablation of the NPY gene in female mice. Specifically, we sought to determine whether basal hormone secretions, preovulatory hormone surges or pituitary responsiveness to GnRH stimulation are compromised in NPY knockout (NPY-KO) mice, in order to assess the importance of NPY under these physiological conditions.

Materials and Methods

Animals

NPY-KO mice were generously provided by Dr. Richard Palmiter from the University of Washington, Seattle, Wash., USA. The NPY-deficient mice were previously generated using homologous recombination techniques and maintained on a C57BL X 129SV background [16]. 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 Jackson Labs (Me., USA), and used as wild-type (WT) controls in experiments. Animals were housed 5 to each cage in a moisture- and temperature-controlled room, with standard pelleted mouse chow and water available ad libitum. Lighting was maintained on a 14:10 cycle, with lights on at 05.00 h and off at 19.00 h. In all experiments, animals were anesthetized with methoxyflurane (Metofane, Pitman-Moore Inc., Washington Crossing, N.J., USA), and blood samples were obtained via cardiac puncture. Serum samples were derived by centrifugation and stored at –70°C.

Reagents

β-Estradiol (E_2) and β-estradiol 3-benzoate (E_2B) were purchased from Sigma (St. Louis, Mo., USA). Silicone tubing (0.04 inches internal diameter, 0.085 inches outer diameter) was obtained from Helix Medical (Carpinteria, Calif., USA) while silicone type A medical adhesive was supplied by Dow Corning (Midland, Mich., USA). Capsules containing E_2 for implantation were prepared by mixing a certain amount of E_2 in a fixed volume of sterile adhesive, and then fitting the evenly mixed 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 μg of E_2.

Radioimmunoassays

The GnRH radioimmunoassay (RIA) was performed as previously described [19] using the EL-14 GnRH antibody, generously provided by Dr. Martin Kelly at Oregon Health Sciences University, Portland, Oreg., USA. The intraassay coefficient of variation for the GnRH RIA was 9.3%. In preparation for the GnRH RIA, hypothalamic tissue samples containing the mediobasal hypothalamus and preoptic areas were dissected immediately after sacrifice, frozen on dry ice and stored at –70°C until use. Each of the mediobasal hypothalamus and preoptic area tissues was added to 1 ml of homogenization solution (800 μl of 100% ethanol and 200 μl of 0.1 *N* HCl). Mediobasal hypothalami were homogenized at 4°C. After centrifugation at 1,500 rpm for 20 min, supernatants were transferred to a SpeedVac system (SVC 200H, Savant Instruments Inc., New York,

N.Y., USA) and dried overnight. Tissue pellets were resuspended in 1 ml of PBS (pH 7.4) (each sample) for later GnRH RIA.

The LH and follicular-stimulating hormone (FSH) RIAs were performed using reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases, including the LH RP-3 and FSH RP-2 reference preparations. The intraassay coefficients of variation for the LH and FSH assays were 8.9 and 5.5%, respectively. Progesterone was assayed with the Immuchem Prog ^{125}I kit (ICN Pharmaceuticals, Costa Mesa, Calif., USA), and E_2 was assayed using the KE2D1 kit (Diagnostic Products Corp., Los Angeles, Calif., USA).

Ovarian Histology

Ovaries were removed at autopsy and fixed overnight in a 4% paraformaldehyde solution. The tissues were then embedded in paraffin, cut into 20-μm sections on a sliding microtome, and stained with hematoxylin/eosin for subsequent light microscope analysis. The number of corpora lutea present in representative sections throughout the ovaries was tabulated for each of the genotypes.

