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IMMUNOCYTOLOGICAL EVIDENCE OF LH-RF IN HYPOTHALAMUS AND MEDIAN EMINENCE : A REVIEW

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SUMMARY

1. The author reviews reports about the immunocytological demonstration of LH-RF in the hypothalamus and describes the materials and methods used by different groups of workers.

2. The different authors are in agreement about the localization of LH-RF axons and axonal endings.

The hypothalamo-infundibular pathway, which is the principal LH-RF neurosecretory pathway, and the accessory extra-hypophyseal pathways in guinea-pig, dog, cat and primates, and the distribution of LH-RF in the median eminence of ram, birds (cock, duck) and amphibians (toad, xenopus, triton) are described.

3. Data concerning the identification of LH-RF producing perykaria are conflicting :

a) In mice, the results of ZIMMERMAN *et al.* (1974) are not considered specific ;

b) In mice and rat, NAIK's data do not seem convincing ;

c) In mammals (guinea-pig, dog and primates : BARRY *et al.*, LEONARDELLI *et al.*) and in amphibians (DOERR-SCHOTT and DUBOIS) the results have not yet been confirmed. According to the latter, LH-RF perykaria are localized either in the mediobasal hypothalamus (primates) or in a region extending between the preoptic area and the caudal part of the tuber cinereum (guinea-pig, dog, cat) or in the posterior nuclei of the septum (xenopus, buffo).

The differences these authors observed by immunocytology in the hypothalamic perykaria of primates and other mammals agrees well with the differences in gonadotrophin release soon after deafferentation of the mediobasal hypothalamus in these two groups of mammals.

Criticisms of this type of identification of LH-RF perikarya are discussed.

The existence of « free » LH-RF which is not visualized as opposed to « bound » LH-RF demonstrated by immunohistochemistry is evoked to explain negative results obtained in attempts to demonstrate immunoreactive LH-RF perykaria in such species as the rat.

ABRÉVIATIONS

- Ab : Antibody
 IgG : Immunoglobulin G
 LH-RF : Luteinizing Hormone Releasing Factor
 BSA : Bovine Serum Albumin
 HSA : Human Serum Albumin

INTRODUCTION

At the end of 1972, an antigen immunologically related to LH-RF was demonstrated for the first time by immunofluorescence in axonal endings of the Median Eminence (M.E.). This study on the guinea-pig was made in our laboratory by LEONARDELLI *et al.* (1973).

At the beginning of 1973 BARRY *et al.*, using the same method, observed reacting cells in the hypothalamus of castrated male guinea pigs given colchicine by intracisternal way.

During the years 1973-1974, these authors published seriate reports describing this entity of pericaryons and nervous fibres in the guinea-pig in various physiological or experimental conditions. Recently, the localisation of LH-RF in other species (dog, cat, primates) has been reported (BARRY and DUBOIS, 1973 *a, b*; BARRY and DUBOIS, 1974 *a, b, c, d, e, f*; BARRY and DUBOIS, 1975; BARRY *et al.*, 1973, 1974, 1975; LEONARDELLI and DUBOIS, 1974 *a, b*; LEONARDELLI *et al.*, 1974).

Concurrently, MAZUCCA and DUBOIS (1974) in guinea-pig, KORDON *et al.* (1974), KING *et al.* (1974) in rat, BAKER *et al.* (1974) in guinea-pig and rat, ALPERT *et al.* (1975), SETALO *et al.* (1975) in rat, using immunoenzymohistochemistry, obtained identical results on the M.E. but were unsuccessful in identifying hypothalamic pericaryons synthetizing LH-RF. The report of ZIMMERMAN *et al.* (1974) using mice was strongly criticized by SETALO *et al.* (1975).

Preliminary results in cattle, sheep and pig were given by the author (DUBOIS, 1973 *a*), who described the exact arrangement of LH-RF in the M.E. of ram (DUBOIS, 1973 *b*). In 1974, relations between LH-RF, SRIF (ALPERT *et al.*, *loc. cit.*) and between LH-RF, SRIF and neurophysin (DUBOIS and BARRY, 1974) in the same area were also reported.

LH-RF was localized in the M.E. in birds by de REVIERS and DUBOIS (1974) studying cock and CALAS *et al.* (1973) studying duck.

In the lower vertebrates, DOERR-SCHOTT and DUBOIS (1974, 1975 *a, b*) reported the presence of LH-RF in the M.E. and the pars nervosa of toad, and recently localized immunoreactive cells in the encephalon of the same species. A complete distribution of the LH-RF system (pericaryons and axonal fibres) in the *Xenopus* has since been given; it seemed to be a very good model for this study. Immunoreactive axonal endings have also been seen in neotenic triton (GABRION and DUBOIS, unpublished).

PELLETIER *et al.* (1974) in rat and CALAS *et al.* (1974) in duck demonstrated reacting axons in the M.E. by ultrastructural immunocytoenzymology.

Recently, NAIK (1975 *a, b*) reported immunoreactive LH-RF neurons (pericyons and axons) in the hypothalamus of rat and mice using immunocytological methods at structural and ultrastructural levels.

MATERIALS AND METHODS

I. — Antisera

Antisera were usually obtained from rabbits. Their preparation differed with the authors (table 1). As a general rule, LH-RF (or a residue with terminal free amino-group) was linked to a protein carrier (HSA, Thyroglobulin, IgG) using glutaraldehyde, toluene diisocyanate, bis diazotized benzidine or carbodiimide.

The immunogen complex was emulsified with Freund's complete adjuvant and injected to rabbits repeatedly.

Antisera to be used were saturated with the protein carrier in order to retain only the anti LH-RF Ab.

