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Immunocytological study of the chronology of pituitary cytogenesis in the domestic pig (*Sus scrofa*) with special reference to the functioning of the hypothalamo-pituitary-gonadal axis

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Summary. This paper reports an immunofluorescent study of the pituitary in the fetal pig (*Sus scrofa*). Fifteen antisera were used against most of the hormones present in the pituitary. Five types of hypophysial endocrine cells were observed in the anterior and intermediate lobes. We determined the sequential appearance of these various cell types in the fetus. The first hormones found at 33 days were ACTH, β-MSH, β- and γ-LPH and α- and β-endorphin ; α-MSH appeared at 40 days and STH at 45 days. The glycoprotein hormones, LH (45 days), TSH (50 days) and FSH (60 days), appeared between 45 and 60 days. The density and staining of the gonadotropes increased up to 80 days, at which time they reached values similar to those of the adult. Prolactin was not found until 80 days.

An anti-LHRH antiserum was used to study LHRH neuron differentiation between 30 and 70 days of pregnancy. The first immunoreactive perikaryons were found at 40 days but the immunoreactive fibers did not reach the median eminence until about 60 days. However, we observed differentiated capillary loops in the palisade layer of the median eminence only in the 70-day fetus.

These results when compared with actual data on the differentiation of the reproductive function in the pig fetus permitted us to define an overall pattern of the differentiation and functioning of the fetal neuroendocrine hypothalamo-pituitary-gonadal system in the porcine species. This pattern includes (i) autodifferentiation and autofunctioning of the gonads and (ii) autodifferentiation of the pituitary with (iii) later assumption by the hypothalamus followed by a phase during which the whole reproductive system functions.

Introduction.

Since the early 1960's, fluorescein-labeled antibodies have been used in mammalian pituitary to identify five different cell types which can be distinguished by the hormones found in their cytoplasm. Moreover, these techniques permit early detection of the endocrine cells in the hypophysial primordia during fetal development, when the levels of synthesized hormones are still too low to be detected by cytochemical methods.

LHRH-containing fibers (Leonardelli *et al.,* 1973) and then immunoreactive perikaryons (Barry *et al.,* 1973) were identified by anti-LHRH in the hypothalamus and median eminence of guinea-pig. These results, at first highly contested, were finally confirmed in the same species by other authors (Silverman, 1976 ; Setalo *et al.,* 1976a ; Hoffman *et al.,* 1978 ; Weindl and Sofroniew, 1978).

Since then anti-LHRH-reacting perikaryons have been identified in many species (see Barry, 1979) ; some authors have tried to find them in fetuses (mice : Gross and Baker, 1979 ; guinea-pigs ; Barry and Dubois, 1974b ; humans : Bugnon *et al.,* 1976a, 1978 ; Paulin *et al.,* 1977).

Not only has the time of appearance of these cell types been studied, but some work has also been done on their functional differentiation. Gross and Baker (1979) found a close temporal relationship between the appearance of GnRH and LH in mice. These data suggest the existence of a functional hypothalamo-pituitary axis at the end of fetal development. On the other hand, Paulin *et al.* (1977) studying humans, concluded that the hypothalamus does not influence the differentiation of gonadotropes during gestation but that it could be responsible for functional variations in the LH activity of those cells. This contradiction arose because two different processes, more or less separated in time — (i) the cytological differentiation of gonadotropes and (ii) their complete functional maturation — must be distinguished.

Material and methods.

1. *Pituitary.* — Twenty pituitaries were taken from pig fetuses ranging in age from 30 (day 0 was the day heat appeared) to 112 days, parturition occurring regularly at 114 days (table 1).

2. *Hypothalamus.* — We used 8 fetal hypothalami (table 2).

TABLE 1

Age and sex of fetuses used to study the pituitary

Age (days)	30	30	33	37	40	40	45	45	50	50
Sex	♂	♀	♀	♀	♀	♀	♀	♀	♀	♂
Age (days)	50	55	60	60	70	80	90	100	110	112
Sex	♂	♂	♀	♀	♂	♂	♀	♂	♂	♂

TABLE 2

Age and sex of fetuses used to study the hypothalamus

Age (days)	30	33	37	40	45	50	60	70
Sex	♀	♀	♂	♀	♀	♂	♀	♂

3. *Fixation.* — After washing the vascular layer with heparinized physiological serum, the 30 to 60-day old fetuses were fixed by intracardiac perfusion with sublimate Bouin-Hollande without acetic acid for 5 min. The 70 to 112-day fetuses were perfused through both carotids simultaneously. After dissection, the sampled organs were immersed in the same fixative for 7 days.

4. *Sex.* — After dissection, we determined the sex of the 30 to 40-day fetuses by morphological observation of the gonad ; the sex of fetuses 45 days or older was determined by examination of the external genitals.

