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Differential Effects of Two Alleles of the *dy* Locus on the Pituitary-Testicular Axis of Mice

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Testicular function was studied *in vivo* and *in vitro* in adult male *dy/dy* and dy^{2J}/dy^{2J} dystrophic mice. The results demonstrate that testicular function in *dy/dy* mice is more affected. The basal levels of pituitary hormones measured were normal in dystrophic mice, except for the presence of hyperprolactinemia in *dy/dy* mice. In *dy/dy* mice testicular weight was diminished and a deficient transduction of the gonadotropic signal is present *in vivo*, accompanied by reduced efficiency of 17-hydroxylase and 17-hydroxysteroid dehydrogenase. In dy^{2J}/dy^{2J} mice the signal transduction is normal and the reduction in enzyme efficiency is limited to 17-hydroxysteroid dehydrogenase. The *in vitro* HCG-induced increases in production of testosterone (T) and estradiol (E_2) were reduced in *dy/dy*/mice, and the data indicate a reduction of enzyme activity rather than in efficiency. In dy^{2J}/dy^{2J}/mice, HCG-induced T synthesis was increased, HCG-induced E_2 synthesis was normal, but basal media E_2 levels were reduced, with the *in vitro* efficiency of aromatase being suppressed under both basal and HCG-stimulated conditions, when compared to their normal littermates.

Key words: Muscular dystrophy, Testes, Testosterone, Progesterone, Estradiol, Hydroxyprogesterone, LH receptors.

A seldom studied aspect of muscular dystrophies is the occurrence of endocrine alterations in patients with these diseases. Changes in the circulating levels of LH, FSH and prolactin have been observed (3, 7, 20, 22, 29, 31-33, 36, 39, 45, 55), as well as in the function of gonadotrophs, lactotrophs and somatotrophs (51). Alterations in thyroid function have also been reported in dystrophic patients (21, 48,

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49). Moreover, these patients present with clinical diabetes, reduced adrenal androgen production and hypogonadism that is characterized by low steroidogenic response to HCG, vacuolization of Sertoli cells and altered spermatogenesis (4, 9-11, 16, 17, 29, 31, 36, 45, 55, 56). ULLOA-AGUIRRE *et al.* (52) proposed that gonadal failure in patients with muscular dystrophies was of hypothalamic origin. Using animal models, GnRH-stimulated gonadotropin release was studied *in vitro*, it was found to be altered in both *dy/dy* and dy^{2J}/dy^{2J}/mice (58), thus supporting the afore mentioned hypothesis, as does recent data in humans (31). MAHLER and PARIZEL (29) proposed that the presence of hyperprolactinemia might be in part responsible for the gonadal failure seen in many patients with muscular dystrophies.

The present study is an attempt to determine if the alterations in the hypothalamic-pituitary-testicular axis, observed in *dy/dy*/and dy^{2J}/dy^{2J}/mice, are due solely to a defect in the hypothalamus, or if additional alterations are present in the pituitary-testicular portion of the axis.

Materials and Methods

Adult (> 3 month old) dystrophic mice, homozygous for two different recessive mutant alleles of the *dy* locus (*dy/dy* and dy^{2J}/dy^{2J}), were obtained from The Jackson Laboratory. Normal heterozygous mice (*Dy/dy* and Dy/dy^{2J}) were used as controls. Both types of mutant mice are maintained on sub-strains of similar genomic background (C57BL/6J-dy and C57BL/6J-dy^{2J}). All animals were kept under controlled temperature (22 ± 2 °C) and lighting conditions (12 h light/24 h). They had free access to commercial food and tap water.

Blood samples were obtained by cardiac puncture within one minute after induction of ether anesthesia, and mice were sacrificed by cervical dislocation. Testes were removed, decapsulated, and each cut into two fragments of similar weight. Plasma was stored frozen at − 20 °C until assayed for hormone levels. Two hemitestes were rapidly frozen in a solid CO_2: acetone mixture, and kept frozen at −70 °C until assayed for testicular LH receptors (LH-R) and steroid levels. The two other hemitestes were incubated in Krebs-Ringer bicarbonate buffer (glucose = 1 mg/ml) with 0 or 12.5 mIU HCG/ml, for 4 h at 32 ± 1 °C, according to previously described protocols (18, 54, 59). Media was stored frozen at −20 °C until assayed for steroid levels.

