

## Evidence that hypophysis and hypothalamus constitute a single entity from the primary stage of histogenesis

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### Summary

To identify the origin of the primordial pituitary cells in early stages of amphibian embryos before the formation of Rathke's pouch or its equivalent, transplantation experiments were carried out on *Bufo*. Wild-type embryos at the open neurula stage were employed as donors and albino embryos at the corresponding stage as recipients. Since brain and pituitary cells of wild-type animals contain a great number of melanin granules, whereas albino cells are devoid of them, the melanin granules enabled the fate of transplants to be traced.

The anterior part of the neural ridge (ANR) was found to be the almost exclusive source of the secretory cells in the epithelial pituitary gland (pars distalis, pars intermedia and pars tuberalis). Transplantation of either the central part of the anterior neural plate (NP) or the ANR revealed that the pituitary primordium and the anlage of the posterior hypothalamus (infundibulum) are closely apposed at the open neurula stage. As morphogenetic movement proceeds, each of the two entities takes a separate migration route. The former part (NP) takes a ventrocaudal route to become the diencephalic floor, and the latter part (ANR) takes a rostroventral route to

acquire morphological contact with the anterodorsal wall of the foregut before differentiating into the epithelial hypophysis. At the end of the tail-bud stage, they meet again beneath the diencephalic floor and establish a hypothalamo-hypophyseal connection. Some of the ANR cells are incorporated into the preoptic region of the anterior hypothalamus. None of the NP cells develop into epithelial pituitary cells.

We conclude that the epithelial pituitary gland is not stomodeal, but placodal, in origin and that the hypothalamo-hypophyseal complex may be regarded as a single entity from an ontogenetic viewpoint.

Abbreviations: ANR, anterior part of neural ridge, NP, neural plate; ACTH, adrenocorticotrophic hormone; GH, growth hormone; LHRH, luteinizing hormone-releasing hormone; MSH, melanophore stimulating hormone, POMC, proopiomelanocortin; PRL, prolactin, TSH, thyrotrophic hormone.

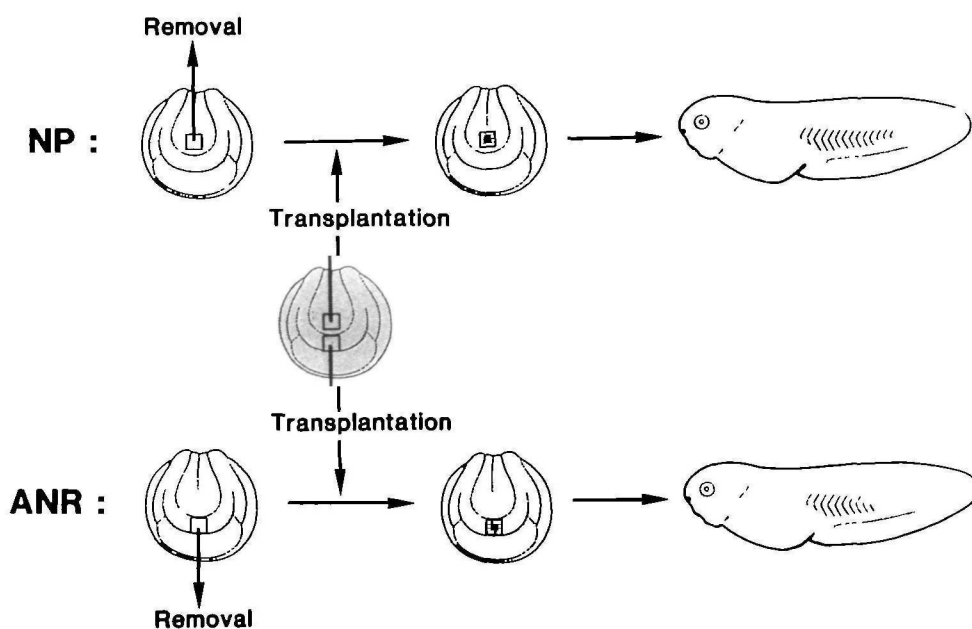
Key words: amphibia, hypophysis, infundibulum, neural ridge, pituitary.

### Introduction

Since the original description by Rathke (1838), it has been widely accepted that the endocrine cells of the epithelial hypophysis (pars distalis, pars intermedia, pars tuberalis) are derived from the stomodeal ectoderm. Only a few authors have expressed doubts about this. Among them was De Beer (1924), who claimed that the pituitary anlage arises before the appearance of the stomodeum in Amphibia. More recently, Takor and Pearce (1975) proposed a new hypothesis for the chick embryo, that the adenohypophyseal anlage is derived from the neuroectoderm, and not from the stomodeum. Pearson et al. (1983) presented another hypothesis about the development of pituitary cells in reptiles, that some of the cells in the embryonic gut epithelium (endoderm) join the stomodeal ectoderm and differentiate into peptide-secreting cells (PRL cells, GH cells, ACTH cells, MSH cells) in the epithelial hypophysis, while the glycoprotein-secreting cells (TSH cells and

GTH cells) arise in the stomodeal ectoderm. However, the evidence presented by these authors was based on purely morphological observations of microscopic sections of normal embryos. Moreover, most work on the developmental biology of the hypophysis has focused on relatively late stages of development (i.e., after the formation of Rathke's pouch). Experimental studies are necessary before drawing a final conclusion as to the developmental origin of pituitary cells in the early stages of embryos.