Basal Hormone Levels and Responses to Ovariectomy

Estrous cycles were monitored in female WT and NPY-KO mice by daily inspection of vaginal cytology as described by Bingel and Schwartz [20]. Mice were selected for serum sample collection after they had exhibited at least two consecutive estrous cycles. For basal level hormone measurements, WT (n = 11) and NPY-deficient mice (n = 12) were sacrificed between 12.00 and 13.00 h of metestrus. The LH, FSH, estrogen and progesterone levels in the sera, and hypothalamic GnRH content, were measured by respective hormone RIAs. Additional groups of WT and NPY-KO mice were anesthetized via methoxyflurane inhalation and bilaterally ovariectomized (OVX). On day 5 or 10 after OVX surgery, these WT and NPY-KO mice (n = 5 for each group) were anesthetized and blood samples obtained via cardiac puncture between 12.00 and 13.00 h. Serum LH, FSH and hypothalamic GnRH were measured by RIAs.

Pheromone-Induced LH Surges

Female mice were individually housed in cages. After exposure to male urine-soaked mouse bedding, WT and NPY-KO mice exhibiting proestrous-like vaginal cytology were anesthetized and killed between 18.30 and 19.00 h via cardiac puncture and exsanguination. Only blood samples from animals showing natural uterus ballooning were used [20]. Serum samples from these animals were stored at –70°C for subsequent LH and FSH RIA. The time of sacrifice was chosen on the basis of previous experiments [21] which showed that the ascending phase of the LH surge in intact, pheromonally stimulated mice occurs between 17.00 and 18.00 h, and that the LH peak occurs at approximately 19.00 h; the same study demonstrated that LH levels return to baseline in these animals by 21.00–22.00 h. In preliminary experiments, we similarly found that LH levels are at or near the minimum level of detectability at 17.00 and 21.00 h of proestrus in both the WT and NPY-KO genotypes (data not shown).

E_2/E_2B-Induced LH Surges

Mice of both genotypes were submitted to OVX and subsets of both animal groups were given estrogen treatments. OVX was performed on the mice at 09.00 h under methoxyflurane anesthesia, and 1-cm Silastic brand capsules (0.04 inches internal diameter, 0.085 inches outer diameter) containing 1.5 μg of E_2 mixed into silicone type A medical adhesive were placed subcutaneously (s.c.) under one flank. Controls received empty capsule implants. At 09.00 h on day 6 following surgery and E_2 capsule implantation, each mouse received a s.c. injection of 1 μg of E_2B in sesame oil. Between 18.30 and 19.00 h on day 7, mice were sacrificed via cardiac puncture and exsanguination. Control groups received either control capsule (without E_2) implantation and vehicle s.c. injection, control capsule implantation and E_2B injection, or E_2 capsule implantation and vehicle injection. Sera were collected for LH and FSH RIA.

Pituitary Responses to GnRH Stimulation

A GnRH stimulation protocol was used as previously described to assess pituitary responsiveness to an intermediate dose of the decapeptide [19]. Female WT and NPY-KO mice received subcutaneous implantation of an E_2 capsule (1.25 μg, 1 cm). At 09.00 h on day 6, each mouse received a s.c. injection of 1 μg of E_2B. Between 08.00 and 09.00 h of day 7, each animal received a s.c. injection of GnRH (200 ng/kg in saline; n = 11 for both NPY-KO and WT animal groups) or saline vehicle (n = 5 for both NPY-KO and WT animal groups). Ten minutes after GnRH or saline injection, mice were exsanguinated via cardiac puncture, and the resultant sera samples were stored at –70°C for subsequent LH and FSH RIA. To test the LH release responses to GnRH in the absence of estrogen replacement, additional groups of OVX WT and NPY-KO mice underwent control implantation and injection procedures, and were otherwise treated and sampled in the same manner as the animals receiving estrogen treatments.

Statistical Analysis

To assess differences in hormone levels between the two genotypes, mean and standard error values for a given hormone were calculated for each of the two genotypes, and compared by means of Student's t tests. Hormone responses to OVX were analyzed by one-way ANOVA followed by post hoc comparisons using Tukey's test. A two-way ANOVA with post hoc comparisons was used to assess differences in GnRH-stimulated LH values among genotype groups that received control or estrogen pretreatments. For all statistical tests, differences were considered to be significant with a p value ≤ 0.05.

