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Leydig cells — further immunocytochemical evidence for their neuroendocrine nature

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In mammals somatic cells secrete peptides regulating spermatogenesis and cell-cell interactions. In man and rodent testes a number of marker substances for neuroendocrine cells were demonstrated. Substance P, Neuron specific enolase, Mono-amine synthesizing enzymes (Thyrosine hydroxylase, *L*-Amino acid decarboxylase), Chromogranin A+B etc. Using immunocytochemical methods we were also able to demonstrate immunoreactivity for several other substances: Neurofilament protein 160, Neurofilament protein 68, Microtubule-associated protein (MAP-2), Calmodulin, Glutamate, Aspartate, Nitric oxyde synthase. Positive reactions for these antigens were found in the cytoplasm of the Leydig cells (LC) in three rodent species: hamster, mouse, guinea-pig. The uniformity in the localization of the investigated substances suggests that the revealed neuroendocrine markers and neuroactive peptides seem to be a basic equipment of mammalian LC. These findings provide further evidence for neuroendocrine nature of LC.

Key words: Leydig cells, neuroendocrine markers, immunocytochemistry, rodents.

Introduction

The gonadotrophins LH and FSH are the main endocrine modulators of the normal physiological functions of the testis, i. e. gametogenesis and steroidogenesis. In addition, steroids and peptides syntesized in the testis have been supposed to can mediate or modulate the actions of gonadotrophins [16]. By immunocytochemistry, a number of locally produced substances indentical or related to neurotransmitters or neurohormones, have been identified in the Leydig cells of the human and rodent testes: POMC-derived peptides [5, 12], the tachykinin substance P and neuron specific enolase [16,17, 1, 2], monoamine synthesizing enzymes thyrosine hydroxylase, aromatic *L*-amino acid decarboxylase and dopamine-β-hydroxylase, chromogranin A+B [8, 15, 4], serotonin [21].

On the other hand, it was established that the Leydig cells contain different growth factors or binding sites for them and neuroactive substances which possibly modulate testosterone production [14, 19].

Taking into account the considerations mentioned above, the present investigation was undertaken with the aim to verify the occurrence of other neuronal and

neuroendocrine markers in the Leydig cells of the three rodent species —mouse, hamster and guinea-pig): neurofilament protein 160 (NFP 160), neurofilament protein 68 (NFP 68), calcium-binding protein calmodulin, microtubule associated protein (MAP-2), glutamate, aspartate, the receptor for nerve growth factor (NGF-R) and nitric oxide synthase (NOS). We will try to get some information about the significance of those substances as autocrine/paracrine factors involved in the regulation of testicular functions in mammals.

Material and methods

Testes of sexually mature laboratory animals: mouse, golden hamster and guinea-pig, were studied. Tissue blocks were fixed in Bouin's fluid for 24 h at room temperature and then they were embedded in paraffin. Histological sections were mounted on chrom-gelatin precoated slides. For the immunocytochemical demonstration of the corresponding antigens, the peroxidase-antiperoxidase (PAP) technique with the avidin-biotin-peroxidase complex method according to D a v i d o f f and S c h u l z e [7] was applied.When polyclonal antibodies were used, the sections were treated with 1,2 % H_2O_2 in absolute methanol to inhibit endogenous peroxidase activity and then with 2 % normal rabbit or swine serum (to block the non-specific binding sites).

The sections were incubated with the primary antibodies or monoclonal antibodies for 48 h at 4 °C in a humid chamber. The following antibodies were used: 1) Mouse monoclonal antibodies directed against NFP 160 (Sigma, 1:200), NFP 68 (Sigma, 1:200),Calmodulin (Sigma, 1:200), MAP-2 (Sigma, 1:200), NGF-R (Sigma, 1:100); 2) Rabbit polyclonal antisera: anti-aspartate (Sigma, 1:200); anti-glutamate (Sigma, 1:1000); anti-nitric oxide synthase-I (Dr Mayer, Graz, Austria, 1:1000). The second antibodies, biotinylated anti-mouse IgG or biotinylated anti-rabbit IgG (Dakopatts) and ABC-complex (Vector 1:250), the mouse PAP-complex (Dakopatts 1:100) or rabbit PAP-complex (Dakopatts, 1:200) were applied.