Recently, two groups of authors have postulated that the neural primordium plays a crucial role in the developmental process of pituitary cells. Eagleson et al. (1986) demonstrated by radiolabelling and transplantation experiments that the cells in the anterior portion of the neural ridge give rise to ACTH cells in the anterior hypophysis. The present authors provided evidence that the central part of the neural plate, which is the anlage of the posterior hypothalamus, is essential for the differentiation of pituitary POMC (i.e., ACTH



**Fig. 1.** Experimental design. Either the ANR (anterior part of the neural ridge) or the NP (central part of the anterior neural plate) was taken from wild-type open neurulae and transplanted to albino embryos at the same developmental stages (stages 18-19). The counterparts of the host embryos had been removed in advance. The host embryos were fixed at each developmental stage.

and MSH) cells (Kawamura and Kikuyama, 1987). In the present study, morphogenetic movement of primordial pituitary cells was traced by transplanting these two embryonic portions taken from open neurulae of the wild-type toad, with melanin granules as a visible intrinsic marker, into albino embryos at the same developmental stage. Here, the origin of primordial pituitary cells is described for the first time in amphibian embryos at the open neurula stage. A preliminary report of this work was presented elsewhere (Kawamura and Kikuyama, 1990).

## Materials and methods

### Animals

Wild-type eggs and albino eggs of the toad *Bufo japonicus* were collected from a pond in Tokyo and maintained in the laboratory at 10°C before use. Developmental stages were determined according to the normal table of Iwasawa (1987). The animal hemisphere (presumptive ectoderm) of wild-type eggs of this species contains a huge number of melanin granules, which will be inherited by the skin cells, neurons and pituitary cells until the late developmental stages (even after the metamorphic climax). In contrast, the neurons and the pituitary cells of albino embryos are totally devoid of melanin granules, although melanophores develop in the skin, sclera and meninges as a result of migration and differentiation of neural crest cells. Accordingly, the melanin granules in the presumptive neurons and pituitary cells in wild-type embryos serve as an intrinsic cell marker, which can be identified readily by light microscopy when the cells are transplanted into albino embryos.

### Operation

The experimental design is summarized in Fig. 1. Eggs at the open neurula stage (stages 18-19) were dechorionated manually in Holtfreter's solution (Rugh, 1962) supplemented with penicillin G potassium (100 U/ml) and streptomycin sulfate (100 µg/ml). The central region of the anterior neural plate (NP) or the anterior portion of the neural ridge (referred

to as the anterior neural ridge, ANR) was taken from wild-type eggs with fine stainless steel needles and transplanted to the corresponding sites in albino eggs of comparable developmental stages, from which the counterpart cell mass of the host embryos had been removed in advance. Unless otherwise stated, the whole embryonic layer was used for the transplants. The embryos were maintained in Holtfreter's solution at 15°C until stage 30 and then transferred to tap-water. In some cases, the ANR was dissociated manually into three layers; the outermost layer consisted of a single cell layer, the middle layer consisted of a bilayer and the innermost layer was a mesh-like endoderm (Fig. 2). Either the outermost layer or the middle layer was employed for the transplants.

### Histology

Heads of the host animals were fixed in Bouin's fluid at each developmental stage and embedded in Paraplast Plus (Monograph, St Louis) by routine methods. Sagittal sections were stained lightly with hematoxylin-eosin, observed and photographed using an optical microscope (Nikon Optiphot). Some of the sections were stained immunohistochemically with anti- $\alpha$ MSH (Imai and Imai, 1986), anti-bullfrog ACTH (Tanaka et al., 1992) or anti-bullfrog PRL (Yamamoto and Kikuyama, 1982) and FITC (fluorescein isothiocyanate)-conjugated anti-IgG (Cappel).

