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CYTO-IMMUNOLOGICAL STUDY OF THE ONTOGENESIS OF THE GONADOTROPIC HYPOTHALAMO-PITUITARY AXIS IN THE HUMAN FETUS

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SUMMARY

Using anti-LH-RH immune serum, LH-RH producing neurons were characterized in the hypothalamus from the 13th week of fetal life up to birth. The immunoreactive perikarya are concentrated in mediobasal, premammillary and anterior hypothalamus (lamina terminalis and rostral commissure region) and their axons contribute to the tubero infundibular tract.

They terminate close to the blood vessels of the Mantelplexus before and after the apparition of the intra eminential loops (at the 16th week); immunoreactive fibres terminate also around the capillaries of the vascular organ of the lamina terminalis and near ependymal epithelium.

In the pituitary, gonadotropic and thyrotropic cells were immunocytologically demonstrated by anti-FSH absorbed with-HCG and LH, and anti-LH, anti-HCG and anti-TSH absorbed or not with α LH.

Only α subunits were identified from the 8th to the 12th week. From the 13th week, β TSH subunits appear in the thyrotrophs. From the 15th week, β FSH and β LH subunits responsible for biological activity were revealed in the same gonadotropic cells; the role of the human fetal hypothalamus in the differentiation of gonadotropic cells is discussed.

INTRODUCTION

The problems concerning the ontogenesis of the human gonadotropic hypothalamo-pituitary axis are of great complexity. They deal with:

1. The differentiation and the activation of gonadotropic pituitary cells, of the cholinergic and monoaminergic pathways, and of the hypothalamic LH-RH producing neurons.

2. The development of morphological and physiological neurovascular relations between the hypothalamus and the anterior pituitary.

3. The development of the different regulating mechanisms of the "feedbacks".

These various aspects have been studied in fetuses of different ages by numerous methods such as:

1. morphological and cytological studies [1, 8, 9, 15, 16, 22].

2. biological and immunological dosages of the gonadotropic hormones, in the pituitary [18, 27], in the blood [18, 27] or produced *in vitro* by pituitary cultures [25, 30, 32, 34].

3. radioimmunological [35] or biological [24, 30] dosages of the LH-RH in the hypothalamus.

4. study of the effect of the LH-RH in fetal pituitary cultures [32, 34].

More recently, cyto-immunological techniques have turned out to be interesting for the study of the hormonal production of the cells of the human fetal pituitary [1, 9, 10, 11, 15, 16, 19, 32].

For our part we have been carrying out research for many years dealing with the morphological and cytological aspects of the development of the human hypothalamo-pituitary complex. We have applied cyto-immunological techniques not only to the study of the various cell types of the pituitary, such as corticomelanotrophs [9, 10, 11, 16], somatotrophs, gonadotrophs, thyrotrophs [15, 16], but also to the hypothalamic LH-RH producing neurons [13, 14] and somatostatin producing cells [17] during the fetal period in the human.

Cytoimmunological techniques provide many advantages in the field of fetal neuroendocrinology. They enable, due to their sensibility, the early detection of very small quantities of hormones; moreover they represent the only means that permit a topochemical localization of the pituitary glandular cells or hypothalamic neuroglandular cells that produce hormones.

This study is composed of two parts: The first one deals with our research into the anti-LH-RH immunoreactive neurons and their fibres which are present as early as the thirteenth week of the human fetal life. We shall describe the morphology and the localization of these neurons, the pathway and the destination of their fibres, particularly those of the hypothalamo-infundibular "tract", as well as the variations in the amount of immunoreactive material. The second part deals with the immunocytological study of the maturation of the pituitary cells producing glycoproteic gonadotropic (FSH and LH) and thyrotrophic hormones (TSH). The comparison of these two types of results about the hypothalamus and the pituitary has enabled us to put forward original data concerning the maturation and the functional aspect of the human fetal gonadotropic axis.

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Table 1. Fetuses studied

Fetal age in weeks	Sex	Weight in grammes	Crown rump length in mm.	Delay between death and fixation
10	F	—	—	8 h
12‡	F	70	100	1/2 h
13‡	M	100	105	1/2 h
16*	F	240	130	1/2 h
16‡	F	250	135	3 h
16‡	F	250	135	3 h
17‡	F	270	155	3 h
19	F	310	160	10 h
21‡	M	450	195	12 h
22‡	M	500	180	1 h
23	M	480	180	12 h
24†	M	880	—	1/2 h
25	M	920	220	10 h
36	M	1700	—	2 h
40	M	3500	—	12 h
40	M	2600	—	12 h
40	F	2900	—	3 h

* brain fixed in Zamboni fluid and embedded in araldite
† brain treated according to Falck and Hillarp technique
‡ fetuses whose pituitary was also studied.

MATERIAL AND METHODS

Our research was carried out on three newborn infants and 30 fetuses whose ages ranged from 6 to 36 weeks. In some of them, we only studied the pituitary: in others, the hypothalamus; both were studied in six of them. The fetal age was determined according to the estimated date of fecondation*, the body weight and the crown rump length. All the subjects were spontaneously aborted alive or extracted alive by hysterotomy.

1. *Immunocytological study of LH-RH producing neurons*

A. Fetuses

The brains were dissected with delays varying from ½ h to 12 h after death. The hypothalamus of the 24 week old male fetus was lyophylized, treated with paraformaldehyde vapours, according to the Falck and Hillarp technique, embedded in paraffin and cut sagitally from 5 to 10 μm. The hypothalamus of one of the 16 week old female fetuses was cut in small pieces, which were fixed in Zamboni fluid [formaldehyde and picric acid in phosphate buffer (PAF)] for 5 h, embedded in araldite, and cut at 3 μm. All the other hypothalamus were fixed in Bouin Hollande (without acetic acid) for 7 days, then embedded in paraffin and cut sagitally from 5 to 10 μm.

One section out of three was treated, for 1–3 h, in a moist chamber by immuno-fluorescence (I.F.) or immunoenzymatic (I.E.) technique in PBS buffer pH 7.2, 0.01 M, with the specific rabbit anti LH-RH γ globulins diluted to 1/20. After washing in buffer, the anti-LH-RH was revealed with sheep anti-rabbit γ-globulins conjugated either with fluoresceine isothiocyanate (Pasteur Institute, final dilution 1/50) or .peroxydase (Pasteur Institute final dilution 1/10). The substrate of the latter was diaminobenzidine and hydrogen peroxyde.