Development of the peroxidase activity was performed with a solution containing 0,2 % 3,3'-diaminobenzidine-4 HCl (Sigma) and H_2O_2 at a final concentration of 0,01 %.

As controls, sections were used in which primary, secondary or tertiary antibodies were replaced by PBS.

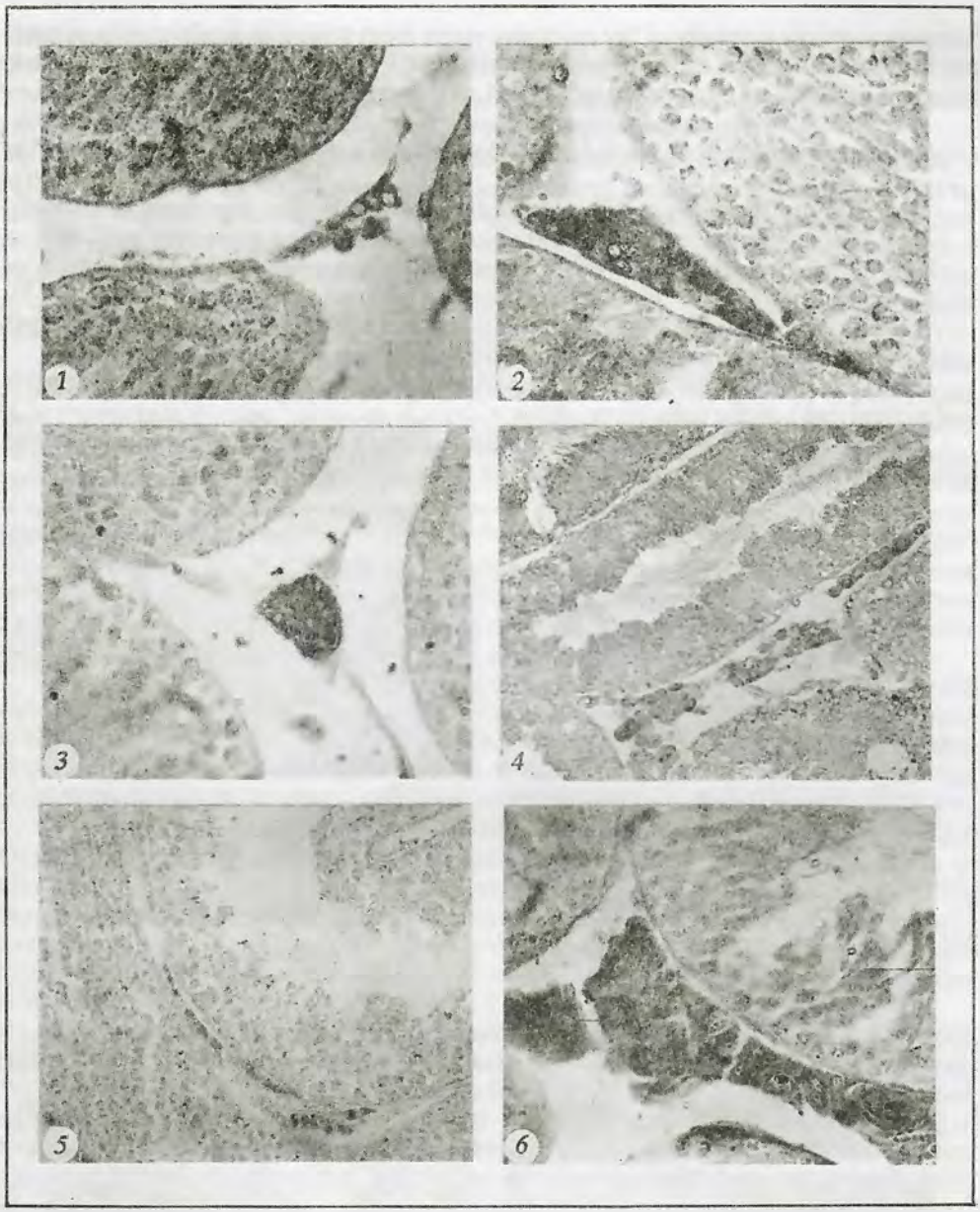
The staining intensity of every section was estimated at pluses (points) by two examiners independent of one another.

