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—Original—

Developmental Hormonal Profiles in *rdw* Rats with Congenital Hypothyroidism Accompanying Increased Testicular Size and Infertility in Adulthood

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Abstract. Congenital hypothyroid mutant male *rdw* rats have enlarged testes in adulthood with dwarfism accompanied by infertility. To explain how *rdw* rats acquire enlarged testes in adulthood, we compared age-matched normal (N) rats at various developmental stages for blood levels of hormones, thyroxine (T4), follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T), and investigated whether T4 therapy (*rdw*+T4) from 3 weeks of age (w) until adulthood could induce recovery of fertility in *rdw* rats, as well as how *rdw*+T4 affected hormonal patterns. Testes weights of *rdw* rats were higher than those of N rats at 19 w in adulthood though it was low during development. Serum T4 values in *rdw* rats were markedly lower than those in N rats but steadily increased up to 19 w. The serum FSH values in *rdw* rats were lower than those in N rats at all ages, and neither serum LH nor T value was significantly different at any age. The testes weight of *rdw*+T4 rats was significantly higher than that of N rats at 13 w with recovered growth, and was higher than that of *rdw* rats at 19 w. When they were mated with proestrous females after 16 w, all females became pregnant and gave birth to a normal number of pups. The T4 and FSH values of *rdw*+T4 rats were significantly higher than those in *rdw* rats, but similar to those in N rats in adulthood. The results suggest that even low levels of circulating thyroid hormone (TH) in *rdw* rats stimulate the development of their testes, probably through Sertoli cells, resulting in the enlarged adult testes without fertility, and that a sufficient circulating TH level from the immature stage plays a pivotal role in restoring mating activity, probably through FSH-mediated action towards adulthood.
Key words: Congenital hypothyroidism, Male *rdw* rats, Serum FSH, Testis enlargement, Infertility, Thyroxine therapy

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Apart from the well-established actions of gonadotropins, the role of the other hormones in the control of the development of male reproductive organs is not well understood [1]. Hypothyroidism is the most common disorder known to affect these organs, and has been shown to be associated with a wide range of reproductive abnormalities of ovarian function in mammals, including humans [2–6]. In males, as hypothyroidism has hardly any metabolic effect on the adult testis and causes minimum effects on testis morphology, the testis was long regarded as one of the least sensitive organs to thyroid hormone [7–9].

In contrast to the lack of effect on animals in the adult period, thyroid hormone seems to affect the rat testis in the development period. Increased testis weight occurs in adulthood accompanied by fertility and an increase of sperm number when neonatal transient hypothyroidism is induced in rats by anti-thyroid hormones such as 6-propyl-2-thiouracil (PTU) [10–12], or polychlorinated biphenyl (PCB), which is a typical environmental hormone [13]. Chronic hypothyroidism in rats given PTU for long periods after birth until the adult age causes atrophy of the testis and induces infertility [14]. Thus, the size or weight of the testis usually seems to correspond well with male sexual activity in neonatally transient or chronic hypothyroidism as well as in seasonal breeders such as rams [15] and minks [16].

It was observed serendipitously that in congenital hypothyroid mutant *rdw* rats, the actual testis weight was significantly increased but the rats remained infertile in adulthood, although the body weight of the mutants was half or less than that of their normal siblings after maturation [17]. This observation contradicted that of previous reports on transient hypothyroidism [10–12] and chronic hypothyroidism in rats [14].

Adult *rdw* rats of an infertile mutant were reported to have impaired sexual behavior and functions of the epididymis and testes [18]. In that report, when *rdw* rats were treated with thyroxine (T4) for a long time starting from the adult age, the sexual behavior and conception rate only partially recovered although the rate of *in vitro* fertilization recovered dramatically when spermatozoa derived from T4-treated *rdw* rats were used [18]. In transient hypothyroid rats, the circulating levels of hormones involved in reproduction were presented as a function of developmental stage in an earlier study [19], however, in male *rdw* rats, the blood hormone levels as a function of developing stage have not yet been reported.

