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·*Original Article*·

Expression of Neuropeptide Y gene in mouse testes during testicular development

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Abstract

Aim: To elucidate the distribution and regulation of Neuropeptide Y (*NPY*) gene expression in testes under physiological and pathophysiological conditions, such as testicular development, fasting and experimental cryptorchidism. **Methods:** In the first experiment, C57BL/6J male mice at the ages of 3 days as well as 2, 3, 5 and 8 weeks were used. In the second and third experiments, adult C57BL/6J male mice were subjected to fasting for 48 h and experimental cryptorchidism for 72 h. The *NPY* transcripts were detected by isotopic *in situ* hybridization histochemistry. **Results:** The *NPY* transcripts were exclusively expressed in the interstitial cells regardless of the age groups and experimental conditions. The *NPY* mRNA levels were found to increase significantly with age (from the neonatal to prepubertal period [$P < 0.01$] and from the prepubetal to postpubertal period [$P < 0.01$]). Food deprivation for 48 h resulted in a significant increase in the *NPY* mRNA levels in the arcuate nucleus of the hypothalamus ($P < 0.01$), but not in the testes. Experimental cryptorchidism for 72 h failed to regulate the *NPY* gene expression in the testes. **Conclusion:** *NPY* gene expression is distributed in Leydig cells and increases in line with testicular development. The regulation of testicular *NPY* is different from that of hypothalamic *NPY*. (*Asian J Androl 2006 Jul; 8: 443–449*)

Keywords: Neuropeptide Y; testes; *in situ* hybridization histochemistry; steroidogenesis; androgen; testicular development; cryptorchidism

1 Introduction

One of the most important roles that the testes play is steroidogenesis, such as the testosterone production. Leydig cells are responsible for testosterone production in mammals. Testosterone production depends on the stimulation of these cells by luteinizing hormones, which are secreted into the peripheral circulation from the anterior pituitary in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus. Several local factors are involved in the regulation of testicular function [1–3]. These factors include neuropeptides, such as opioids, growth hormone-releasing hormones, corticotropin-releasing hormones, and pituitary adenylate cyclase-activating polypeptide (PACAP) [4, 5]. These neuropeptides are synthesized in the testes and might play a role in the testicular function in an autocrine and/or paracrine manner.

A previous study demonstrates that an orexigenic neuropeptide, Neuropeptide Y (*NPY*), which is predominantly synthesized in the hypothalamic arcuate nucleus,

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is also expressed in the testes, especially in Leydig cells [6]. In the central nervous system, both the physiological roles and the regulation of *NPY* have been well documented. For instance, the central administration of *NPY* stimulates food intake and weight gain [7]. *NPY* directly stimulates the release of gonadotropin at the pituitary level or potentiates the release of gonadotropins from the pituitary in response to GnRH in the hypothalamus [7]. Fasting significantly stimulates the synthesis of *NPY* in the arcuate nucleus of the hypothalamus and the effects of fasting on the upregulation of hypothalamic *NPY* are largely mediated by a decrease in the amount of circulating leptin during fasting because the infusion of leptin can completely reverse the fasting effects [7]. In contrast to hypothalamic *NPY*, the regulation of testicular *NPY* as well as its physiological role are still poorly understood. As a first step toward better understanding the role of testicular *NPY*, the present study was conducted to elucidate the distribution and regulation of *NPY* gene expression in mouse testes under physiological and pathophysiological conditions using *in situ* hybridization histochemistry.

2 Materials and methods

2.1 Animals

Seventy-four C57BL/6J male mice were used. They were group-housed (3–4 mice/cage) in plastic cages (30 × 20 × 12 cm) and maintained on a 12 h : 12 h light : dark cycle (light off at 09:00) at a constant temperature (22ºC) throughout the study. Food and water were available *ad libitum* except during the fasting experiment. All procedures were approved by the ethics committee of animal care and experimentation at our university (University of Occupational and Environmental Health, Kitakyushu, Japan).

