Immunohistochemical Demonstration of a Testicular Substance Related to Luteinizing Hormone–Releasing Hormone

Abstract. *A substance related to luteinizing hormone–releasing hormone was demonstrated, by immunohistochemical procedures, in the cytoplasm of interstitial cells within the rat testes. In many seminiferous tubules, nuclei of spermatogonial cells were also immunopositive. Both cytoplasmic and nuclear fractions of testicular homogenates contain immunoreactive compounds, and this report identifies which cell types contain this substance. The localization of a peptide hormone within the nucleus of a target cell population may indicate its mode of action.*

The elucidation of the structure of luteinizing hormone–releasing hormone (LHRH) and its subsequent synthesis led to attempts to use this compound and its analogs to promote fertility. However, it was soon found that long-term administration of LHRH or its agonists has antigonadal effects, causing testicular atrophy and suppression of spermatogenesis (*1*), decreases in the weights of the ventral prostate (*2*) and ovaries (*3*), and pre- and postcoital contraception (*4*). At the molecular level, long-term treatment with LHRH leads to reductions in the numbers of receptors for follicle stimulating hormone, luteinizing hormone, and prolactin in the ovaries (*3, 5*) and testes (*3, 6*), with concomitant decreases in steroidogenesis. It is now clear that LHRH agonists can also act directly on the gonads, and specific saturable high-affinity LHRH binding sites in the testes have been described (*7*).

Although the extremely low levels of circulating hypothalamic LHRH are probably insufficient to activate the testicular binding sites, these sites may mediate the action of an LHRH-like substance present in the testes and ovaries (*8*). Immunopositive staining for LHRH has also been found in the adrenals (*9*). (Many other brain peptides, including somatostatin, substance P, the endorphins and enkephalins, and thyroid hormone–releasing hormone, are distributed in a variety of organs throughout the body.)

We have investigated the cellular and intracellular distribution of an immunoreactive LHRH-like substance within rat testes. Radioreceptor assay and radioimmunoassay techniques can be used to measure the amounts of the LHRH-like substance in homogenates, but do not indicate the location of the substance within a specific cell type or within components of these cells. Therefore, we used immunohistochemical procedures (*10*) to examine histological sections of the rat testes.

Nine young adult male rats (CD strain from Charles River Breeding Laboratories, weighing approximately 200 g) were anesthetized with pentobarbital. After thoracotomy and cardiac perfusion with Bouins' fixative, the testes were hemisectioned, dehydrated in ascending concentrations of ethanol, and embedded in paraffin. Sections were cut at 8 μm and mounted on subbed slides. After the tissues were rehydrated, a rabbit antiserum against LHRH (R743) was applied at a dilution of 1:250. The recognition sites of antiserum R743 extend from tryptophan at position 3 to proline at position 9 of the LHRH molecule, as revealed by cross-reaction with various fragments of LHRH. Twenty-four hours later the slides were washed in tris-buffered saline containing 1 percent normal sheep serum, and the antiserum was conjugated to peroxidase-antiperoxidase with sheep antiserum to rabbit immunoglobulin G. A reaction product was formed by incubating the slides in 0.05 percent 3,3′-diaminobenzidene hydrochloride and 0.01 percent H_2O_2 in tris buffer, *p*H 7.6. For controls, the primary antiserum was replaced with (i) normal rabbit serum, (ii) primary antiserum absorbed with synthetic LHRH for 24 hours, or (iii) tris-buffered saline. The tissues were then dehydrated and examined with a light microscope.

The reaction product was clearly visible in the cytoplasm of the interstitial cells of Leydig (Fig. 1) and in the nuclei of spermatogonia within the seminiferous epithelium of some, but not all, of the seminiferous tubules (Fig. 2). The nuclei of the Leydig cells were negative; the cytoplasm of the spermatogonial cells was also negative whether or not their nuclei stained. In other seminiferous tubules, nuclei of spermatogonial cells located in the same position as the positive nuclei described above were not stained. No reaction product was observed within the interstitial blood vessels or connective tissue elements. Both the nuclei and cytoplasm of Sertoli cells and of the more mature cells of the germ-cell line located closer to the lumina of the seminiferous tubules were negative. No reaction product was observed in tissues that had been incubated in the absorbed antiserum, normal sheep serum, or tris-buffered saline.

These data confirm our earlier finding of a testicular LHRH-like substance, as indicated by radioimmunoassay with antiserum R743 (*8*). We now report that this substance is located only in certain cell types, and that it is distributed within different intracellular components of these different cell types. The immunopositive material is probably not the decapeptide LHRH. Since R743 is directed toward the amino acid sequence from positions 3 through 9 of LHRH, any compound within the testes having this sequence of amino acid residues would be detected by this antiserum. We have also assayed testicular extracts with other LHRH antiserums having different recognition sites (*8*). Antiserums that require either the COOH- or NH_2-terminus of the LHRH decapeptide for binding did not detect any LHRH immunoreactivity in testicular extracts, and therefore it is unlikely that the testicular LHRH-like substance is the free decapeptide.

Electron microscopy will be required to determine with which organelles, or population of organelles, the LHRH-like substance observed in the cytoplasm of Leydig cells is associated. The observed staining may indicate that the LHRH-like substance is synthesized in the cytoplasm of these cells.