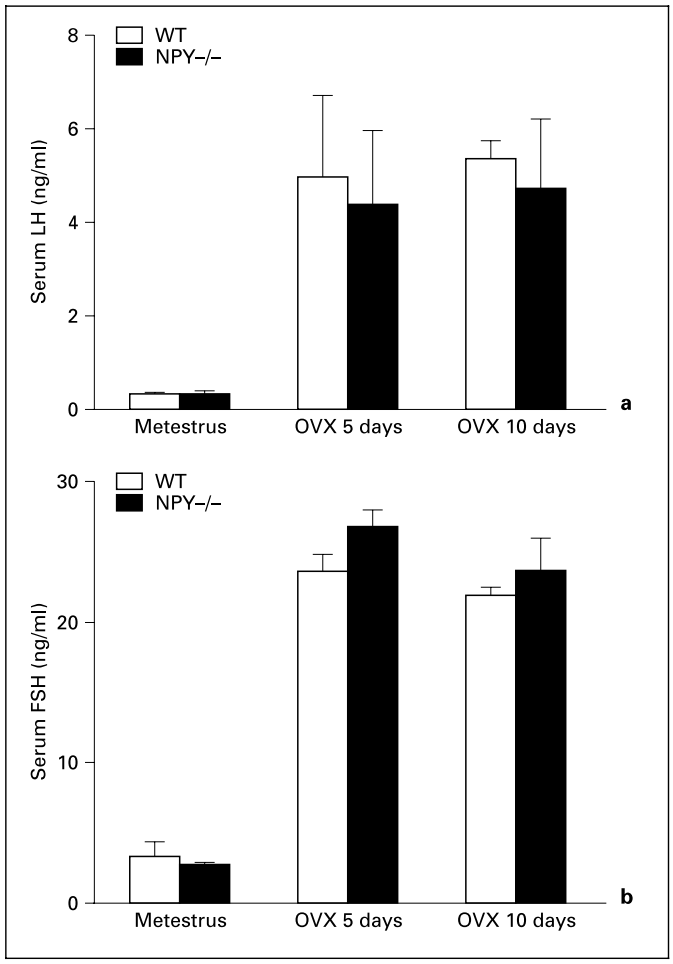
Results

Reproductive Characteristics of NPY-KO versus WT Mice

Table 1 summarizes several basic characteristics of reproductive function in the female NPY-KO and corresponding WT mice. No significant differences were observed in the average body weight or date of vaginal opening in NPY-KO versus WT animals, or in the litter size and gender ratio of the offspring. Histological inspection of the ovaries from both genotypes revealed no gross differences in the number or appearance of corpora lutea or ovarian follicles at any stage of maturation.

Basal Hormone Levels and the Effects of OVX

Hormone analyses in metestrous WT and NPY-KO mice revealed no significant differences between the two



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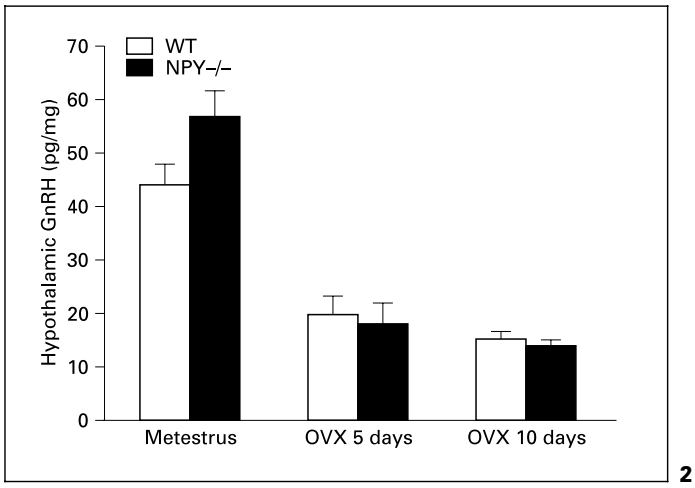

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Fig. 1. Serum LH (**a**) and FSH (**b**) levels at 12.00 h of metestrus and changes after OVX. As shown in the graph, at 12.00 h of metestrus, there was no significant difference in serum LH and FSH levels between NPY-deficient mice and WT controls (WT, n = 11; NPY–/–, n = 12). Five or ten days after OVX, the mice showed a dramatic increase in serum LH and FSH concentrations; again, there was no significant difference between the two groups (n = 5 for each of the WT and NPY–/– groups).