TABLE I

Antisera used in immunocytological studies of LH-RF

Origin	Reports	Immunogen
1. DUBOIS M. P.	BARRY <i>et al.</i> LEONARDELLI <i>et al.</i> DOERR-SCHOTT <i>et al.</i> MAZUCCA <i>et al.</i> de REVIERS <i>et al.</i>	LH-RF/HSA/glutaraldehyde LH-RF/HSA/Toluene diisocyanate
2. KERDELHUE B. (KERDELHUE <i>et al.</i> , 1973)	KORDON <i>et al.</i> CALAS <i>et al.</i>	LH-RF/guinea-pig Ig./glutaraldéhyde
3. NISWENDER G. (NETT <i>et al.</i> , 1973)	ZIMMERMAN <i>et al.</i>	Tyrosine and/or histidine residue of LH-RF/BSA/Bisdiazotized Benzidine
4. ARIMURA A. (ARIMURA <i>et al.</i> , 1973, 1975)	SETALO <i>et al.</i> PELLETIER <i>et al.</i>	Glycine ¹⁰ LH-RF/BSA/carbodiimide
5. DERMODY W. C. (DERMODY <i>et al.</i> , 1973)	BAKER <i>et al.</i>	LH-RF
6. HIRSCH M.	NAIK	Not specified (unpublished)
3. NISWENDER G. 4. ARIMURA A.	KING <i>et al.</i>	Cf. 3., 4.

2. — Material

Species studied are given in table 2.

TABLE 2
Species studied by various authors

I. — Mammalians		
Guinea-pig	BARRY <i>et al.</i> (1)	1973
	BARRY <i>et al.</i> (2)	1973
	BARRY and DUBOIS	1973 <i>a, b</i>
	BARRY and DUBOIS	1974 <i>a, b, c, d, e, f</i>
	LEONARDELLI <i>et al.</i>	1973
	LEONARDELLI and DUBOIS	1974 <i>a, b</i>
	LEONARDELLI <i>et al.</i>	1974
	MAZUCCA and DUBOIS	1974
	BAKER <i>et al.</i>	1974
	Hamster	LEONARDELLI <i>et al.</i>
Rat	BARRY and DUBOIS	1973 <i>a</i>
	LEONARDELLI <i>et al.</i>	1973
	BAKER <i>et al.</i>	1974
	KING <i>et al.</i>	1974
	KORDON <i>et al.</i>	1974
	PELLETIER <i>et al.</i>	1974
	ALPERT <i>et al.</i>	1975
	NAIK	1975 <i>a, b</i>
SE TALO <i>et al.</i>	1975	
Mouse	ALPERT <i>et al.</i>	1975
	ZIMMERMAN <i>et al.</i>	1975
	NAIK	1975 <i>a, b</i>
Cat, dog	BARRY and DUBOIS	1975
Cattle (sheep, ox, pig)	DUBOIS M. P.	1973
	DUBOIS and BARRY	1974
Primates	BARRY <i>et al.</i>	1975
II. — Birds		
Duck	CALAS <i>et al.</i>	1973
	CALAS <i>et al.</i>	1974
Cock	de REVIERS and DUBOIS	1974
III. — Amphibians		
Toad	DOERR-SCHOTT and DUBOIS	1974
	DOERR-SCHOTT and DUBOIS	1975 <i>a</i>
Xenopus	DOERR-SCHOTT and DUBOIS	1975 <i>b</i>

Preparation of samples.

a) *Photonic microscopy.*

Tissues were fixed by various ways and embedded into paraffin (or polywax 1000) or frozen and cut in cryostat for the immunocytological studies.

b) *Electronic microscopy.*

After fixation, tissue was embedded in araldite and serial fine sections mounted on grids were directly treated by immunocytological methods according to MORIARTY and HALMI (1972);

TABLE 3
Methods of preparing tissue for immunocytological study used by various authors

Authors	Fixatives		Sectioning and Embedding	
	Photonic microscopy	Electronic microscopy	Photonic microscopy	Electronic microscopy
BARRY'S GROUP (BARRY, DOERR SCHOTT, DUBOIS, LEONARDELLI, de REVIERS) MAZUCCA and DUBOIS (1974)	Bouin-Holland fluid (without acetic acid) added with sublimate (0.5 p. 100 W/V) ZAMBONI (ZAMBONI and de MARTINO, 1967)		Paraffin Cryostat Cryostat	
CALAS <i>et al.</i> (1973)	Paraformaldehyde (4 p. 100 in phosphate buffer 0.1 M, pH 7.2) perfusion : 5 mn			
CALAS <i>et al.</i> (1974)		ParaF. (<i>id.</i>) Perfusion : 10 mn Postfixation : 1 h		« Vibratome » (TC2 Sorvall) : reaction on thick cuts-Postfixation (glutaraldehyde 4 p. 100 + OsO ₄ 2 p. 100). Araldite
SETALO <i>et al.</i> (1975) KORDON <i>et al.</i> (1974)	ZAMBONI (<i>loc. cit.</i>) a) Bouin-Holland fluid (Cf. BARRY'S group) b) ParaF. 4 p. 100 (in NaCl 0.9 p. 100) c) Picroformol. Perfusion + postfixation : 4 h		Polywax 1000 Paraffin Paraffin Paraffin	

TABLEAU 3 (suite)