2.2 Experiments

In the first experiment, 24 C57BL/6J male mice at the age of 3 (prepubertal period), 5 (pubertal period), and 8 weeks (postpubertal period) (n = 8 in each age group) were used. To compare the developmental profile of testicular *NPY* with hypothalamic *NPY*, 18 C57BL/6J male mice at the age of 3 days (neonatal period), 2 and 3 weeks (prepubertal period) (n = 6 in each age group) were used. They were killed with excessive carbon dioxide at 13:00 and thereafter the testes were rapidly removed, frozen on dry ice and stored at –80ºC until they were used. In the second experiment, sixteen 10-week-old C57BL/6J male mice were used. Food deprivation started at 13:00 in the fasting group (n = 8). The same number of mice was used in the control group. Drinking water was available throughout the period of food deprivation. The mice were killed with excessive carbon dioxide 48 h after the initiation of fasting. Thereafter, the testes and the brains were removed, frozen on dry ice and stored at –80ºC until they were used. In the third experiment, sixteen 10-week-old C57BL/6J male mice were used. They were deeply anesthetized using pentobarbital (50 mg/kg) and then cryptorchidism was unilatelarally induced through a mid-abdominal incision (n = 8). The left testis was drawn from the scrotum into the abdominal cavity and then was sutured to the parietal peritoneum using 5-0 bicryl sutures. The sham-operated animals (n = 8) were handled similarly except that both testes were left intact. Three days after the surgery, the mice were killed with excessive carbon dioxide. The testes were removed and halved. One was frozen on dry ice and stored at –80ºC. The other was fixed in 4% formaldehyde solution.

2.3 In situ hybridization histochemistry

Frozen sections of the testes and brains were cut at 12 μm on a cryostat, mounted onto gelatin-coated slides (Matsunami Glass, Osaka, Japan) and stored at –80ºC until they were used. The probes used were oligodeoxynucleotides complementary to mRNA coding for *NPY* (5'-GGA GTA GTA TCT GGC CAT GTC CTC TGC TCG CGC GTC-3'). The specificity of each probe has been described previously [8]. We also checked the specificity of signals using a sense *NPY* probe and a 100-fold excess of unlabeled *NPY* probe (a competitive hybridization study). The probe was 3'-end labeled using terminal deoxynucleotidyl transferase and [^{35}S]dATP. The *in situ* hybridization procedures have all been described previously [8]. In brief, sections were fixed in 4% formaldehyde solution for 5 min and incubated in saline containing 0.25% acetic anhydride (vol/vol) and 0.1 mol/L triethanolamine (TEA) for 10 min, then dehydrated and delipidated in chloroform. Hybridization was carried out overnight at 37ºC in 67.5 μL hybridization buffer containing 50% formamide and 4 × standard saline citrate (SSC) (1 × SSC = 150 mmol/L NaCl and 15 mmol/L sodium citrate), 500 μg/mL sheared salmon sperm DNA (Sigma, St. Louis, MO, USA), 250 μg/mL baker's yeast total RNA (Roche, Mannheim, Germany), 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02%

bovine serum albumin) and 10% dextransulfate (500 000 mol wt, Sigma, St. Louis, MO, USA) under a Nescofilm coverslip (Bando Chemical IMD, Osaka, Japan). Total counts of 1.0×10^6 count per minute (CPM)/slide were used. After hybridization, sections were washed for 1 h in four changes of 1 × SSC at 55ºC and for a further 1 h in two changes of SSC at room temperature. Hybridized sections were apposed to autoradiography films (Hyperfilm, Amersham, Buckinghamshire, UK) for 7 days (hypothalamus), 14 days (testes; 3, 5 and 8 weeks old) and 21 days (testes; 3 days, 2 and 3 weeks old). Slides were then dipped in nuclear emulsion (Ilfold K-5, Cheshire, UK) and exposed for 28 days. All slides from different groups were processed simultaneously and were exposed to the same film in each region to minimize the effect of variations in hybridization and wash conditions.