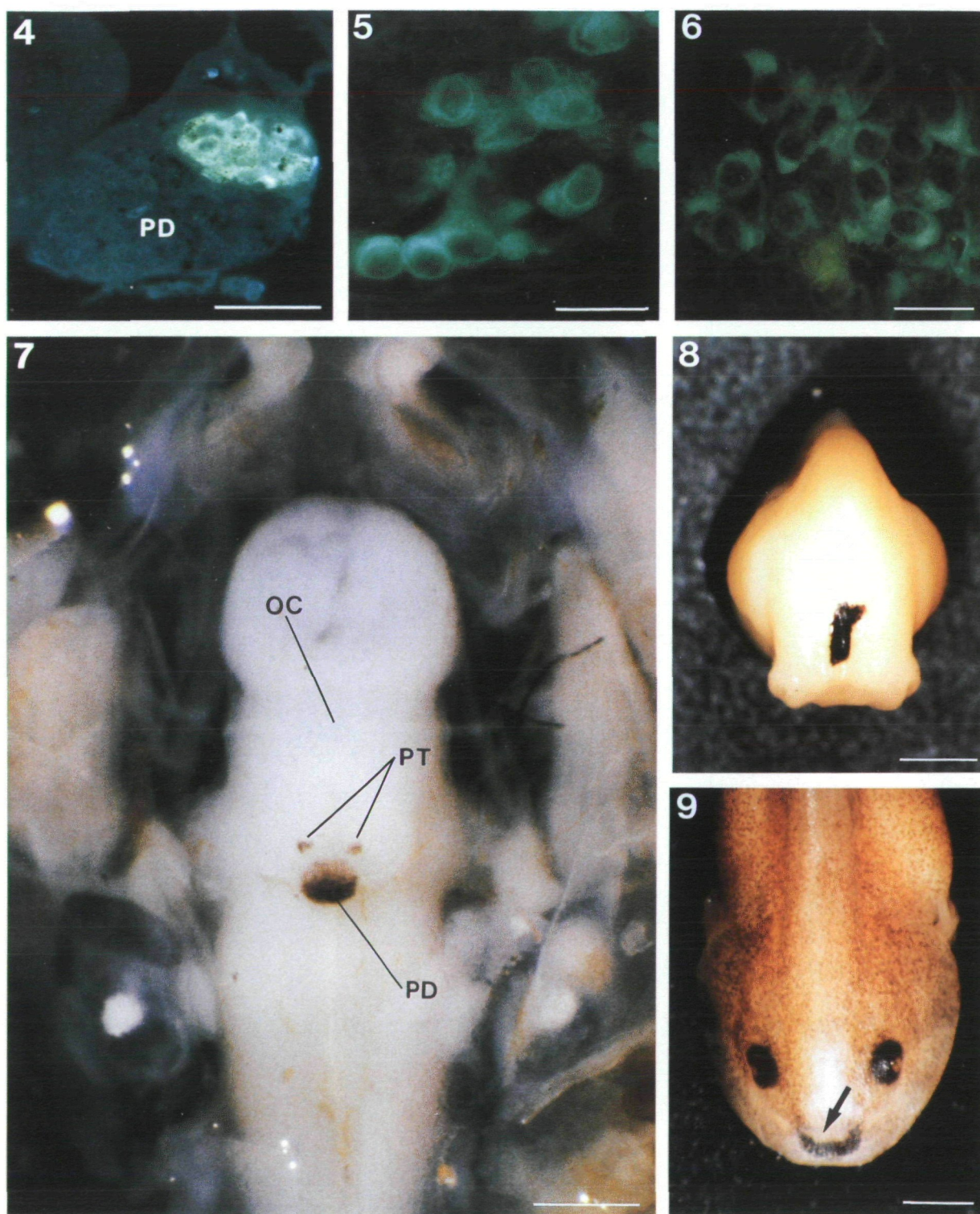
### Scanning electron microscopy

Donor embryos were fixed in ice-cold 2.5% acrolein in 0.1 M sodium phosphate buffer at pH 7.2 and post-fixed in 2%  $\text{OsO}_4$  solution. They were conductive-stained with tannic acid- $\text{OsO}_4$ , dehydrated with ethanol, dried by the critical point method using liquid  $\text{CO}_2$ , coated with palladium/gold and viewed with a Hitachi S-2400A scanning electron microscope operated at 25 kV.

## Results

The transplants were easily identifiable both macroscopically and microscopically. The developmental fates of ANR and NP will be described separately.





**Fig. 4.** Immunohistochemical staining of the pars intermedia of the hypophysis of ANR origin with anti- $\alpha$ MSH. PD, pars distalis of the pituitary gland. Scale bar=50  $\mu$ m.

**Fig. 5.** Immunohistochemical staining of the pars distalis of the hypophysis of ANR origin with anti-bullfrog ACTH. Scale bar=20  $\mu$ m.

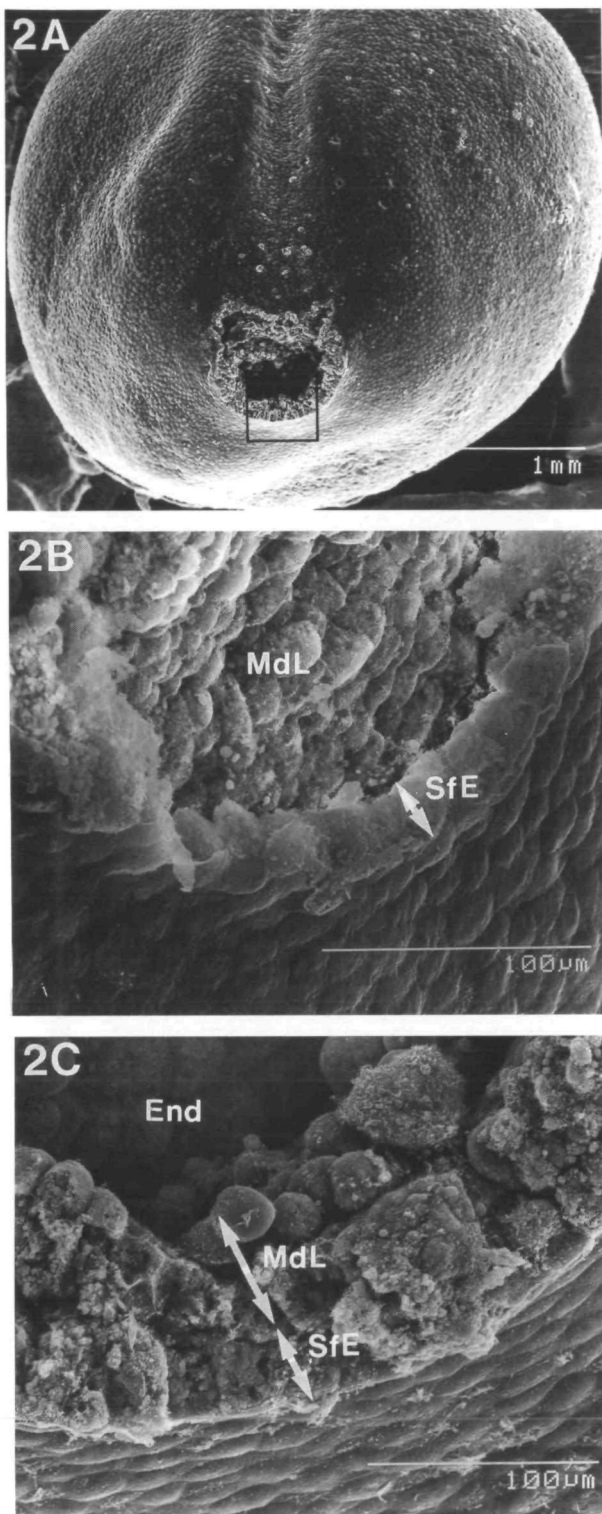
**Fig. 6.** Immunohistochemical staining of the pars distalis of the hypophysis of ANR origin with anti-bullfrog PRL. Scale bar=20  $\mu$ m.

**Fig. 7.** Ventral view of an albino tadpole that received an ANR graft from a wild-type embryo. Note that the pars distalis and pars tuberalis are heavily labelled with melanin granules. The ventral side of the cephalic cartilage is removed. OC, optic chiasma; PD, pars distalis of the pituitary gland; PT, pars tuberalis. Scale bar=500  $\mu$ m.

**Fig. 8.** Frontal view of an albino embryo (stage 25=tail-bud stage) that received a transplant of the outermost layer of the ANR from a wild-type embryo at stage 18. The graft constitutes the primordium of the upper lip. Scale bar=500  $\mu$ m.