B. Immune serum (I.S.)

Preparation. Synthetic LH-RH† was conjugated with human serum albumin by glutaraldehyde according to the Barry and Dubois's technical scheme [29]. After preparation, the conjugate was divided into unit doses corresponding to 0.4 mg of synthetic LH-RH that were stored frozen at −20°C until use. Immunization carried out on four rabbits, was performed by injecting, one unit dose emulsified in the same vol. of Freund's complete adjuvant‡ and divided into 40 intradermal injections in the back and in the roots of the limbs. These injections were repeated every 10 days. An intravenous dose of the conjugate alone was given one month after the fifth injection; Each animal received 2.4 mg of LH-RH. Blood was collected every 10 days after the last injection.

Purification. The antibodies reacting with human serum albumin were precipitated by adding human serum albumin and discarded by centrifugation. γ globulins were isolated from the supernatant by a double precipitation with ammonium sulfate at 50% saturation, dialysed for 48 h against NaCl gr/l and stored frozen until use.

Criteria of specificity. Every rabbit has produced antiserum giving significant binding of LH-RH by radioimmunoassay to a dilution varying from 1/30,000 to 1/40,000 according to the rabbit.§ Immunocytological tests were performed on hypothalamus of the fox, cat, dog, guinea pig, [12] and human fetus; immuno-reactive axons and perikarya (but only for the guinea pig and human fetus) were seen, even when anti LH-RH was diluted up to 1/200. All the specific immunocytochemical stainings were abolished by prior addition of LH-RH to the immune serum (0.2 mg/ml of pure serum). No extinction of the staining was observed by adding somato-satin, or TRH (up to 1 mg/ml) or human serum albumin. No staining was obtained in the five species on sections treated with anti-human serum albumin or anti-ACTH, instead of anti-LH-RH or with fluoresceine or peroxydase conjugated anti-rabbit γ globulins alone.

2. *Immunocytological study of gonadotrophs*

A. Fetuses

Pituitaries, excised within two h of death, were fixed either uncut or cut, according to size, in Zamboni fluid [picric acid and formaldehyde in phosphate buffer (PAF)], dehydrated, embedded in araldite and

* By subtracting fifteen days from the date of the last menstrual period.
† Roussel laboratories.
‡ Difco laboratories.
§ Dosage made by Mr. Kerdelhue, Laboratoire des Hormones polypeptidiques (C.N.R.S. gif sur Yvette).

Table 2. Fetuses studied

Age in weeks	Sex	Weight in grams	Crown rump length in mm.
6	—	—	—
8	M	—	—
8	—	—	—
8	—	—	—
8	F	—	—
9	—	—	—
10	M	—	—
11	F	—	—
12*	F	70	100
13*	M	100	105
15	M	200	—
15	F	210	—
16	F	240	130
16*	F	250	—
16*	F	250	—
17*	F	270	155
21*	F	450	195
22*	M	500	180
26	M	710	—

* fetuses whose hypothalamus was also studied.

cut into serial semi-thin sections of one or two μm. After removing araldite with sodium methoxide, the sections were treated either by I.F. or I.E. with various immune sera directed against human hormones.

B. Immune sera*

We used anti-HCG, anti-human LH, and anti-human TSH, all of them crude, and anti-FSH absorbed with HCG. These antisera were submitted to tests of inhibition with the corresponding antigens on semi-thin sections. Moreover we have absorbed anti-LH and anti-HCG with α LH† (1 mg/ml to make them β LH specific) and we have also done the same with anti-TSH to make it β TSH specific by saturation of the anti-α-subunit antibodies.

Anti-LH and anti-TSH were used diluted to 1/100; anti-HCG and anti-FSH absorbed with HCG were used diluted to 1/50. Each of the antisera both unabsorbed and absorbed was applied on at least 2 semi-thin sections of each of the pituitaries.

Table 3. Tests of inhibition

I.S. \ Antigens	Human-LH 300 μg/ml	Human-TSH 300 μg/ml	Human-FSH 300 μg/ml
anti-HCG	−	+	+
anti-LH	−	+	+
anti-TSH	+	−	+
anti-FSH absorbed with HCG	+	+	−

− total inhibition of the immunocytological reactions
+ lack of total inhibition of the immunocytological reactions.

* Immune sera furnished by the National Institute of Health.

† Furnished by the Dr. Bangham, (Medical Research Council London).

RESULTS

1. *LH-RH peptidergic system*

a. *Morphology of the neurons.* Immunoreactive material, specifically reacting with anti-LH-RH immune serum, has been revealed in the perikarya and fibres in each of all the hypothalamus from the thirteenth week up to birth. The great amount of this material enables the morphological, numerical and topographical study of the perikarya as well as the aspect and trajectory of the fibres. The neurons present important variations in the intensity of the immunocytochemical reaction, probably corresponding to differences in the charge of intracytoplasmic immunoreactive material. They are of great size, ovoid or pyriform, sometimes of unipolar or bipolar aspect with a thin and varicose axon and eventually with a wide and thick dendrite (Fig. 1, photos 4 and 5).

Immunoenzymatic technique with peroxydase conjugate brings out the granular aspect of the immunoreactive intra-cytoplasmic material (Fig. 1 photos 6 and 7). Sometimes, the Golgi area appears unstained near the nucleus, and is frequently directed towards the departure of the axon. With Herlant's tetrachrome applied after I.F., immunoreactive neurons present a cyanophyl and granular cytoplasm [13].

b. *Topography and abundance of the neurons.* In order to establish the topography of these neurons and estimate their number, we have studied one section out of three by immuno-cytology. We have observed great variations in the number of the neurons according to the fetuses which are most likely due to their LH-RH physiological charge but also to the variations in the quality of the immunocytological results in a particular biological material. The histological quality of this material depends on the variations in the delay between death and fixation. Moreover we consider that some difficulties in the staining are due to the fixation (duration, size of blocks varying with the fetal age) and to the thickness of the sections, both of them taking part in the penetration of globulins in nervous structure containing LH-RH.

We have observed from about ten to more than one hundred and fifty immunoreactive neurons according to the hypothalamus. In the thirteen week old male fetus, we have revealed more than 40 of them, and more than one hundred in each of the sixteen week old female fetuses. They are still numerous at the ninetenth week. They seem to be less numerous from the twenty first week up to birth but their number increases during the perinatal period and we have counted more than sixty of them in some of the newborn infants.