Testicular LH-R levels were measured using radioreceptorassay (RRA) procedures described previously (1, 25). The ^{125}I-HCG (CR-121, NIH) used in these studies had a maximum binding ability of 53.4 %, and a specific activity of 6.52 μCi/μg. The concentration of protein in testicular membrane preparations used for determination of LH-R was measured by a modification of Lowry's method (32), using bovine serum albumin as the standard.

Plasma LH, FSH and prolactin levels were measured by RIA as previously described (12, 43, 44). The levels of plasma, testicular and incubation media testosterone (T) levels were determined using RIA procedures described before (1, 59). The measurements of testicular and incubation media progesterone (P_4) and 17-hydroxyprogesterone (OHP) levels, as well as those of incubation media estradiol (E_2) levels, were done using solid-phase RIA procedures. Since these kits use standard curves based on human serum, parallelism between standard curves for each hormone and curves made with pooled aliquots from each type of sample analyzed was determined (table I).

Data from the RRA and RIA were obtained using the RRAPLOT, RRADOSE, RIAPLOT and RIADOSE programs (2). Data were evaluated by either Student's t-test or two-way analysis of variance (AN-

(ANOVA) using the SPSS-X software on an IMB mainframe (40-42). For the ANOVA, data were tested for normality of distribution by the Kolmogorov-Smirnov test and for homogeneity of variance by Barlett's test, and log- or square-root transformed as needed (42, 47).

Results

It should be noted that the locomotive impairment is not similar in both types of dystrophic mice. When not prodded to walk fast, dy^{2J}/dy^{2J} mice would move normally most of the time, whereas *dy/dy* mice stumbled constantly even when moving slowly. This difference in the severity of muscular dysfunction between both types of mice resembles the difference observed between patients with Duchenne and Becker dystrophies.

Both types of dystrophic mice had significantly lower body weight than their controls (fig. 1). However, in *dy/dy* mice the decrease was dramatic. Also, absolute testicular weight was decreased in *dy/dy* but not in dy^{2J}/dy^{2J} mice (fig. 1; whenever possible, non-significant data is not shown in figures). Due to the small size of *dy/dy* mice, it was not possible to measure all hormones in the plasma obtained from them, therefore reliable statistics were only obtained for prolactin levels. A dramatic increase in prolactin was measured in *dy/dy* mice, whereas of the samples measured, one had elevated gonadotropins and the other had normal levels (fig. 1). In dy^{2J}/dy^{2J} mice, where all three hormones were measured, no significant differences in the levels of LH, FSH or prolactin were detected in comparison to Dy/dy^{2J} mice (data not shown). Although testicular T levels were decreased in both *dy/dy* and dy^{2J}/dy^{2J} mice (fig. 3), only in the latter were the circulating levels lower than in normal mice (fig. 2). Moreover, in *dy/dy* mice the release of T into the circulation, as estimated by the plasma to testicular T ratio, was greater than in normal mice, whereas no differences were observed between dy^{2J}/dy^{2J} and control mice (fig. 2). While the testicular levels of progestins were normal in dy^{2J}/dy^{2J} mice, in *dy/dy* mice the testicular P_4 levels, but not the OHP levels, were significantly higher than those of their normal siblings (fig. 3). In both types of dystrophic mice, the efficiency of the conversion of testicular P_4 to T, as judged by the ratios of the corresponding hormone levels in the testis, was

