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Developmental origin of the rat adenohypophysis prior to the formation of Rathke's pouch

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SUMMARY

In amphibians, it has already been shown that the adenohypophysis originates from the anterior neural ridge. During the migration and morphogenesis of this organ, the anterior neural ridge transiently forms a Rathke's pouch-like structure by attaching itself to the rostral tip of the foregut, and finally gives rise to the adenohypophysis by detaching from the foregut and becoming connected to the infundibulum of the hypothalamus. In order to identify the origin of the adenohypophyseal cells in mammalian embryos prior to the formation of Rathke's pouch (RP), we labeled the rostral end of the neural plate and the adjacent area focally with DiI at the open neurula stage (9.5 dpc). After a 48-hours culture of the whole embryos, strongly labeled cells were detected in the RP only when DiI was applied to a small area situated just anterior to the rostral end of the neural plate. By explanting the labeled RP for a further 7 days, we confirmed immunohistochemically that the labeled cells developed into the secretory cells of the adenohypophysis. The developmental origin of the adenohypophysis is identified for the first time in the early mammalian embryo before the formation of RP.

Key words: Hypophysis, Neural plate, Neural ridge, Neuroectoderm, Pituitary, Rathke's pouch, Rat

INTRODUCTION

Since the earliest report by Rathke (Rathke, 1838), it has been widely accepted that the epithelial hypophysis has its developmental origin in an extraneural part of the embryo, i.e. the stomodeal ectoderm. However, in amphibians and birds, several investigators have re-examined the embryonic origin, with special reference to the initial stage of development, and have discovered that the adenohypophysis has its origin in the neuroectoderm. In *Xenopus*, Eagleson et al. transplanted radiolabeled tissue into open neurulae and concluded that the cells in the anterior portion of the neural ridge gave rise to Adrenocorticotropin (ACTH)-producing cells in the anterior hypophysis (Eagleson et al., 1986). Furthermore, Kawamura and Kikuyama traced the fate of the anterior neural ridge grafted from wild-type embryos to albinos, and found that the anterior neural ridge underwent a series of morphogenetic processes, beginning with a backward movement beneath the forebrain and ending by attaching to the infundibular stalk (Kawamura and Kikuyama, 1992; Kawamura and Kikuyama, 1998). During the course of this morphogenetic movement, the adenohypophyseal primordium formed a Rathke's pouch-like protrusion oriented towards the forebrain floor with its caudal end attached transiently to the rostral tip of the foregut. In birds, similar results were obtained by Couly and Le Douarin who used an artificial quail-chick chimera to visualize the fate of the tissue of interest (Couly and Le Douarin, 1985; Couly and Le Douarin, 1987).

In mammals, the origin of the pituitary cells has been presumed to be a part of the rostral part of the epidermal ectoderm that is destined to become the stomodeal roof. Although this presumption has been widely accepted, it is based on purely morphological observation of the microscopic sections of normal embryos; no direct evidence has been provided even up to the present day. In order to fill this gap, we have traced the origin of the presumptive Rathke's pouch (RP) back to the open neurula stage of rat embryos by means of a focalized application of DiI. To this end, whole-embryo cultures and successive organ cultures were prepared.

MATERIALS AND METHODS

Whole culture of rat embryos

Embryos of Sprague-Dawley rats at the open neurula stage were obtained from anaesthetized mothers at 9.5 dpc. The uteri were removed, and each embryo was transferred into Tyrode's solution. The placentae and the embryonic membranes were left intact. The

embryos were processed immediately for microinjection as described below. Both injected and non-injected control whole embryos were put into culture within 1 hour of the removal from the uterus, according to the method of New (New, 1990) and Osumi-Yamashita et al. (Osumi-Yamashita et al., 1994). After incubation for 48 hours, when RP had formed completely, the RP and the neighboring tissue were examined under a fluorescence microscope as whole-mount or serial cryosections. Some of the RPs were explanted to an organ culture (Fig. 1).

Labeling with DiI

A saturated solution of DiI C_{18} (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes) was made in 100% dimethylformamide and diluted 30-fold with 100% dimethylformamide after centrifugation at 4°C. The working solution was stored at 4°C and allowed to warm to room temperature before injection. Microinjection was performed by using glass micropipettes filled with the dye solution (Osumi-Yamashita et al., 1994). Focalized application was performed on the following points of the ectoderm: (1) the rostromedial end of the neural plate; (2) the rostromedial portion of the neural ridge; and (3) extraneural plate ectoderm apposed just anterior to the rostromedial end of the neural plate (Fig. 2). These three regions were presumed to be the embryonic origin of RP by analogy with the results reported for amphibians and birds. Special care was taken not to cross the boundary of the neural plate. Each embryo was examined under a fluorescence microscope, and only appropriately labeled embryos were transferred to a whole-embryo culture.