Authors	Fixatives		Sectioning and Embedding	
	Photonic microscopy	Electronic microscopy	Photonic microscopy	Electronic microscope
ZIMMERMAN <i>et al.</i> (1974)	Bouin		Paraffin	
BAKER <i>et al.</i> (1974)	Bouin		Paraffin	
PELLETIER <i>et al.</i> (1974)		<p>a) Glutaraldéhyde (2.5 p. 100 in cacodylate buffer)</p> <p>b) Glutar. 1 p. 100 ParaF. 3 p. 100 (in the same buffer)</p>		Araldite Araldite
ALPERT <i>et al.</i> (1974)	10 p. 100 neutral buffered formalin		Paraffin	
NAÏK (1975 a)	a) ZAMBONI (<i>loc. cit.</i>) b) Bouin		Paraffin Paraffin Paraffin	
NAÏK (1975 b)		1/4 p. 100 buffered glutar.		Araldite
KING <i>et al.</i> (1974)	a) Glutaraldéhyde (3 p. 100 in cacodylate buffer 0.1 M, pH 7.4 added with 0.5 p. 100 CaCl ₂ and 5 p. 100 sucrose) b) Bouin		Paraffin Paraffin	

others fixed samples were cut into thick sections with « Vibratome », stained with reactants, postfixed in osmic acid and embedded in araldite.

Table 3 shows the methods employed by various authors for preparing tissues.

3. — *Immunocytological staining*

In all cases, the indirect reaction was used (GOLDMAN, 1968). Both methods, immunofluorescence and immunocytoenzymology, were used.

Using the later one, specific anti LH-RF Ab. was localized on antigenic structures of the preparations with sheep anti-rabbit IgG Ab linked with peroxydase either by covalence or by immunological binding (immunoglobulin-enzyme bridge technique of MASON *et al.*, 1969), or with soluble rabbit [antiperoxydase-peroxydase] complex according to STERNBERGER *et al.*, 1970.

As a general rule, the substrate used in these immunochemical tests was diaminobenzidine, with or without osmic acid poststaining.

RESULTS

There is a general agreement in the localization of LH-RF axons and axonal endings but fundamental divergencies concern the localization of LH-RF neuronal pericaryons.

I. — *Axonal ways*

In guinea-pig, most of the axons coming from the LH-RF-producing cells are incorporated in a preoptico infundibular neurosecretory tract (BARRY, *loc. cit.*). These axons terminate in close contact with the capillaries of the primary portal plexus by means of long radiating collaterals.

Some axons (or collaterals of axons) form accessory extra hypophyseal neurosecretory pathways : immunoreactive axons may be seen in various parts of the hypothalamus, epithalamus, telencephalon and mesencephalon, particularly in the supraoptic crest, the vascular organ of the lamina terminalis (less developed and smaller in guinea-pig than in rat), the amygdalic complex, the supramammillary area and the capsule of the mammillary body, beyond the terminal part of the anterior crura of the fornix, in the floor of the mesencephalodiencephalic junction, as far as the epithalamoepiphyseal area, beyond the subcommissural organ and near the habenula.

It seems that the axons terminate in the area of the supraoptic crest, in the vascular organ of the lamina terminalis and in the epithalamoepiphyseal area. These immunoreactive axons probably play a very important role in intersegmental regulations ; these mechanisms remain to be studied. The morphological substrate for these activities seems to be « neurosecretory synapses » recalling the arrangements already described for the extrahypophyseal Gomori ⊕ neurosecretory pathways (LEGAIT and LEGAIT, *cit.* by BARRY, 1961).

Some of these synapses appear to be of axosomatic type. Physiologically, their role might be to inform certain centers of the state of activity of LH-RF producing neurons.

Stereotaxic lesions performed at different levels show typical accumulation

of intraaxonal immunoreactive material above the lesion. This accumulation is less spectacular than that seen after stereotaxic interruption of catecholaminergic and serotonergic axons.

Immunoreactive LH-RF is already seen in the dorsal labium of the M.E. of 45 day guinea-pig foetus. The amount increases up to term, decreases immediately after birth and increases again a week or two later.

In adult female, after puerperium and before the first post-partum ovulation, considerable depletion of fluorescent material can be seen.

During the first post-partum cycle and in cyclic females, significant modifications can be observed in the quantity of specifically immunoreactive material along the distal part of the « preoptico infundibular LH-RF neurosecretory pathway ». A sudden drop to minimum concentrations occurs during prooestrus and preovulatory period of oestrus followed by a progressive increase during post-ovulatory period of oestrus and post-oestrus. A minimum concentration occurs towards the end of dioestrus with some individual variations.

2. In rats and mice, there is less LH-RF than in guinea-pig. In rat, the stain forms a crown around and above the peripheral portion of the pars tuberalis and the part lying between the infundibulum and the hypothalamus, and only occasionally in other portions of the external layer of the M.E. In mice, LH-RF occurs in the peripheral region of the M.E. and is located along the proximal border of the external layer.

3. In sheep, swine and cattle, the axonal endings reach close to the pars tuberalis or the capillary loops of the intercalar plexus that penetrate into the M.E.

The ram is choice material for such a study because of quasi schematic LH-RF distribution (DUBOIS, 1973 *a, b*); namely, the relation between the LH-RF fibers and the palissadic layer of the M.E. seems different in this species when compared with guinea-pig or rat. On horizontal M.E. sections, the palissadic layer of the external area is localized at the posterior half of the M.E. and the upper part of the nervous pituitary stalk. In the anterior half, very dense LH-RF axonal endings terminate close to the pars tuberalis and come into contact with the small capillaries of the intercalar plexus. In the posterior half, where the palissadic layer is inserted between the pars tuberalis and the fibrous layer of the M.E. middle zone, the major part of the LH-RF fibres remain in the fibrous layer, with dense axonal endings close to the capillary loops (short or long) of the portal plexus; only some of them perpendicularly enter the palissadic layer and insert themselves between its fibres to reach the small capillaries of the intercalar plexus placed at the precise border separating the pars tuberalis from the nervous tissue of the M.E.

