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GNRH induces activation of Leydig-like cells in *Pleurodeles waltlii*. A morphometric study

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Summary. The ultrastructure of the interstitial cells of the glandular tissue of *Pleurodeles waltlii* was studied in testis of animals obtained in early breeding season (January) under gonadotropic releasing hormone (GNRH) treatments and controls. These cells (parenchimal or Ledyig-like cells) displayed the structural characteristics of steroid-producing cells.

GNRH administration for 24 hours induced a significant decrease of both medial volume and volume density of lipid droplets. On the other hand, cell volume, nucleus, mitochondria, mitochondrial cristae and tubules of smooth endoplasmic reticulum were increased. The surface density of mitochondrial cristae was also increased.

Key words: Testis - Leydig cells - Urodeles - GNRH - Morphometry

Introduction

The organization of urodele testis is markedly different from the other vertebrates and from mammals in special. Male gonads consist of a variable number of lobes, depending on the age of the animal. Each lobe contains numerous seminiferous units called lobules (terminology after Lofts, 1974). The spermatogenic wave passes linearly from lobule to lobule in a caudo-cephalic sequence. The process is synchronized so that all the germinal cells in a given lobule are at the same stage of development at a given time. As the different stages of sperm development are cephalo-caudally orientated along the length of each individual lobe, successive zones containing spermatogonia, spermatocytes, spermatides, spermatozoa and glandular tissue, can be recognized.

Interstitial cells develop from fibroblast-like stromal cells immediately surrounding the germinal lobules. However, their differentiation does not coincide with the initiation of the spermatogenic sequence but rather with the terminal stages of spermatid changes and sperm discharge from the lobules. The stromal cells surrounding the spermatogenic lobules in *Pleurodeles waltlii* acquire the morphological characteristic of active steroid-secretory cells only after the wave of spermatogenesis has passed and the adjacent seminiferous lobule has extruded its spermatozoa (Ucci, 1982).

Glandular cells of *Pleurodeles waltlii* testis have been reported as cells containing a big, rounded nucleus and a well developed, tubular shaped, smooth endoplasmic reticulum (SER). Lipid droplets and mitochondria containing tubular cristae are abundantly present (Picheral, 1968). The same ultrastructure has been described in other urodela (Ucci, 1982; Bergmann et al., 1982). These cells have also been reported in both birds (Rothwell, 1973) and mammals (Andersen, 1978).

Glandular cells of urodela testis are capable of steroid synthesis, as has been demonstrated by histochemical and biochemical approaches (Certain et al., 1964; Picheral, 1970; Joly, 1971; Tso and Lofts, 1977).

The stimulatory effect of GNRH on male behaviour may be a seasonal phenomenon, because GNRH stimulation of sexual behaviour has only been observed during the early half of the breeding season (October to January). At the end of the breeding season, an injection of GNRH is completely unable to stimulate this behaviour (Moore et al., 1982).

McCreery and Licht (1983) reported that continuous intravenous injections of synthetic mammalian GNRH induce an increase in plasma of both FSH and LH, showing higher effect after 24 h of treatment.

This report describes the ultrastructure and morphometry of glandular cells of *Pleurodeles waltlii* testis under GNRH treated and control conditions.

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Materials and methods

Adult males urodele *Pleurodeles waltlii* at early breeding season were used in this work. Five animals were injected with 20 μg/animal of synthetic mammalian GNRH (Peyva lab., Spain) dissolved in 0.1 ml sterile physiological saline. Control animals were injected with 0.1 ml of sterile physiological saline.

Animals were sacrificed by decapitation 24 hours after treatment and their testis were removed. The caudal part of testis was immediately fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (ph 7.2) for 2 h at 4°C. After fixation, samples were rinsed three times in 0.1M cacodylate buffer (ph 7.2) containing 4% sucrose. Postfixation was carried out for 1 h at 4°C in 1% osmium tetroxide in the same buffer and embedded in Epon 812.

Thick sections were stained with toluidine blue. Thin sections were stained with lead citrate and uranyl acetate and examined under a Philips E.M. 300.