5. *Antibodies and immunofluorescence.* — After the pituitaries, embedded in paraffin and cut into 5-μ thick sections, had been mounted with gelatinous water (0.5 p. 100) they were treated by indirect immunofluorescence using immune sera (1/200 for 3 h) against the gonadotropins or their β subunit (LH, FSH, β-LH, β-FSH (*), the β unit of thyrotropin β-TSH), prolactin (PRL), somatotropin (STH), corticotropin (ACTH (1-24) and (17-39)), melanotropins (α- and β-MSH), lipotropins (β- and γ-LPH) and α- and β-endorphins.

An anti-LHRH antibody was used (1/100 for 3 h) for the hypothalami. Table 3 regroups the antigenic sources used to obtain the antisera. The anti-gammaglobulin antibodies of rabbit, raised in sheep, were provided by the Institut Pasteur (Paris).

TABLE 3

Antigenic sources used in preparing the antisera

Antigen	Source and reference
LH	Ovine (CNRS, M_2)
FSH	Ovine (CNRS, M_2)
β-LH	Porcine (Dr. Courte, INRA)
β-TSH	Bovine (antisérum : Dr. Pierce, Los Angeles)
PRL	Bovine (NIH, PB_2)
STH	Human (Kabi, Stockholm)
Porcine ACTH (17-39)	Synthetic (Ciba-Geigy, Bâle)
ACTH (1-24)	Synthetic (Ciba-Geigy, Bâle)
α-MSH	Synthetic (Ciba-Geigy, Bâle)
Bovine β-MSH	Synthetic (Ciba-Geigy, Bâle)
β-LPH	Porcine (Dr. Graaf, Budapest)
γ-LPH	Porcine (Dr. Graaf, Budapest)
α-endorphin	(Dr. Guillemin, San Diego)
β-endorphin	(Dr. Guillemin, San Diego)
LHRH	Synthetic (Cyclo Chemical Ltd, Los Angeles)

6. *Specificity.* — The specificity of the antibodies was systematically checked on the pituitaries and the hypothalami (i) by omitting the first antibody, (ii) by replacing that antibody by nonimmune rabbit serum or (iii) by previously

(*) Anti-β-FSH : HCG-saturated anti-ovine FSH (20 000 IU/ml undiluted antiserum).

incubating the first antibody in the presence of an homologous or heterologous antigen. The specificity of these same antisera had been confirmed by studies on the pituitary in many vertebrate species (fish : Follenius and Dubois, 1978 ; amphibians : Moriceau-Hay *et al.,* 1979 ; rodents : Tramu and Dubois, 1972 ; Chatelain *et al.,* 1979 ; cattle : Dubois, 1969 ; Dacheux and Dubois, 1976 ; pigs : Dacheux, 1978, 1981 ; sheep : Dubois and Mauléon, 1969 ; primates : Girod and Dubois, 1976 ; humans : Begeot *et al.,* 1978 ; Dubois *et al.,* 1978) and the hypothalamus (Barry *et al.,* 1973 ; Dubois and Barry ; 1974 ; Barry *et al.,* 1975 ; Paulin *et al.,* 1977 ; Tramu *et al.,* 1977 ; review in Barry, 1979).

Results.

The different tests we carried out showed that staining specificity was satisfactory (table 4).

1. *Chronology of cell appearance* (table 5). — No immunopositive cells were found in 30-day pituitaries with the antisera used. The first corticotropes which appeared at 33 days were situated in the most anterior part of the pituitary and reacted to anti-ACTH (1-24) and (17-39), anti-β-MSH, γ-LPH and α- and β-endorphins. The same cells reacted with anti-β-LPH, and some other more posterior cells were also positive (Pl. I, 1, 2, 3). The intermediate lobe showed no

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PLATE I

Fetal pig pituitary. Apparition of corticotropes.

1. — 33-day fetus, section treated with anti-β-LPH : anterior region of the LA is rich in immunoreactive cells. More posteriorly, cell density is lower. ($\times$ 130).

2. — 33 day fetus, adjoining section treated with anti-ACTH (17-39) : only the anterior part of the LA shows immunoreactive cells. ($\times$ 130).

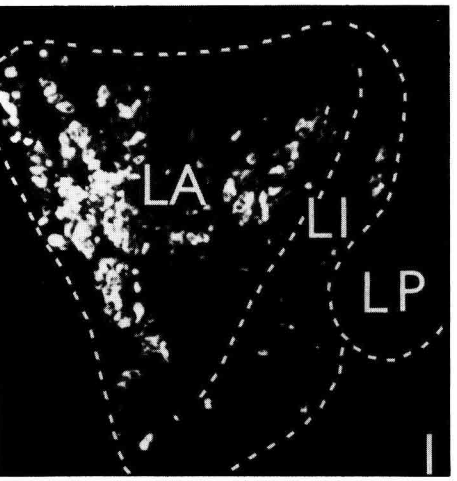
3. — 33-day fetus : cells of the anterior region of the LA identified by anti-β-endorphin. ($\times$ 540).

4. — 37-day fetus : section treated with anti-β-LPH : at this time, this antibody identifies more posterior cells. ($\times$ 130).