4. In dog and cat (BARRY and DUBOIS, 1975), there is a well defined *tubero infundibular tract* passing by the dorsal labium of the M.E.; some of its axons reach the nervous lobe of the pituitary gland. The *preoptico infundibular tract* is less dense; its radiated collaterals electively end around the capillaries of the intercalar plexus but they stop at a distance from the long capillary loops.

5. In primates (BARRY *et al.*, 1975), reacting axonal fibres appear to constitute an hypothalamo or tubero infundibular tract; its periportal capillary endings are seen as radiated collaterals similar to those observed in different species (BARRY *et al.* 1973; BARRY and DUBOIS, 1973 *a*), but less numerous than in guinea-pig.

6. In birds, such as cock or duck, LH-RF fibres are plentiful in the external

area of the M.E. ; they usually have a radial arrangement in the palissadic layer and a longitudinal one in the reticular layer. They terminate at the primary capillaries of the portal system and are scarce in the internal layer of the M.E.

7. In amphibians, LH-RF is dense in the M.E. of toad, but its distribution is unsystematic ; LH-RF fibres in this species appear to reach the nervous lobe of the pituitary.

In the encephalon of *Xenopus*, the whole system of axons has been very well demonstrated from the pericaryons to the pituitary.

In the neotenic triton, axonal endings constitute a thin bundle arriving at the pituitary under the layer of ependymal cells that border the infundibular pouch.

II. — *Pericaryons*

1. ZIMMERMAN *et al.* (1974) thought to have demonstrated LH-RF in the pericaryons of certain cells of arcuate nucleus and ependymal tanocytes of the M.E. in mice ; their results were rejected as being unspecific by SETALO *et al.* (1975) who failed, as did KORDON *et al.* (1974), to find immunoreactive pericaryons in rat hypothalamus.

2. NAİK (1975 *a, b*) also reported he had localized immunoreactive LH-RH neurons (axons and pericaryons) in the hypothalamus at the microscopic as well as the ultrastructural level, in both rat and mice. According to this author, most of the neurons are scattered in the hypothalamus preoptic and medial prechiasmatic areas (where BARRY *et al.* (1973) found them in guinea-pig), ventro medial, arcuate nuclei and ventrolateral premamillary body. In our opinion, the pericaryon patterns given in the NAİK's report were not sufficiently demonstrated : namely, the pictures of the arcuate nucleus resembled those that KORDON *et al.* (1974) found to be artefactual. At an ultrastructural level, while the pictures of immunoreactive axons in the M.E. seemed significant and agreed with results of PELLETIER *et al.* (*loc. cit.*) we were not convinced by the pictures showing a positive reaction (according to the author) in arcuate nucleus pericaryons.

3. Using our anti LH-RF antisera, on guinea-pigs, BARRY on one hand, LEONARDELLI on the other, describe reacting pericaryons in various states :

- a) in untreated guinea-pig foetus between days 58 and 62 of gestation ;
- b) in adult castrated males, given colchicine as an inhibitor of axoplasmic flow by the intracisternal way ;
- c) in adult castrated males treated diversely : methanol or melatonin in methanolic solution in lateral ventricles ;
- d) in normal animals given serotonin (at antioviulatory doses) by intraperitoneal or intracisternal ways. In this case, posterior LH-RF producing neurons cannot be seen, but anterior neurons are accumulating reacting material ;
- e) in animals given metaraminol or diethylthiocarbamate which decreases hypothalamic dopamine and noradrenaline ; anterior LH-RF producing neurons are not affected whereas LH-RF increases in the posterior ones of the ventral tuber ;
- f) sulphirid, an antioviulatory drug, augments the content of anterior neurons in LH-RF ; its action is blocked by catecholamine inhibitors ;
- g) atropine also tends to increase the charge of LH-RH producing neurons.

Reacting pericaryons described by BARRY or by LEONARDELLI in guinea pig are located in a large field, including the preoptic and the suprachiasmatic areas, surrounding the supraoptic recessus of the IIIrd ventricle, and reaching the dorsal and median septal nuclei in front and, behind, the caudal part of the tuber cinereum; a few cells reach the premammillary area. This topography extends forward the hypophysiotropic zone of HALASZ in rat (SZENTAGOTAI *et al.*, 1968).

According to these authors, these cells compose three groups: a principal one in the prechiasmatic and suprachiasmatic areas; a pre and pericommissural group, and a third group in front at a distance from the rostral commissura. The cells have a diameter of 20-25 μ and they are ovoid or pyriform in shape. They are usually scattered but may be found in small groups of 3 to 5. Under the experimental conditions, the total number is not more than a few hundred per animal.

When the cells are stained by phenolic thionin after immunofluorescent detection, the pericaryons show a basophilic cytoplasm, a round vesicular nucleus (\varnothing 10-12 μ) and very distinct centrally located nucleolus (\varnothing 3 μ).

4. As in guinea-pig, the prepuberal stage *in dog and cat* (BARRY and DUBOIS, 1975, *loc. cit.*) favours the evidence of immunoreactive LH-RF neurons that does not need any experimental process for this purpose.

a) *In cat*, only a few pericaryons are usually conspicuous, whereas they are numerous in dog.

b) *In this species*, they are divided in two groups:

- in front in the preoptic and anterior hypothalamic areas and the septal and precommissural regions,
- in the rear side (about 40 p. 100 of the whole) in the tuberal and premammillary zones. Some of these neurons may be seen in the periventricular and dorsomedian areas and even in the rostral mesencephalic region (between the roots of the common ocular nerves or before the peduncular ganglion).