In order to explain how congenital hypothyroid *rdw* rats acquire enlarged testes in adulthood in spite of their retarded growth and infertility, the present experiments were designed to examine: 1) the blood levels of hormones involved in reproduction in *rdw* and age-matched normal (N) rats at various developmental stages from immaturity through adulthood; 2) whether thyroid hormone therapy from an immature age (3 weeks of age) until adulthood could induce recovery of fertility; and 3) how such thyroid hormone therapy affects hormonal patterns.

Materials and Methods

Animals, blood sampling and organ collection

rdw rats and normal littermates or age-matched normal (N) rats derived from the Wistar-Imamichi strain were produced by mating adult F1 males and females as previously reported [17, 20–22]. The mutants were identified based on low body weight and retarded development of the ears at about 2 weeks of age (w). At 6 w, the mutant and N rats were weaned, and the animals were subjected to the following experiments before or after weaning as indicated.

The present study was approved by the Ethics Committee for Care and Use of Laboratory Animals for Biomedical Research of the Graduate School of Agriculture Science, Tohoku University and Utsunomiya University.

Thyroxine treatment

Thyroxine (L-Thyroxine, T4; Sigma Chemical Co., St. Louis, Mo.) was dissolved in 2 N NaOH and diluted in saline solution (final pH 8.0 to 8.3) to 20 $\mu g\ ml^{-1}$. *rdw* rats were treated daily from 3 to 6 w by abdominal injection of T4 solution at a dose of 10 μg per 100 g body weight, and were given T4 after 6 w ad libitum in tap drinking water supplemented with T4 solution at a dose of 20 μg per 100 g body weight.

Mating and fertility tests

Five male *rdw* rats or 6 *rdw* rats treated with T4 (*rdw*+T4) from 16 to 19 w were subjected to mating

and fertility tests as described in earlier reports [18]. Mature virgin female rats with normal estrous cycles were used as mating partners. In the evening, each male rat was paired with a female in proestrus and the two were kept together overnight. The females were checked for sperm by smear examination the next morning and kept for more than 3 weeks for examination of pregnancy and parturition.

Blood sampling and testes collection and hormone analysis

Animals were sacrificed for the collection of blood and excision of testes for subsequent examination. *rdw* and N rats (5–6 animals per group) were sacrificed at 2, 4, 7, 10, 13, 16, 19 and 31~45 (>30) w and *rdw*+T4 rats (5–6 animals per group) were sacrificed at 4, 7, 13 and 19 w in the afternoon under anesthesia with a combination of ketamine (100 mg per kg of body weight) and xylazine (5 mg per kg). Serum was immediately separated by centrifugation and stored in a freezer until use. The left testis was put into neutralized formalin solution for fixation.

Histology of rat testis

Typical testes from the left side at 19 w of *rdw*, N and *rdw*+T4 rats were chosen, fixed in neutralized formalin solution, subjected to paraffin embedding, cut into 7-μm sections, and stained with haematoxylin-eosin. The sections of the testis were microscopically compared among the 3 groups with respect to spermatogenesis in the seminiferous tubules.

Measurement of serum hormones

The concentration of total serum T4 (ng ml^{-1}) was measured with a solid-phase radioimmunoassay method using a commercially available kit (T4-RIABEAD); Dainabot Co., Ltd. Tokyo, Japan. Intra- and interassay coefficients of variation were 2.5 and 4.0%, respectively. The sensitivity was 0.2 μg per 100 ml. Cross-reactivities with L-triiodothyronine and diiodothyronine were 0.6 and 0.1%, respectively. The concentrations of serum FSH and LH (ng ml^{-1}) were measured using double-antibody radioimmunoassay kits obtained from Amersham Life Science Ltd (Amersham, Bucks). Intra- and interassay coefficients of variation were 4.2 and 11.1% for FSH and 6.5 and 10.9% for LH, respectively. Testosterone was measured by an enzyme- immunoassay in microtitre plates based on the double antibody technique. This procedure was originally developed for a progesterone assay [23] and was adopted for the testosterone assay by using testosterone– 3-(O-carboxymethyl) oxime as the tracer and using testosterone-1α-hemisuccinated-BSA as the antigen for production of antibody. The standard curve was derived for 0.4 to 100 ng testosterone ml^{-1} and the ED50 of the assay was 10 ng ml^{-1}. The intra- and interassay variations were less than 5% and 10%, respectively.