Morphometry

For the morphometric study of Leydig cells, photographs at three levels of magnification were used.

For determination of absolute nuclear volume and volume fraction (Vv%) cell nuclei were used (150 per animal) on 1 μm toluidine blue-stained thick section at 1,000 ×.

Determination of diameter, mean volume, absolute volume and volume fraction (Vv%) of lipid droplets and mitochondria was made using 30 micrographs per group at 15,000 ×. The same magnification was used to obtain the shape factor of nucleus, lipid droplets and mitochondria using the Fischmeisters's shape coefficient ($F = 3\pi^3/8 \times Na/p^2$, where Na is the number of profiles and p the perimeter) as a measure of sphericity (F = 1 for a sphere).

Determination of diameter, volume fraction (Vv%) and surface density of mitochondrial cristae and SER tubules was made on 30 micrographs per groups at 45,000 ×. Volume fractions in micrographs at 15,000 × were determined by a superficial analysis method and in micrographs at 45,000 × by a point counting method (Weibel, 1969). All measurements were made by an automatic image analysis with an «IBAS» (Kontron, FRG) analyzer. The statical comparison was performed by the Student's test. The difference between two mean values was considered significant if probability of error (p) was found to be less than 0.05%.

Results

Morphological observations

Glandular tissue of *Pleurodeles waltlii* testis is composed by a homogeneous tissue of spheric lobules containing steroid-producing cells. These cells have a rounded nucleus with one or two nucleoli (Fig. 1). In the cytoplasm, mitochondria with tubular cristae are present. Cytoplasmic lipid droplets were reduced in treated cells (Fig. 2), compared with control cells (Fig. 1) The qualitative appearance of SER profiles were striking in GNRH-treated in comparison with controls.

Morphometric study

Steroid-secreting cells of *Pleurodeles waltlii* testis measured about 360 μm and showed the morphometric characteristics summarized in Table 1.

After GNRH treatment (Table 2), the nucleus showed a significant volume increase relative to control but not the other morphometric parameters. Lipid droplets presented a significant reduction of diameter and volume. Although the shape factor was unchanged, the cytoplasm and cell volume density were reduced 4 times after GNRH treatment with a significance degree of 99.9% ($p = 0.01$).

Mitochondria followed the opposite behaviour to lipid droplets. They increased their diameter and volume having a significant difference at $p = 0.001$ in comparison to control. The relative volume occupied by mitochondria in GNRH-treated cells was 3 folds higher than in controls and, at the same time, there was a proportional increase of mitochondrial cristae. The surface density of cristae grew up to 56% from 42% in control testis, showing a significant difference at $p = 0.001$.

The smooth endoplasmic reticulum did not change its surface density relative to the cell but showed a significant ($p = 0.001$) increase of relative volume density, and there was a reduction on cisternal diameter at a $p = 0.01$ of significance.

The treatment of urodela testis with GNRH for 24 h produced an increase in cell size if we consider the cell volume as a measurement of cell size (Table 3). This phenomenon was general for all the organelles (Table 3), being more significant for mitochondria and mitochondrial cristae, which showed 3-4 fold increase in volume. The opposite pattern was presented by lipid droplets which reduced their volume 3 folds relative to control.

Table 1. Morphometric parameters of Leydig cells of control group

	NUCLEUS	LIPID DROPLETS	MITOCHONDRIA TOTAL	MITOCHONDRIA CRISTAE	S.E.R.
DIAMETER	6±0.1 (a)	0.7±0.01 (a)	0.5±0.01 (a)	48±0.7 (b)	54±0.9 (b)
VOLUME μm^3	102±8	0.2±0.02	0.07±0.005		
SHAPE FACTOR	0.9±0.01	0.9±0.1	0.8±0.02		
Vv % CELL	29±2	26±2	4.4±0.05	1.3±0.01	29±2
Vv % CYTOPLASM		33±3	6±0.4	2±0.1	36±2
Sv %				42±1 (c)	55±2