The high percentage of immunoreactive pericaryons in the infundibular and premammillary regions agrees with the data related to the hypophysiotropic area (HALASZ *et al.*, 1962), the effects of surgical deconnexion of the mediobasal hypothalamus in rodents (HALASZ and GORSKI, 1967) and the persistence of infundibular immunoreactive LH-RF after a stereotaxic lesion in guinea pig (BARRY *et al.*, 1973).

According to the data suggesting a double mechanism for the hypothalamic control of pituitary gonadotropin secretion (BARRACLOUGH, 1966), one could assume that the rostral group of LH-RF neurons in guinea-pig, cat and dog would regulate cyclic release of gonadotropins, whereas the posterior one (very evident in dog) would control basal (or tonic) secretion.

5. In primates (*Cynomolgus*, *Cynocephalus*, *Cercopithecus*, *Papio* and *Macacus*) in normal conditions, BARRY *et al.* (1975) observed most immunoreactive pericaryons electively localized in the *mediobasal* hypothalamic area (particularly in the infundibular nucleus and the premammillary zone, more rarely in the ventromedian and paraventricular areas).

So, the immunoreactive pericaryons producing LH-RF have a very different topography: in primates, they chiefly are rostral though, in guinea-pig, they may be seen from the preoptic area (BARRY *et al.*, 1973) or even from the paraolfactive region (BARRY *et al.*, 1974).

In this respect, the surgical deconnexion of the mediobasal hypothalamus produces very different results in rodents (HALASZ and GORSKI, 1967) as compared to primates (KNOBIL, 1974) : the former maintain basal gonadotropins secretion but exhibit neither cyclic LH surge nor ovulation. On the contrary, in the latter, the isolated mediobasal hypothalamus is compatible with cyclic gonadotropins release and is able to assure the negative feed-back towards oestrogens.

This supposition agrees well with recent data showing the generality of oestrogen-sensitive sites localized in the mediobasal hypothalamus of rhesus monkey (FERIN *et al.*, 1974).

6. Comparative endocrinology has put forward new arguments in favour of these results.

DOERR-SCHOTT and DUBOIS (1974), studying hypophysectomized toad, found a dense group of cells reacting with anti LH-RF Ab. These cells were localized in an accurate field next to the medioseptal nucleus in the posterior telencephalon. They were in the septum, behind the lateroseptal nucleus, and especially numerous at the posterior border of the telencephalic ventricles. The way the fibres led out from these cells indicated that they encountered the hypothalamo-hypophyseal tract (these fibres did not have the staining properties of the tract) ; they divided either next to the pars tuberalis or in the M.E.

Recently, these results were repeated by the same author on normal adult xenopus with a more demonstrative pattern of LH-RF neurons (pericaryons and axons).

DISCUSSION

As stated above, all the results from different groups concerning LH-RF axonal ways and endings are in good agreement. On the contrary, data are conflicting as regards the pericaryons.

1. SETALO *et al.* (1965) rejected the results of ZIMMERMAN *et al.* (1974) as not being specific : they observed staining of ependymal cells (as seen by ZIMMERMAN *et al.*, on their material) with a non-specific serum (as purified rabbit IgG) as well as with anti LH-RF rabbit Ab. Similarly, BAKER *et al.* (1974) did not detect LH-RF in the ependyma of any of the three species investigated : rat, mouse or guinea-pig. We agree with these conclusions.

2. Above we criticized NAÏK's results in rat and mice (*loc. cit.*) ; no other author up to now has been able to demonstrate LH-RF pericaryons in these species in such conditions.

3. There is a strong opposition to BARRY and to LEONARDELLI who identified immunoreactive cells chiefly in hypothalamus of guinea-pig. They are the only ones to study this along with other authors, principally using rats or mice (whose the demonstration of hypothalamic LH-RF neurons is not convincing). Neither BARRY's, LEONARDELLI's, nor DOERR-SCHOTT's results have been reproduced yet ; but, no other workers used amphibians or tried to place guinea-pig in the same physiological or experimental conditions as BARRY or LEONARDELLI did in order to observe significant patterns of LH-RF neurosecretion in pericaryons.

There were two principal objections to their results :

a) In questioning BARRY's and LEONARDELLI's data, KORDON *et al.* (1974) argued from their own negative results in rat (BARRY and LEONARDELLI were also unsuccessful in this species...) and from the evidence of many non specifically reacting cells and fibres in the arcuate nucleus, the septum and around the rostral commissura when they used antiguinea-pig antiglobulin. Thus, according to KORDON *et al.* (1974), the demonstration of fluorescent cells, as seen by BARRY and by LEONARDELLI, would be an artefactual reaction due either to a non specific adsorption of the reactants or to insufficiently saturated anti-HSA antibodies belonging to the anti LH-RF HSA antiserum used by BARRY and by LEONARDELLI. Further more they criticized the condition in which tissue was prepared for study by these authors (paradoxally, they did not contest the validity of results obtained in the same conditions by BARRY or LEONARDELLI in the M. E...).

This criticism is based only upon arguments drawn from their own failure and not on objective evidence :

— first, as regard the preparation of tissue for study, the evidence of results published in reports of BARRY and LEONARDELLI cannot be denied nor can the excellent correspondance with similar data on LH-RF in M.E. from other authors (including those of KORDON *et al.*...).

— Further more, neither BARRY nor other workers of this group ever used γ -globulin as a carrier for LH-RF to immunize antisera producing rabbits.