Statistical analysis

All of data were expressed as mean ± SEM. Statistical analyses of differences between *rdw* and N rats and among *rdw*, N and *rdw* + T4 rats were performed using Student's t test and one way-ANOVA, respectively. Differences were considered significant at $P<0.05$.

Results

Body and testicular weight

Body weight was already lower in *rdw* rats than in N rats at 2 w and a significant difference of body weight was maintained until 19 w and later. The body weight of *rdw* rats was less than half of that of N rats after 7 w (Fig. 1). The testes weight of *rdw* rats was significantly lower than that of N rats at 2–13 w. The testicular weight of *rdw* rats increased at 10 w, caught up with that of N rats at 16 w and was significantly higher than that of N rats at 19 w (3.9 ± 0.1 g vs. 2.9 ± 0.1 g; *rdw* vs. N) The testicular weight became smaller and not significant with that of N rats after >30 w (3.3 ± 0.2 g vs.3.4 ± 0.4 g; *rdw* vs. N) (Fig. 1)

Sexual and mating behavior

No sexual behavior or interest in proestrous females was observed in *rdw* rats (0/5), in contrast with N rats, which displayed full sexual activity before 19 w, with vaginal sperm in paired females the next morning (5/5).

Measurement of serum hormones

T4 levels in *rdw* rats were markedly lower than those in N rats at all examined ages ($p<0.01$). The levels were lowest at 2 w, and slightly but significantly increased from 2 to 19 w ($p<0.01$: 2w vs. 4w; 4w vs. 7w; 7w vs. 16w; 7w vs. 19w). The

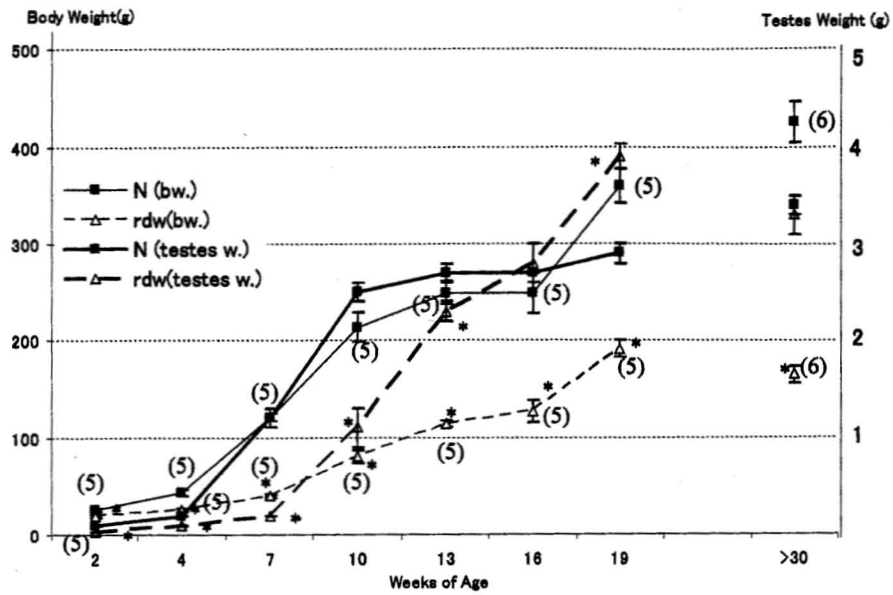

Fig.1. Mean ± SEM values of body weights (g) of *rdw* (dotted thin line with triangle) and normal (N) (solid thin line with square) rats and of testes weight (g) of *rdw* (dotted bold line with triangle) and N (solid bold line with square) rats at 2, 4, 7, 10, 13, 16, 19 and >30 weeks of age. Numbers in the parenthesis show the number of rats used in each group. * significant difference in *rdw* vs. N ($P<0.01$).

