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Modulation of the hypothalamopituitary axis in Mini rat: Further studies with hypophysiotropic somatostatin and gonadotroph cells.

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Abstract

In transgenic Mini rat, in which the expression of intrinsic growth hormone (GH) gene is suppressed by the antisense GH transgene are characterized by definite somatic growth retardation at puberty. Compared to age-matched wild-type Wister (non-Mini) rat, Mini rat showed low blood GH levels and GH cell depletion in the anterior pituitary (AP). The AP and periventricular hypothalamic nucleus (PeVN) were processed for immunohistochemical analysis with antisera to Luteinizing hormone (LH) and somatostatin (SOM), respectively. Although LH-immunoreactive (IR) cells showed the decrease in number in Mini rat from 4 to 8 weeks of age, there was the significant increase in non-Mini rat. Interestingly, SOM-IR cells in the PeVN showed the marked increase in number in Mini rat in contrast to the decrease in non-Mini rat during the same periods. The testis weights were not significantly different between Mini and non-Mini rat at 4 weeks of age, whereas at 6 and 8 weeks of age the value of Mini rat was significantly smaller than non-Mini rat. It was concluded that the onset and development of LH-testicular axis need intrinsic GH regulation, which is involved the SOM cell interaction from PeVN, and the SOM plays a crucial role in the development of pituitary-testicular maturation during the puberty.

Introduction

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The transgenic Mini rat is carrying an antisense RNA transgene which directly suppresses the transcription of intrinsic rat growth hormone (GH) gene. This antisense gene is mainly expressed in the anterior pituitary (AP) [1, 2] and indirectly suppresses the AP formation and GH cell number. Hence, Mini rat has mainly affected the development of the AP and the number of total GH cells during the puberty [3]. In addition, the changes of GH in the transgenic animal are reported to affect hypothalamic-pituitary-gonadal (HPG) axis which dues to the reduction of GH-releasing hormone (RH) and luteinizing hormone (LH) RH neurons in the arcuate nucleus and eventually the increase of somatostatin (SOM) neurons in the periventricular hypothalamic nucleus (PeVN) [4,5].

With respect to the SOM release pattern in sexual dimorphism was reported in which testicular development and production of sex steroids may have strongly affected hypothalamic SOM-immunoreactive (IR) cells in the PeVN [6]. Male rat and mice have higher SOM mRNA levels in the PeVN nucleus and higher SOM-IR in the median eminence, compared to female [7, 8, and 9]. That sexually dimorphic is associated with the onset of GH secretion pattern at the puberty. In this point, 40–70% of SOM neurons with androgen receptor (AR) in the PeVN decided the sex differences of the GH secretion in the male rat [10]. Such sex differences on HPG axis may depend on the exposure of sex steroids during the puberty. However, the exact role of the HP axis by the hypothalamic regulation of both LH and GH production remains uncertain.

Therefore, the purpose of this study was to investigate the effect of GH action on the alteration of gonadal development with respect to SOM-IR cells and LH-IR cells by applying immunohistochemical and morphometric methods of the hypothalamus and the AP.

Materials and Methods

Transgenic rat (ARGHGEN-1Nts, Mini rat) was donated by the Nippon Institute for Biological Science (NIBS) of Japan. Wild rat (Wistar rat, non-Mini rat) was used as a control. All methods were conducted according to the standards of humane animal care. Animals were housed in stable temperature and humidity conditions with lights on between 6 a.m. and 8 p.m. and food and water available ad libitum.