According to BARRY and our own experience, our anti LH-RF/HSA antisera or anti HSA Ab., used without HSA saturation, gave variable unreliable information which did not relate in any way to specific patterns found with anti LH-RF Ab. saturated by HSA or with complete absence of immunofluorescence using anti HSA Ab saturated by HSA. This is particularly true in the areas where KORDON *et al.*, found non specific reacting cells with their own antiserum, chiefly in the area of the arcuate nucleus. Moreover, anti LH-RH/HSA Ab. saturated by HSA or anti HSA antisera were not able to reveal reacting cells or fibers in fields where anti LH-RF/HSA Ab. saturated by HSA did.

However, from our own experience, non specific fluorescent cells may have been seen [with anti (LH-RF/HSA) Ab. saturated by HSA] in some parts of ovine, bovine or porcine encephalon, but *this fluorescence has never been inhibited when the antibody was saturated by LH-RF, so that immunoreactive fibers of the M.E. were not evident.*

On the contrary, reactions observed by BARRY or by LEONARDELLI and given as being specific on their material became negative after saturation by the LH-RF of the antiserum they used (after previous neutralisation of anti HSA Ab.).

— Using anti LH-RF antisera prepared by KERDELHUE, BARRY repeated the same trials in guinea-pig (per. comm.) and did not observe aspecific reactions with his usual method as KORDON *et al.*, did.

b) As regards the antigen we used to obtain anti LH-RF antisera (binding of LH-RF decapeptide with HSA by gluteraldehyde or toluene diisocyanate), SETALO *et al.* (1975) questioned the specificity of our antibodies, because the LH-RF decapeptide lacks a reactive free amino group that would be necessary for such conjugations.

Moreover, the purity of our LH-RF preparation not having been given in our reports, they thought « ... it is possible that some impurities of breakdown products of LH-RF with free aminogroups could have been coupled with the carrier protein :

in such a case, the antibody would have been induced against such a product ... ».

As concerns the second point, table 4 gives the characteristics of the product we used.

TABLE 4
Biochemical characterisation of LH-RF

I. — <i>Aminoacid analysis</i>		<i>Short column run</i>	
Serine	0.97	Tryptophan	1.16
<i>p</i> -Glutamic	1.06	Histidine	0.93
Proline	0.99	Arginine	0.91
Glycine	2.12		
Leucine	1.02		
Tyrosine	0.87		
Histidine	1.02		
Arginine.....	0.97		

II. — *Bioassay* (Dr. David Coy ⁽¹⁾)

(Stimulation *in vivo* of LH release by the sample : serum LH levels measured by RIA were compared with those obtained from dose of standard LH-RF).

Activity of the sample : 109 p. 100 (68-176 p. 100 at 95 p. 100 confidence limits).

⁽¹⁾ Dr. SCHALLEY's Group : Veterans Administration Hospital, North Carolina.

In regards to the first point, HABEED and HIRAMOTO (1968) reported that, if glutaraldehyde preferably reacts with free amino groups (to 80-90 p. 100), it also reacts with the imidazol ring of histidine (30-40 p. 100) and the phenolic ring of tyrosine (20 p. 100) when tyrosine is linked with glycine ; such a condition is met by LH-RF (C₂-histidine, C₆-tyrosine-C₆-glycine). In our tests, ¹²⁵I-LH-RF was incorporated at a 23 p. 100 level.

SCHICK and SINGER (1961), using toluene diisocyanate, called up the reactions of the isocyanate group as well with (OH)⁻ as with (—NH²) : serine with its primary alcohol group and the phenol (OH) of tyrosin may be related to this type of conjugation In our tests, ¹²⁵I-LH-RF was incorporated at a 30 p. 100 level.

CONCLUSION

Determination of the chemical formula of LH-RF (MATSUO *et al.*, 1971 ; SCHALLY *et al.*, 1971 ; SIEVERTSSON *et al.*, 1971) and its synthesis have made possible the preparation of specific antibodies. By the use of these, the hypothalamic structures involved in the synthesis, transport and transfer (to blood or cerebrospinal fluid) of LH-RF can be demonstrated by immunofluorescence or by cytoimmunoenzymology.

The presence of axons carrying LH-RF, particularly in the distal regions of the hypothalamus, can easily be shown in vertebrates (with the exception of reptiles and fish). However, visualization of specifically immunoreactive perykaria raises many difficulties. Only in certain species, in particular physiological states and under favourable experimental conditions is it possible to observe reasonable numbers of specifically immunoreactive perykaria when proper controls are used.

The results presented are the work of a single school and therefore need confirmation. This work shows that the distribution of perykaria varies with the species. It is most marked in the mediobasal hypothalamus in the primates, but there are two groups of perykaria, anterior (in the septopreoptic area and anterior hypothalamus) and posterior (tubero-infundibular and premamillary) in the guinea-pig, dog and cat, whereas they are concentrated in the posterior septal zone in *Xenopus* and the common toad.

These different distributions correspond, at least in rodents and primates, with the differences observed after surgical deafferentation of the mediobasal hypothalamus in these animals (HALASZ and GORSKI, 1967; KNOBIL, 1974).

In the guinea-pig, dog and cat, the distribution corresponds to the hypophysiotrophic area of HALASZ (HALASZ, PUPP and UHLARIK, 1962) but extends beyond it, particularly anteriorly. In these species the division of perykaria into two groups of cells which react differently to pharmacological agents leads one to suppose, in conformity with the hypothesis of a double mode of control of the secretion of anterior hypophyseal gonadotrophin (BARRACLOUGH, 1966), that the anterior group is involved in the cyclic discharge of gonadotrophins whereas the posterior group controls the basal or tonic secretion.