**Fig. 9.** Dorsal view of an albino tadpole (stage 34) that received a transplant of the outermost layer of the ANR from a wild-type embryo at stage 18. This layer developed into the upper lip (indicated by an arrow), but not to the pituitary gland. Melanin granules in the sclera are produced by the host cells. Scale bar=1 mm.





**Fig. 2.** Scanning electron micrographs of the donor embryos immediately after removal of the embryonic layers of the ANR. In some cases either the outermost layer or the middle layer of the ANR was used for transplantation. (A) frontal view at low magnification; (B) high power view of the area marked by a rectangle after removal of the outermost layer, (C) high power view of the area marked by a rectangle after removal of the outermost layer and the middle layer. End, endoderm; MdL, middle layer; SfE, surface ectoderm.

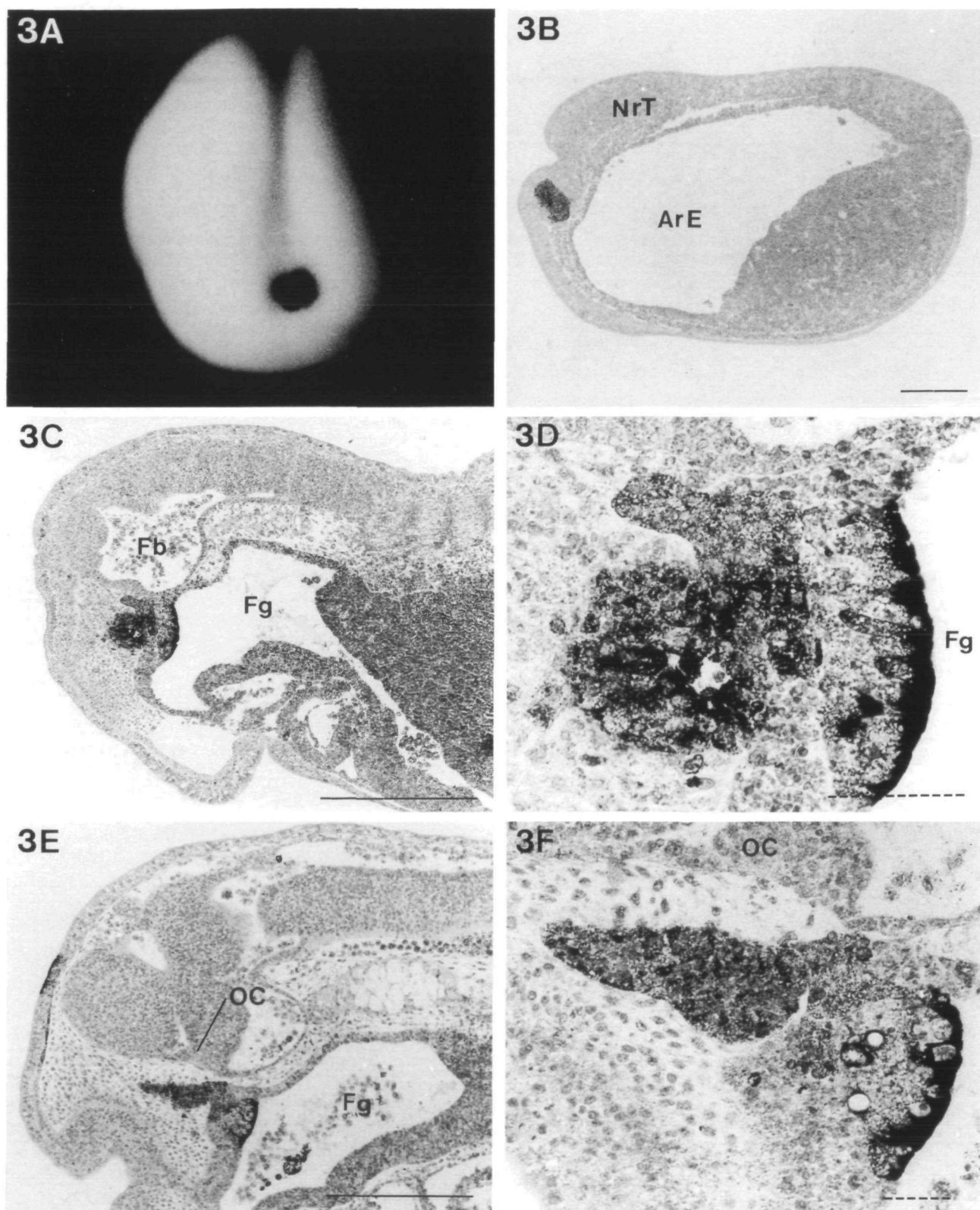
#### *Developmental fate of ANR*

Successive stages of morphological movements of ANR (anterior part of neural ridge) are shown in Fig. 3. Fig. 3A shows a host embryo immediately after the operation (stage 19=open neurula). Shortly after the operation (stage 22=neural tube closed, beginning of hatching, Fig. 3B), the graft commenced movement in a rostroventral direction, and most of the cell mass of the graft soon became hardly visible from the outside due to the deepening of the neural groove and the lateral folding of the neural ridge. At stage 25 (tail-bud stage, Fig. 3C,D), sagittal sections revealed that the transplants had lost contiguity with the surface ectoderm and were situated beneath the rostral end of the neural tube. The transplants consisted of a cone-shaped cell mass. At this stage, they did not have morphological contact with the neural tube at the light microscopic level. No labelling (melanin granules) was found in the neural tube. As the segmentation of brain proceeded, the transplants became more elongated along the body axis of the embryo. Deep labelling was found in the rostral end of the foregut endoderm (stage 28=beginning of the external gill formation, Fig. 3E,F). At stage 29 (external gills growing, Fig. 3G,H), the dorsal surface of the graft flattened to fit the forebrain floor and began to divide into a minor rostral part and a major caudal part. In 21 out of 30 specimens, the rostral part was macroscopically observed to be incorporated into the diencephalic floor before stage 32 (stage of the branchial mantle formation) and finally constituted a part of the prechiasmatic region. The caudal part of the graft became rounded to form the epithelial hypophysis (stage 34=formation of the branchial mantles completed, Fig. 3I). At the same time, the infundibulum developed at the connection between the hypophysis and the diencephalic floor. Microscopic observation of the histological sections revealed that most of the cells in the pars distalis and all of the cells in the pars intermedia and pars tuberalis were deeply labelled with melanin granules (Fig. 3J). Immunostaining with anti- $\alpha$ MSH confirmed that all of the  $\alpha$ MSH-positive cells possess melanin granules (Fig. 4). It was also confirmed that all of the immunoreactive ACTH cells and PRL cells are labelled with melanin granules (Figs 5 and 6). The infundibulum and the median eminence were not labelled. The pars distalis and the pars tuberalis were easily discernible on the dorsal aspect of whole-mount specimens owing to the deep labelling with melanin granules (Fig. 7). When the dissociated layers were employed as transplants, only the middle layer developed into the pituitary gland. The outermost layer was found to be the primordium of the upper lip (Figs 8 and 9).