Table 1. Levels of serum thyroxine (T4), follicle stimulating hormone (FSH) and luteinizing hormone (LH) in normal (N) and *rdw* rats

Hormone (ng/ml)	Weeks of Age	2	4	7	10	13	16	19	>30
T4	*rdw*	10.5 ± 0.5[b] (5)[d]	13.3 ± 2.0[b] (5)	22.0 ± 4.2[b] (5)	23.5 ± 0.5[b] (5)	22.5 ± 0.5[b] (5)	37.0 ± 1.9[b] (5)	33.8 ± 1.2[b] (5)	≤10.0[b] (6)
	N	48.8 ± 2.0[c] (5)	47.3 ± 2.0[c] (5)	68.2 ± 3.7[c] (5)	51.9 ± 3.7[c] (5)	80.0 ± 12.0[c] (5)	57.0 ± 3.8[c] (5)	78.0 ± 2.2[c] (5)	40.0 ± 2.9[c] (6)
FSH	*rdw*	12.7 ± 1.6[b]	13.3 ± 0.8[b]	18.6 ± 5.5[b]	14.9 ± 1.4[b]	13.3 ± 0.6[b]	16.2 ± 0.5[b]	15.6 ± 0.5[b]	18.9 ± 1.1[b]
	N	13.2 ± 1.0[b]	15.7 ± 1.8[b]	35.8 ± 0.9[c]	33.2 ± 7.4[b]	22.9 ± 1.7[c]	30.5 ± 1.7[c]	28.3 ± 1.0[c]	42.0 ± 4.1[c]
LH	*rdw*	2.1 ± 0.5[b]	1.8 ± 0.2[b]	1.8 ± 0.2[b]	1.5 ± 0.1[b]	1.2 ± 0.3[b]	2.2 ± 0.3[b]	2.9 ± 0.9[b]	2.6 ± 0.6[b]
	N	2.8 ± 0.8[b]	1.6 ± 0.3[b]	1.5 ± 0.2[b]	1.6 ± 0.3[b]	1.8 ± 0.1[b]	1.5 ± 0.1[b]	2.0 ± 0.2[b]	2.2 ± 0.1[b]
Testos-terone	*rdw*	0.03 ± 0.02[b]	0.1 ± 0.1[b]	0.5 ± 0.1[b]	0.8 ± 0.4[b]	1.7 ± 0.5[b]	2.5 ± 0.5[b]	2.1 ± 0.5[b]	0.9 ± 0.3[b]
	N	0.01 ± 0.01[b]	0.1 ± 0.0[b]	1.3 ± 0.7[b]	0.9 ± 0.2[b]	2.3 ± 0.3[b]	2.3 ± 0.6[b]	1.8 ± 0.3[b]	1.7 ± 0.4[b]

[a]Data are expressed as mean ± SEM.

[b-c]Values with different superscripts for *rdw* and N are significantly different ($P<0.01$).

[d]Values in parentheses shows numbers of rats.

levels decreased to minimal values again after >30 w (ns: 2w vs.>30w) (Table 1). The serum levels of FSH in N rats were minimal at 2 w and 4 w, increased to peak values at 7 w, and then stayed at a constant level until 19 w and increased at >30 w, while the levels in *rdw* rats were maintained at basal values from 2 w to 19 w and at >30 w and were significantly lower than those in N rats at 7 w, 13–19 w and >30 w ($p<0.05$). The serum levels of LH were not significantly different between *rdw* and N rats from 2 w to 19 w or at >30 w. The serum testosterone levels in both *rdw* and N rats were minimal at 2 w and 4 w, increased in parallel from 7 w in both groups and were maximal at 13 w in N rats and 16 w in *rdw* rats. There was no significant between the levels in the two groups from 2 to 19 w and at >30 w (Table 1).