We have established the topography of the anti-LH-RH immunoreactive neurons from our observations in the hypothalami where they were the more numerous. We have observed, in areas of highest concentration, up to six cells for each 5 micron section

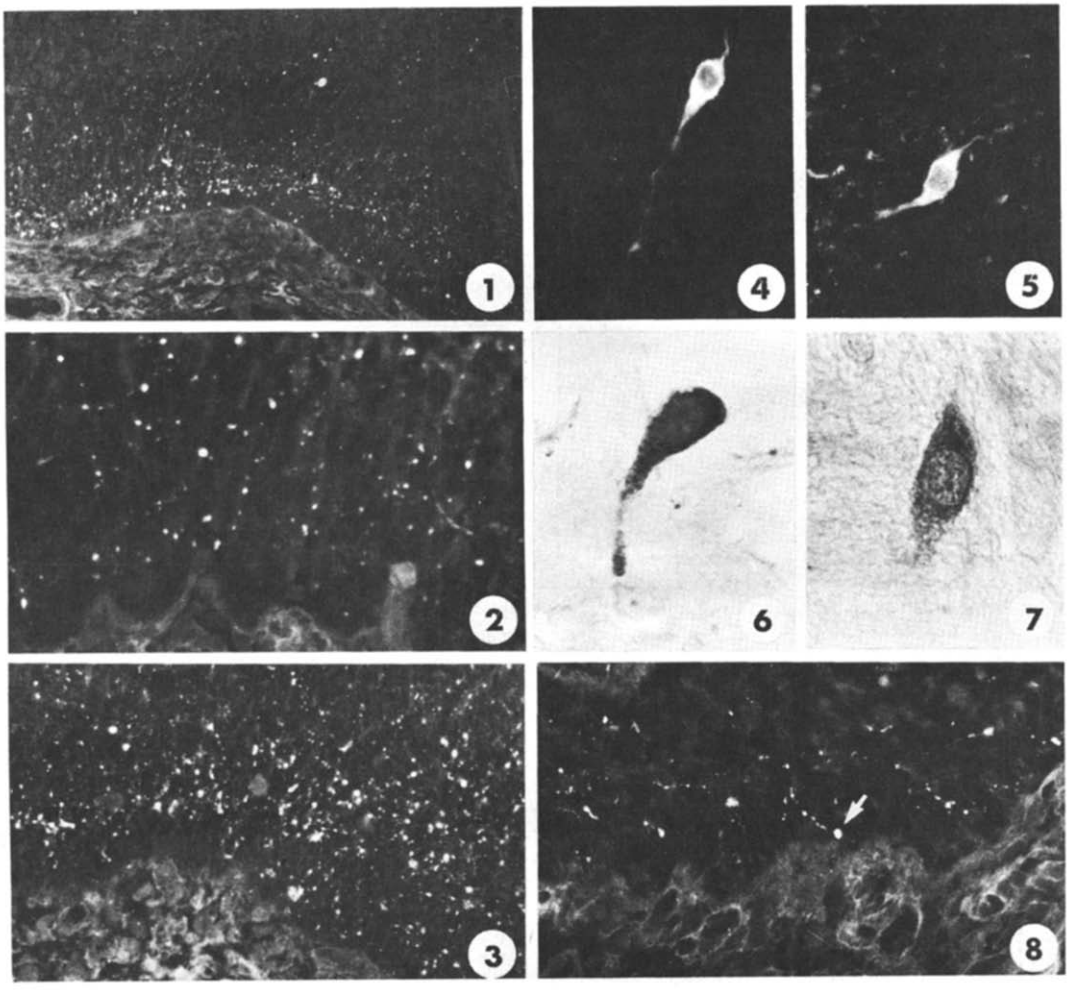

Fig. 1. Immunoreactive fibres and perikarya with anti LH RH (16 week old female fetuses). Immunofluorescence (I.F.) or immunoenzymology (I.E.). Semi-thin sections of PAF fixed hypothalamus (photos 1, 2, 3, 6 and 8). 5 μ sections of paraffin embedded tissue after Bouin Hollande fixation (photos 4, 5 and 7). Photos 1–3: nodular aspect, abundance and orientation of the I.F. fibres in the posterior lip of the median eminence in 3 different semi-thin sections; low magnification: photo 1; higher magnification: photos 2, 3. Note the direction of the fibres and of their radiating collaterals near the periphery of the M.E. and the blood vessels of the Mantelplexus.

studied. These LH-RH producing neurons are never grouped in a nucleus, as such, but scattered in several preferential areas of dispersion in some median areas of the anterior, middle and posterior hypothalamus (Fig. 4). They are numerous in the anterior hypothalamus and are scattered before and above the rostral commissure and even more above it in the septal region. They can be found in great number, in several fetuses, in the lamina terminalis where they are preferentially located in the external cellular layer behind the capillary net of the vascular organ that is very developed in the human [20, 21]. In the mediobasal hypothalamus, they remain in the lateral walls of the infundibulum and in the posterior lip of the median eminence (the pars caudalis tuberi being much developed in the human) where there originates an important bundle of hypothalamo-infundibular fibres. They are less numerous just behind the optic chiasma (the pars oralis tuberi being little developed in the human). Finally, in the posterior hypothalamus, some of them are dispersed between the premammillary recess and the mammillary bodies, though they are less numerous above and behind these structures.

Immunoreactive fibres are difficult to reveal in sections of paraffin embedded tissue, particularly when these sections are thick (10 μm); this explains their apparent small number, though they can be observed as early as the 13th week. Our studies on 3 μ semi-thin sections of araldite embedded tissue have revealed the very great number of fibres in the various areas where we had previously observed only a small number of them in sections of paraffin embedded tissue. Most of the time these fibres appear to be very thin, with a nodular aspect, though exceptionally they appear to be widened, with a varicose aspect. They form various contingents: the most important of these