#### *Developmental fate of NP*

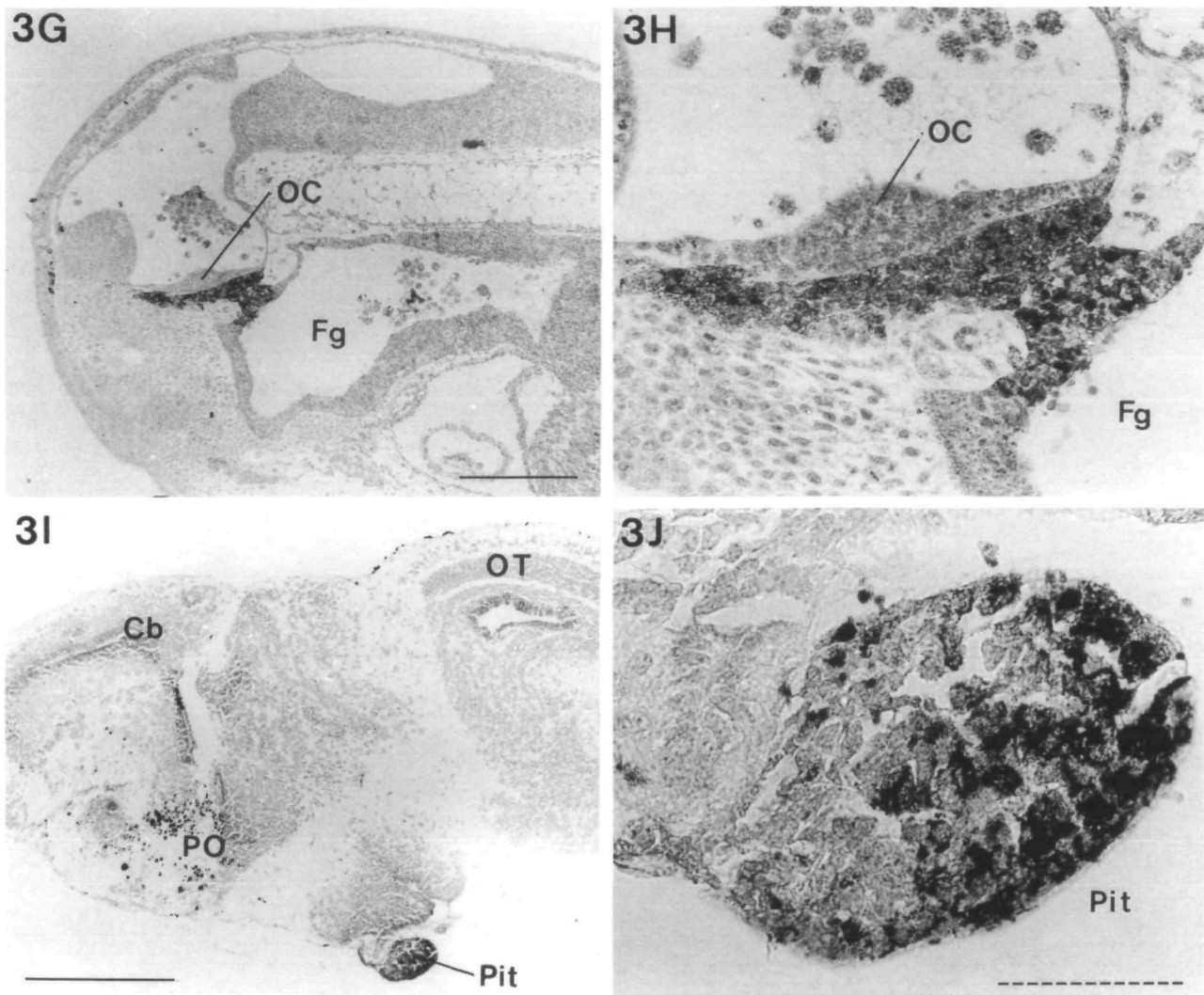
Fig. 10A shows a host embryo immediately after the operation (stage 19). The NP (central part of the neural plate) underwent a caudoventral movement in accordance with the deepening of the neural groove and the lateral folding of the neural ridge. As a result, the transplant soon became invisible from the outside. In





sagittal sections, the transplants were identifiable as small areas of epithelial tissue situated a little caudally to the ANR (stage 22, Fig. 10B). After closure of the neural tube, the transplants were integrated into the neural tube as a caudal portion of the forebrain floor

(stage 25, Fig. 10C,D). Before this stage, the transplants had been completely segregated from ANR descendants, although the NP was closely apposed to the ANR at the time of the operation. Around the time when the forebrain completed segmentation into the