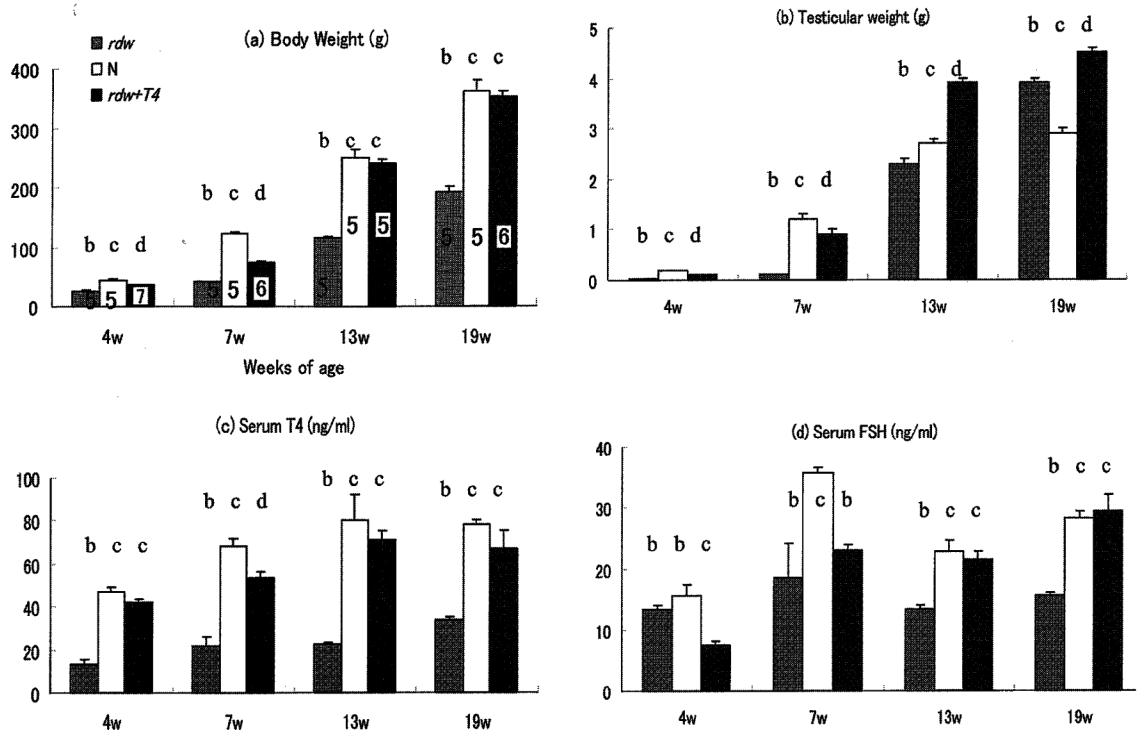

Fig.2. Mean ± SEM values of weights (g) of (a) body and (b) testes at 4, 7, and 13 and 19 weeks of age (w) in *rdw* rats (gray column), normal (N) rats without any treatment (white column) and rats with T4 therapy from 3 w (*rdw* +T4) (black column) and levels of serum (c) thyroxine (T4) and (d) follicular stimulating hormone (FSH) at 4, 7, and 13 and 19 weeks of age (w) in *rdw* rats (gray column), normal (N) rats without any treatment (white column) and rats with T4 therapy from 3 w (*rdw* +T4) (black column).
b-d: Values with different superscripts in *rdw*, N and *rdw*+T4 are significantly different ($p<0.01$). Numbers in columns show the number of rats used in each group.