At 4, 6, and 8 weeks of age, male Mini rat (n = 5) and non-Mini rat (n = 5) were deeply anesthetized with sodium pentobarbital (40 mg/kg i.p.), weighed, and immediately decapitated. The AP, hypothalamus, and testis were fixed in 10% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h. These specimens were then dehydrated in a graded series of ethanol and xylene and embedded in paraffin. The tissues were cut into sequential 6 m-thick sections. These tissue sections were dewaxed in xylene and rehydrated in a graded series of ethanol solutions. Sequential sections of the AP and hypothalamus were processed for immunohistochemistry to investigate LH- and SOM-IR expression. For LH and SOM immunohistochemistry, sections were incubated in 10% normal goat serum in 0.02 M phosphate-buffered saline (PBS) with 0.2% Triton X-100 for 30 min at room temperature. Tissues were treated with 0.3% H_2O_2 dissolved in 0.02 M PBS to inactivate endogenous peroxidase activity. AP sections were incubated with anti-rabbit LH antibody (1:3000 dilutions, NIDDK) for 1 h at 35°C. Hypothalamus sections were incubated with anti-rabbit SOM antibody (1:2000, DAKO) overnight at 4°C. All treated sections were processed with peroxidase-labeled protein A (Organon Teknika, USA). Subsequently, all sections were treated with 0.006% diaminobenzidine and 0.003% hydrogen peroxide in 0.02 M PBS for 5–10 min at room temperature. For identification of the stages of spermatogenesis and acrosomal formation on the testis, glycosylated substrates on the testis were stained by periodic acid-Schiff (PAS) reaction.

A morphometric study for LH-IR cell was carried out according to our previous reports [3] Statistical analysis was performed using Sigma stat for Windows. The data of testis weight, GSI and LH-IR cell numbers were analyzed by two-way analysis of variance (ANOVA) and post hoc tests with Tukey-Kramer's test. Analyses were considered to be statistically significant at $P < 0.001$.

Results

The majority of LH-IR cells in the AP were localized in the specific region, named sex zone, near the pituitary stalk. These cells from both groups of animals were oval in shape with a slightly eccentrically situated nucleus (Fig1. 2A-F). It was of particular interest that the LH immunoreactivity in Mini rat was much stronger than that in non-Mini rat at 4 weeks of age (Figs. 1A, D). It seemed that the intensity of immunoreactivity in Mini rat decreased gradually with age during 4 to 8 weeks of age (Figs. 1A-C). The microphotographs of LH-IR cells at 8 weeks of age were compared at the same magnification (Figs. 1C and F). The number of these cells in the AP in Mini rat was apparently more than that in non-Mini rat, because the absolute size of the AP cells and the AP itself was small compared to non-Mini rat as shown in Table 1A.

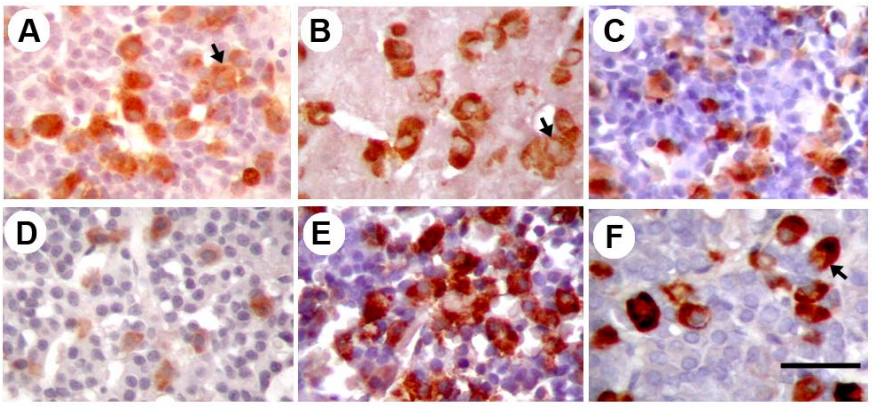

Figure 1. *Representative microphotographs of LH-IR cells in the AP of Mini and non-Mini rat. A-C are from Mini rat and D-F are from non-Mini rat at 4, 6 and 8 weeks of age, respectively. Note that the number of LH-IR cells was significantly increased in Mini rat at 4 weeks of age (A). Arrows indicate the appearance of* signet-ring type *of LH-IR cells. Scale bar = 30 µm.*

Table 1. *Mean ± SEM number of LH-IR cells (×10^5) and Mean ± SEM size of LH-IR cells in the AP of Mini and non-Mini rat at 4, 6 and 8 weeks of age were shown in A and B, respectively.*

(A) Mean ±SEM number of LH-IR cells (× 10^5) in the AP of Mini rat and control rats at four, six and eight weeks of age.