Fig. 2. Hypothalamic GnRH content at metestrus and changes after OVX. As shown in the graph, at 12.00 h of metestrus, there was no significant difference in hypothalamic GnRH content between NPY-deficient mice and WT controls (WT, n = 10; NPY–/–, n = 13). Five or ten days after OVX, the mice showed a dramatic decrease in hypothalamic GnRH content; again, there was no significant difference between the two groups (n = 5 for each of the WT and NPY–/– groups).

Table 1. Litter size and gender ratio of mouse offspring

	Litter size	Male/female	Litters	Mice
WT	6.15 ± 0.17	1.045	21	123
NPY–/–	6.82 ± 0.24	0.985	39	266

Data were obtained from observations of 39 NPY-deficient and 21 WT litters and compared for significance. There was no significant difference between the two genotypes in terms of the aspects listed above.

genotypes in serum levels of E_2 (WT: 6.96 ± 1.18 pg/ml, n = 11; NPY-KO: 8.33 ± 1.5 pg/ml, n = 12; p = 0.48) and progesterone (WT: 54.86 ± 6.42 pg/ml, n = 11; NPY-KO: 58.96 ± 7.84 pg/ml, n = 12; p = 0.70). There was no significant difference between the two genotypes in serum LH and FSH levels at metestrous (fig. 1). Moreover, serum concentrations of LH and FSH in the two genotypes rose to the same extent by 5 and 10 days following OVX (fig. 1). Hypothalamic GnRH concentrations were also not different in metestrous WT versus NPY-KO mice (fig. 2). The tissue GnRH concentrations were significantly diminished following OVX; however, these decreases were similar at both the 5- and 10-day post-OVX time points for both genotypes (fig. 2).

Pheromone-Induced Gonadotropin Surges

As found in other strains of mice [19], continuous exposure of female mice of either genotype to soiled bedding of males was followed by the appearance of proestrous-like vaginal cytology. In both the WT and NPY-KO females treated in this manner, LH surges were evident at 18.30–19.00 h of the presumptive proestrus (fig. 3a). The levels of LH reached in these animals were significantly elevated compared to corresponding values in metestrous WT and NPY-KO mice (fig. 3a). The amplitude of the LH

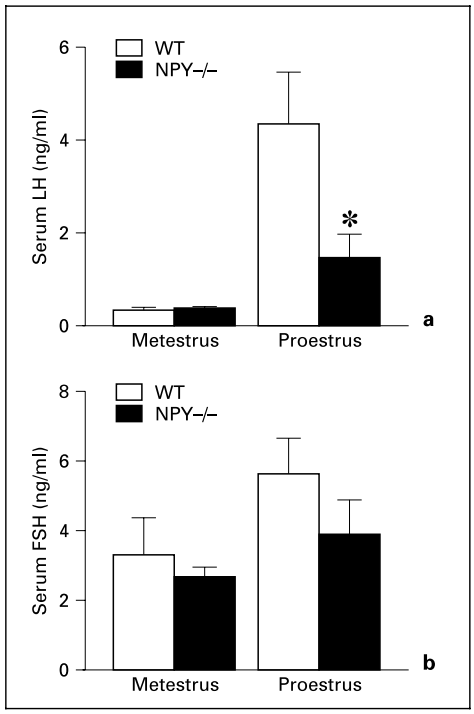

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Fig. 3. Male pheromone-induced preovulatory LH surges and the corresponding FSH levels. Between 18.30 and 19.30 h of proestrus, mice of both genotypes were sacrificed, and serum LH and FSH levels were measured by RIA. Both WT and NPY-deficient mice in metestrus were sacrificed during the same period and serum LH and FSH was measured for comparison. **a** The amplitude of the male pheromone-induced preovulatory LH surges in NPY-deficient mice was significantly lower than that of the WT mice (WT, n = 10, NPY–/–, n = 12; * p = 0.028). **b** There was no significant difference between the two genotype groups in serum FSH levels during the same period.