A quantitative image analysis of the developed films was performed using the MCID image analysis system (Imaging Research, Ontario, Canada). In the hypothalamus, four sections containing anatomically matched levels of the arcuate nucleus (Bregma –2.56 to –3.14 mm) were selected for each mouse by referring to a standard atlas of the mouse brain [9]. In the testes, transverse sections at the maximum diameter were selected for quantitative analyses. The optical density was measured in four sections of the testis and the arcuate nucleus and then it was averaged for each mouse. In the testes, the optical density was examined in the area of the interstitial cells on developed films using the MCID image analysis system. The optical density was presented as an arbitrary unit. Because it depends on a pattern of gene expression and exposure time, the optical density in control in each site showed substantial differences (*cf*. the hypothalamus and testes). We initially quantified the optical density in the area of interstitial cells on developed films using the MCID image analysis system. We also counted the total number of grains in the interstitial cell division of emulsion-dipped cells and confirmed the correlation of the data in both an MCID image analysis and a grain count. Therefore, the present data showing the optical density reflect the total copies of *NPY* transcripts for the same area of interstitial cell division. Because no statistically significant changes in the number of interstitial cells were observed with age, an increase in the level of the testicular *NPY* gene expression would reflect an increase in the number of *NPY* transcripts per interstitial cell. Quantitative analyses were done by an observer who was unaware of the age groups and treatments of the samples.

2.4 Hematoxyline-eosin staining

After fixation, the testes were embedded in paraffin blocks. Sections measuring 5 μm in thickness were cut transversally from each block with a microtome, mounted on slides, deparaffinized in xylene, and dehydrated with graded ethanol. At the maximum diameter of the testes, the sections were then stained with hematoxylin and eosin.

2.5 Statistics

All data are presented as the mean ± SE. Statistical analyses were carried out using one-way fractional ANOVA followed by the Bonferroni post-hoc test. $P < 0.05$ was considered to be statistically significant.

3 Results

In situ hybridization histochemistry revealed that the *NPY* transcripts were exclusively present in the interstitial cells, presumably the Leydig cells regardless of the age group and experimental conditions (Figure 1A–C). Few *NPY* transcripts were observed in the seminiferous tubules (Figure 1A–C). The signals were completely abolished with the addition of a 100-fold excess of unlabeled *NPY* probe (Figure 1D). No signals were obtained with a sense probe for the probe complementary to the *NPY* probe (data not shown). Significant age differences

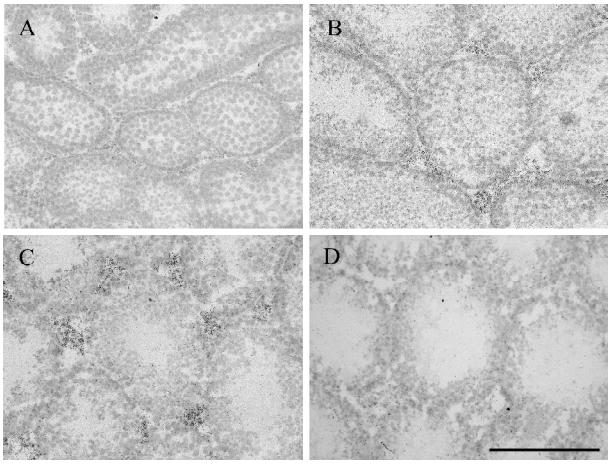

Figure 1. Representative photomicrographs of emulsion-dipped section showing the Neuropeptide Y (*NPY*) transcripts in the mouse testes at the ages of 3 (A), 5 (B) and 8 weeks (C) and with the addition of a 100-fold excess of unlabeled probe in the testes at the age of 8 weeks (D). Sections were hybridized to a ^{35}S-labeled oligodeoxynucleotide probe complementary to mRNA for *NPY* and counter-stained using eosin. Scale bar represents 100 μm.

were observed in the total areas of the coronary sectioned testes. Because the diameter of the testes increased significantly with age (3 days *vs.* 2 and 3 weeks, $P < 0.01$; 3 weeks *vs.* 5 weeks, $P < 0.01$; 5 weeks *vs.* 8 weeks; $P < 0.01$), the levels of *NPY* mRNA in the testes were represented as the optical density/area. Representative film autoradiographs showing the developmental profile of the *NPY* gene expression are presented in Figure 2A. The levels of *NPY* mRNA increased significantly at ages of 5 and 8 weeks in comparison to those at ages of 3 weeks ($P < 0.01$; Figure 2B). The distribution pattern of the *NPY* gene expression showed no age-related differences (Figure 2A). In a different set of animals in earlier developmental stages, the levels of *NPY* transcripts increased significantly with age (optical density; 3 days: 1 853 ± 306, 2 weeks: 4 272 ± 307, 3 weeks: 4 866 ± 851 [arbitrary unit]; 3 days *vs.* 2 and 3 weeks, $P < 0.01$). Similar to the peripubertal developmental stages, no *NPY* transcripts were observed in the seminiferous tubules in earlier developmental stages (data not shown).