Ages (weeks)	Mini rats	non-Mini rat
4	2.28 ± 0.05 (n=5)	1.55 ± 0.03 (n=5)
6	1.71 ± 0.01 (n=5)	3.66 ± 0.01 (n=5)
8	1.47 ± 0.03 (n=5)	4.39 ± 0.11 (n=5)

The number in parenthesis shows a number of animals examined.

(B) Results of two-way ANOVA of the above data

	df	F
Group	1, 24	141.54*
Age	2, 24	27.58*
Group × Age	2, 24	88.22*

*ANOVA: analysis of variance, df; degree of freedom, F: F value P: * p < 0.001*

SOM-IR cells were exclusively confined to the PeVN along the third ventricle in Mini and non-Mini rat (Figs. 2A-F). These cells were round to oval in shape with centrally or slightly eccentrically situated nucleus. SOM-IR fibers were also found and sparsely distributed around cell bodies. It should be noted that the number of SOM-IR cells in the PeVN of Mini rat was much more increased during 4 to 8 weeks of age compared to age matched non-Mini rat (Figs. 2A-C).

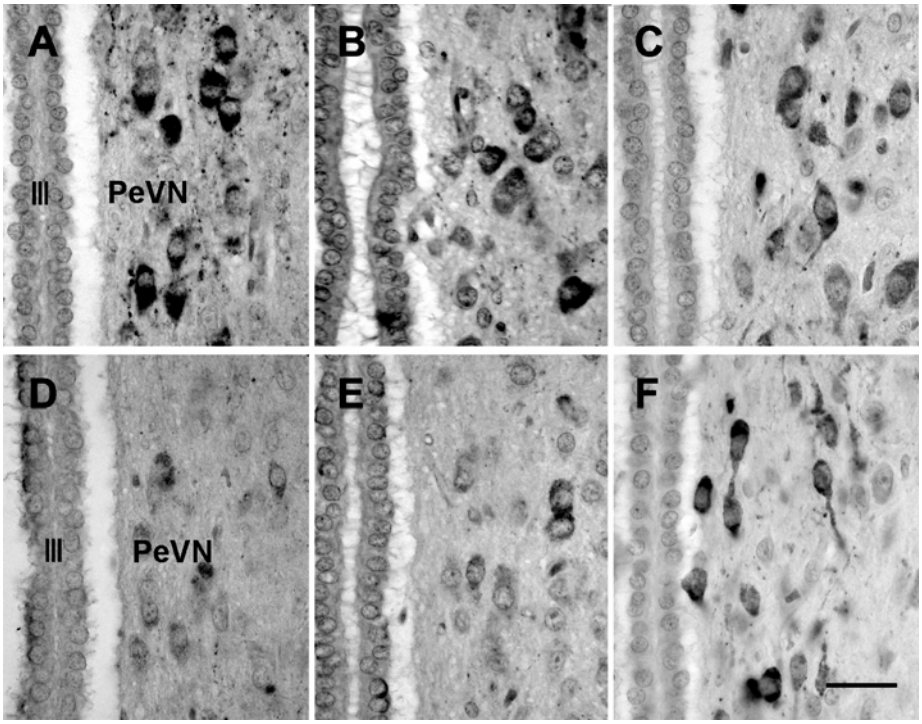

***Figure 2**. Representative microphotographs of SOM-IR cells in the PeVN of Mini and non-Mini rat. A-C are from Mini rat and D-F are from non-Mini rat at 4, 6 and 8 weeks of age, respectively. Note that SOM immunoreactivity in the PeVN of Mini rat was much stronger at 4, 6 and 8 weeks of age than non-Mini rat. PeVN; periventricular hypothalamic nucleus, III; 3rd ventricle. Scale bar = 30 m.*