Results

Leydig cells of the rodent testes under study present a moderate to strong immunoreactivity for NFP 160, NFP 68, MAP-2, glutamate, aspartate, calmodulin, NGF-R and NOS.

In all cases immunoreactivity was localized within the cytoplasm of the Leydig cells. It is important to note that the cytoplasm of a great number of cells was stained while that of the remaining steroidogenic cells of the same cluster or in other groups is negative (Figs 4,9).

Secondly, differences in immunostaining intensity among testicular material from hamster, mouse and guinea-pig were observed. Most of the Leydig cells in mouse testis possess a stronger immunoreactivity for all tested antigens (Figs. 2,6,8). In



Figs. 1, 2. MAP-2 immunoreactivity in hamster (Fig. 1) and in mouse (Fig. 2) Leydig cells (LC). Differences in staining intensity among the LC in the same group are observed. ×400
Figs 3,4. Calmodulin immunoreactivity. In hamster testis (Fig. 3) a stronger reaction than in mouse LC (Fig. 4) is present. ×200
Figs 5,6. Neurofilament protein 160 expression in LC of guinea-pig (Fig. 5) and mouse (Fig. 6) testes. ×160, ×400 respectively

hamster testis the intensity of the reactions varies from strong to moderate. In guinea-pig there were large groups of positive cells, but in other clusters of steroidogenic cells only a small number of them showed posititve staining (Figs 5,10).

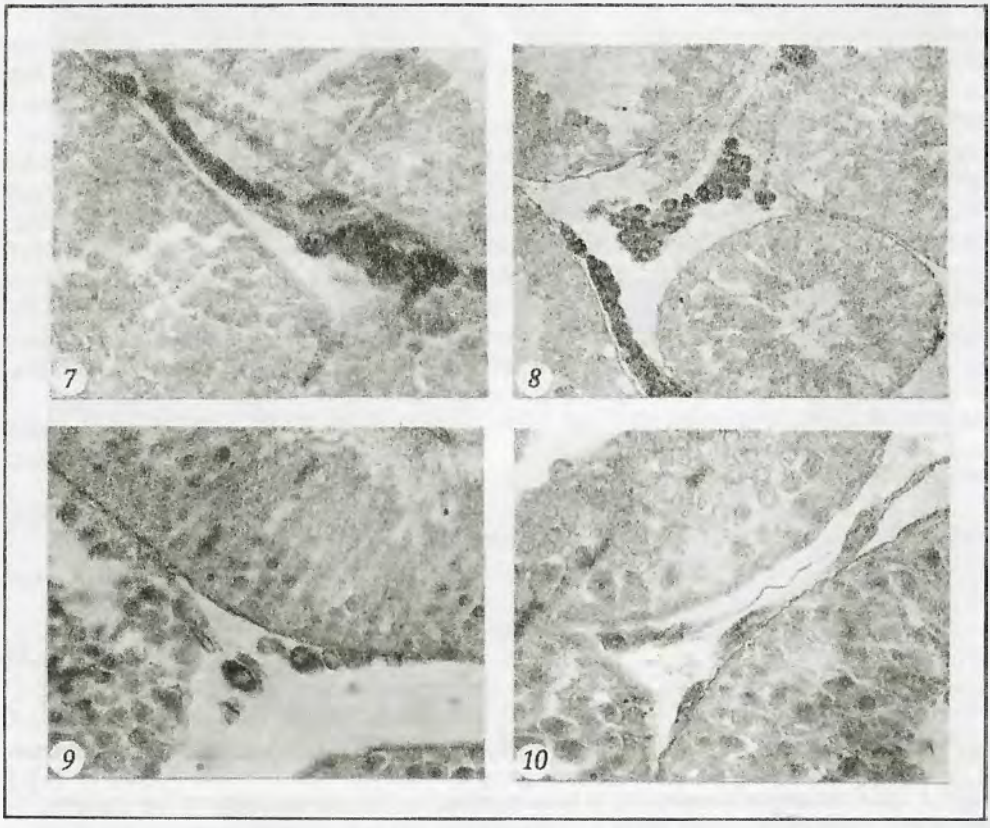
The MAP-2 reaction was stronger when the double amplification technique was applied and the staining product had predominantly a perinuclear distribution (Figs 1,2).