Thyroid hormone therapy

Body and testicular weight: T4 treatment of *rdw* rats caused remarkable recovery of body growth to almost normal levels after 13 w. The testes weight of *rdw* rats treated with T4 was significantly increased over that of N rats after 13 w and over that of *rdw* rats with enlarged testes at 19 w (Fig. 2).

Male fertility: Sexual behavior, including interest in proestrous females, was observed in all rats and all rats exhibited full sexual activity before 19 w, with vaginal sperm in paired females the next morning (6/6). All paired female rats were confirmed as pregnant and bore a normal number of pups (14.5 ± 0.5).

Hormone levels: Serum T4 levels of T4 treated *rdw* rats were restored to normal euthyroid levels after 4 w except at 7 w (Fig. 2). Serum FSH levels were restored by T4 treatment in *rdw* rats to normal levels after 13 w (Fig. 2).

Testicular histology: The weight of the left testis (body weight) was 2.0 g (126 g), 1.8 g (450 g) and 2.3 g (380 g) in *rdw*, N and *rdw* + T4 rats, respectively when the rats were autopsied at 19 w. The microscopic appearance (× 200) of the size of seminiferous tubules in sections differed among the 3 kinds of samples and reflected the weight of testis, indicating that the tubule size of *rdw* and of *rdw*+T4 rats was larger than that of N rats (Fig. 3).

Discussion

The growth of *rdw* rats was clearly suppressed throughout the lifetime of the rats. The depressed

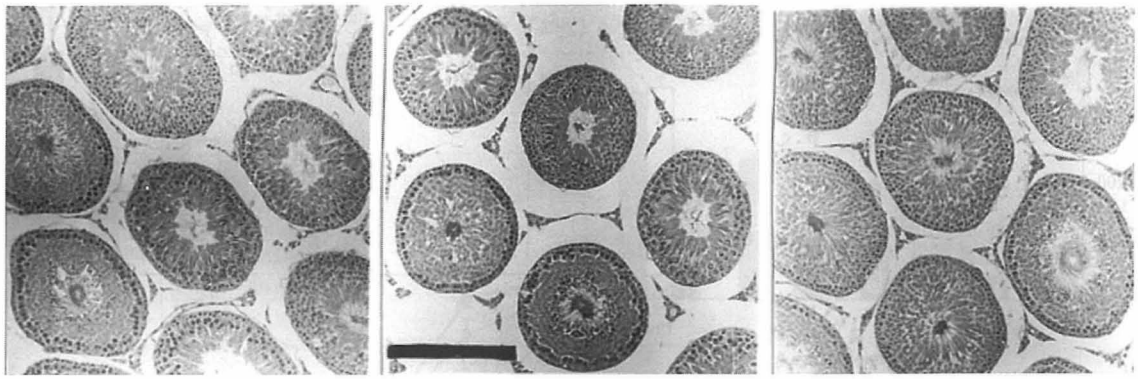

Fig.3. Appearances (× 200) of seminiferous tubules in testis chosen typically in (a) *rdw*, (b) normal (N) and (c) *rdw*+T4 rats at 19 weeks of age. Weight of a testis was 2.0 g, 1.8 g and 2.3 g in *rdw*, N and *rdw* + T4 rat, respectively when autopsied. Each testis was fixed in neutralized formalin solution, subjected to stain with haematoxylin-eosin. The diameter of seminiferous tubules reflected the weight of testis indicating that the size of *rdw* and of *rdw*+T4 rats is larger than that of N rats. A black bar shows a scale of 100 nm.