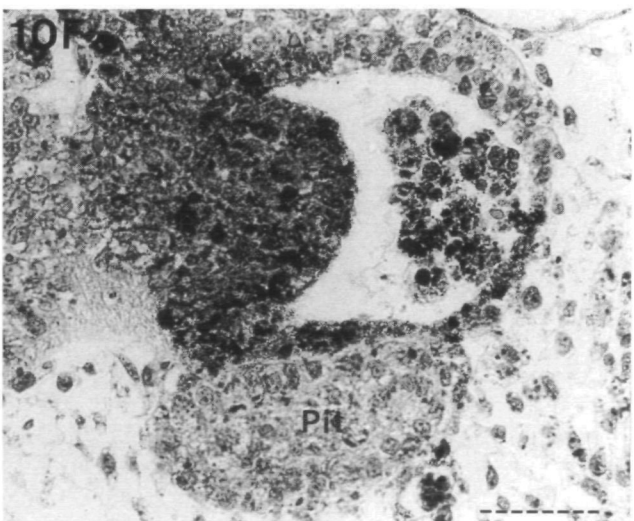
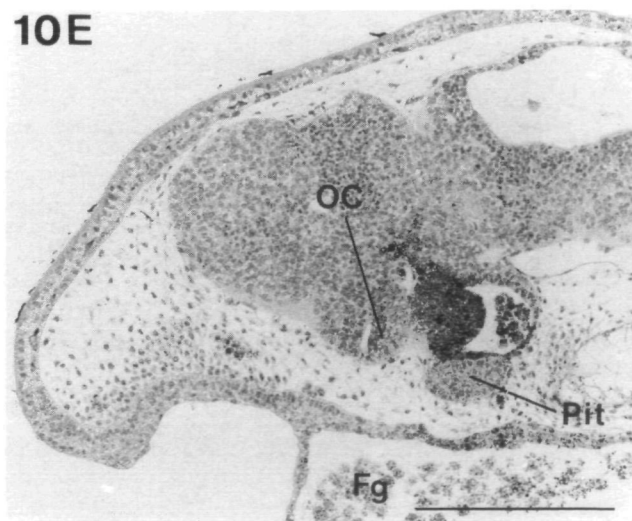
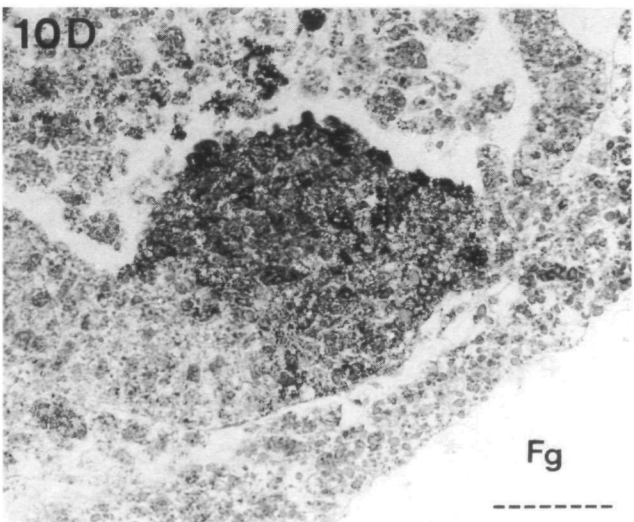
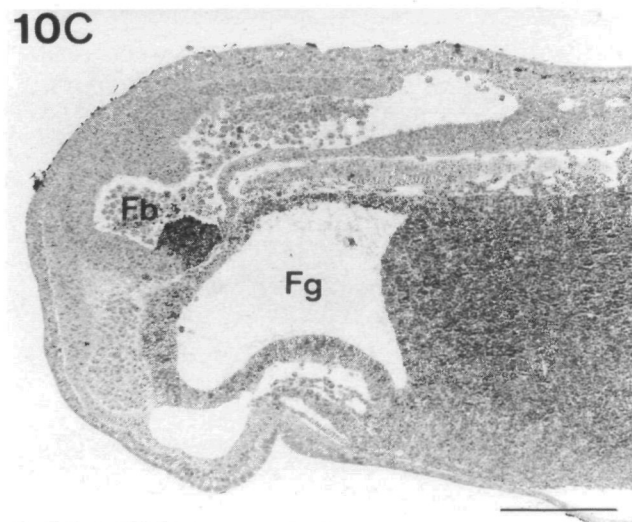
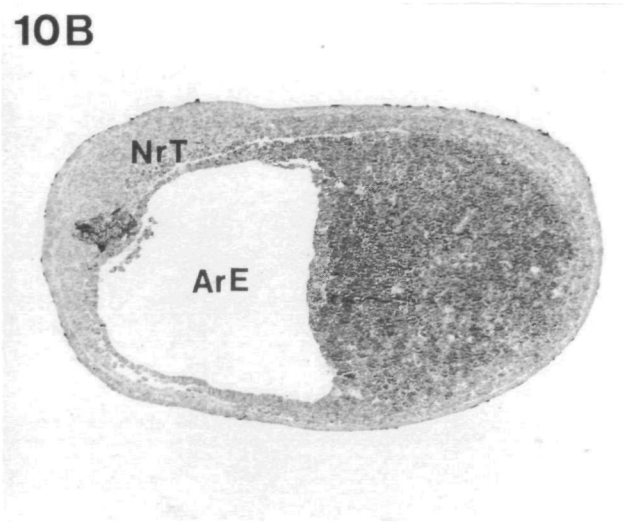
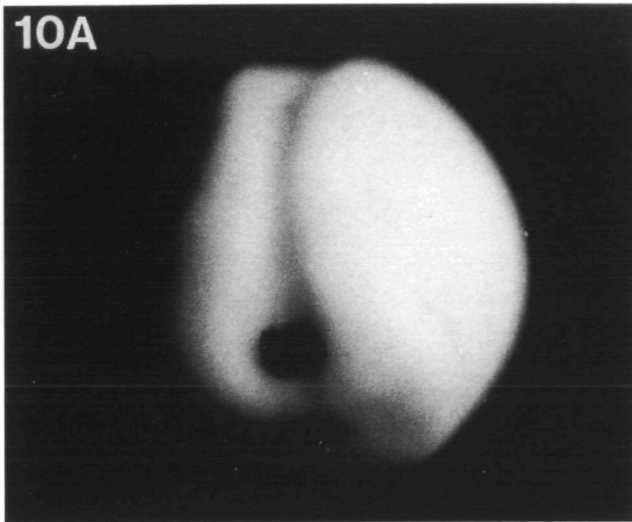
**Fig. 3.** Successive photographs of the host embryos which received the ANR. Fig. 3A shows a frontal view of the whole embryo immediately after the operation (stage 19=open neurula). (3B; stage 22=shortly after closure of the neural tube, beginning of hatching) The ANR commences morphogenetic movement in a rostroventral direction. (3C,D; stage 25=tail-bud stage) The graft is situated beneath the rostral end of the neural tube. (3E,F, stage 28=beginning of the external gill formation) The ANR graft is elongated along the body axis and connected caudally to the rostroventral end of the foregut. The connecting part of the endoderm is deeply labelled with melanin granules. A part of the frontal epidermis is also labelled (cf. Figs 8 and 9). (3G,H; stage 29=external gills growing) The graft is flattened to fit the bottom of the forebrain and becomes more elongated. The rostral part of the graft begins to segregate. (3I,J, stage 34=formation of the branchial mantles completed) By this stage, the morphogenesis of the pituitary gland has been completed. Note that the anterior and intermediate lobes of the hypophysis and the preoptic region of the brain are deeply labelled with melanin granules. The melanin granules in the meninges are not derived from the graft, but are synthesized by the melanophores that developed from the neural crest cells in the host embryos. Fig. 3B to J are micrographs of sagittal sections, stained lightly with hematoxylin and eosin. ArE, archenteron; Cb, cerebrum; Fb, forebrain; Fg, foregut; NrT, neural tube; OC, optic chiasma; OT, optic tectum; Pit, pituitary gland; PO, preoptic region. Solid bar=300  $\mu$ m. Dashed line bar=50  $\mu$ m.