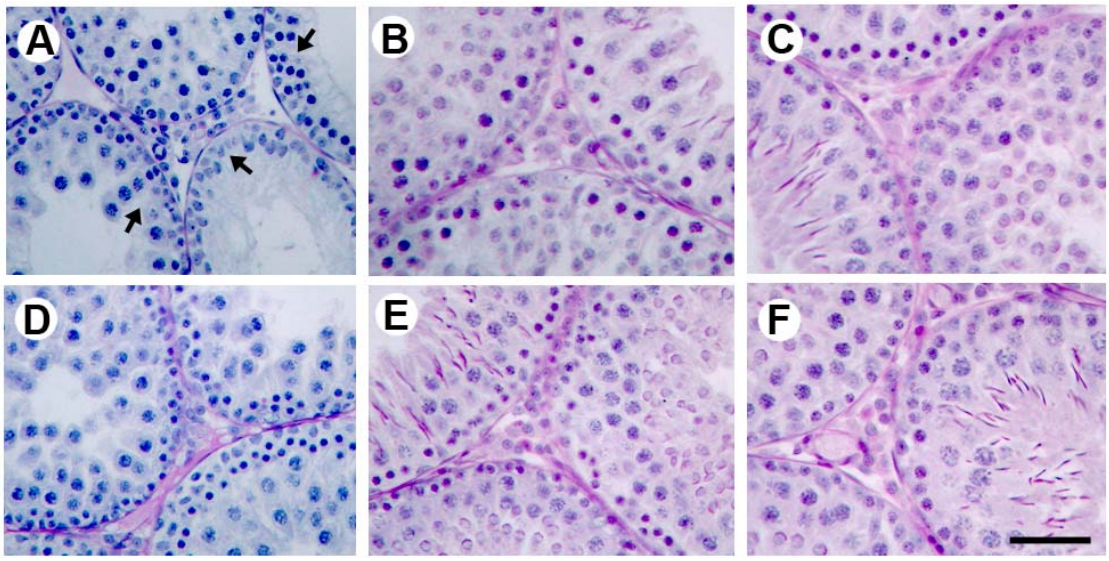

Figure 3. *Photographs of spermatogenesis in Mini (A-C) and non-Mini rats (D-F) showing morphological differences in each stage at four, six and eight weeks of age. Note that diminution of seminiferous epitherium height and reduction of number of spermatids (arrows) are found in Mini rat at four weeks of age. Scale bar = 50 m.*

Table 2. *(A) Age related changes in mean ± SEM body, testis weights and the GSI of Mini rats and control rats at 4, 6 and 8 weeks of age*

	Testis weight (mg)		**GSI**	
Age (weeks)	**Mini-rat** (n= 5)	**Non-Mini rat** (n= 5)	**Mini-rat** (n= 5)	**Non-Mini rat** (n= 5)
4	272±12	250±8	3.88±0.15	3.61±0.14
6	483±17	838±21	5.00±0.28	4.74±0.12
8	876±26	1,256±27	6.92±0.19	5.24±0.10

(B)Results of two-way ANOVA of testis weight

	df	**F**
Group	**1, 24**	**180.08***
Age	**2, 24**	**672.53 ***
Group × Age	**2, 24**	**55.68***

ANOVA: analysis of variance, df: degree of freedom, F: F value
*P: * p < 0.001*

The stage of spermatogenesis and acrosomal formation was indicated by PAS reaction with hematoxylin counter stain (Figs. 3A-F). Qualitative assessment revealed that the diminution of seminiferous epithelium height and reduction of the number of spermatids (arrows) are found in Mini rat at 4 weeks of age (Figs. 3A and D). Although there was no morphological disorder in both Mini and non-Mini rat at 6 to 8 weeks of age, spermatids and spermatozoa were detected to be elongated. Additionally in all stages of spermatogenesis, any gross disturbances in the spermatozoa were never observed during the same periods (Figs. 3B-C, 3E-F).

In a morphometric study and statistical analysis, the mean ± S.E.M. number of LH-IR cells in the AP was shown in Table 2A. Particularly, the number of LH-IR cells in Mini rat was about 2.28 ($\times 10^5$) at 4 weeks of age in contrast to 1.55 ($\times 10^5$) in non-Mini rat (Table 1A). Statistical analysis with 2-way ANOVA revealed a significant decrease from 4 to 8

weeks of age. However, LH-IR cell number in non-Mini rat showed a significant increase during the same periods (Table 1B). These values of LH-IR cells indicated significant differences between Mini and age-matched non-Mini rat at 4, 6 and 8 weeks of age.