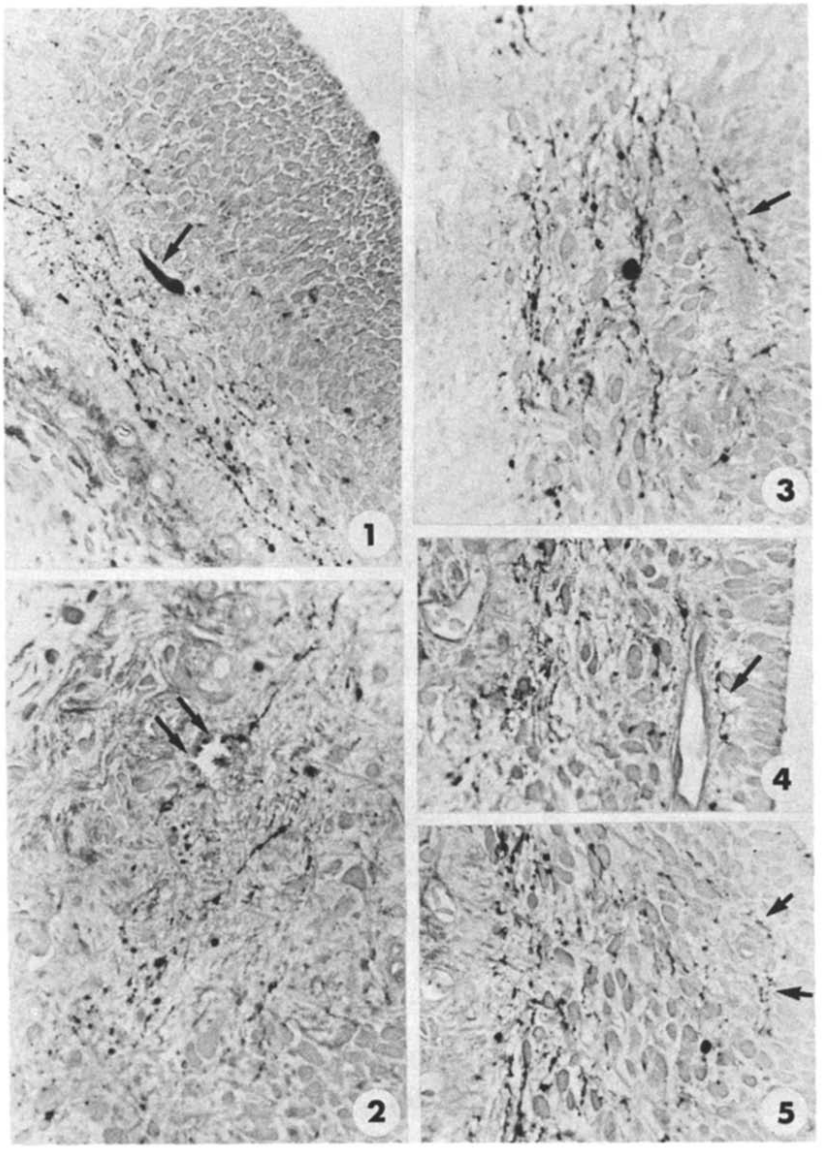

Fig. 2. Immunoreactive (I.R.) fibres with anti-LH-RH I.S. in the lamina terminalis (L.T.) (16 week old female fetus). Immunoenzymatic techniques: in semi-thin section after PAF fixation. Photo 1: fibre bundle aspect in the external layer of the L.T. Note a cell (arrow). Photo 2: Endings (arrows) close to a capillary of the vascular organ of the L.T. Photos 3 and 4: perivascular fibres (arrows) near the ependymal epithelium; tangential (photo 3) or transversal (photo 4) section of the capillary. Photo 5: aspects of I.R. fibres (arrows) in the ependymal epithelium.

go from the hypothalamus to the infundibulum, particularly into the posterior lip of the median eminence, whereas the fibres are few in number in the anterior and lateral areas of the median eminence (Fig. 1, photos. 1, 2, 3). These fibres give rise to radiating collaterals which go perpendiculary to the stem axis, towards the blood vessels of the Mantelplexus where they terminate against the capillaries by widened endings filled with immunoreactive material (Fig. 1, photo. 8). From the sixteenth week, some fibres terminate close to the intra-eminential loops of the primary plexus. These fibres are also present in the lamina terminalis; we have observed only a few of them in sections after paraffin embedding; in semi-thin sections of lamina terminalis of the sixteen-week-old fetus, we have shown clearly an important bundle of fibres parallel to the main axis of the lamina terminalis, in its external layer. This gives rise to collaterals that terminate close to the deep capillaries of the vascular organ. A few of them terminate with widened endings in the ependymal epithelium (Fig. 2).

Some fibres make their way towards the epithalamus and hypocampic area, probably coming from LH-RH producing neurons of the anterior hypothalamus. Others go towards the mesencephalon probably arising from neurons of the premammillary areas.