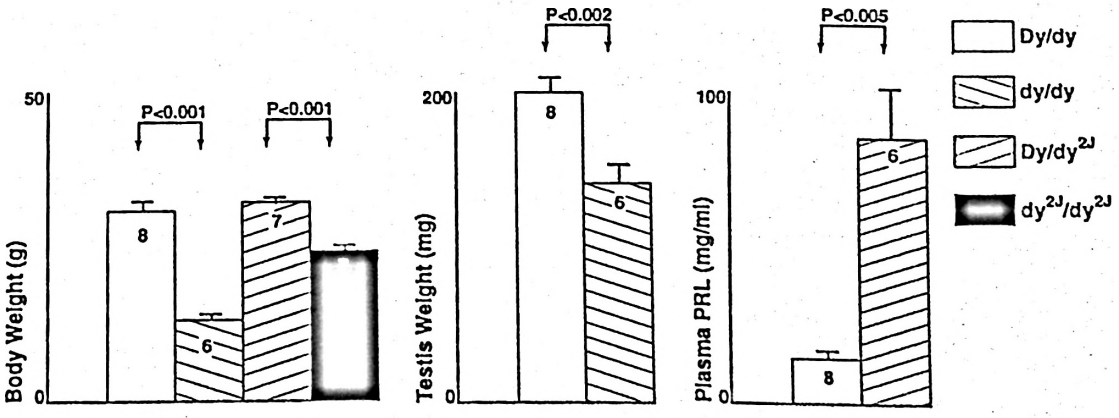

Fig. 1. *Body and testes weights, and circulating levels of prolactin, in mice.*
Values are represented as mean ± SEM for the number of mice indicated. Statistics were obtained using Student's t-test.

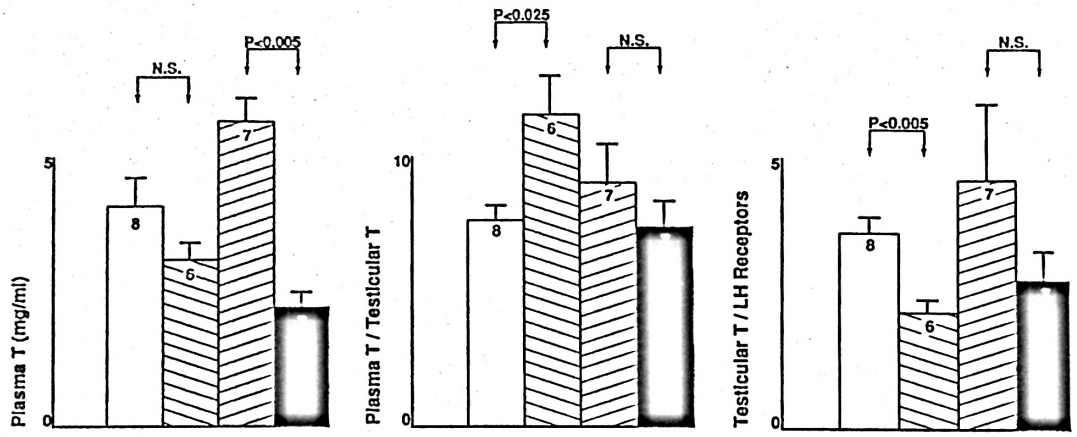

Fig. 2. *Plasma testosterone (T) levels, plasma T to testicular T ratio, and testicular T to LH-R ratio.* Values are represented as mean ± SEM for the number of mice indicated. Statistics were obtained using Student's t-test. Simbols as in fig. 3.