5. — 37-day fetus, adjoining section treated with anti-ACTH (17-39) : The LI presents immuno-reactive cells occurring in smaller numbers than with anti-β-LPH antibody. ($\times$ 130).

6. — 40-day fetus : first cells identified by anti-α-MSH in the LI. ($\times$ 540).

EM : median eminence ; LA : anterior lobe of pituitary ;
LI : intermediate lobe of pituitary ; LP : posterior lobe of pituitary ;
The dashed lines outline the different lobes of the pituitary.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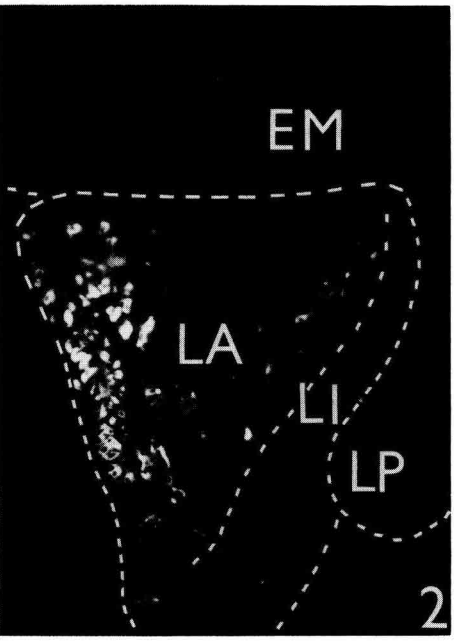
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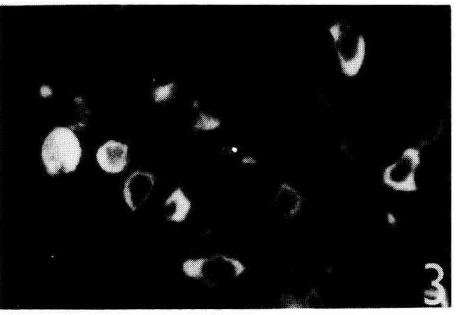
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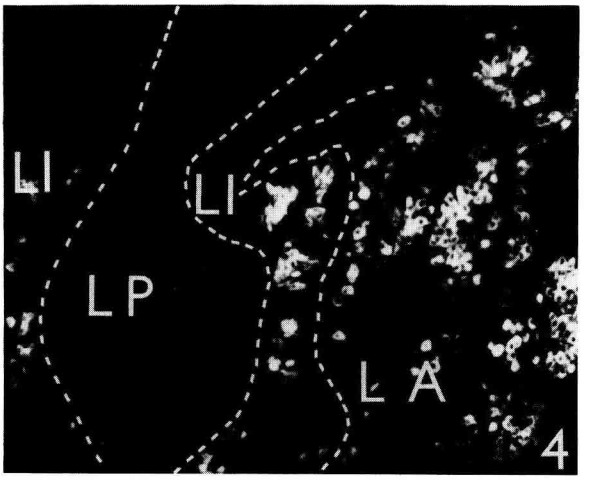
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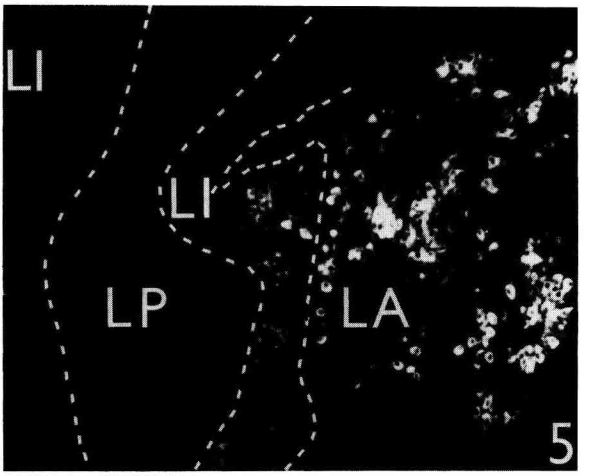
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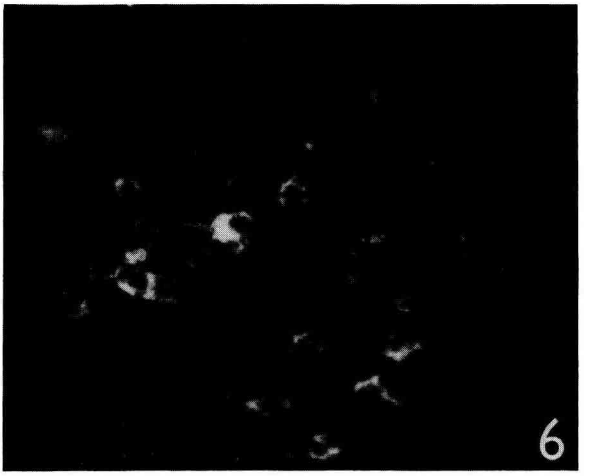
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TABLE 4