As previously reported [10], food deprivation for 2 days resulted in a significant increase in the *NPY* mRNA levels in the arcuate nucleus of the hypothalamus ($P < 0.01$; Figure 3A), whereas the level and the distribution pattern of *NPY* mRNA remained unchanged in the testes (Figure 3B, 4A, B). Experimental unilateral cryptorchidism for 3 days failed to regulate the *NPY* mRNA levels and the distribution pattern in comparison to both the contralateral testes of the cryptorchidism group and both testes in the sham-operation group (Figures 3C, Figure 4C, D). Hematoxylin-eosin staining revealed more atrophic germ cells and smaller tubes in the testes of the cryptorchidism group than in both the contralateral testes and the testes of the sham-operation group (data not shown).

4 Discussion

The present study provides evidence showing *NPY* transcripts to be exclusively distributed in the interstitial cells of the testes in mice. The levels of *NPY* mRNA increased significantly from neonatal through prepubertal, pubertal and postpubertal periods. As previously reported, food deprivation significantly upregulated the *NPY* gene expression in the arcuate nucleus of the hypothalamus,

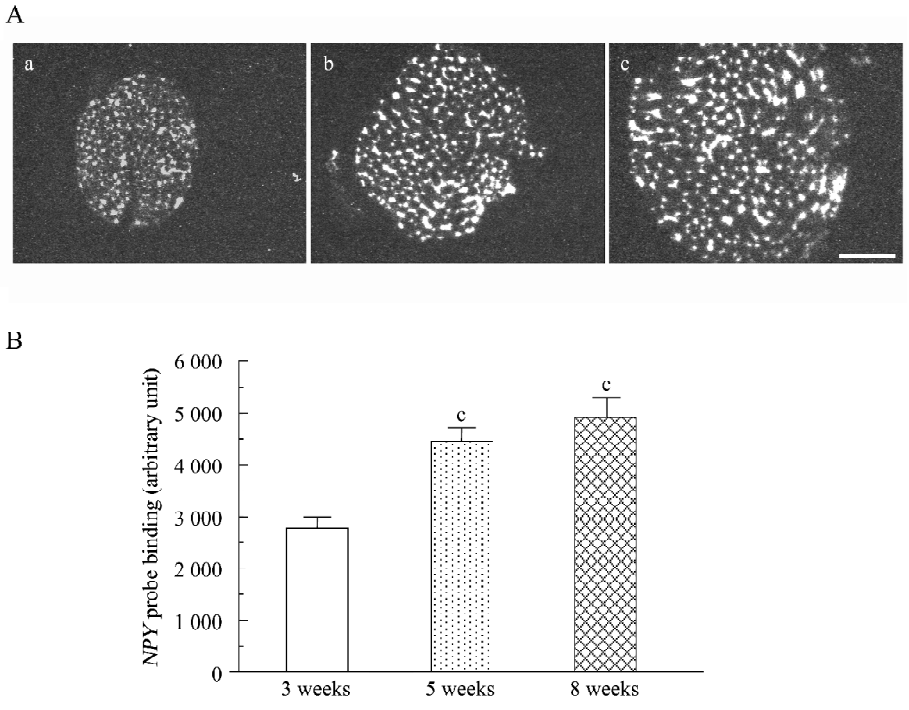

Figure 2. Representative autoradiographs (A) and developmental profile during peripubertal period (3, 5, 8 weeks old) (B) showing that Neuropeptide Y (*NPY*) gene expression in the testes of 3 (a), 5- (b) and 8-week-old mice (c). Sections were hybridized to a ^{35}S-labeled oligodeoxynucleotide probe complementary to mRNA for *NPY*. The scale bar represents 1 mm. Number of mice used was eight in each age group. Values represent the mean ± SE. $^{c}P < 0.01$ *vs.* 3-week-old mice.