growth rate of these congenital hypothyroid rodents corresponded well with the findings of earlier reports [24, 25]. The repression of growth in hypothyroid rats has been shown to be due to a deficiency of growth hormone (GH) in the circulation from the infantile to juvenile stages and in adulthood [19, 20, 22]. The testicular weight of the *rdw* rats was clearly lower than that of N rats at postnatal 12–13 w, but the testicular weight increased rapidly within several weeks afterwards until it surpassed that of N rats in spite of body growth retardation. The *rdw* rats were infertile and failed to show sexual behavior with respect to the proestrous females even at an advanced age. These observations were in accord with our previous findings on testicular size in *rdw* rats [17, 18]. With respect to testicular histology, the size of seminiferous tubules in the sections of *rdw* rats was larger than that of N rats. Therefore, the enlargement of the testis in *rdw* rats may be caused by the increase of the volume of seminiferous tubules and an increase in germ cell numbers in a testis [26]. In a previous report, we showed that the function of spermatozoa in *rdw* rats is subnormal, as the rate of occurrence of cytoplasmic droplets from caudal epididymal spermatozoa is more than 10 folds higher in *rdw* than in N rats [18].

Mating behavior was lacking in *rdw* rats at 19 w, when N rats already showed full mating activity. The lack of sexual behavior may have been due to weaker sperm quality, since spermatozoa from adult *rdw* rats lack *in vitro* fertilization ability [18].

Serum testosterone levels were not different between *rdw* and N rats, although the values were sometimes variable. It was in agreement with an earlier report showing that transient neonatal hypothyroidism induced testis enlargement in rats at the adult stage in an euthyroid phase after cessation of PTU treatment and that the testosterone levels were not different from that in the euthyroid phase [12]. Transient PTU treatment resulted in a 70% increase in the adult Leydig cell number, but these Leydig cells had decreased volume, LH receptor number, and steroidal potential *in vitro* [27]. The present results on circulating testosterone levels suggest that a similar mechanism may exist in chronic hypothyroid *rdw* rats. Serum LH levels were not different between chronic hypothyroid and euthyroid rats throughout life, and the patterns of LH levels were different from those of rats in which neonatal transient hypothyroidism were induced, in which LH levels were lowered throughout life even after recovery from the PTU-induced hypothyroid state [19].

The same study demonstrated that serum levels

of pituitary hormones such as GH and prolactin were reduced and that those of thyroid stimulating hormone (TSH) were increased during neonatal hypothyroidism, but that they recovered to euthyroid ones after PTU treatment was stopped [19]. The discrepancy that serum LH was not suppressed in the hypothyroid state is characteristic of *rdw* rats. The FSH levels of *rdw* rats were markedly lower than those of N rats at 7 w and later. Earlier reports showed that FSH levels reduce during the immature stages in chemically induced hypothyroid rats [19, 28, 29], and that FSH levels in transient hypothyroid rats are permanently reduced through even much later euthyroid periods of life [19]. The duration of the hypothyroid state resulting from transient hypothyroidism induced by PTU was almost the same as that caused by congenital hypothyroidism in *rdw* rats, i.e. about 20 days during the postnatal period [10]. Therefore, neonatal hypothyroidism for about 3 weeks may affect FSH secretion from the pituitary gland throughout life, and an especially significant reduction of FSH levels was apparent after 7 weeks of age in *rdw* rats. This corresponds well with the finding of a previous report in which hypothyroidism was induced in rats approaching puberty [19, 28] and suggests that thyroid hormone deficiency may seriously affect FSH secretion in male rats [30, 31]. The depressed serum level of FSH may be the cause of delayed maturation of Sertoli cells in hypothyroid rats [28, 32, 33]. Serum T4 levels were remarkably lower in *rdw* rats than in N rats at all ages. However, the levels were slightly increased even in *rdw* rats to detectable values from 4 to 19 weeks of age. This means that *rdw* rats are hypothyroid but not in the phase of deprivation. Chronic hypothyroidism caused by PTU treatment from birth to 90 days was reported to severely suppress testicular growth [14]. In transient neonatal PTU-treated rats, serum T4 levels increase from 30 days of age until normal levels are reached after about 20 days [19] and this elevated T4 may accelerate the increase of Sertoli cell number [27, 34]. Even in congenitally hypothyroid *rdw* rats, the depressed levels of secreted T4 may be sufficient to gradually stimulate Sertoli cells, resulting in increased testicular size. Testicular weight decreased again along with a decrease of serum T4 to non-detectable levels in older adult *rdw* rats after >30 w. This may indicate a correlation of testicular weight increase with the level of secretion of thyroid hormone.