Immunofluorescence observed in the pituitary with immunserum previously incubated with various homologous or heterologous antigens

Antibody	Antigens					
	Without antigen	β-LH ov	LH ov	TSH ov	STHh	PRL ov
β-LH por.	+ I	−	−	+		
LH ov	+ I-II	+ −	−	+ −		
FSH ov	+ I-II	+	+ −	+ −		
FSH ov + HCG	+ I	+	+ −	+ −		
β-TSH bov	+ II	+	+	−		
STH h	+ III				−	+
PRL ov	+ IV				+	−

Antibody	Antigens					
	Without antigen	ACTH (1-24)	α-MSH	β-MSH	α-endorphin	ACTH (1-39)
ACTH (17-39)	+ V-VI	+				−
ACTH (1-24)	+ V-VI	−	+	+		
β-MSH	+ V-VI	+	+	−		
α-MSH	+ VI	+	−	+		
β-LPH	+ V-VI	+		+	+	
γ-LPH	+ VI-V	+	+	+		
α-endorphin	+ V-VI	+			−	
β-endorphin	+ V-VI	+			+	

+ positive reaction ; − negative reaction ; + − lower reaction than control.
I : gonadotropes ; II : thyreotropes ; III : somatotropes ; IV : prolactotropes ; V : corticotropes of the anterior lobe ; VI : corticotropes of the intermediate lobe.

positive cells, and anti-α-MSH gave no positive response. At 37 days, the corticotropes of the anterior lobe responded to the seven antisera studied. Those of the intermediate lobe reacted maximally to anti-β-LPH but rather weakly to other antibodies (Pl. I, 4, 5). Only very few of these cells exhibited a slight

TABLE 5

Summary of the main stage of cytological differentiation in the fetal pig pituitary

Date (days)	Process observed
30	No positive labeling
33	First corticotropes (anterior-posterior gradient)
40	First anti-α-MSH labeling
45	First somatotropes
45	First, very rare, gonadotropes
50	First, very rare, thyreotropes
80	Gonadotropes reach maximal level and density
80	First lactotropes
114	Parturition ; numerous lactotropes

reaction to anti-α-MSH. At 40 days, the intermediate lobe cells were labeled by anti-α-MSH (Pl. 1, 6). It thus seems that the corticotropes differentiate according to an anterior-posterior gradient, the first hormone to appear being β-LPH. At 45 days, very few gonadotropes and some somatotropes were revealed by LH and β-LH and STH, respectively ; after a period of high levels between 50 and 70 days, the somatotropes were very weakly stained up to the end of pregnancy. At 50 days, the first thyrotropes appeared, and at 60 days anti-FSH and HCG-saturated FSH reacted positively on a small number of cells (Pl. II, 1, 2, 3). From 60 to 80 days, the number of gonadotropes increased markedly, and from 80 days their contents and density, which seemed constant, were similar to those observed in adults. At 80 days, we observed the first lactotropes. Fetuses of 100, 110 and 112 days showed a large number of these cells which had a high staining intensity at 110 and 112 days (Pl. II, 4).

2. *Hypothalamus.* — The staining specificity of the anti-LHRH tested in each hypothalamus was satisfactory (Pl. III, 1, 2).

Fetal hypothalami of 30, 33 and 37 days showed no positive labeling. At 40 days, numerous perikaryons with variable contents were seen in the most anterior (paraolfactory and rostral precommissural) areas. Practically no immunoreactive fiber was visible at that time. At 45 days, the situation was similar but there were fewer cells.

In figure 1 showing a 50-day fetus, the situation was somewhat different. Cells were also visible in the *lamina terminalis.*

At 60 days (fig. 2), the distribution was different. Fewer perikaryons were visible but some were present in the rostral limbic and septal areas ; one perikaryon was seen in the rostral mesencephalic area. The fibers were no longer limited to the *lamina terminalis* but extended towards and into the median eminence which was hardly yet vascularized (Pl. III, 5).

At 70 days, cell number and content were still lower. Fibers were seen in the median eminence where the capillary loops were well differentiated in the palisade layer (Pl. III, 7).