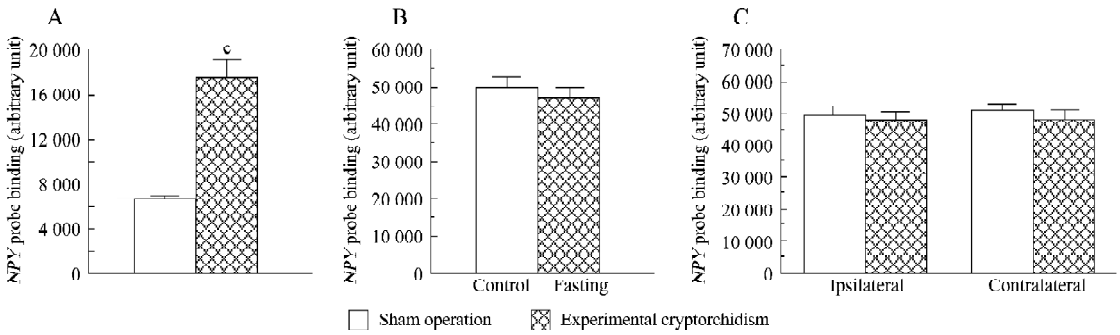

Figure 3. The effects of food deprivation for 48 h on Neuropeptide Y (*NPY*) transcripts prevalence in the arcuate nucleus of the hypothalamus (A) and the testes (B). The effects of experimental cryptorchidism for 3 days on *NPY* transcripts prevalence in ipsilateral and contralateral testes (C). Experimental cryptorchidism was performed in the left testis and the same number of mice had a sham-operation. Number of mice used was eight in each group. Values represent the mean ± SE. $^{c}P < 0.01$ *vs*. sham-operated mice.

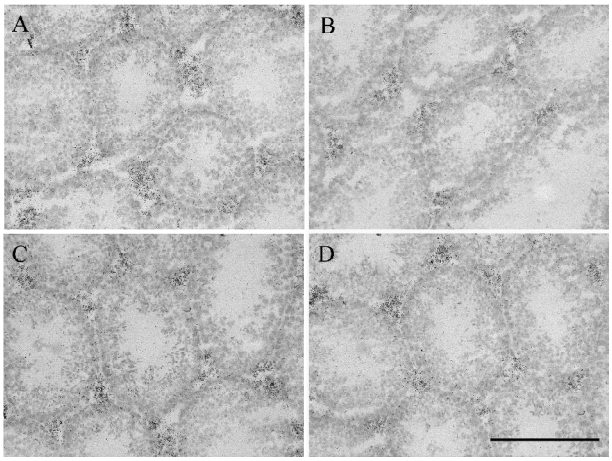

Figure 4. Representative photomicrographs of emulsion-dipped slides showing the Neuropeptide Y (*NPY*) transcripts in the mouse testes of food deprivation (B), the ipsilateral testes of experimental cryptochidism (D), and their respective controls (A and C). Sections were hybridized to a ^{35}S-labeled oligodeoxynucleotide probe complementary to mRNA for *NPY* and counter-stained using eosin. Scale bar represents 100 μm.

but not in the testes [7]. These findings suggested that the *NPY* gene expression might be distributed in the Leydig cells of the testes, and the regulation of testicular *NPY* gene expression might be associated with testicular development and that it might be distinctive from *NPY* gene expression in the hypothalamus.