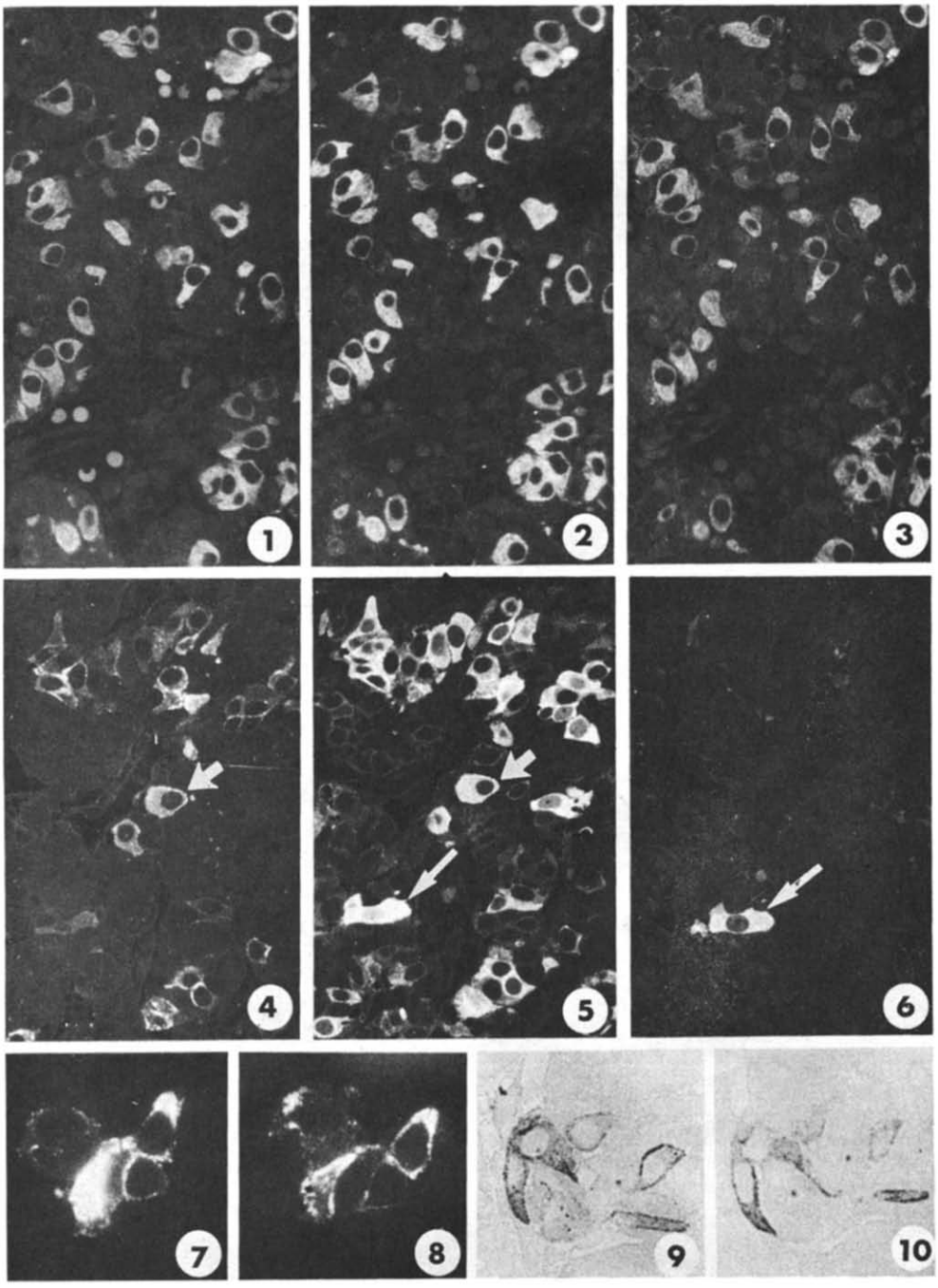

Fig. 3. Immunoreactive (I.R.) gonadotrophs and thyrotrophs in the human fetal pituitary (16 week old female fetuses). Semi-thin sections; PAF fixation. Immunofluorescence (I.F.) (photos 1–8). Immunoenzymology (I.E.) (photos 9, 10). Photos 1–3: indifferent staining by anti HCG (photo 1) by anti-LH (photo 2), by anti-TSH (photo 3) of the glycoproteic hormone containing cells (gonadotrophs and thyrotrophs) in serial semi-thin sections. Anti α subunit antibodies are responsible for this staining that does not permit a differentiation between thyrotrophs and gonadotrophs. Photos 4–6: Many glycoproteic hormone containing cells are stained by the anti-TSH immune serum (photo 5) but only one thyrotroph cell (long arrow) is revealed by immuneserum (I.S.) specific for β TSH subunit in the adjacent semi-thin section (photo 6). The β LH specific I.S. reveals only the cells that are gonadotrophs (photo 4) (small arrow). Photos 7 and 8: adjacent sections of the same gonadotrophs indifferently revealed with β LH specific I.S. (photo 7) or one specific for β FSH (photo 8). Photos 9 and 10: the same results as those above with I.E. technique. With β LH specific I.S. (photo 9) and β FSH specific I.S. (photo 10).

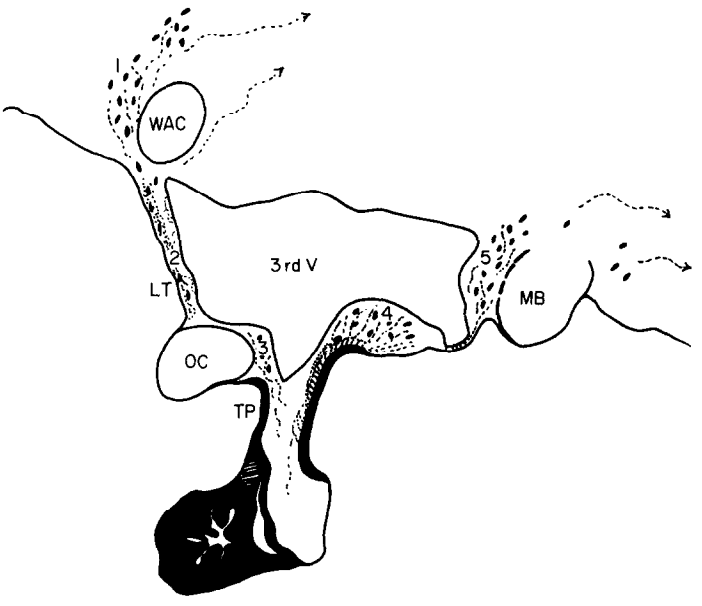

Fig. 4. Topography of the LH-RH producing perikarya and their fibres in the human fetal hypothalamus. Perikarya are localized in several hypothalamic areas such as: The septopreoptical area: (the septum, before and above the white anterior commissure (1), in the lamina terminalis and its surroundings (2)). The mediobasal area: behind the optic chiasma (3) but also above the posterior lip of the median eminence (4). The premammillary area (5). Fibres are grouped in various bundles which are essentially in the hypothalamo-infundibular area. The most important of them makes its way in the floor of the posterior lip and terminates close to the vessels of the Mantelplexus. The extra hypophyseal fibres form themselves into a contingent in the lamina terminalis where they terminate around the capillaries of the vascular organ in contact with or inside ependymal epithelium (+). Some fibres make their way towards the hypothalamus and others towards the mesencephalon (arrows).

2. *Gonadotropic and thyrotropic cells*

All our results are summarized in Table 4. In the sixth week, no cell could be revealed by the various antisera. As early as the 8th week of the development, a few cells, scattered in the epithelial cords, were revealed by anti-HCG, anti-LH, and anti-TSH but no staining was observed by the same antisera absorbed with α LH (specific for the β LH or the β TSH subunits).

The examination of the homologous fields of consecutive semi-thin sections treated by the anti-TSH, anti-HCG, and anti-LH has shown that the same cells were indifferently revealed by the three I.S. which do not permit any immunocytological distinction of gonadotrophs and thyrotrophs. From the 8th to the 12th week, these cells increased in number but immunocytological results stayed the same (Fig. 3, photos 1–3). At the 13th week, among the cells stained by the I.S. directed against the total hormones, it was possible to isolate a few thyrotrophs by revealing them electively by anti-TSH absorbed with α LH (specific for β TSH).

It was not before the 15th week that gonadotrophs could be revealed either by anti-HCG, I.S. or anti-LH I.S. previously absorbed with α LH, and by anti-FSH previously absorbed with HCG and LH.

The study of serial sections treated by one or another of these I.S. confirmed that the same cells contain the two β subunits of LH and FSH simultaneously (Fig. 3, photos. 7–10).

DISCUSSION

1. *LH-RH neurons*

We shall remark once again that the value of our results is strictly dependant on the cytoimmunological techniques we used, on the numerous verifications of the specificity of our anti-LH-RH I.S. and the elective staining, with I.F. and I.E. (all this being on sections of paraffin embedded tissues, fixated with Bouin Hollande, also on lyophylised material treated according to the Falck and Hillarp technique, and finally on semi-thin sections of PAF fixated material embedded in araldite). These controls enable us to consider that our observations are a good immunological proof of

Table 4. Cytoimmunological results as a function of fetal age

Immune sera \ Age in weeks	6	8	13	15	22
anti-HCG	−	+	+	+	+
anti-LH	−	+	+	+	+
anti-HCG absorbed with α LH	−	−	−	+	+
anti-LH absorbed with α LH	−	−	−	+	+
anti-FSH absorbed with HCG	−	−	−	+	+
anti-TSH	−	+	+	+	+
anti-TSH absorbed with α LH	−	−	+	+	+

the presence of LH-RH in the human hypothalamus at the 13th week of fetal life (at least in the male). Beside this, from a cytoimmunological point of view, this LH-RH can be seen with a granular aspect in the perikarya. It is thus elaborated in them and transported in the crinophore axons (and maybe in the dendrites) of the neurones. The preferential localization of the LH-RH producing neurons in various anterior, mediobasal and posterior areas of the hypothalamus shows the large extent of the regions implied in the production of LH-RH and therefore in the control of the gonadotropic axis.