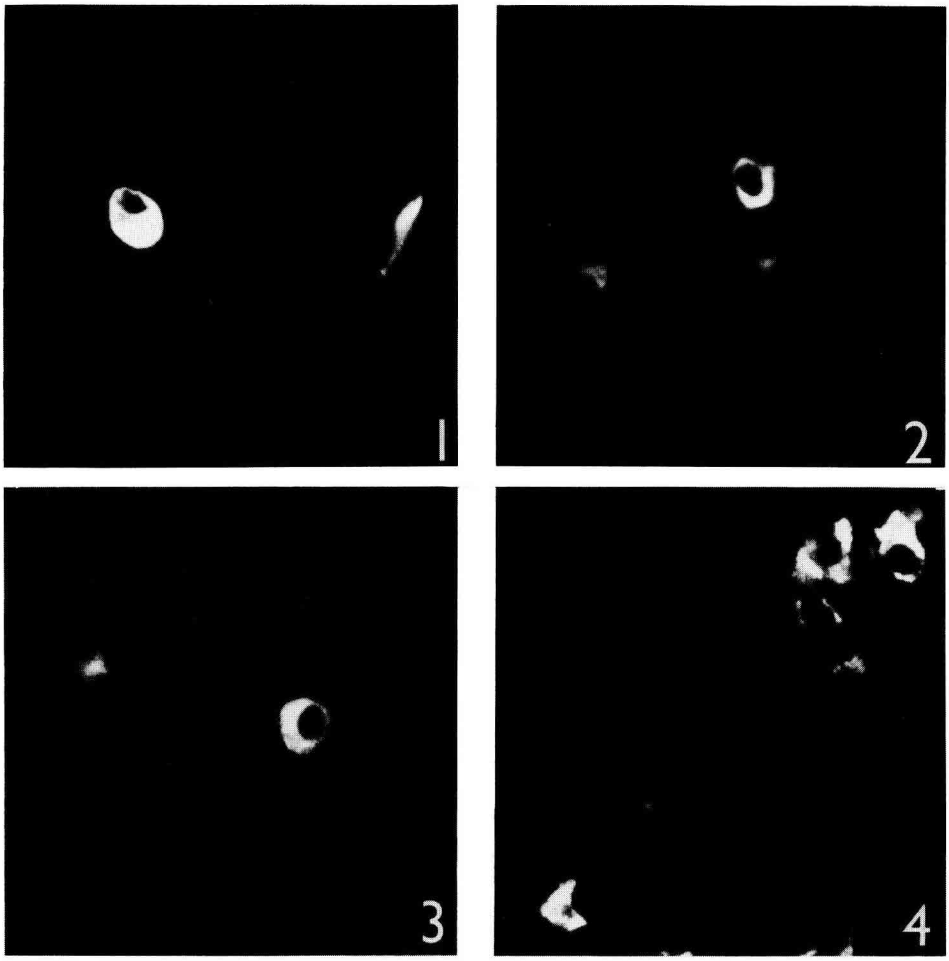


PLATE II

Fetal pig pituitary.

1. — 60-day fetal pituitary : cells identified by anti-β-LH. (× 625).
2. — 80-day fetal pituitary : cells identified by HCG-saturated anti-FSH. (× 625).
3. — 80-day fetal pituitary : cells identified by anti-β -TSH. (× 625).
4. — 90-day fetal pituitary : cells identified by anti-PRL. (× 625).

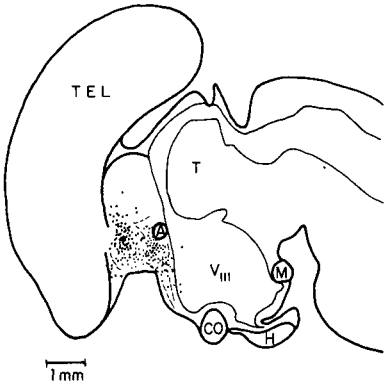


FIG. 1. — *Topography of LHRH neurons at 50 days* (× 5). The dashed lines are the fibers ; each point represents one perikaryon (only neurons observed on 20 p. 100 of the serial sections are shown). A : anterior white commissure ; CO : optic chiasma ; H : anterior pituitary ; M : mamillary bodies ; T : thalamus ; TEL : telencephalon.

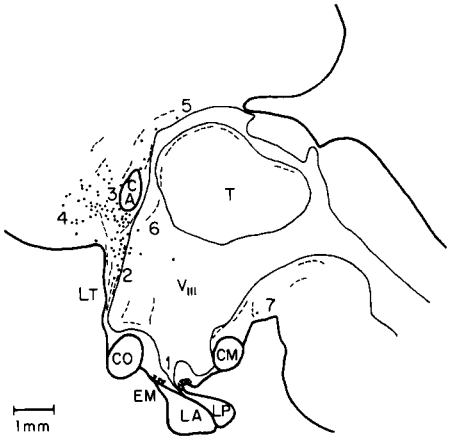


FIG. 2. — *Topography of LHRH neurons at 60 days* (× 5). The dashed lines are fibers ; each point represents one neuron (only neurons observed on 20 p. 100 of the serial sections are shown). 1 : mediobasal hypothalamus ; 2 : preopticoterminal region ; 3 : precommissural region ; 4 : paraolfactory region ; 5 : rostral limbic region ; 6 : septoepithalamic region ; 7 : rostral mesencephalic region. CA : anterior white commissure ; CM : mamillary body ; CO : optic chiasma ; EM : median eminence ; LA : anterior lobe of pituitary ; LP : posterior lobe of pituitary ; LT : lamina terminalis ; T : thalamus ; V_{III} : third ventricle.

Discussion.