(C) Results of two-way ANOVA of GSI

	df	**F**
Group	1, 24	26.44*
Age	2, 24	89.92*
Group × Age	2, 24	11.25*

ANOVA: analysis of variance, df: degree of freedom, F: F value
*P: * $p < 0.001$*

The mean ± SEM weight of testis and the Gonad-somatic index (GSI) is shown in A. The testis of Mini rat showed dwarfish phenotype at 4 to 8 weeks of age, and the body weight exhibited 1.8-fold growth rate compared to 3.5-fold in non-Mini rat. In addition, testis weights exhibited 3.2-fold growth rate compared to 5.0-fold in non-Mini rat. Statistical analysis with a two-way ANOVA revealed significant main effects of group, age, and group × age interaction in B and C. Post hoc analysis with Tukey-Kramer's test revealed that the GSI in Mini rat showed a significant and gradual decrease between 4 and 8 weeks of age. However, the GSI ratio indicated higher value in Mini rat at 8 weeks of age compared to age matched non-Mini rat in C.

Discussion

We examined age-related changes of LH-IR cells in the AP of Mini rat in which expression of the endogenous GH gene of the AP was disturbed by the carrying antisense GH gene. Despite only a moderate reduction of blood GH levels in Mini rat [1], the morphological alterations in LH-IR cells that we indicated in a previous study were much more severe than we expected. In fact, the AP volume and the number and size of the GH-IR cells in the Mini rat were significantly smaller than those in the non-Mini rat [3]. However, based on our data showing the number and size of the LH-IR cells in Mini rat were significantly higher than those in non-Mini rat at 4 weeks of age. This strongly suggests that LH-IR cell number and size and gonadotroph production may activate the function in Mini rat at puberty earlier than in non-Mini rat. We also reported the effects of neonatal monosodium L-glutamate (MSG) treatment on AP development in dwarf animals [4, 11]. However, MSG-treated animals had reduced numbers and sizes of LH cells in the AP compared to control animals [4, 12]. To our understanding, this is the first report that evaluates morphological and developmental changes in the LH-IR cells in the AP of Mini rat.

In an earlier study in transgenic mice, inserted human GH showed a remarkable increase in SOM level in the PeVN and an extreme decrease in AP volume [5]. This result also clearly shows that developmental modulation of the AP during puberty brings a functional disorder of SOM-IR cell distribution in the PeVN. A recent study reported colocalization with androgen receptors and SOM-IR cells in the PeVN, which suggests that SOM cells have male-specific function in the PeVN [13]. Indeed, castration of male rat decreases both hypothalamic SOM peptide and mRNA content [14], both of which can be restored by testosterone treatment [9, 15 and 16]. In support of this concept, we speculate that the androgen receptor of SOM in PeVN contributes to hypothalamo-pituitary axis maturation, which is synchronized with blood sex steroid levels from Leydig cells in the testis.

The disorder of testicular maturation indicated that Mini rat had reduced seminiferous tubule diameters and unsynchronized spermatogenesis at 4 weeks of age (Figs.3A). However, testicular weight and GSI results indicated higher levels in Mini rat than in non-Mini rat at 4 weeks of age (Table 1A). Most of the testis volume is governed by seminiferous tubules; therefore, reduced seminiferous tubule diameter is likely to be the cause of the reduced testis size in Mini rat during 6 to 8 weeks of age (Figs. 3B-C). Otherwise, histological observations did not reveal any spermatogenesis arrests that might explain the smaller diameter of the seminiferous tubules in Mini rat. Thus, either the number of spermatogonia or the rate of their division is disturbed carrying of the antisense GH gene or by a 60% reduction in GH level in the blood. The GH receptor gene is expressed in rat Leydig cells during puberty [17]. Isolated Leydig cells were shown in culture medium to promote testosterone expression after GH treatment [18]. These observations suggest that there could be important developmental interactions between GH- and LH-producing cells in the AP.

Furthermore, presence of the pituitary-testicular axis during postnatal life might stimulate the development of SOM-IR

neurons in PeVN, while the functional onset of the hypothalamic-pituitary axis during the neonatal period might use SOM-IR neurons in PeVN to modulate both cross talks. Elucidation of the key role of SOM release patterns from the median eminence via the PeVN may make it possible to find inhibitory mechanisms using the expression of steroid receptors in Mini rat.

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