telencephalon and diencephalon, descendants of the ANR (primordium of the epithelial hypophysis) re-established morphological contiguity with the diencephalic floor where the cells derived from NP were situated, such that the ANR resumed morphological contact with the NP (stage 30=external gills fully developed, Fig. 10E,F). At advanced stages, derivatives of the NP were found in the dorsal infundibular nucleus, but not in the epithelial hypophysis. Labelling was found also in the median eminence (stage 31=be-

ginning of the branchial mantle formation, Fig. 10G,H).

## Discussion

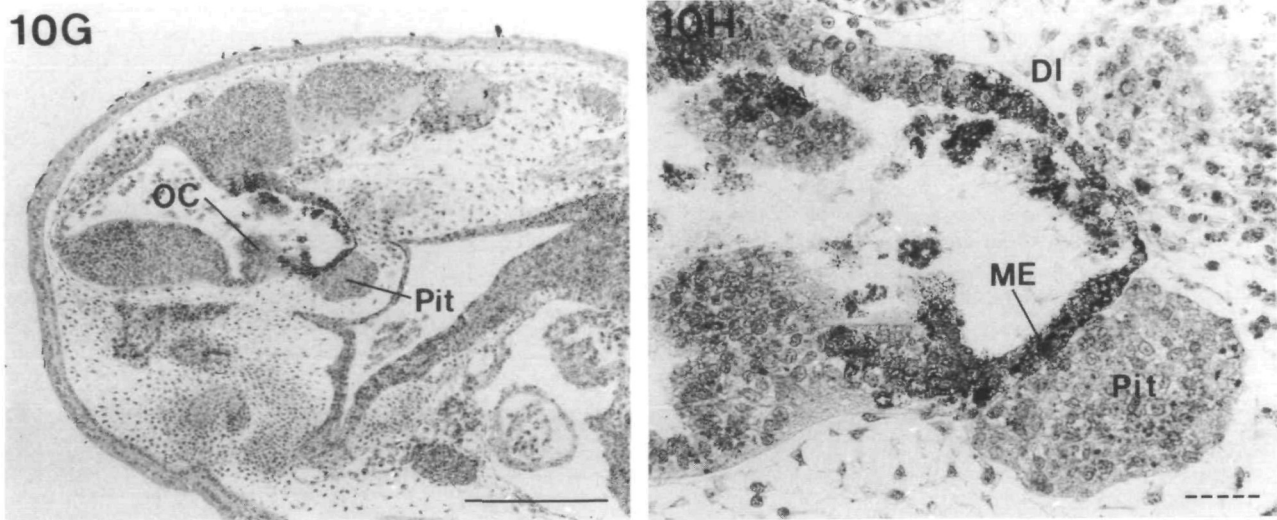
Although many studies have been published regarding the developmental morphology of the pituitary gland, little information was available concerning the location of the pituitary anlagen in amphibian embryos before



the formation of Rathke's pouch or its equivalent. The present study revealed that the epithelial hypophysis is derived from the anterior part of the neural ridge (ANR). Homotopical transplantation of ANR with an intrinsic cell marker resulted in labelling of the entire

epithelial hypophysis. At first, the ANR assumed a cone-shaped morphology under the forebrain floor, and the caudal part of the ANR derivatives acquired morphological contact with the anterior wall of the foregut. At this stage, this tissue can be regarded as





**Fig. 10.** Successive photographs of the host embryos that received NP. Fig. 10A shows a frontal view of the whole embryo immediately after the operation (stage 19=open neurula). (10B, stage 22=shortly after closure of the neural tube, beginning of hatching) The NP derivative is situated a little caudally to the ANR. (10C,D; stage 25=tail-bud stage) The NP is integrated into the neural tube as a caudal portion of the forebrain floor. (10E,F; stage 30=external gills fully developed) The descendants of the NP re-establish morphological contact with the adenohypophysis (10G,H; stage 31=beginning of the branchial mantle formation) The derivatives of the NP constitute the dorsal infundibular nucleus and the median eminence. Fig. 10B to H are micrographs of sagittal sections, stained lightly with hematoxylin and eosin. ArE, archenteron; DI, dorsal infundibular region, Fb, forebrain; Fg, foregut; ME, median eminence; NrT, neural tube; OC, optic chiasma; Pit, pituitary gland. Solid bar=300  $\mu$ m. Dashed line bar=50  $\mu$ m.