Fetal pituitary. — Our results on the chronology of the appearance of the various hypophysial cell types agree with those of other authors who have studied the same process (besides those already cited : Bugnon *et al.*, 1976b, c ; Setalo and

Nakane, 1976 ; Baker and Yu, 1977 ; Watanabe and Daikoku, 1979 ; Danchin *et al.*, 1981). However, individual factors may play a role ; one of us (Dubois, 1977) in a previous study observed an earlier appearance (at 30 days) of corticotrope immunoreactivity.

During early differentiation of the corticotropes according to an antero-posterior gradient between 33 and 40 days we observed, as other authors, that the first immune serum labeling the cells was anti-β-LPH (Chatelain *et al.*, 1979), while anti-α-MSH was the last to reveal the intermediate lobe cells (Baker and Jaffe, 1975 ; Dupouy and Dubois, 1975 ; Bugnon *et al.*, 1976a). These observations are compatible with the existence of a common precursor having some peptide sequences which would be inaccessible to antibodies until they had cleaved into different hormonal molecules (Mains *et al.*, 1977 ; Chatelain *et al.*, 1979 ; Chrétien *et al.*, 1979 ; Hakanson *et al.*, 1980).

The somatotropes appeared at 45 days. Their low content after 70 days might be due either to a decrease in STH synthesis, which would explain the insignificant role of the pituitary in fetal growth (Jost and Cohen, 1966 ; Jost *et al.*, 1966 ; Stryker and Dziuk, 1975), or to increased STH release because fetal plasma assays show a strong increase of that hormone beginning at 70 days (Atinmo *et al.*, 1976).

When the first gonadotropes appeared at 45 days and the first thyrotropes at 50 days, we did not observe any dissociation between the α and β units of glycoprotein hormones as reported in humans (Dubois *et al.*, 1975 ; Bugnon *et al.*, 1976d).

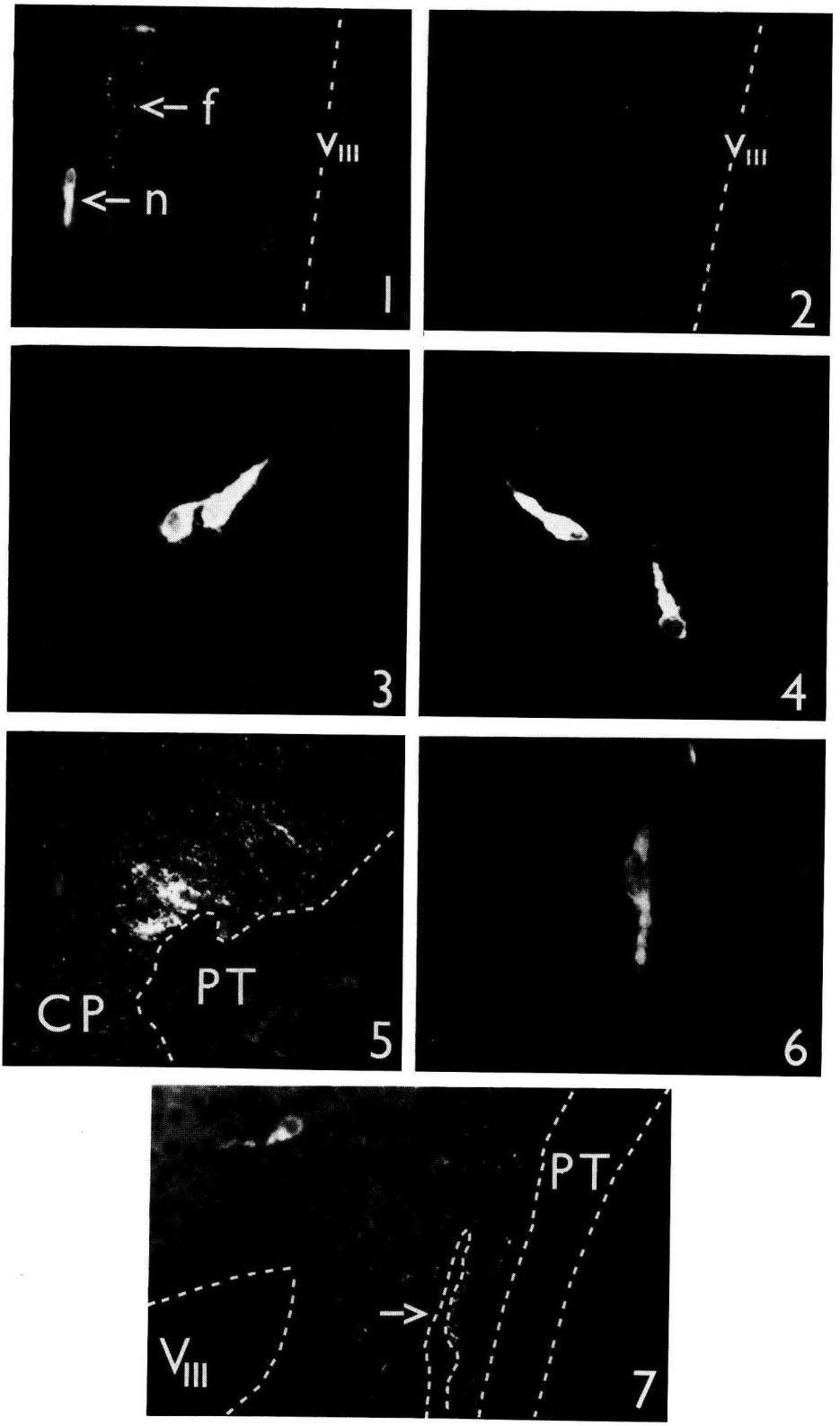
However, a first phase of fetal gonadotrope development occurred between 45 and 70 days during which cell density increased gradually ; a second phase from 80 days showed constant cell density and maximal fluorescence.