The completely different behaviour of the primate hypothalamus after mediobasal deafferentation agrees with the accumulation of LH-RH immunoreactive neurons and the oestrogen-sensitive sites in this region (KNOBIL, 1974; FERIN *et al.*, 1974).

In the visualization of immunoreactive axons, immunocytochemistry allows one to follow the effects of lesions of the LH-RF pathways after deafferentation of whole or part of the hypothalamus. It also demonstrates, in axosomatic synapses, unsuspected connections between the hypothalamus and various other parts of the brain. These links underline the *neuromodulator* role of LH-RF which is more than just the excretion of a gonadotrophin-releasing substance into the pituitary portal vessels.

The difficulties encountered in the visualization of perykaria synthesizing LH-RF in certain species, such as the rat, have yet to be explained. One must also question whether the immunohistochemical techniques used do not remove some of the neuropeptide from the structures which contain it. Perhaps a larger or smaller proportion of a *free* fraction (as opposed to a *bound* fraction probably occurring in cytoplasmic granules readily visible in nerve endings and therefore easily immobilized), varying in amount in different species, is lost at different times during the technical procedures used.

Perhaps there are also masked forms of LH-RF which are not susceptible to immunocytochemical detection.

Further research is needed in order to define the topographical difference in the distribution of LH-RF neurons in the various classes and species of vertebrate and to explain these differences.

RÉSUMÉ

MISE EN ÉVIDENCE DU LH-RF DANS L'HYPOTHALAMUS
ET L'ÉMINENCE MÉDIANE PAR IMMUNOCYTOLOGIE : REVUE

1. Après un historique des travaux consacrés à la détection immunocytoologique du LH-RF, sont exposées les conditions d'obtention des antisérums utilisés par les différents groupes de recherches, les espèces étudiées, les modalités techniques de la réaction immunocytoologique en microscopie photonique et électronique selon les différents auteurs.

2. Les résultats séparent ce qui a trait à la mise en évidence des fibres nerveuses à LH-RF et des extrémités axonales d'une part, des péricaryons d'autre part.

A. — En ce qui concerne le premier point, les résultats présentés par les différents auteurs sont dans l'ensemble concordants.

a) La voie neurosecrétrice preoptico-infundibulaire à LH-RF du cobaye a été bien étudiée par BARRY *et al.*, ainsi que les voies accessoires extrahypophysaires, s'achevant principalement au niveau de la crête supraoptique, de l'organe vasculaire de la lame terminale et de l'aire épithalamoépiphytaire. Les variations physiologiques de la charge en LH-RF de l'éminence médiane (E.M.) où s'achève la majorité des fibres à LH-RF sont exposées.

b) Dans l'E.M. du Rat et de la Souris, l'accumulation de LH-RF est relativement moins importante.

c) La répartition de LH-RF dans l'E.M. du Bélier a été étudiée plus spécialement par l'auteur qui en précise la disposition quasi schématique : le LH-RF s'accumule au contact direct des capillaires du plexus intercalaire jouxtant la pars tuberalis hypophysaire dans la partie rostrale de l'E.M., mais au niveau de la partie caudale, il n'atteint ceux-ci que par l'intermédiaire de fibres perpendiculaires espacées traversant la couche palissadique de la zone interne ou s'accumule autour des anses courtes et longues de ce même plexus.

d) La distribution des fibres et extrémités axonales à LH-RF chez le Chien, le Chat, les primates, les oiseaux (coq, canard), les amphibiens (crapaud, xénope, triton) est mentionnée.

B. — La mise en évidence des péricaryons aboutit à des résultats contestés.

a) Chez la Souris, les résultats de ZIMMERMAN *et al.* (1974) sont rejetés comme dénués de spécificité ;

b) ceux de NAÏK (1975) chez le Rat et la Souris apparaissent non convaincants ;

c) les résultats obtenus par le groupe de BARRY et LEONARDELLI chez le Cobaye, le Chien, le Chat et les primates et par DOERR-SCHOTT sur les amphibiens n'ont pas encore été confirmés. Pour ces auteurs, la répartition des pericaryons apparaît variable selon les espèces : dans l'hypothalamus médiobasal chez les primates, séparés en deux groupes, antérieurs et postérieurs, au sein d'une zone allant de l'aire préoptique à l'extrémité caudale du tuber cinereum chez le Cobaye, le Chien et le Chat, au niveau des noyaux postérieurs du septum chez le Crapaud et le Xénope. Ces résultats obtenus chez les mammifères s'accordent avec ceux tirés de la déconnexion chirurgicale de l'hypothalamus médiobasal dans ces mêmes espèces.

Les critiques opposées à cette identification des péricaryons synthétisant le LH-RF sont discutées.

Enfin, l'existence d'un LH-RF « libre » ou « masqué » s'opposant au LH-RF « lié » est évoquée pour expliquer les difficultés éprouvées dans la mise en évidence du décapeptide chez certaines espèces, dont le Rat.

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Abbreviations

- ME : Median eminence
PT : Pars tuberalis
PL : Palissadic layer
DL : M.E. dorsal labium
VL : M.E. ventral labium
SCL : Short capillary loop
CV : Capillary vessel
LV : Lateral ventricle.

PLATE I

Ram. Median Eminence. Parasagittal plane
(Immunofluorescence ; poststaining by Evans Blue)

Strong heaping up of LH-RF axonal endings (arrows) close to pars tuberalis in rostral pars of dorsal and ventral labia, in front of the anterior limit of the palissadic layer (Mag. \times 250).