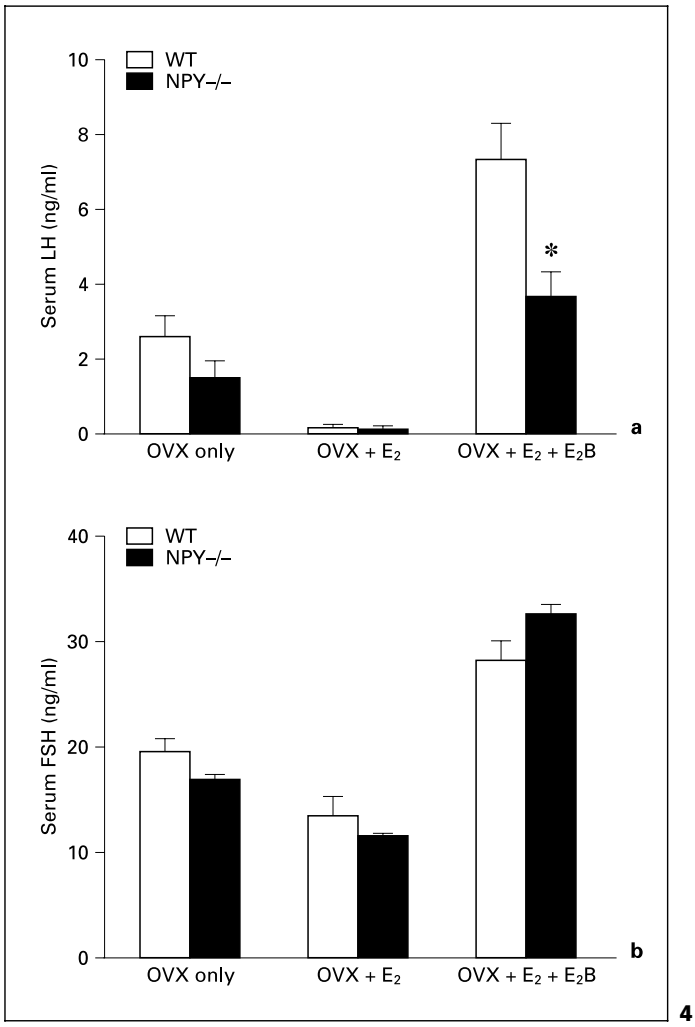

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Fig. 4. Serum LH and FSH levels in estrogen-induced gonadotropin surges. Detailed information about estrogen treatment is given in Materials and Methods. **a** Seven days after OVX, serum LH levels increased, with no significant difference between WT and NPY-deficient mice (WT and NPY–/–, n = 10–12). E_2 capsule implantation at OVX exerted a negative effect on serum LH levels, which did not differ between NPY-deficient and WT mice (n = 10–11 for both groups). In estrogen-induced LH surges, the amplitudes of LH surges in NPY-deficient mice were dramatically attenuated (WT, n = 10, NPY–/–, n = 12; * p = 0.0063). **b** The same serum samples were measured for FSH. Estrogen treatment induced a significant increase in serum FSH levels (compared with OVX only, $p < 0.05$), while there was no dramatic difference between NPY-deficient mice and WT controls.

rise, however, was reduced by 66% in the NPY-KO mice compared to the WT animals (1.47 ± 0.42 ng/ml in NPY-KO vs. 4.33 ± 1.12 ng/ml in WT mice; n = 10–12 per group; p = 0.028). Additional animals in both groups were killed at earlier (16.00–18.00 h) and later (19.40–21.00 h) time points, and RIAs of samples from these animals revealed no premature or delayed elevations of LH in serum. All of the latter values were found to be near or below the detectable limit of the LH assay (0.02 ng/ml). Serum FSH values were not significantly different between the two genotypes in metestrous animals, and pheromone stimulation did not result in a significant increase in FSH secretion in either group (fig. 3b).