PLATE III

Fetal hypothalamus.

1. — 60-day fetus : cells and fibers identified by anti-LHRH. (× 210).
2. — Adjoining section treated with anti-LHRH previously saturated by 1 mg of LHRH per ml of pure antibody. (× 210).
3. — 40-day fetus : LHRH neurons in the paraolfactory region. (× 390).
4. — 50-day fetus : LHRH neurons in the paraolfactory region. (× 390).
5. — 60-day fetus : LHRH fibers in the palisade layer of the median eminence ; no capillary loop is visible. (× 210).
6. — 70-day fetus : LHRH neuron in the preoptic terminal. (× 390).
7. — 70-day fetus : well-differentiated capillary loops are visible (arrow).

CP : palisade layer ; f : fiber ; n : LHRH neuron ; PT : pars tuberalis ;
V_{III} : third ventricle. ;
The dashed lines outline the anatomical regions.


f
n
V_{III}
1
V_{III}
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CP
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PT
V_{III}
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According to Smith and Dortzbach (1929), Melampy *et al.* (1966) and Liwska (1975), 80 days is an important stage in the evolution of hypophysial LH concentrations. Also, Colenbrander *et al.* (1977) showed that circulating LH, which is undetectable between 49 and 80 days, augments significantly from 80 days. Moreover, Elsaesser *et al.* (1976) and MacDonald (1979) measured high quantities of plasma LH during the last three weeks of pregnancy (see MacDonald, 1979).

Thus, as we also noted using immunofluorescence, the 70 to 80-day period is an important stage in the functional differentiation of gonadotropes.

We observed prolactin cells only from 80 days, thus agreeing with the data of authors who noted that these cells always differentiate late, if not last.

Fetal hypothalamus. — Figure 2 shows a very anterior distribution of anti-LHRH immunoreactive perikaryons. This topography, very similar to that reported in guinea-pigs (Barry *et al.*, 1973 ; Setalo *et al.*, 1976a ; Jennes and Stumpf, 1980), cats (Barry and Dubois, 1974a, 1975) and rats (Naik, 1975a, b ; Setalo *et al.*, 1976b ; Ibata *et al.*, 1979), was obtained in young fetuses (60 and 70 days) and may therefore be different from adult distribution.

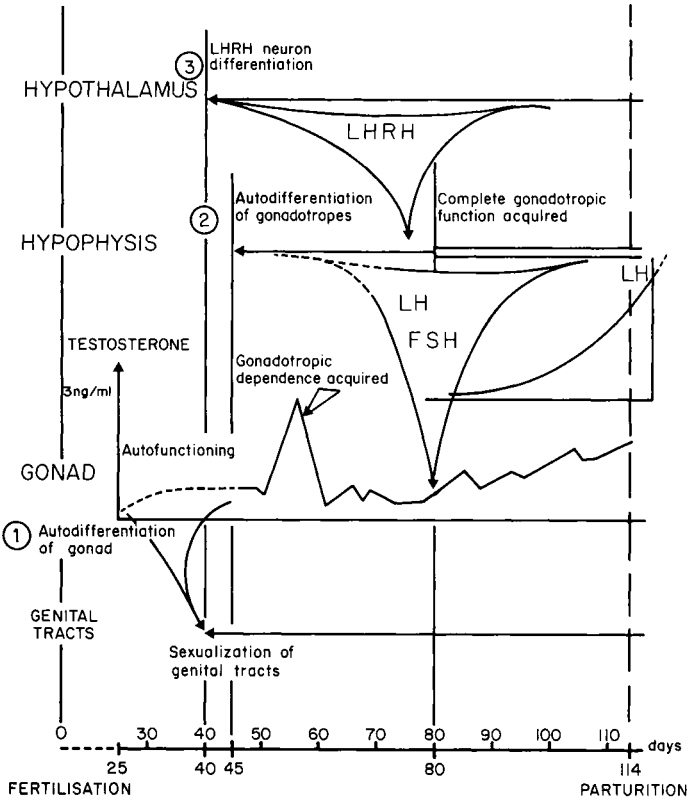

FIG. 3. — *Diagram showing how the neuroendocrine hypothalamo-pituitary-gonad system is triggered in the fetal pig* (Sus scrofa).