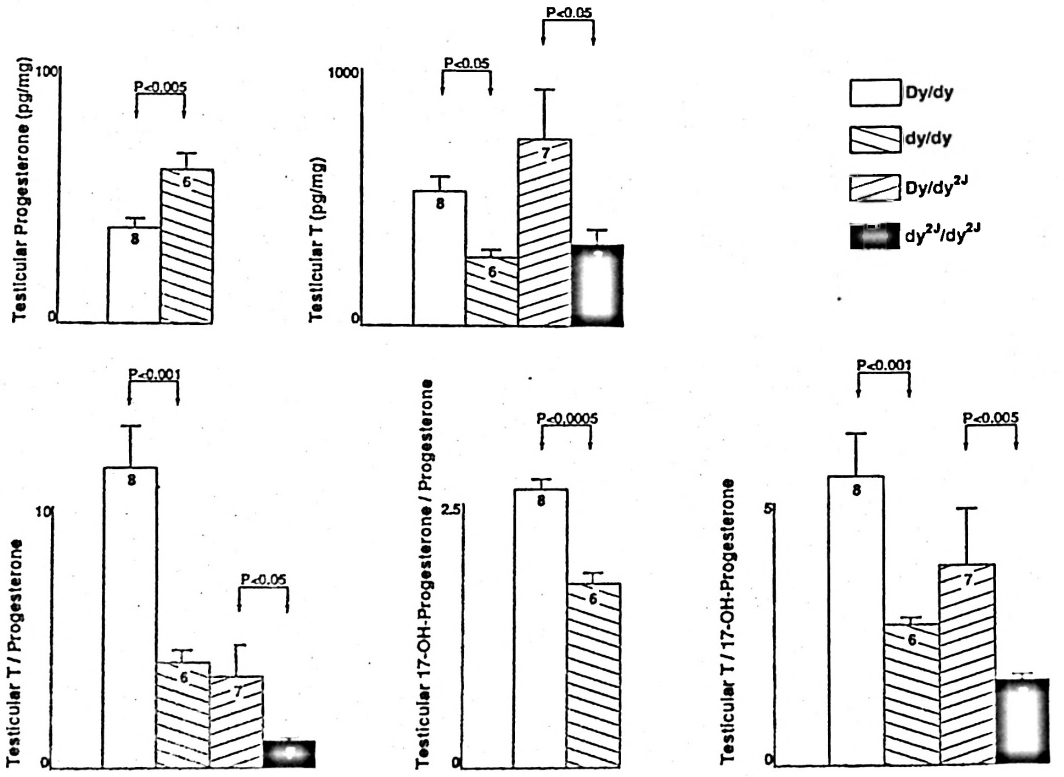

Fig. 3. *Testicular progesterone (P4) levels, testicular testosterone (T) levels, testicular T to P4 ratio, testicular 17-OH-progesterone (OHP) to P4, and testicular T to OHP ratio in mice.* Values are represented as mean ± SEM for the number of mice indicated. Statistics were obtained using Student's t-test.

reduced when compared to their respective normal siblings. In *dy/dy* mice, this was due to a reduction in the efficiency of the conversion of testicular P_4 to OHP, and of OHP to T. However, in dy^{2J}/dy^{2J} mice, this was due only to a decreased efficiency in the conversion of OHP to T (fig. 3). No significant decrease in the concentration of testicular LH-R was observed in dystrophic mice when compared with normal controls (data not shown). However, the efficiency of the transduction of the gonadotrophic signal, deduced from the testicular T to LH-R ratio, was reduced in the Leydig cells of *dy/dy* mice, but not in those of dy^{2J}/dy^{2J} mice, in comparison to that determined in normal mice (fig. 2).

The differences in the numerical values observed between the two groups of normal mice (*Dy/dy* and Dy/dy^{2J}), can sometimes occur when the testis are incubated on different occasions. In the present study corresponding normal and mutant littermates were each incubated in the same assay, but each mutant was assayed in a different incubation. Incubation of testes fragments with or without HCG, showed that, although there was an elevation in media T levels in response to HCG stimulation in all mice, *dy/dy* did not respond as well as *Dy/dy* mice, and dy^{2J}/dy^{2J} responded better than Dy/dy^{2J} (table II). In these samples, no differences in the basal or HCG-stimulated levels of P_4 or OHP could be measured between normal and dystrophic mice. When the incubation media levels of E_2 were determined, it was observed that, in *dy/dy* mice, basal E_2 levels were similar to those of normal mice, but the HCG-dependent increase in these levels was of a lesser magnitude. In dy^{2J}/dy^{2J} mice, the inverse was true, with basal E_2 levels being lower than those of control mice, and the HCG-stimulated ones being similar to those of their normal siblings. Under *in vitro* conditions, the overall efficiency of the conversion of P_4 to T was not different between normal and dystrophic mice, nor was there a difference in the efficiency of the conversion of P_4 to OHP detected. In *dy/dy* mice, incubation with HCG caused a significant decrease in the efficiency of the conversion of media OHP to T, but this effect was not observed in dy^{2J}/dy^{2J}, *Dy/dy* and Dy/dy^{2J} (table II). In dy^{2J}/dy^{2J} mice, the efficiency of the conversion of media T to E_2, under basal and HCG-stimulated conditions, was lower than the equivalent determined in normal mice. No differences in these last parameters were detected between *dy/dy* and *Dy/dy* mice.