Effects of Estrogen on Gonadotropin Levels

OVX WT and NPY-KO mice that received E_2-containing capsules s.c., but not additional injections with E_2B, exhibited suppressed levels of LH compared to OVX animals which received blank capsules. OVX WT mice treated with both an E_2 capsule and a s.c. E_2B injection

LH surges (7.33 ± 0.97 ng/ml; n = 10), reached levels that were significantly increased in comparison to both the untreated OVX WT controls (n = 10; p = 0.0009) and the OVX WT animals treated with an E_2-filled capsule alone (n = 11; p = 0.0003) (fig. 4a). By contrast, NPY-KO mice receiving both the E_2 capsule and s.c. E_2B injection exhibited LH levels that only minimally exceeded those in OVX NPY-KO mice not receiving estrogen treatment (3.58 ± 0.74 ng/ml, n = 12, vs. 1.77 ± 0.31 ng/ml, n = 12; p = 0.034). Moreover, E_2 capsule and s.c. E_2B injection produced LH surge levels in NPY-KO mice that were less than 50% of those observed in their WT counterparts (NPY-KO: 3.58 ± 0.74 ng/ml, n = 12; WT: 7.33 ± 0.97 ng/ml, n = 10; p = 0.0063) (fig. 4a). Attenuation of LH levels at 18.30–19.00 h in the NPY-KO mice did not result from an advanced or delayed LH surge, as values from animals killed prior to 18.00 h or later than 19.40 h were invariably less than 0.02 ng/ml, regardless of genotype (data not shown). FSH surges were also evident in animals receiving both E_2 capsules and E_2B injections (fig. 4b), although no difference in the magnitude of FSH surges was observed in NPY-KO versus WT mice. Mean serum FSH levels in the surging animals reached values that were significantly greater than those in OVX mice, and in OVX mice receiving only E_2 capsule implants (fig. 4b) (OVX + E_2 + E_2B vs. OVX only group, p = 0.04; OVX + E_2 + E_2B vs. OVX + E_2 group, p = 0.009).

LH Secretory Responses to GnRH

OVX mice received an E_2 capsule implant and E_2B injection, and were subsequently treated with a s.c. injection of 200 ng/kg GnRH or saline vehicle at 10.00 h on the morning following the day of E_2B injection. The serum LH levels following GnRH injections were significantly greater than corresponding values following saline injections in both the NPY-KO and WT mice (fig. 5a). However, the magnitude of the LH response to GnRH was significantly lower in the NPY-KO animals (3.00 ± 0.41 ng/ml, n = 11) than that observed in WT mice (4.88 ± 0.56 ng/ml, n = 10) (p = 0.013). In OVX mice that did not receive any estrogen treatments, the GnRH treatments did not produce any significant increase in LH levels in either genotype compared to values observed following saline injections (fig. 5b). The GnRH injections did not produce significant changes in FSH levels, regardless of whether or not animals were primed with E_2 and E_2B (data not shown).