The very precocious appearance of LHRH neurons agrees with studies in humans using either immunofluorescence (Bugnon *et al.,* 1977a, b, 1978 ; Paulin *et al.,* 1977) or hypothalamic LHRH assay (Winters *et al.,* 1974). However, as in humans (Paulin *et al.,* 1977), we noted that relations with the pituitary are only established much later, i.e. between 60 and 70 days.

Differentiation and triggering of the hypothalamo-pituitary-gonad system. — All the data obtained on the differentiation of the porcine hypothalamo-pituitary-gonad system have been regrouped in figure 3. Three main stages may be distinguished chronologically.

1. *Early gonadal autodifferentiation independent of pituitary differentiation which occurs later.* — The sex of the gonad can be determined at 26 days by ultrastructural observation (Pelliniemi, 1975), and the future testis, but not the ovarian primordium, already contains testosterone (Raesides and Sigman, 1975). At 30 days, fetal sex can be determined by macroscopic observation of the gonad after dissection ; at 42 days the external genitals are differentiated (Patten, 1948).

Moreover, the work of Meusy-Dessolle (1974) and Colenbrander *et al.* (1978) shows that after a phase of very active testicular functioning (3 mg/ml of plasma testosterone at 55 days), fetal testosterone drops rapidly between 55 and 60 days and remains low until about 80 days, reflecting the appearance of testicular dependence on gonadotropins which are not detected in fetal blood until 80 days (Colenbrander *et al.,* 1978).

Sexualization of the genital tract is due secondarily to early testosterone secretion.

2. *Hypophysial gonadotrope autodifferentiation independent of LHRH stimulus.* — Five types of cells have been seen to differentiate in isolated early *in vitro*-cultured primordia of the pituitary (rats : Nemeskery *et al.,* 1976 ; Watanabe and Daikoku, 1976 ; Begeot *et al.,* 1979 ; birds : Ferrand *et al.,* 1980). The same results were obtained after early encephalectomy *in utero* in rat (Chatelain *et al.,* 1976, 1979). Moreover, in human anencephalic fetuses, Begeot *et al.* (1978) and Dubois *et al.* (1978) observed the onset of corticotrope differentiation.

Gonadotropes differentiate in the pig at 45 days, well before the LHRH fibers reach the median eminence (between 50 and 60 days) and the capillary loops of the portal system are established (between 60 and 70 days).

3. *Hypothalamic assumption of reproductive system control.* — The establishment of relations between hypothalamic and hypophysial cells (between 60 and 70 days) triggers the hypothalamo-pituitary-gonad system, as shown by increases in plasma LH (Elsaesser *et al.,* 1976 ; Colenbrander *et al.,* 1977 ; MacDonald *et al.,* 1979) and plasma testosterone (Meusy-Dessolle, 1974 ; Colenbrander *et al.,* 1978) beginning at 80 days.

Thus, it is our opinion that although LHRH does not induce gonadotrope differentiation, it is probably requisite to the acquisition of complete cell maturation and to good cell functioning.

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Résumé. Une étude immunocytologique de l'hypophyse de fœtus de porc domestique (*Sus scrofa*) a été réalisée à l'aide de quinze antisérum dirigés contre la plupart des hormones présentes dans l'adénohypophyse, afin de déterminer la séquence d'apparition des cinq types de cellule endocrine de cet organe au cours du développement fœtal. Les premières hormones identifiées sont, à 33 jours, l'ACTH, la β-MSH, la β- et γ-LPH et l'α- et β-endorphine ; l'α-MSH apparaît à 40 jours et la STH à 45 jours. Entre 45 et 60 jours les hormones glycoprotéiques LH (45 jours), TSH (50 jours) et FSH (60 jours) apparaissent. La densité et l'intensité du marquage des cellules gonadotropes augmentent jusqu'à 80 jours date à laquelle elles atteignent des valeurs voisines de celles observées chez l'adulte. La prolactine n'a été observée qu'à partir de 80 jours.

Un antisérum anti-LHRH a été utilisé pour l'étude de la différenciation des neurones à LHRH dans l'hypothalamus entre 30 et 70 jours de la gestation. Les premiers péricaryons immunoréactifs ont été trouvés à 40 jours mais les fibres immunoréactives n'atteignent l'éminence médiane que vers 60 jours. Cependant nous n'avons observé de boucles capillaires bien différenciées qu'à partir de 70 jours.

Ces résultats, comparés à ceux obtenus sur la différenciation de la fonction de reproduction chez le fœtus de porc, permettent de dresser un schéma général, dans l'espèce porcine, de la différenciation et du fonctionnement du complexe neuroendocrine hypothalamo-hypophyso-gonadique au cours de la vie fœtale. Ce schéma comprend : (i) autodifférenciation et autofonctionnement précoce de la gonade, (ii) autodifférenciation des cellules gonadotropes hypophysaires avec (iii) une prise en charge ultérieure par l'hypothalamus, suivie par une phase durant laquelle l'ensemble du système reproducteur fœtal fonctionne.

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