Table I. *Characterization of solid-phase radioimmunoassays.*

Comparison between the statistical parameters of the curves derived from sample pools and those of the standard curves they were assayed with. m = slope, Y^I = Y intercept and r = correlation coefficient.

Hormonal Sample	m	Y^I	r
a) Testes progesterone	−1.849	0.473	−1.000
Standard curve	−1.855	0.472	−0.999
b) Media progesterone	−1.637	0.153	−1.000
Standard curve	−1.635	0.158	−0.994
c) Testes 17-OH--progesterone	−1.958	0.407	−1.000
Standard curve	−1.956	0.406	−0.998
d) Media 17-OH--progesterone	−1.944	0.259	−1.000
Standard curve	−1.941	0.263	−0.999
e) Media estradiol	−1.825	4.044	−1.000
Standard curve	−1.831	4.047	−1.000

Discussion

Although for many years dy^{2J}/dy^{2J} and specially *dy/dy* mice had been used as models for Duchenne/Becker muscular dystrophies (15), the possible limitations of these animal models were suggested by the

Table II. *Testicular incubation media progesterone (P_4), 17-OH-progesterone (OHP), testosterone (T), and estradiol (E_2).* Values are represented as mean ± SEM for the number of mice indicated in parenthesis. Points with the same letter in superscript are not statistically diferent ($P > 0.05$) among them (Student-Newman-Keuls procedure of the multiple range test). Data for *dy/dy* versus *Dy/dy*, and dy^{2J}/dy^{2J} versus Dy/dy^{2J} were analyzed in separate statistical groups.

Hormone	*Dy/dy*		*dy/dy*		Dy/dy^{2J}		dy^{2J}/dy^{2J}	
	control	HCG	control	HCG	control	HCG	control	HCG
P_4 (pg/mg testis)	0.57 ± 0.04^{a} (8)	33.66 ± 2.54^{b} (8)	0.67 ± 0.03^{a} (6)	30.44 ± 4.20^{b} (5)	0.80 ± 0.15^{x} (7)	16.64 ± 2.08^{y} (7)	0.74 ± 0.13^{x} (7)	20.32 ± 2.28^{y} (7)
OHP (pg/mg testis)	2.3 ± 0.2^{a} (8)	88.6 ± 5.6^{b} (8)	2.2 ± 0.3^{a} (6)	119.3 ± 36.5^{b} (6)	1.6 ± 0.2^{x} (7)	38.2 ± 7.4^{y} (7)	1.4 ± 0.1^{x} (7)	53.8 ± 8.3^{y} (7)
T (ng/mg testis)	1.6 ± 0.2^{a} (8)	49.7 ± 5.2^{c} (8)	1.6 ± 0.2^{a} (6)	32.8 ± 3.5^{b} (6)	1.5 ± 0.3^{x} (7)	22.6 ± 2.3^{y} (7)	1.5 ± 0.2^{x} (7)	46.5 ± 8.7^{z} (7)
E_2 (pg/g testis)	182 ± 44^{a} (8)	1185 ± 93^{c} (8)	115 ± 48^{a} (6)	532 ± 119^{b} (6)	219 ± 26^{y} (7)	926 ± 91^{z} (7)	123 ± 24^{x} (7)	1049 ± 88^{z} (7)
T/P_4	2.9 ± 0.4^{b} (8)	1.5 ± 0.1^{a} (8)	2.5 ± 0.3^{b} (6)	1.2 ± 0.2^{a} (5)	2.1 ± 0.4^{xy} (7)	1.4 ± 0.1^{x} (7)	2.5 ± 0.4^{y} (7)	2.3 ± 0.3^{y} (7)
OHP/P_4	4.1 ± 0.4^{b} (8)	2.7 ± 0.1^{a} (8)	3.3 ± 0.3^{ab} (6)	2.8 ± 0.2^{a} (6)	2.4 ± 0.4^{x} (7)	2.2 ± 0.1^{x} (7)	2.3 ± 0.4^{x} (7)	2.6 ± 0.2^{x} (7)
T/OHP	707 ± 68^{bc} (8)	558 ± 42^{ab} (8)	786 ± 113^{c} (6)	336 ± 63^{a} (6)	877 ± 83^{xy} (7)	661 ± 73^{x} (7)	1123 ± 111^{y} (7)	921 ± 135^{xy} (7)
E_2/T	115 ± 22^{b} (8)	25 ± 3^{a} (8)	71 ± 32^{ab} (6)	15 ± 2^{a} (6)	166 ± 26^{z} (7)	41 ± 2^{x} (7)	85 ± 18^{y} (7)	25 ± 3^{w} (7)