The LH-RH peptidergic system is present in the 13 week old fetus, and the study of the trajectory and the destination of these fibres raises problems dealing with the peptidergic system and its capacities of control over the fetal pituitary and perhaps over the nervous structures, by its extra hypophyseal fibres. In the 13 to 16 week old fetuses, the presence of the endings of the hypothalamo-infundibular fibres against or close to the Mantelplexus vessels (the only vessels present at this period) shows the possibility of hypophysiotropic action of the LH-RH by neurohemocrin pathways thanks to the portal blood vessels. From the 16th week onwards, when the formation of the intra-eminential loops takes place, (corresponding to an increase in the complexity of the intra-eminential neurovascular structures), our observations show the presence of only a few LH-RH endings in their perivascular spaces while there still remain many endings of LH-RH radiating collaterals in the periphery of the median eminence and in the stem near the vessels of the Mantelplexus. In semi-thin sections of PAF fixed median eminence of a 16 week old female fetus, we confirm the great number of immunoreactive fibres and endings especially in the floor of the posterior lip which is well developed in the human.

The functional signification of the fibres of the lamina terminalis and their endings against the deeply situated vessels of the vascular organ and in the ependymal epithelium is difficult to explain. Hypotheses have been raised after similar observations in other species [ref. in 7]. For the moment, the destination of the other extra hypophyseal fibres (of the epithalamus and mesencephalon) remains unknown in the human fetus. It raises the problem of the eventual existence of a neuro transmission system which would modulate the functioning of other neurons [ref. in 7]. Be that as it may, it is quite sure that the LH-RH peptidergic hypothalamo-infundibular system is already present at the 13th week of fetal life.

From an anatomical point of view, our results in the human fetus concur with those established in other adult primates [4–7]. This is particularly true for the intra-hypothalamic repartition of the immunoreactive cells and for the trajectory and endings of their fibres. Besides this, all of our results concur with the experiments of total disconnection of the mediobasal hypothalamus, where a hypothalamic control of the LH production of these primates is undisturbed even after section [28].

For some authors [31] the human fetal hypothalamus has an "LH RH like" activity on hypophysis cultures only when they, the hypothalami, are taken from 19 week old female fetuses or from 24 week old male fetuses. However, hypothalamic extracts would not have an LH-RH biological activity in female rats in pro-oestral period with blocked ovulation, before the 16th week of fetal life [24].

Finally, immunoreactive LH-RH could be revealed in the brain of four and a half week old human fetus [35]. This last study does not disagree with our observations since a small number of cells, or weakly immunoreactive cells, may not have been revealed in our young fetuses of 10 and 12 weeks, or the brains studied by Winters *et al.* could have contained maternal or even placental LH-RH [23] which could explain this early appearance of LH-RH. Furthermore our results can be compared with those obtained in other mammal fetuses [2, 3] whose neurons were also easily stained in some females during the period of important ovarian growth and during the postnatal period.

2. *Gonadotropic and thyrotropic cells*

Our results show that the production of pituitary glycoproteic hormones begins with the 8th week of fetal life. It is not possible to differentiate the gonadotrophs from the thyrotrophs, from the 8th week up to the 12th week, for all these cells are indifferently revealed by one or another of our anti-HCG, anti-LH or anti-TSH immune sera, whereas none of them is revealed by our I.S. specifically directed against the β subunits of these very hormones. These facts lead us to think that these differentiating cells must contain only α subunits (revealed by the antibodies directed against total hormones) of one or another of the pituitary glycoproteic hormones during the period going from the 8th up to the 12th week of fetal development.

At the 13th week, our β TSH specific I.S. reveals some cells of this population, whereas the anti-β FSH and anti β LH I.S. do not reveal any of them. We can say then, that the first differentiation of the thyrotrophs, which are, in all probability, able to synthetize an active TSH, takes place at this time. The time these cells appear concurs with other cytoimmunological studies of the human fetus [1]. It also agrees with the results of some hormonal dosages [30], and with the period of development and organisation in vesicules of the thyroïd, which can capture iodine as early as the thirteenth week.

At the 15th week, cells which were not revealed so far by β TSH specific I.S. can be stained either by β LH or β FSH specific I.S. This shows us that from this period on, gonadotrophs are fully differentiated and can produce active LH and FSH as well. So there is a single kind of gonadotropic cell which can produce both FSH and LH in the human

fetus [32] as well as in the adult [33]. This last result, dealing with the complete maturation of gonadotrophs not later than the 15th week and obtained with I.S. directed against human hormonal antigens, is a provisory one until verifications are made with the 13 week old female fetuses and with the 14 week old male and female fetuses. Be that as it may, the differentiation of LH and FSH producing cells seems to occur after the differentiation of thyrotrophs. The period when the gonadotrophs appear has been estimated differently according to the authors who used I.S., prepared (except for HCG) with nonhuman hormonal antigens (ovine, bovine or porcine) that were sometimes made specific for the human β LH subunit but never for the β FSH subunit.

P. M. Dubois and M. P. Dubois[19], using anti-ovine LH I.S., anti-porcine β LH and α LH I.S., and anti-HCG I.S. state that α LH can be revealed in cells at the 8th week but that β LH is only seen at the 16th week in female fetuses and at the 20th week in male fetuses. In 1974, Pasteels *et al.*[32], using anti-ovine LH I.S. and anti ovine-FSH (the latter being directed against human FSH and LH) detected LH as early as the 10th week and FSH at the 8th week. Baker[1] reveals cells thanks to an anti HCG at the 10th and a half week and at the 13th week only with an anti-ovine β LH.

If we compare these results with ours, our own studies look more significant since they have been carried out on a material which is particularly favorable to cyto-immunological detection and with an I.S. directed against human hormones, particularly against human β LH and β FSH subunits.

In 1969, Levina, using biological dosages, revealed an FSH activity in pituitary extracts of 13 to 14 week old female fetuses and of 21 week old male fetuses, but after noting a biological LH activity in the female fetus at the 18th week, noticed that LH is revealed by immunological techniques as early as the 13th week in both female and male. In 1976, Kaplan and Grumbach [27] using radioimmunological dosage, detected, in the pituitary, LH and FSH from the 68th day, and in the serum, FSH and LH from the 84th day and 99th day respectively. Groom [25] by incubating pituitaries detects LH and FSH in the 8th week old fetus but Hagen [26] states that he reveals a great quantity of glycoproteic α subunits in the pituitary extracts of nine and a half week old to 16 week old fetuses and that β FSH and β LH subunits appear near the 16th week in both sexes.