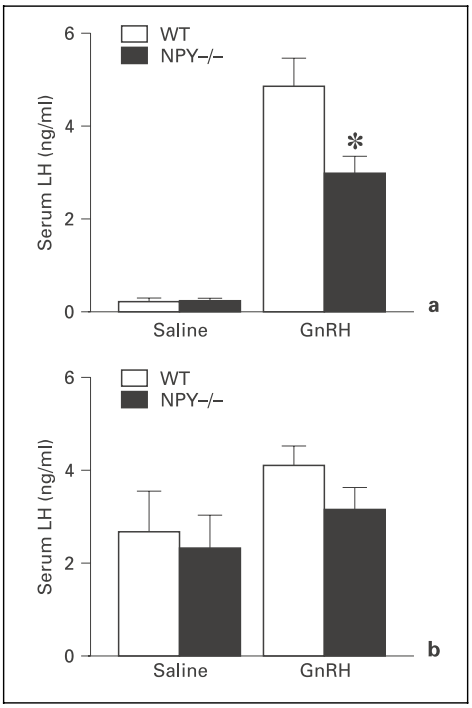

Fig. 5. GnRH-induced LH releases. **a** OVX mice received the same estrogen treatment that was able to induce LH surges. On the morning of the day on which the LH surge was to occur, a s.c. application of GnRH (200 ng/kg) induced a marked LH release in both WT and NPY-deficient mice 10 min after GnRH injection. However, the induced LH secretions in NPY-deficient mice were significantly lower than those in WT mice (NPY–/–, n = 11, WT, n = 10; * p = 0.013). Saline had no effect (WT and NPY–/–, n = 5). **b** In mice receiving OVX only without estrogen treatment, GnRH was not effective in inducing LH release in either genotype.

Discussion

Previous studies have provided evidence that NPY is critically important in the control of pulsatile GnRH release [14, 22, 23], sexual maturation [24–26] and the release of preovulatory gonadotropin surges [3, 4, 13, 26]. In a recent analysis of NPY-KO mice, however, it was found that the absence of NPY production does not lead to any gross impairment of fertility or growth. We have therefore investigated this apparent discordance between data obtained in the NPY-KO phenotype and the prevailing opinions that NPY has an important neuroendocrine role in reproduction. In the present studies, we have more closely scrutinized the reproductive endocrinology of NPY-KO mice, in order to determine whether significant endocrine abnormalities are present which may not be overtly manifested as a lack of fertility. Our results

reveal that NPY-KO mice do exhibit a substantial and specific deficit in their reproductive hormonal secretions; they release preovulatory and estrogen-induced LH surges that are greatly diminished in amplitude compared to those of their WT counterparts.

There is considerable support for the idea that NPY exerts actions at both the hypothalamic and pituitary levels to facilitate release of GnRH and LH surges, respectively. The peptide stimulates GnRH release in vitro and in vivo in a steroid-dependent manner [27, 28], and likewise facilitates GnRH-stimulated LH secretion in vitro and in vivo, especially under conditions which normally lead to LH surges. That these actions are important in the generation of LH surges derives support from numerous observations, i.e. that NPY mRNA levels [29], NPY tissue content in median eminence [2], NPY release in median eminence [30] and NPY levels in the portal vasculature [1] are all acutely increased in association with the initiation of GnRH and LH surges. Moreover, intracerebral administration of NPY antiserum blocks the release of LH surges [31], as does passive immunoneutralization in the peripheral circulation [1].

Given the foregoing body of evidence, we hypothesized that ablation of the NPY gene would be accompanied by a major diminution of the LH surge. Our observations in both pheromone-stimulated and estrogen-primed NPY-KO mice have revealed that such an attenuation is indeed characteristic of the NPY-deficient animal. Our findings in the NPY-KO mice thus confirm the role of NPY for normal amplification of preovulatory LH surges. The extent of the attenuation that we observed in these mice, moreover, is comparable to the degree to which an NPY Y1 receptor antagonist blunts the LH surge in proestrous rats (i.e. 65%) [6]. Our results are therefore also consistent with the assumption that activation of the Y1 receptor subtype may account for NPY facilitation of the surge, a hypothesis which remains to be tested directly by comparing amplitudes of LH surges between strains of mice bearing different deletions of specific NPY receptor subtype genes.

Despite the substantial attenuation of LH surge amplitude, NPY-KO mice did not exhibit any significant reduction in corpora lutea number, pregnancy rate or litter size. It thus appears that ovulation can occur normally in these mice in response to LH surges of amplitudes reduced up to 35% with respect to those observed in WT mice. This phenomenon has long been known in female rats, in which as little as 15% of the spontaneous LH surge is apparently sufficient to trigger ovulations [32]. The reasons for such suprathreshold LH surge under normal conditions are not clear, but may reflect the special characteristics of rodents housed under relatively neutral laboratory conditions. Relatively little environmental stress, as well as ad libitum feeding, may confer a maximally fertile state to the normal laboratory rodent by providing unlimited metabolic energy for reproduction. One consequence of this reproductive neuroendocrine state may be a maximally activated GnRH and LH surge mechanism, which under normal laboratory conditions is far in excess of the threshold for ovulation induction.