being an equivalent of Rathke's pouch in mammals. At the advanced stages of development, the ANR derivatives made contact with the diencephalic floor and finally developed into the epithelial hypophysis. The connection between the epithelial hypophysis and the diencephalon, namely the infundibulum, was found to be derived from the central part of the neural plate. Accordingly, the hypophyseal primordium and the anlagen of the infundibulum are closely apposed at the open neurula stage. As morphogenetic movement proceeds, they take separate paths (the pituitary anlagen take a rostral path and the infundibular primordium a ventrocaudal path), which finally meet together near the rostradorsal wall of the foregut. Although the present experiment does not provide crucial evidence for the ectodermal origin of the pituitary gland, it can be concluded with certainty that the epithelial hypophysis is derived from the middle layer of the ANR. Indirect evidence for the neuroectodermal origin of the pituitary gland has been presented by a few authors. Japundžić et al. (1985) presented such a hypothesis based on the fact that pituitary cells in a certain mutant strain of rat exhibit neuronal and melanocytic phenotypes. Takor and Pearse (1975) postulated a neuroectodermal derivation of all the endocrine cells of the adenohypophysis in the chick, and claimed that the whole hypothalamo-hypophyseal complex is a neuroectodermal derivative of the ventral neural ridge (VNR) (see also Levy et al., 1980). Although the hypothesis of Takor and Pearse was indirectly supported from a more integrative viewpoint (the concept of a diffuse neuroendocrine system, or the

APUD theory; Pearse, 1968, 1987), this was based essentially on purely morphological observations of histological sections of normal specimens. It must be kept in mind that histological observation of successive stages of specimens may lead to false conclusions unless the cells under study have a stable cell marker. From this standpoint, the present study provides crucial evidence that the anlage of the epithelial hypophysis is related topologically to the neuronal primordium, although it cannot be concluded to be "neuroectodermal", because the transplants in the present experiment were not necessarily confined to the ectodermal layer (mesodermal cells may be included in the graft). However, it can be concluded that the endocrine pituitary anlage is placodal, as are some of the sensory cells and ganglion cells. In this connection, the work by Eagleson et al. (1986) on *Xenopus* is relevant. These authors performed a similar transplantation experiment with radiolabelled grafts and concluded that the ANR (these authors adopted the terminology of VNR, ventral neural ridge, by analogy with the nomenclature for avian embryos) gives rise to ACTH cells and brain POMC cells, but not to other endocrine cells in the pituitary gland, including MSH and prolactin cells. In our case, almost all the parenchymal cells in the epithelial hypophysis, including MSH, ACTH and PRL cells, were shown to originate in the ANR. Since Eagleson et al. (1986) did not specify the cell layer of the ANR (VNR) employed for transplantation, the apparent discrepancy in the results may be due to the difference in the cell layer in the ANR. Another possibility is that the transplants may deteriorate at the

time of radiolabelling in vitro. It is also probable that only limited cells in the graft were radiolabelled.

The present authors reported that the pituitary POMC cells (ACTH cells and MSH cells) do not develop if contact between the NP and pituitary primordium is prevented by removal of the NP (Kawamura and Kikuyama, 1987). This implies two possibilities. One is that the pituitary POMC cells are derived from NP cells. The other is that the NP plays an indirect role in the development of pituitary POMC cells, while these cells originate in a region other than the NP. In the present study, we have demonstrated conclusively that almost all the epithelial pituitary cells, including POMC cells, originate in the ANR and that the NP cells do not develop into epithelial pituitary cells. Accordingly, the second possibility seems the more plausible. Since the NP was found to differentiate into the connecting part of the brain and hypophysis, this hypothesis implies that some inductive stimuli from the embryonic brain are necessary for the differentiation of pituitary POMC cells. In the avian embryo, Couly and Le Douarin (1985, 1987) provided evidence for a common origin of the hypothalamic and pituitary cells by interspecific transplantation experiments.

It is well established in the field of endocrinology that the hypothalamus and the pituitary gland are intimately related functionally. The present study provided further evidence for an ontogenetic relationship between the two. Recent immunohistochemical studies have thrown light on the relationship by demonstrating the presence of a neural-neuroendocrine marker, NSE (neuron-specific enolase) in the anterior pituitary gland (Van Noorden et al., 1984). It is also noteworthy that embryonic cells in the pituitary primordium and brain are simultaneously induced to transcribe the POMC gene, possibly as a result of reciprocal brain-pituitary interactions (Hayes and Loh, 1990). The present study showed that the hypophyseal cone (amphibian equivalent of Rathke's pouch in mammals) segregated into rostral and caudal parts. The caudal part developed into the epithelial hypophysis, whereas the rostral part was incorporated in the preoptic region of the hypothalamus. This indicates that there is a group of cells that originate in the ANR and are incorporated into the brain to become neural cells after the formation of the neural tube has been completed. Another example of migration of a cell group into the brain tissue of developing amphibia has been demonstrated recently (Murakami et al., 1991). In the premetamorphic newt (*Cynops pyrrhogaster*), LHRH-immunoreactive cells are not observed in the brain, but in the nasal organ. After metamorphosis, LHRH-immunoreactive neurons appear in the preoptic area. Unilateral removal of nasal placode at the tail-bud stage results in the development of LHRH neurons at metamorphosis in the preoptic area exclusively on the side that the nasal placode was left intact. Indirect evidence that in mice LHRH neurons migrate from the nasal organ into the brain has also appeared (Schwanzel-Fukuda and Pfaff, 1989). Considering that the nasal placode is also of neural ridge origin (Couly and Le Douarin, 1985), it may be a

generally occurring phenomenon that some of the cells of neural ridge origin are incorporated into the brain through the ventral side or the rostral end of the forebrain after the neural tube formation.

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