All values mean±S.E.; (a) in micrometers; (b) in nanometers; (c) in relation to the mitochondria

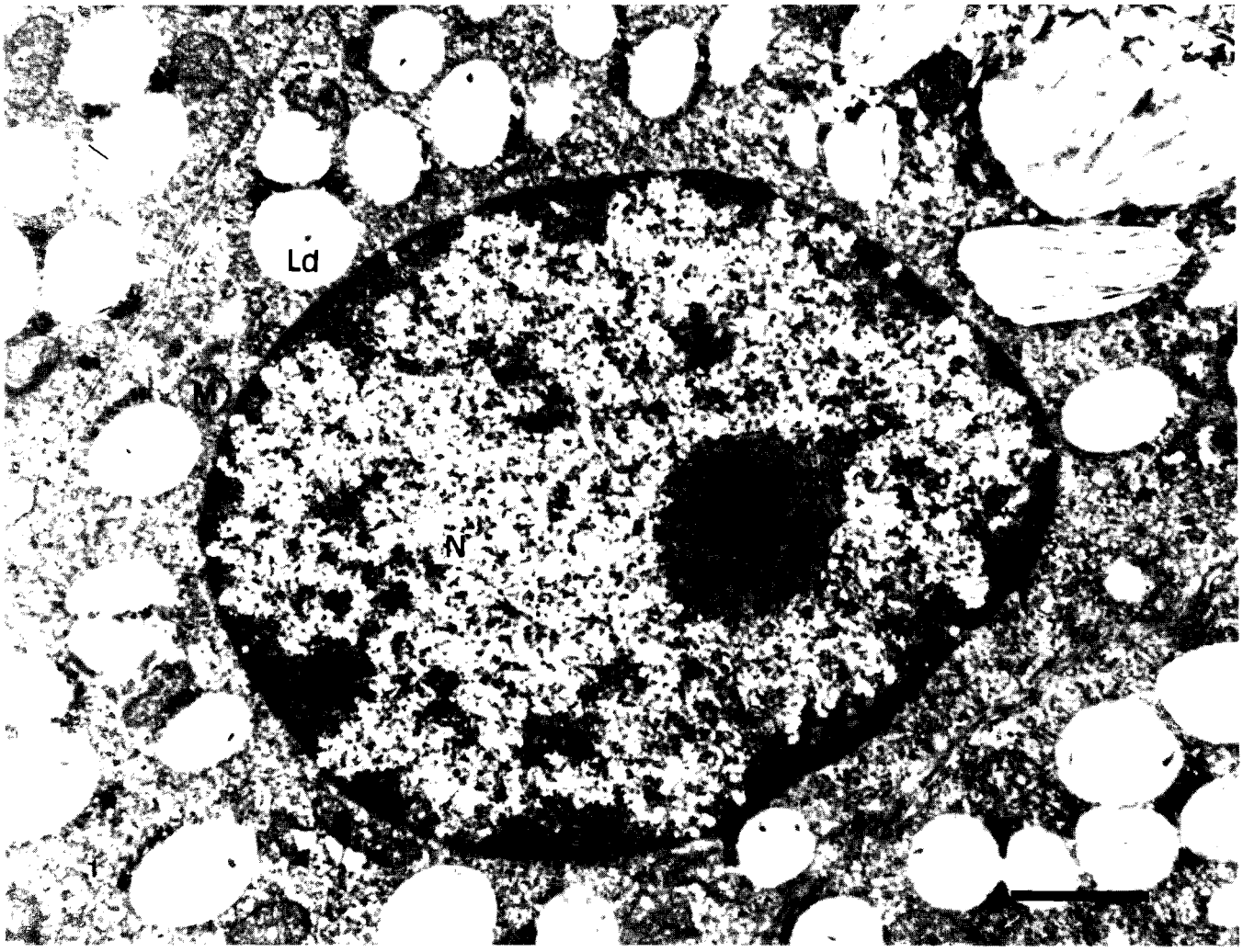

Fig. 1. Fine structure of control Leydig-like cell. 20,000 ×. N = Nucleus; Nu = Nucleolus; Ld = Lipid droplets; M = Mitochondria; Bar = 1μm