Basal serum levels of LH, FSH, E_2 and progesterone were not different from those of WT animals, and responses of the two gonadotropins to OVX were also similar in both genotypes. While we were not able to characterize pulsatile LH in these animals, the lack of any discernible differences in basal or post-OVX hormone levels suggests that the GnRH pulse generator activity is not significantly impaired in the absence of NPY production. Studies in monkeys have implicated NPY in the control of GnRH pulsatility [14, 23] as well as in the regulation of GnRH surge release [22]. In rats, basal LH secretion is stimulated by exogenous NPY in estrogen-primed rats [8] and inhibited by the peptide in OVX animals [33]. The lack of any obvious disruption of LH secretion in the NPY-KO mice could either reflect a species difference in the actions of NPY, or there may in fact be some modest effects on GnRH pulsatility in the mouse which are not discernible in our single samples obtained via cardiac puncture. It is also possible that NPY is only one of several modulators of GnRH pulse generation, and that the relatively normal LH levels in the NPY-KO mouse may reflect the activation of compensatory, developmental responses by these other neuromodulators to the absence of NPY expression.

The average age at which vaginal opening occurred was also comparable in NPY-KO and WT genotypes. While we could not perform a detailed hormonal analysis of pubertal maturation, the lack of any significant difference in time of vaginal opening in mice is nevertheless suggestive of a relatively normal reproductive developmental rate in the absence of NPY. In previous studies however NPY administered to the third ventricle of prepubertal rats resulted in a delay of puberty [12, 34], prompting the conclusion that NPY may play an inhibitory role in the timing of pubertal maturation. As in the case of basal GnRH and LH secretion in the adult, there are several possible reasons for this apparent discrepancy. There may be species differences in the involvement of NPY in puberty, or a subtle advancement of some pubertal landmark may in fact have been present but not detected in these experi-

ments. Alternately, NPY may normally play a significant role in pubertal development in mice, but in the NPY-deficient animal other neuronal groups can substitute for the lack of the peptide and provide compensatory regulatory input to the juvenile GnRH pulse generator.

Gene targeting methods are of good value in analyzing the function of a variety of neuroendocrine peptides and their receptors. Interpretation of results from NPY-KO mice however requires caution in particular since various NPY receptor subtypes (Y1–Y6) may be activated to varying degrees in vivo by NPY as well as by other members of the YY peptide family. Specific involvement of NPY in puberty and in basal hormone secretions may have thus been obscured in these experiments by contributions of other endogenous ligands which may normally subserve the same functions in a redundant manner. Participation of NPY in pubertal maturation and GnRH pulsatility thus remains ambiguous and will only be resolved pending development of conditional NPY-KO animals and/or genotypes bearing multiple ligand or receptor gene deletions.

Our findings in the NPY-KO mice nevertheless demonstrate unequivocally that secretion of normally proportioned LH surges is dependent upon NPY actions. Our observations also suggest that at least some of the actions of NPY in this context are likely exerted at the level of the anterior pituitary gland, since pituitary responsiveness to GnRH was attenuated in NPY-KO mice. Under normal conditions, actions of NPY on gonadotropes are likely mediated by Y1 receptor activation and subsequent stimulation of protein kinase C [6, 7]. Presumably, absence of NPY in the NPY-KO mice leads to a reduction in the level of Y1 receptor activation, diminished PKC stimulation, and an attenuation of signaling events leading to LH secretion. It remains to be determined whether additional hypothalamic defects also contribute to the attenuation of GnRH surges in NPY-KO mice, and subsequently to the attenuation of LH surges in these animals.

The broader biological importance of the amplifying effects of NPY also remains to be clarified. Elimination of this amplifying function clearly results in a major diminishment of the LH surge in both mice and rats, yet the residual amount of LH released is sufficient to sustain a relatively normal level of fertility under laboratory conditions. Future studies may document the importance of the NPY amplification mechanism under less neutral environmental conditions, such as dietary restriction or behavioral stress.

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