3. *General discussion*

The development of the LH-RH hypothalamic system and the establishment of its neurohemal relationship with the vessels take place before the maturation of pituitary gonadotrophs and particularly before the synthesis of the β FSH and β LH subunits which occurs near the 15th week in the same gonadotropic cell type. The observations of a greater number of cells revealed by our anti LH-RH in the 16 week old female fetuses may correspond to the increase of production of LH and FSH in the female fetus at this stage of development [18, 27].

The great sensibility of gonadotrophs to the action of the LH-RH was tested *in vitro* on hypophyseal cultures. An effect on the LH and FSH secretion could be observed at the 13th week [32] as well as on the LH secretion at the 16th week [34].

In order to know more about the role of the pituitary gonadotrophs in the human fetus and about their early control by the hypothalamic LH-RH producing neurons we are carrying out further investigations aimed at discovering eventual differences in the behavior of these neurons. These differences could correspond to the variations in the level of LH and FSH in the blood, the hypophysis and the amniotic fluid according to the fetal sex and age [18].

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REFERENCES

1. Baker B. L. and Jaffe R. B.: The genesis of cell types in the adenohypophysis of the human fetus as observed with immunochemistry. *Am. J. Anat.* **143** (1975) 137–148.
2. Barry J. and Dubois M. P.: Différenciation périnatale de l'hypothalamus gonadotrope chez les Mammifères. In *Endocrinologie Sexuelle de la Période Périnatale Colloque INSERM* (1974) pp. 63–68.
3. Barry J. and Dubois M. P.: Etude en immunofluorescence de la différenciation prénatale des cellules hypothalamiques élaboratrices de LH-RH et de la maturation de la voie neurosécrétrice préoptico-infundibulaire chez le Cobaye. *Brain Res.* **67** (1974) 103–114.
4. Barry J., Girod C. and Dubois M. P.: Topographie des neurones élaborateurs de LRF chez les Primates. *Bull. Assoc. Anat.* **59** (1975) 103–110.
5. Barry J. and Carette B.: Etude en immunofluorescence des neurones élaborateurs de LRF chez les Cébidés. *C.r. hebd. Séanc. Acad. Sci., Paris* **281** (1975) 735–738.
6. Barry J. and Carette B.: Immunofluorescence study of LRF neurons in primates. *Cell Tiss. Res.* **164** (1975) 163–178.
7. Barry J.: Systématisation et afférences des structures hypothalamiques à compétence gonadotrope chez les primates. In *Système Nerveux Activité Sexuelle et Reproduction* (Edited by Masson) (1976) 13–25.
8. Bugnon C. and Gainet M.: Etude des phénomènes de cytogénè se dans l'adénohypophyse foetale humaine. 88 ème Congrès de l'AFAS (1969). In *Ann. Sci. Univ. Besançon* (1969) *3° série Méd.* **6**, 38–39.
9. Bugnon C., Lenys D., Bloch B. and Fellmann D.: Etude cyto-immunologique des phénomènes de différenciation cellulaire précoce dans l'adénohypophyse foetale humaine, *C. R. soc. Biol.* **168** (1974) 4–5. 460–465.
10. Bugnon C., Lenys D., Bloch B. and Fellmann D.: Les cellules corticotropes et mélanotropes de l'adénohypophyse chez le Chat, le Renard, le Rat et le foetus humain: études avec diverses techniques de fluorescence induite, de cyto-immunologie, et à l'hématoxyline plombique, *C. R. Soc. Biol.* (1975) **169** no. 5 1271–1276.
11. Bugnon C., Lenys D., Bloch B. and Dessy C.: Détection cyto-immunologique des cellules corticotropes et mélanotropes dans l'adénohypophyse foetale humaine aux stades précoces du développement. *Bull. Assoc. Anat.* (1975) **59** 166, 571–582.

12. Bugnon C., Bloch B., Fellmann D. and Gouget A.: Etude comparative chez le Cobaye, le Chat et le Chien, des fibres nerveuses hypothalamo-infundibularies immunoréactives à un immunsérum anti LH-RH. *C. r. soc. Biol.* (1976) **170** no. 1 83–87.
13. Bugnon C., Bloch B. and Fellmann D.: Mise en évidence cyto-immunologique de neurones à LH-RH chez le foetus humain. *C. r. hebd. Séanc Acad. Sci., Paris* (1976) **282** 1625–1628.
14. Bugnon C., Bloch B. and Fellmann D.: Etude immunocytologique des cellules et des fibres à LH-RH chez le foetus fumain. *Bull. Assoc. Anat.* (1976) (In press).
15. Bugnon C., Bloch B. and Fellmann D.: Etude cyto-immunologique des cellules gonadotropes du foetus humain *Bull. Assoc. Anat.* (1976) (In press).
16. Bugnon C., Lenys D., Bloch B. and Fellmann D.: Exploration cyto-immunologique sur coupes semi-fines des phénomènes de différenciation précoce de diverses populations cellulaires dans l'adénohypophyse foetale humaine. *C. R. soc. biol.* (1976) (In press).
17. Bugnon C., Fellmann D. and Bloch B.: Etude cyto-immunologique du système peptidergique à somatostatine chez le foetus humain. VIII Congrès de Neuroendocrinologie Expérimentale Tours, 1976. *J. Physiol.* (1976) (In press.)
18. Clements J. A., Reyes F. I., Winter J. S. D. and Faiman C.: Studies on human sexual development—III. Fetal pituitary and serum, and amniotic fluid concentrations of LH, CG, FSH. *J. clin. Endocr. Metab.* **42** (1976) 9–19.
19. Dubois P. M. and Dubois M. P.: Mise en évidence par immunofluorescence de l'activité gonadotrope LH dans l'antéhypophyse foetale humaine. In *Endocrinologie Sexuelle de la Période Périnatale Colloque INSERM* (1974) 37–62.
20. Duvernoy H., Koritke J. G. and Monnier G.: Sur la vascularisation de la lame terminale humaine. *Z. Zellforsch.* **102** (1969) 49–77.
21. Duvernoy H. and Koritke J. G.: Les fibres nerveuses de la lame terminale et leurs relations avec le réseau capillaire. *C.R. Ass. Anat.* **117** (1963) 562–576.
22. Gainet M.: Contribution à l'étude de l'adénohypophyse foetale humaine. Thèse Médecine, Besançon (1969).
23. Gibbons J. M., Mitnick M. and Chieffo V.: *In vitro* biosynthesis of TSH and LH releasing factors by the human placenta. *Am. J. Obstet. Gynec.* (1975) 127–131.
24. Gilmore D. P. and Dobbie H. G.: Absence of gonadotrophin releasing activity in the human foetal hypothalamus before 16 weeks. Proc. Soc. Endocrinol. In *J. Endocr.* **64** (1975) 52p.
25. Groom G. V., Groom M. A., Cooke I. D. and Boyns A. R.: The secretion of immunoreactive LH and FSH by the human foetal pituitary in agar culture. *J. Endocr.* **49** (1971) 335–344.
26. Hagen C.: Identification of human FSH, LH its subunits in foetal and adult pituitary glands. *Acta Endocr.* **80** (1975) (Suppl. 199) 91.
27. Kaplan S. L. and Grumbach M. M.: The ontogenesis of human fetal hormones II Luteinizing hormone (LH) and follicle stimulating hormone (FSH). *Acta Endocr.* **81** (1976) 802–829.
28. Knobil E.: On the control of gonadotropin secretion in the rhesus monkey. *Recent Progr. Horm. Res.* **30** (1974) 1–46.
29. Leonardelli J., Barry J. and Dubois M. P.: Mise en évidence par immunofluorescence d'un constituant immunologiquement apparenté au LH-RH dans l'hypothalamus et l'E.M. chez les Mammifères. *C. r. hebd. Séanc. Acad. Sci., Paris* **276** (1973) 2043–2046.
30. Levina S. E.: Endocrine features in development of human hypothalamus, hypophysis and placenta. *Gen. comp. Endocr.* **11** (1968) 151–159.
31. Levina S. E.: In *Hormones in Development* (Edited by M. Hamburg and E. J. W. Barrington) (1971).
32. Pasteels J. L., Gausset P., Danguy A. and Ectors F.: Gonadotropin secretion by human fetal and infant pituitaries. In *Endocrinologie Sexuelle de la Période Périnatale Colloque INSERM* (1974) 13–36.
33. Phifer R. F., Midgley A. R. and Spicer S. S.: Immunohistologic and histologic evidence that FSH and LH are present in the same cell type in the human pars distalis. *J. clin. Endocrinol. Metab.* **36** (1973) 125–141.
34. Tamura T., Minaguchi H. and Sakamoto S.: Responsiveness of human foetal pituitary to hypothalamic hormones *in vitro*. *Endocr. Jap.* **20** (1973) 545–553.
35. Winters A. J., Eskay R. L. and Porter J. C.: Concentration and distribution of TRH and LH RH in the human fetal brain. *J. clin. Endocr. Metab.* **39** (1974) 960–963.