Table 2. Morphometric parameters of Leydig cells of GNRH-treated group

	NUCLEUS	LIPID DROPLETS	MITOCHONDRIA TOTAL	MITOCHONDRIA CRISTAE	S.E.R.
DIAMETER	6±0.1 (a)(*)	0.4±0.01 (a)(***)	0.6±0.01 (a)(***)	40±0.4 (b) (**)	41±0.5 (b)(**)
VOLUME μM^3	140±10 (*)	0.1±0.003 (***)	0.2±0.01 (***)		
SHAPE FACTOR	0.9±0.01 (n.s.)	0.9±0.01 (n.s.)	0.9±0.01 (**)		
Vv% CELL	29±2 (n.s.)	6.5±0.9 (***)	11±1 (***)	5±0.03 (***)	34±2 (***)
Vv % CYTOPLASM		8±1 (***)	14±1 (***)	6±0.03 (***)	44±2 (***)
Sv %				56±2 (c)(***)	54±2 (n.s.)

All values mean±S.E.; (a) in micrometers; (b) in nanometers; (c) in relation to the mitochondria. Significant differences from control at * $p<0.05$; ** $p<0.01$; *** $p<0.001$ n.s.: not significant.

Table 3. Morphometric parameters for a single cell

	CONTROL	GNRH-TREATED
CELL	356±24	480±36 ***
NUCLEUS	102±8	140±10 **
HETEROCHROMATIN	30±2	52±4 **
LIPID DROPLETS	93±0.1	31±0.4 ***
MITOCHONDRIA TOTAL	16±0.2	52±0.4 ***
CRISTAE	5±0.01	22±0.03 ***
S. E. R.	103±8	±164±18 ***

All values as absolute volume (in μm^3) ±S. E.; significant differences marked as in table 2.