Kanzaki *et al.* [6] demonstrated that *NPY* mRNA was predominantly expressed in the Leydig cells of rats using the reverse transcription polymerase chain reaction (RT-PCR) technique after separating the components of cell types in the testes. The present findings generally correlate with the findings of their study and further clarify the clear distribution and the regulation of testicular *NPY* gene expression *in situ*. However, there is some discrepancy between the study by Kanzaki *et al.* [6] and our present findings. In study of Kanzaki *et al.* [6], a weak *NPY* gene expression was detected in the isolated rat Sertoli cells. In the present study, almost no *NPY* transcripts were detected in the mouse seminiferous tubules. This discrepancy might be a result of differences in the methods used. It is possible that the RNA from contaminating Leydig cells might have been amplified by the highly sensitive RT-PCR method in the study of Kanzaki *et al.*[6]. Because *NPY* transcripts were clearly detectable with a high signal/noise ratio and no signals were obtained with the sense probe and the addition of a 100-fold excess of unlabeled probe, we believe that the distribution of *NPY* transcripts in our *in situ* hybridization are reliable. However, we cannot rule out the possibility that the discrepancy is a result of species differences. Jorgensen *et al.* [11] revealed using immunocytochemistry that *NPY*-immunoreactive nerves are detected in relation to blood vessels and seminiferous tubules in human prenatal and mature testes, whereas Wang *et al.* [12] revealed that *NPY* immunoreactivity is present in the interstitial cells in the testes of rodents.

The regulation of testicular *NPY* gene expression was different from that of hypothalamic *NPY* gene expression. A previous study reveals that the hypothalamic *NPY* levels are low at birth, but thereafter dramatically increase by postnatal day 16, and then subsequently decline to reach adult levels after weaning or by postnatal day 20 [13]. These findings contrast sharply with the developmental changes in the testicular *NPY* mRNA levels ob-

served in the present study. In addition, unlike hypothalamic *NPY*, the testicular *NPY* gene expression remained unchanged following fasting.

The regulatory mechanisms of hypothalamic NPY have been well characterized. Leptin has been shown to be the strongest regulator of fasting-induced *NPY* mRNA upregulation [14]. The decline of leptin levels during fasting is a signal to increase the expression of NPY in the hypothalamus [14]. Leptin-deficient mice, therefore, exhibit significantly elevated *NPY* mRNA levels [15]. The systemic administration of leptin can reverse the fasting, as well as leptin-gene deficient-induced upregulation of *NPY* mRNA in the hypothalamus [15]. Despite the fact that leptin receptor has been shown to be present in the Leydig cells of the testes in rodents [16], it is possible that the regulation of testicular *NPY* mRNA might be independent of the serum leptin levels. However, little information regarding the regulatory mechanisms of testicular *NPY* has been reported.

Based on the distribution of testicular *NPY*, it is possible that *NPY* might play a role in such Leydig cell functions as testosterone production and/or secretion as a local regulator. The present study demonstrates that the *NPY* gene expression significantly increases from the prepubertal up to the postpubertal period, and such an increase was associated with an elevation in the serum testosterone levels [17]. A previous report reveals that NPY might serve as a vasoconstrictor in the testis, probably by acting on the *NPY-Y1* receptors because the local injection of *NPY* causes a major decrease in blood flow in the injected testis [18]. Functional *NPY* receptor, Y1 receptor mRNA as well as protein was indeed found in the testes, particularly in the smooth muscles of the arterioles and small arteries [19]. These findings raise the speculation that testicular *NPY* might play a role in the maintenance of testicular function as a vasoconstrictor.

However, testicular *NPY* expression was independent of the changes in germ cells caused by experimental cryptochidism. A previous study showed that spermatogenesis started to be inhibited within 2 days after experimental cryptochidism [20]. Our study reveals that 3 days after experimental cryptochidism, germ cells became atrophic, whereas no changes in *NPY* gene expression and insterstital cell morphology were observed. It seems that the testicular *NPY* might not be directly involved in spermatogenesis. In particular, it might not have any direct effect on male germ cells.

Although general investigation demonstrates that conventional *NPY* knockout mice show fertile and no significant genotype differences of their testicular histology in comparison to wild-type mice [21], we cannot conclude that testicular *NPY* is unrelated to testicular development and maintenance because of possibilities that compensatory mechanism might exist from other genes. More precise studies focusing on testicular histology and function in *NPY* knockout mice might provide information regarding the role of *NPY* in testicular function.

In conclusion, the present study demonstrates that: (i) *NPY* transcripts were specifically expressed in Leydig cells in the testes; (ii) such expression was increased with the maturation and development of testes; and (iii) the expression was different from that of hypothalamic *NPY*, in the response against nutritional starvation.

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