Examination of embryos

At the end of culture, embryos were assessed for heartbeat, yolk-sac circulation, somite number and gross morphology. Living embryos with normal morphology (119/121 cases) were subjected to further processing at the 30-35 somite-stage. The upper jaws were picked up in Tyrode's solution by using a fine tungsten needle and watchmaker's forceps. Some of the jaws were used for histological studies, and others were transferred to an organ culture before histological examination. Histological samples were observed as whole-mount or serial cryosections. Cryosections were obtained by fixing specimens in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 4 hours, freezing them in OCT compound (Sakura Finetek), and cutting them at a 10 μm thickness in a cryostat. A Nikon epifluorescence microscope equipped with a rhodamine filter set was used.

Organ culture of the RP

Organ cultures of RP were prepared according to Watanabe and Daikoku (Watanabe and Daikoku, 1976) with some modifications. Briefly, RPs were isolated together with the forebrain floor and surrounding mesenchyme, settled on a piece of Millipore filter (Type AA, Millipore Co.), and placed on a stainless steel-grid in a plastic dish (Falcon) containing a 1:1 mixture of BGJb culture medium (GIBCO) and F12 nutrient medium (GIBCO) supplemented with penicillin-streptomycin (10 U/ml-10 μg/ml, GIBCO) and 10% fetal calf serum (MoreGate). Cultures were maintained for one week at 37°C in a humidified atmosphere of 95% air 5% CO_2. The culture medium was changed every 3 days.

Immunostaining

After 7 days of organ culture, tissue explants were pealed off from the membrane filter, fixed immediately in 4% PFA in PBS for 4 hours, frozen and cut at 10 μm. Serial sections were photographed under a fluorescence microscope and treated with rabbit antisera against porcine ACTH (Tanaka and Kurosumi, 1986). In the present study, ACTH was chosen as a marker for a subset of the adenohypophyseal cells, because they appear first in the cultured RP. Immunoreactive ACTH cells were visualized by the streptavidin-peroxidase method.

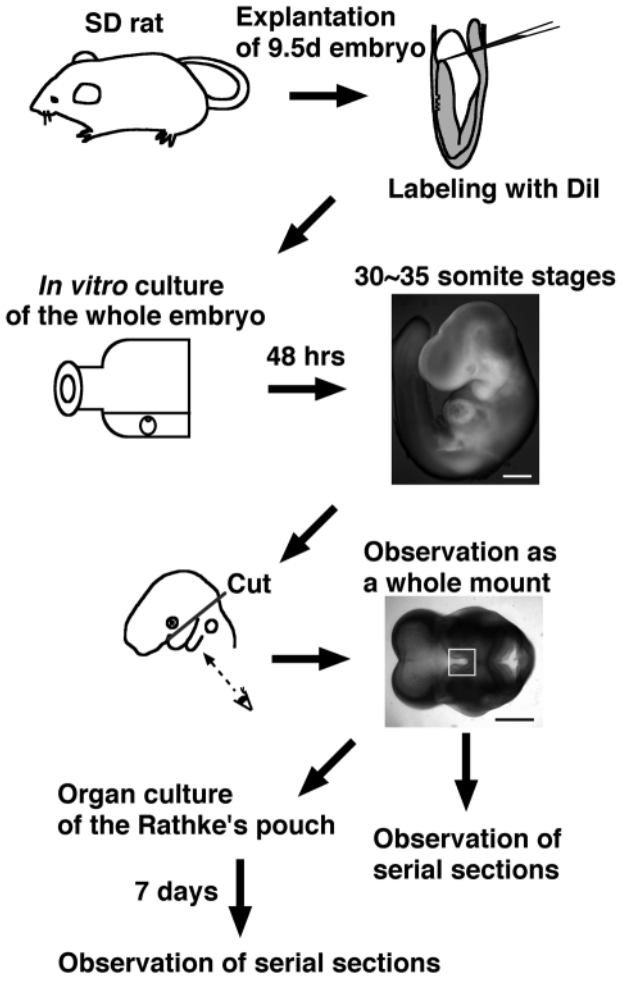

Fig. 1. Experimental procedures. Rat embryos were labeled with DiI at 9.5 dpc. The labeled embryos and nonlabeled control embryos were maintained as a whole-embryo culture for 48 hours and then decapitated to observe the fluorescence. Successfully labeled embryos were dissected to obtain Rathke's pouch. The Rathke's pouches were cultured for another 7 days before histological processing. Scale bars: 500 μm.

Matched pairs of the dark and bright field photographs were analyzed by an image analyzer (ARGUS, Hamamatsu Photonics) to examine the co-localization of DiI and ACTH.