The calcium-binding protein calmodulin showed a similar localization in Leydig cell cytoplasm and a strong to moderate immunostaining was present (Figs 3,4). The positive immunoreactivity for NGF-R showed the presence in the Leydig cells of binding sites for a specific growth factor for the nervous system.

Concerning neurofilament protein 160 and neurofilament protein 68, a stronger reaction was observed in the case of the NFP 160 (Figs 5,6).

Both reactions for glutamate and aspartate were well expressed in mouse, hamster and guinea-pig Leydig cells (Figs 7,8).

In our material we were able to demonstrate immunocytochemically the occurrence of NOS in Leyding cells of the three species studied (Figs. 9,10).



Figs 7,8. Glutamate immunoreactivity in LC of hamster (Fig. 7) and mouse (Fig. 8) testes. In both cases a strong immunostaining is observed. ×400, ×200 respectively
Figs 9,10. NOS immunostaining in hamster (Fig. 9) and guinea-pig (Fig. 10) LC is demonstrated. ×400

Discussion

The present study show that Leydig cells in mouse, hamster and guinea-pig testes possess a great number of neuropeptides and neurotransmitters.

The immunocytochemical data about the existence of neurofilament proteins — NFP 160 and 68, MAP-2, glutamate and aspartate in Leydig cell cytoplasm point to common characteristics with nerve cells. Our findings enlarge the already published results concerning human testis [8]. The MAP-2, a protein typical for nerve cells is also demonstrated in our material as well as in rat Leydig cells [9]. It seems that the neuropeptides expressed in Mammalian Leydig cells are a common basic equipment of testicular steroidogenic cells probably stabilizing their structure and function [15].

In the three species investigated Leydig cells show a positive staining for calmodulin which is a typical component of neuroendocrine cells. It is postulated that Ca^{++}interacts with intracellular Ca^{++}-binding proteins such as parvalbumin, calbindin D-28 K, S-100 protein and calmodulin which then regulate the intracellular calcium concentration and translocation [11]. In the testis, calcium is thought to be involved in the production and secretion of testosterone and in the regulation of spermatogenesis [20].

The positive NGF-R immunoreactivity suggests that rodent Leydig cells possess receptors for this growth factor thus supporting the opinion about neuroendocrine nature of Leydig cells. Possibly, NGF is involved in autocrine regulation of testicular functions as well as other growth factors: EGF, TGF-β_1 [19, 10].

All proteins investigated by us co-exist in rodent Leydig cells, however in different concentration. Their different expression may be species-specific, but most probably it is related to the different functional state of the steroidogenic cells. The finding that in the same tissue section or in the same group, LC show a positive immunocytochemical reaction or remain unreactive, points to the second possibility.

Recently it was established that nitric oxide is a major messenger molecule regulating blood vessel dilatation and serving as a neurotransmitter [13]. NOS is activated by calcium which binds calmodulin as an enzyme cofactor [13]. By immunohistochemistry this enzyme was not identified in liver, heart, spleen, kidney, testis, thymus etc [6]. In our investigations the localization of NOS in the Leydig cell cytoplasm point to a possible action of nitric oxide as an intercellular messenger in steroidogenic process.

The current findings together with already established expression of a great number of bioactive substances by Mammalian Leydig cells provide further evidence for their neuroendocrine nature and raised the question about the functional significance of peptides mentioned above. A modulatory action of substance P, a tachykinin, on in vitro release of testosterone by hamster Leydig cells was earlier reported [3]. But the complex process of spermatogenesis is unlikely to be fully understood until all the factors involved in its control become known. Most probably, the neuropeptides, calcium-binding proteins, growth factors (or binding sites for them) identified immunocytochemically in the Leydig cells play a crucial role in testicular autocrine/paracrine mechanisms [5, 18].

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