The localization of the immunopositive LHRH-like substance within certain nuclei of the more basally located spermatogonial cells suggests that this sub-

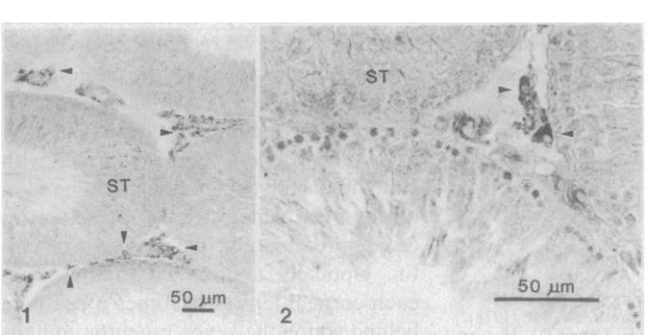

Fig. 1. Low-magnification view of a field of Leydig cells and seminiferous tubules (*ST*) in the rat testis. The Leydig cell cytoplasm is immunopositive (arrowheads), but their nuclei are immunonegative. Fig. 2. High-magnification view showing the immunopositive cytoplasm of the Leydig cells (arrowheads), and immunopositive nuclei of spermatogonia cells (asterisks) within the seminiferous tubules.

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stance may play a regulatory role in initiating or inhibiting spermatogenesis. In the rat, germ cells of the seminiferous tubules are not all at the same stage of spermatogenesis. The heterogeneous staining we observed might reflect the various stages of the cycle of the seminiferous epithelium encountered in random histological sections through the seminiferous epithelium.

It is unlikely that the LHRH-like substance is synthesized in the nuclei of the spermatogonia. The mode of action of this substance could be similar to that of androgens, which are also produced in the interstitial cells. In this paradigm, the LHRH-like substance might be synthesized in the Leydig cells and transported by diffusion, or perhaps in conjunction with a carrier molecule, to the seminiferous tubules. Upon gaining access to the germ cells it may be translocated to the nucleus. The LHRH-like substance could then affect the mitotic rate of the cell in a fashion similar to that of steroids.

The concept of a peptide gaining access to the nuclear compartment is new. Marchisio *et al.* (*11*) have recently demonstrated by immunofluorescence and autoradiographic methods that nerve growth factor can be localized within the nuclei of pheochromocytoma cells. These authors suggest that nerve growth factor may serve to form or modulate nucleation sites for pools of tubulin and actin. The testicular LHRH-like compound might serve a similar function in initiating spermatogenesis within the testes.

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Fluorescence of Photoreceptor Cells Observed in vivo

Abstract. *Most rhabdomeres in the eye of the fly* (Musca domestica) *are fluorescent. One kind of fluorescent emission emanates from a photoproduct of the visual pigment, other kinds may be ascribed to photostable pigments. These phenomena provide not only a means of spectrally mapping the retina but also a new spectroscopic tool for analyzing the primary visual processes in vivo.*

Fluorescence is not the most salient property to be expected from a visual pigment. Rather than waste excitation energy in such a "trivial" process, molecules of visual pigment would be expected to have a high efficiency for photoisomerization, as they have (*1*). When rhodopsin fluorescence was reported (*2*), it was found to have a low quantum efficiency of less than 1 percent. These considerations may explain why fluorescence methods, despite their selectivity, have not been used extensively for studying the primary steps in the visual process. But now more than a century has passed since Helmholtz first reported fluorescence of the vertebrate retina and subsequent studies have ascribed the various fluorescence colors, in part, to intermediates of visual pigment bleaching (*3*).

Using a technique of ommatidial fundus fluoroscopy applied to an intact animal, we show that retinula cells of flies may exhibit various fluorescence colors closely related to the properties both of visual pigments and of the recently discovered accessory photostable pigments contained in the rhabdomeres.

In the compound eye of diurnal insects, the receptor cells are separated from the outside world by transparent components (crystalline cone and cornea) whose total thickness rarely exceeds 0.1 mm. Taking advantage of this situation, which is encountered in no vertebrate eye, we recently devised several techniques for studying photoreceptor cell processes in live animals (*4*, *5*). One of these techniques consists of covering the "waffled" corneal surface with a medium such as nail polish, immersion oil, or even water, whose refractive index approximately matches that of chitin. Optically neutralized in this way, each corneal lenslet becomes a porthole behind which the seven receptor endings of a retinula can be viewed with a microscope (*4*).

We have combined this technique with epifluorescence microscopy (Fig. 1B), using excitations at various wavelengths within the spectral range relevant to fly vision. The basic observations we made under steady-state conditions are illustrated in Fig. 2 and summarized in Table 1. (For the numbering of receptor cells in a fly retinula, see Fig. 1F.)

When excited by blue light (400 to 500 nm), all rhabdomeres R1 to R6 of *Musca domestica* (white-eye) emit red light (Fig. 2A), the emission maximum of which ($\lambda > 620$ nm) was estimated by substituting a pupil spectroscope (Zeiss) for the microscope eyepiece. In contrast, the distal tip of the central rhabdomere R7 may exhibit three different colors: green, black (no fluorescence), or red, depending on the ommatidium.

Under ultraviolet (UV) excitation (300 to 400 nm) all R1 to R6 exhibit a pinkish color (Fig. 2B). By contrast, the three types of R7 and R8 exhibit the following colors: black, black, and pink, respectively (Table 1) (*6*).

To ensure that the emission arose from the rhabdomeres, we cut the eye with a vibrating razor blade and examined the eye stump and eye slice (Fig. 1, C to E). In all cases the characteristic red color of R1 to R6 as well as the green color of some R7's could still be observed under blue excitation. Hence, we conclude that the rhabdomere itself is an extended fluorescent light source, which, through its light-piping property, channels part of the emitted light up to the microscope.

We then examined the retinas of flies deprived of vitamin A obtained by rearing their larvae on a β-carotene and vitamin A–free Sang's synthetic medium (*7*). The fluorescence of all rhabdomeres appeared to be reduced to very low levels, suggesting that the colors observed emanate from the visual pigment, β-carotene, or vitamin A photostable derivatives, or a combination thereof.

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