RESULTS

Almost all of the embryos (119/121 cases) developed normally during 48 hours of whole-embryo culture with regard to the gross morphology and histology of the brain and associated structures. At the end of this culture period, RP became visible under the microscope without dissection. The site of the labeling was confined to a small area with a diameter of approximately 100 μm (Figs 3-5) at the time of application. The fluorescence of DiI did not show signs of diffusion during the whole embryo or successive organ culturing. DiI was spotted as three foci. In the first series of experiments, the rostromedial end of the neural plate (point 1, Fig. 2) was labeled. At the end of the whole-embryo culture period, the

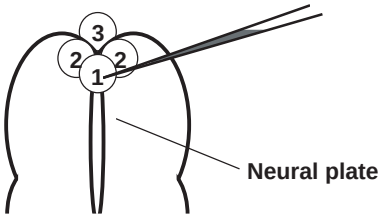

Fig. 2. Site of labeling. DiI was applied as three foci: (1) at the rostromedial end of the neural plate, (2) at the rostromedial portion of the neural ridge, and (3) at an extraneural point anteriorly apposed to point 1. Special care was taken not to cross the boundary of the neural plate.

labeled cells were found exclusively in the forebrain floor outside of the RP (39/41 cases; Fig. 3). In the second series of experiments, labeling of the rostromedial position of the neural ridge (point 2, Fig. 2) also resulted in the staining of the forebrain floor. In this case, the stained cells were situated in a more anterior position than in the former case (8/9 cases; Fig. 4). No labeling was found in the RP and in no case was the endodermal cells or mesenchyme stained. By contrast, in the last series of experiments, when DiI was applied on a point on the extraneural plate ectoderm anteriorly apposed to the rostromedial end of the neural plate (point 3, Fig. 2), the labeling was found on the RP and oral plate in 52/69 cases. Six embryos bore the label on the oral plate but not on the RP. Observation of the cryosections revealed that the stained cells were scattered over the RP (Fig. 5). Tailing of DiI with a strong

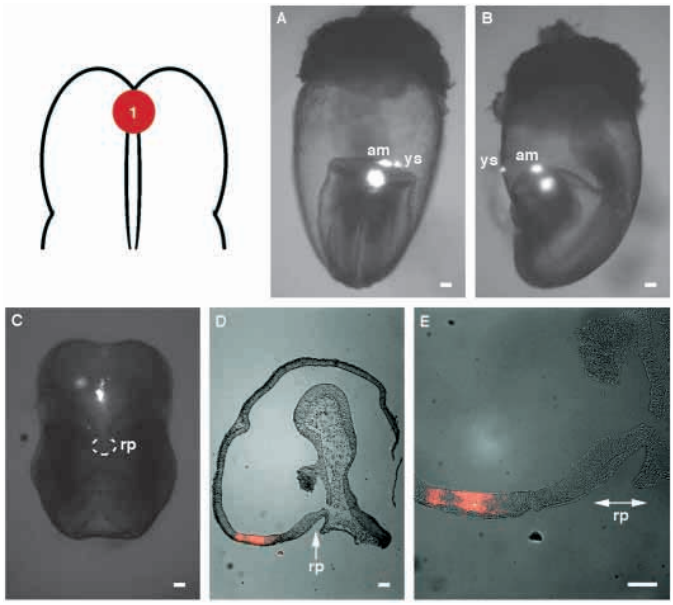

Fig. 3. Fluorescence microscopy of embryos labeled at the rostromedial end of the neural plate (1). (A) Ventral view of an embryo immediately after application of DiI. (B) Lateral view of the same embryo. (C) Ventral view of a decapitated embryo after in vitro culture for 48 hours. The labeling is found on the forebrain floor outside of Rathke's pouch (rp). (D) Sagittal section. Note that the labeled cells are found in the forebrain floor. (E) High-power view of D. *am* and *ys* indicate extra-embryonic DiI placed on the amnion and yolk sac, respectively, at the time of insertion of the glass capillary. Scale bars: 100 μm.