We found that long-term T4 therapy of *rdw* rats resulted in remarkable recovery of full mating capacity with normal growth. The present histological observations revealed that T4 therapy also increased the size of the seminiferous tubules as well as in untreated *rdw* rats. The increase of tubular size by T4 therapy in *rdw* rats may cause testicular enlargement accompanied with normal sexual activity like the transient hypothyroid rat [11, 12, 26]. Long-term T4 treatment from the adult stage caused only partial recovery of mating behavior in *rdw* male rats *in vivo*, although the epididymal spermatozoa succeeded in *in vitro* fertilization. However, T4 treatment of *rdw* rats from an immature stage was found to be effective for inducing recovery of *in vivo* sexual activity [18, 35, 36].

Serum FSH levels were restored to euthyroid levels after maturation of hypothyroid *rdw* rats when long-term T4 therapy was applied. It is possible that the long-term action of thyroid hormone is mediated via FSH secretion from the pituitary gland, resulting in the normalization of the testicular function of hypothyroid *rdw* rats. It has been shown that FSH receptors exist only in the testis, mainly in Sertoli cells, in male animals [37] and that increased endogenous secretion of FSH during development can also stimulate testicular function in rats in adulthood [38]. Thus, the circulating FSH level plays a pivotal role in the normalization of testicular function by thyroid hormone in hypothyroid *rdw* rats.

The long-term T4 treatment in the present experiments did not reduce the testis weight to a normal level. On the contrary, the testis weight of T4-treated *rdw* rats was significantly heavier than the testis weight of non-treated ones. The T4 treatment for *rdw* rats started from 3 weeks of age. Transient hypothyroidism was induced in neonatal rats by PTU treatment until 25 days and stopped therafter [10]. In *rdw* rats treated with T4, the change of hormonal status from a hypothyroid to a euthyroid state is similar to that which occurs in the recovery from transient hypothyroidism. Indeed, the serum T4 levels of *rdw* rats with long- term treatment were not different from those of rats in the euthyroid state. Neonatal hypothyroidism that occurs naturally or is caused by PTU treatment is known to result in an increase in testis size in later adult life. PTU treatment starting 8 days after birth

or later did not increase testis weight regardless of duration of the treatment [39]. Meanwhile, another report showed that PTU treatment starting 2 weeks after birth or later did not increase the weight of the testis [40]. Thus, the critical period of the regulation of normal testicular development by thyroid hormone may be earlier than 3 weeks of age in *rdw* rats. Recently, a missense mutation in thyroglobulin which causes hypothyroidism was found in *rdw* rats [41, 42]. The authors of the former report developed a method for genotyping thyroid gland DNA of *rdw*, heterogenous and wild-type rats by PCR-RFLP using a restriction enzyme, Mbo 1 [41]. By applying this method to neonatal rats, it was possible to select homozygous *rdw* rats intact at birth [43]. When T4 treatment of these rats was started from 2 days after birth and continued until 19 w at autopsy, the testicular weight was observed to be normal without testicular enlargement (Umezu unpublished data).

In conclusion, our findings suggest that: 1) even low levels of secreted circulating thyroid hormones can stimulate testicular development in congenital hypothyroid *rdw* rats in adulthood without restoring fertility; 2) circulating LH levels are not changed in the hypothyroid phase in *rdw* rats; 3) circulating FSH levels play pivotal roles in the normal development of the rat testis by mediating thyroid hormone effects, probably on Sertoli cells; 4) T4 therapy from 3 w can restore mating activity of male *rdw* rats, and 5) the greatest enlargement of testis size in *rdw* rats was obtained in adulthood, indicating that the critical period during which thyroid hormone acts to regulate testis size may be sometime before 3 weeks of age.

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