discovery that Duchenne/Becker muscular dystrophies (D/BMD) were due to lack of or presence of an altered dystrophin molecule, and by the existence of an homologous mouse mutant *mdx/y* (6, 19, 57). Unfortunately, *mdx/y* mice do not present with a clinical syndrome similar to the most common cases of D/BMD. Recently, other animal models that do present with a clinical picture similar to D/BMD, and whose molecular defect was a deficiency in the dystrophin molecule, were described. They are the *xmd/y* dogs, which have canine X-linked muscular dystrophy (13, 14, 53, 57), and the *xmd/y* cats which have feline X-linked muscular dystrophy (8, 57). However, because of the cost and problems in breeding any carnivore, as well as the recent reluctance to use animals usually considered as pets in biomedical research, the use of canine and feline models for muscular dystrophies has been limited. Thus, the question remains whether mice with mutations at the *dy* locus are adequate models for muscular dystrophies.

In humans there are other loci that produce a muscular dystrophy that could be confused with D/BMD. As a matter of fact Muscular Dystrophy II (McKusick, Cat. #253700) is clinically indistinguishable from DMD (34). Furthermore, a dystrophin-related protein (DRP) has recently been described, and is conserved in rodents, humans, pigs, cattle, goats, dogs, chickens and nematodes (24, 26, 27). In adult men and mice, the testis is one of the organs with the highest DRP levels (24, 27). The locus for this protein (*DMDL* in humans, and *dmdl* in mice) has been mapped to human chromosome #6 (6q24), and to mouse chromosome #10 in the same region as that for the *dy* locus. Moreover, this region of mouse chromosome #10 includes the *myb* oncogene whose human homologue (*MYB*) is located on chromosome #6 (6q22-q24) (28, 37, 46). This would indicate that there is a conservation, between the mouse and human genomes, of the segment that includes the *myb* and *dy* loci. Thus, there is a distinct possibility that mice with mutations at the *dy* locus have the murine equivalent of human muscular dystrophy II. Recent data obtained in mice indicates that the alteration in the *dy* locus might be due to a microdeletion or point mutation of the *dmdl* gene, in contrast to the larger deletions observed in D/BMD. Thus, in *dy/dy* or dy^{2J}/dy^{2J} mice DRP is present but with an abnormal structure, whereas dystrophin is absent in *mdx* mice (27). Other interesting features of the DMDL locus are that it is located in a region that encodes also for the estrogen receptor, vasoactive intestinal peptide and the *MAS1* oncogene (28, 37, 46). Furthermore, the locus *Zfa* on mouse chromosome #10 is another locus located in this mouse chromosome that has a chromosome X homologue, just like the *dmdl* locus (35, 38). Although not in the same region, it should be noted that the locus for human prolactin is located at 6p23-21.1 (23).