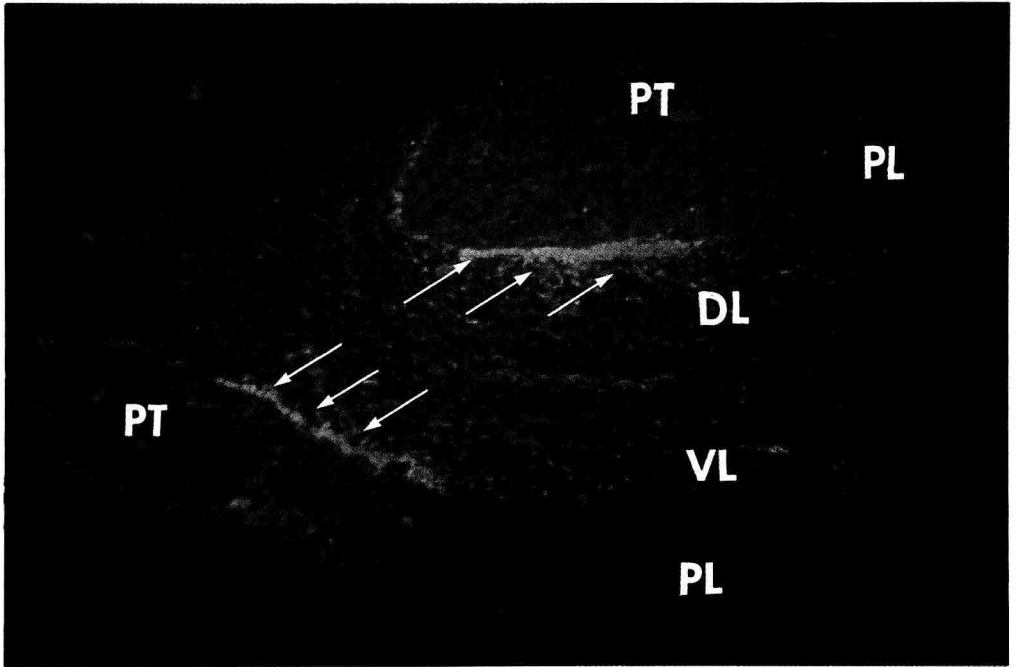


PLATE II

Above : Ram. M.E. horizontal section (immunofluorescence).

Strong accumulation of LH-RF axonal endings in M.E. rostral pars, close to pars tuberalis (in this region, palissadic layer is absent) (Mag. \times 250).

Below : Ram. M.E. frontal section (immunofluorescence)

In palissadic layer LH-RF axons end at a short capillary loop and small capillary vessels (arrows) of intercalar plexus (at the junction separating M.E. from pars tuberalis). Only a few fibres cross palissadic layer (compare with the upper figure) (Mag. \times 1000).

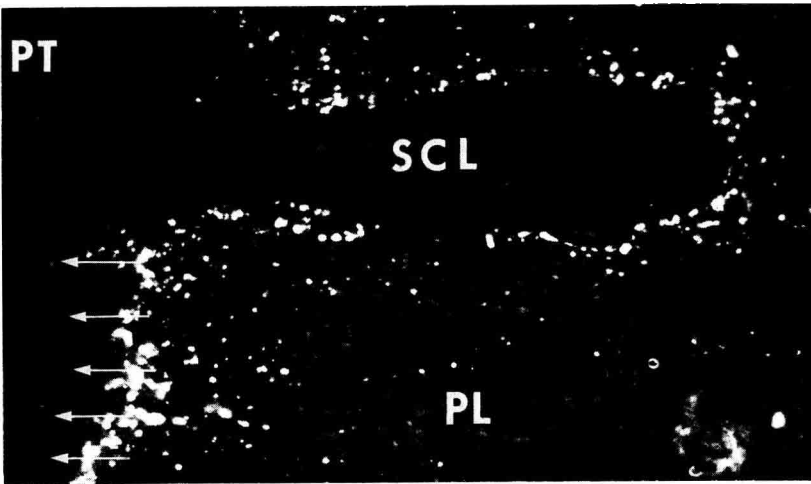
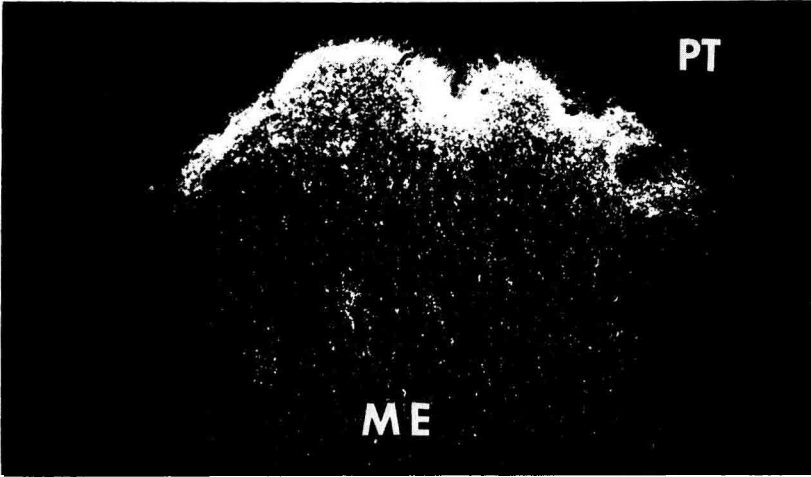


PLATE III

Above : *Cock. M. E.* (Immunofluorescence).

LH-RF axons and axonal endings (de REVIERS & DUBOIS)
(Mag. \times 250).

Below ; *Hypophysectomized buffo. Telencephalon* (Immunofluorescence).

Group of LH-RF perykaria (arrows) in the septum next to lateral ventricle (Mag. \times 1,000). (Courtesy of Dr. J. DÖRR-SCHOTT.)