DISCUSSION

Hagen. Thank you for a very nice paper. I would like to ask you, whether you have studied the specificity of your antisera. Have you for example tried to see whether antiserum against β-FSH is able to bind labelled α-subunit and intact FSH. The reason for asking is, that we have found that all our antisera tested, were able to bind labelled intact hormones as well as subunits.

Bloch. We did not use radioimmunoassay, but as indicated before, we verified the specificity of the immune sera (table 3) by cytoimmunological tests. For example, we controlled the immunocytological staining, observed with anti FSH absorbed with HCG (specific for β FSH), was not abolished by addition of LH. But this staining disappeared after addition of FSH.

In the same way, we verified the specificity of anti LH and anti TSH. In particular, we carefully verified that the staining obtained with anti LH absorbed with α LH (specific for β LH) was not modified by addition of FSH.

Kalra. Did you find LH-RH in the cell bodies of the neurons in the arcuate nucleus?

Bloch. We have detected LRF containing neurons in the mediobasal hypothalamus. It is probable that a part of these neurons belong to the infundibular nucleus but we cannot ascertain it so far. Barry *et al.* (ref. [6] in text) in the adult squirrel monkey, indicated that some LRF neurons are located in the infundibular nucleus.

Kalra. But in the same sample you did observe LH-RH in the perikarya of cells of the anterior hypothalamus?

Bloch. Yes, particularly in the external layer of the lamina terminalis and before and above the white anterior commissure.

Kalra. Did you see any difference in the timing of the appearance of LH-RH activity in the nerve terminals and cell bodies in the hypothalamus during fetal life? In other words, does LH-RH appear in the cell bodies before it does in the nerve terminals?

Bloch. In the younger fetus studied in which we found LRF, at the 13th week, this hormone was present in the nerve endings of the median eminence and in the perikarya; but at earlier stages, it will be perhaps possible to observe LRF preferentially in nerve endings or in perikarya?

Barry *et al.* in the guinea pig fetus (ref. [2] and [3] in text) found that LRF was detectable first in the median eminence at the 48th day of gestation and only after in the perikarya (55th day).

Swaab. I should like to make a general comment on the problem to prove specificity of immunolocalization techniques. The absorption tests you mentioned are not sufficient for this purpose. If no staining is obtained anymore after absorption of the antibody to the antigen it does not prove more than that all the antibodies were directed to the antigen used. It does not exclude *cross reaction.* As an example we can mention our problems with antibodies raised to synthetic arginine-vasopressin (AVP). These antibodies allowed a good tissue localization of AVP. Solid phase absorption of the anti-AVP with AVP prevented any staining. Yet, in rats that lack AVP by a genetical defect, i.e. homozygous Brattleboro rats, a comparable bright immunofluorescence was found. This appeared to be due to cross reaction to the structurally closely related hormone oxytocin. The cross reacting antibodies could be removed from the plasma by absorption to oxytocin (Swaab and Pool, 1975). The same problems were met in the ACTH/α-MSH peptide-family (Swaab *et al.*, 1976). In addition, radioimmunoassay (RIA) cannot help us to characterize the antibodies for immunolocalization procedures. The difference between these two techniques is at least partly due to the quite different dilution in which the antibodies are used in RIA and immunolocalization. Immuno-electronmicroscopy allows observations in the dilution range between those applied in RIA and immunolocalization. Thus we observed that cross reaction with e.g. oxytocin gradually disappeared when dilutions of the antibody against vasopressin were increased (Van Leeuwen and Swaab, 1977). Specificity has thus to be studied in the antibody dilution also used in the immunolocalization technique.

Bloch. The specificity of the immunocytological techniques and of the radio-immunoassay depends on the specificity of the antiserum.

We used our antisera diluted from 1/50 to 1/400; though the dilution was less important than for R.I.A. some immunocytological tests permitted to control the specificity of the stainings obtained either by I.F. or by I.E.: for example, the prior saturation of anti LH RH with LH RH, totally inhibits the staining. Other neuropeptides as TRH, SRIF, oxytocin or human serum albumin are unable to inhibit the staining. No reaction was obtained with fluoresceine or peroxydase conjugate alone. Other antisera as anti ACTH or anti HCG do not reveal any structure in the hypothalamus. Anti somatostatin or anti oxytocin, or anti neurophysin reveal neurons in very different localization and of different aspect.