The results presented here add another organ, the testis, to those which were previously shown to be affected in dystrophic mice (50, 58). Moreover, our results demonstrate a difference in the severity of testicular deficiencies observed in dystrophic mice. Testes from *dy/dy* mice appear to be more affected than those of dy^{2J}/dy^{2J} animals. This difference correlates well with the dystrophic phenotypes of these mice, namely *dy/dy* presenting a DMD-like phenotype and dy^{2J}/dy^{2J} present a BMD-like phenotype. The basal levels of pituitary hormones measured in the present study were normal in dystrophic mice, except for the presence of hyperprolactinemia in *dy/dy* males. This agrees with findings in many dystrophic patients (29-30), although PRL deficiency has been reported in a few cases (33). Testicular weight was diminished in *dy/dy* but not in dy^{2J}/dy^{2J} mice. Testicular atrophy was reported in dystrophic men already by Steinert (as cited in 55). This

type of patient may also have deficient basal and/or gonadotropin-stimulated T production with or without the testicular atrophy (20, 29, 31, 45, 55). Studies *in vivo* with *dy/dy* mice, provide evidence for deficient transduction of the gonadotropic signal, accompanied by reduced efficiency of 17-hydroxylase and 17-hydroxysteroid dehydrogenase. In contrast, in dy^{2J}/dy^{2J} mice gonadotropic signal transduction appeared normal and the reduction in enzyme efficiency was limited to 17-hydroxysteroid dehydrogenase. In the *in vitro* studies the HCG-induced increases in producion of T and E_2 were reduced in *dy/dy* mice. The data suggested a reduction in enzyme activiy rather than in its efficiency. In contrast, in dy^{2J}/dy^{2J} mice, the HCG-induced T synthesis was greater than in normal controls, the HCG-induced E_2 synthesis was normal, and basal media E_2 levels were reduced. In these mice, the *in vitro* efficiency of aromatase was lower under both basal and HCG-stimulated conditions. Thus, although both types of mice have testicular deficiencies, dy^{2J}/dy^{2J} mice are less severely affected than those with the *dy/dy* genotype.

Several conclusions are apparent from the above data. Dystrophia Muscularis in the mouse is definitively associated with testicular dysfunction. It is possible therefore, that patients with the human equivalent, Muscular Dystrophy II, may also exhibit a similar type of dysfunction. This cannot be confirmed at the present time because the sporadic literature on reproductive alterations in dystrophic patients deals with those having myotonic dystrophy. Reproductive data on patients with D/BMD or muscular distrophy II are unavailable. Whether testicular dysfunction is a characteristic of muscular dystrophies in general, regardless of the specific disease, requires additional studies. Another consideration worthy of study is the possibility that the chromosomal association of the *DMDL/dmdl* locus with other loci such as *Zfa*, *MYB*, *MAS1*, and the ones for PRL, vasoactive intestinal peptide and estrogen receptors may have some physiological relevance.

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Resumen

Se estudia la función testicular *in vivo* e *in vitro* en ratones machos *dy/dy* y en distróficos dy^{2J}/dy^{2J}, demostrándose que ésta se encuentra más afectada en ratones *dy/dy*. Los niveles basales de hormonas hipofisarias son normales, a excepción de la hiperprolactinemia en ratones *dy/dy*. El peso testicular es menor en los ratones *dy/dy*, y la transducción del mensaje gonadotrópico es deficiente, manifestándose por una reducción en la eficiencia de la 17-hidroxilasa y de la 17-hidroxiesteroide deshidrogenasa. También es menor la producción *in vitro* de testosterona (T) y de estradiol (E_2), dependiente de HCG, probablemente debido a cambios en la actividad enzimática. En los ratones dy^{2J}/dy^{2J}, la síntesis de T dependiente de HCG se encuentra potenciada, mientras que la de E_2 no cambia, aunque sus niveles basales disminuyen. La eficiencia *in vitro* de la aromatasa se encuentra disminuida en ratones distróficos.

Palabras clave: Distrofia muscular, Testículo, Testosterona, Progesterona, Estradiol, Hidroxiprogesterona, Receptores de LH.

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