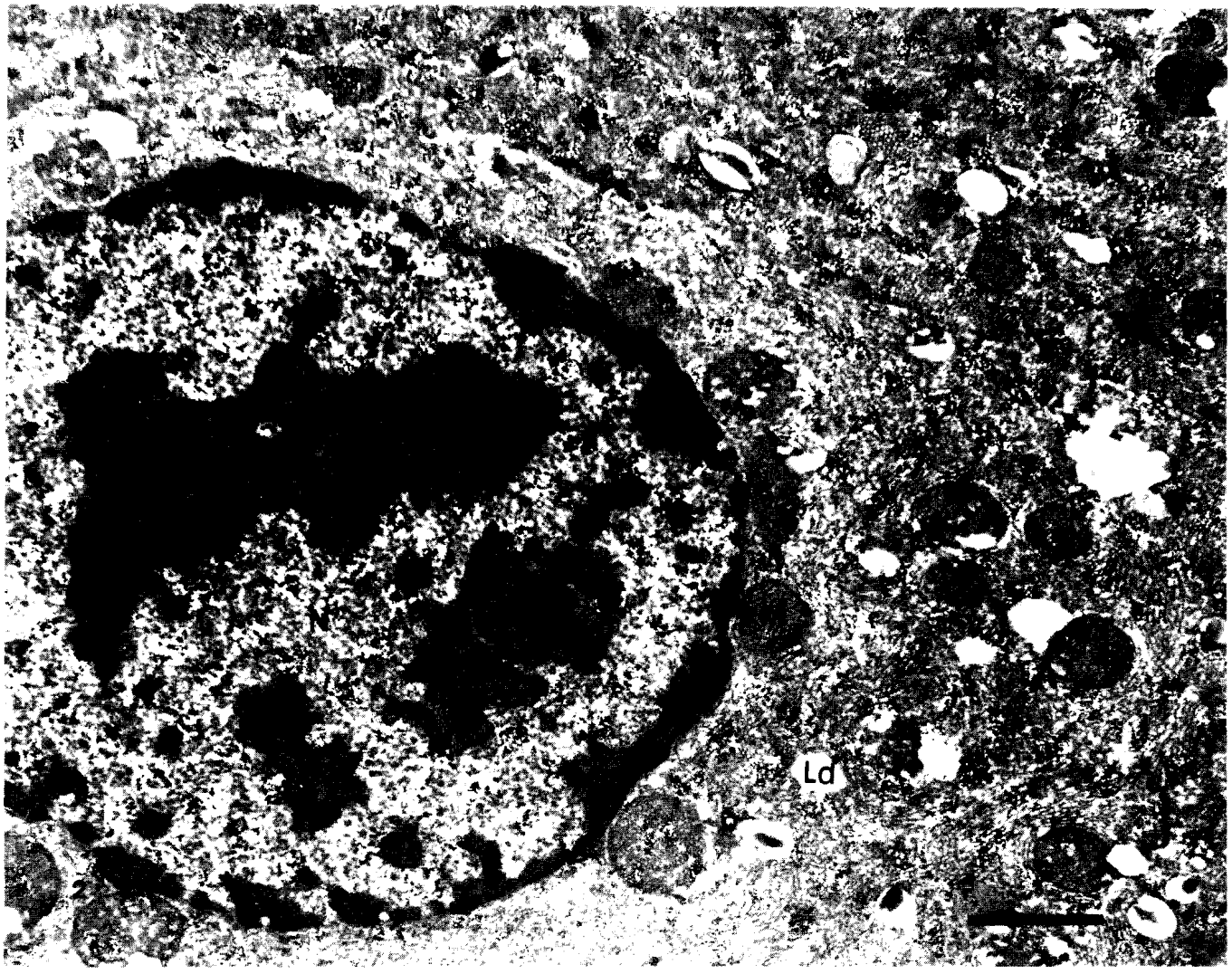

Fig. 2. Leydig-like cell after 24 h of GNRH treatment. 20,000 ×. SER = Smooth endoplasmic reticulum. Other abbreviations as in figure 1. Bar = 1m.

Discussion

The glandular tissue of *Pleurodeles waltlii* testis is structural and physiologically homologous to interstitial tissue of mammalian testis. It is morphologically characterized by the presence of Leydig-like cells containing lipid droplets, mitochondria with tubular cristae and abundant SER (Picheral, 1968). This amphibian glandular tissue is able to use progesterone and 17α hydroxiprogesterone for the production of testosterone (Ozon, 1965).

The increasing steroidogenic activity has been correlated with the nuclear hypertrophy (Connell and Connel, 1977). Our results show significant nuclear volume differences between control and GNRH treated cells. The higher activity developed by GNRH in these cells produces an apparent loss of nuclear information as we can suppose from heterochromatin increase.

Lipid droplets contain cholesterol as a reserve pool of material for the synthesis of steroid hormones (Armstrong, 1968). It has been established that the amount of lipid droplets is roughly inverse to the secretory activity of the cell (Hsü et al., 1985). We have observed a clear decrease in size, mean and absolute volumes and in Vv% of lipid droplets in GNRH treated cells.

Mitochondria contain hydrolases which are involved in cholesterol breakdown, playing an important role in steroid biosynthesis (Toren et al., 1964; Christensen and Gillim, 1969). Our results induce us to consider that GNRH stimulates the first steps of steroid synthesis via a higher mitochondrial hydrolase activity. Similar responses have been observed in other vertebrates after ACTH treatment (Berchtold, 1970; Mazzochi et al., 1979).

Enzymes involved in the synthesis of steroid hormones are mostly localized in SER (Christensen and Gillim, 1969; Tamaoki et al., 1971). A high development

of SER characterizes steroid secretory cells during the period of synthesis. In this sense, we have observed an increase of volume occupied by SER in the cytoplasm as well as a decrease in tubular diameter.

GNRH induces changes in Leydig-like cells of *Pleurodeles waltlii* testis that appear as a clear stimulation of steroid synthesis inducing the cell hyperactivity. The physiological mechanism of GNRH on these Leydig-like cells, probably via hypophisys, will be matter of forthcoming studies.

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