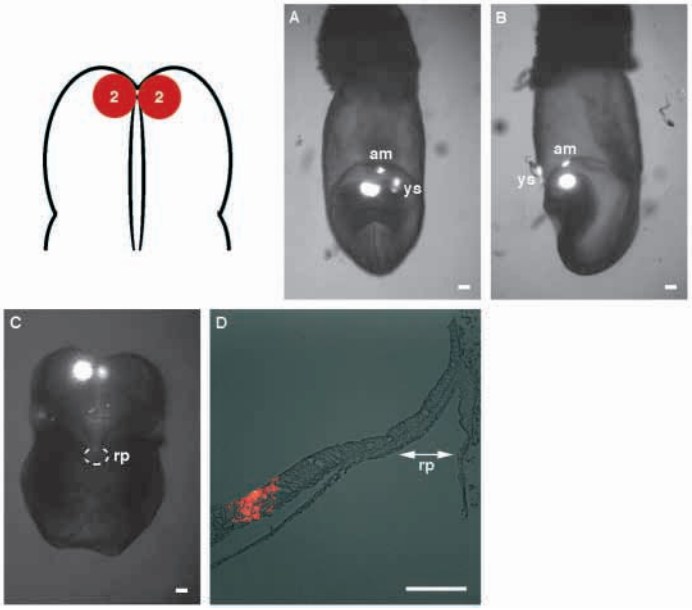

Fig. 4. Fluorescence microscopy of embryos labeled at the rostromedial portion of the neural ridge (2). (A) Ventral view of an embryo immediately after application of DiI. (B) Lateral view of the same embryo. (C) Ventral view of a decapitated embryo after in vitro culture for 48 hours. The labeling is found outside of Rathke's pouch (rp). (D) Sagittal section. Note that the labeled cells are found in a more rostrally situated position of the forebrain floor than in Fig. 3. *am* and *ys* indicate extra-embryonic DiI placed on the amnion and yolk sac, respectively, at the time of insertion of the glass capillary. Scale bars: 100 μm.

fluorescence was also observed along the midline of the palate epithelium. In 2, 1 and 11 cases in the respective series, the labeling became hardly detectable at the end of the whole-embryo culture period.

To see if the labeled cells in the RP developed into functional secretory cells of the adenohypophysis, we removed the RP after photographing the fluorescence and transferred it to an organ culture. Seven days later, when the adenohypophysis of matched normal embryos became immunopositive with anti-ACTH, the pituitary gland taken from the labeled embryos was processed for immunostaining with anti-ACTH. Overlapping of the positive signals of DiI and immunoreactive ACTH was observed in a subset of cells (Fig. 6).

DISCUSSION

In the present study, three focal areas near the anterior end of the neural plate were labeled with a carbocyanin dye at the open neurula stage to determine the localization of presumptive adenohypophyseal cells. The developmental stage of the experimental materials was thought to be comparable with that employed in similar studies on amphibians (Eagleson et al., 1986; Kawamura and Kikuyama, 1992) and birds (Couly and Le Douarin 1985; Couly and Le Douarin, 1987). At this stage, the morphology of the neural primordium of mammalian embryos is different from that of lower vertebrates in that the mammalian neural ridge consists of a pair of lateral ridges situated on both sides of the neural plate. In other words, the

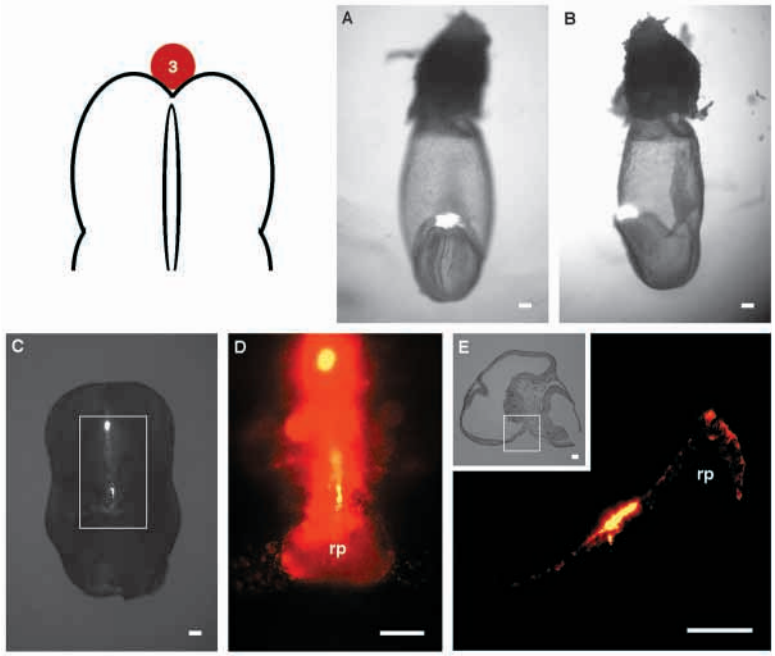

Fig. 5. Fluorescence microscopy of embryos labeled at an extraneural plate area anteriorly apposed to point 1 (3). (A) Ventral view of an embryo immediately after application of DiI. (B) Lateral view of the same embryo. (C) Ventral view of a decapitated embryo after in vitro culture for 48 hours. (D) High-power view of C. The labeling is found on Rathke's pouch (rp, 52/69 cases). (E) Sagittal section. Scale bars: 100 μm.

anterior part of the neural ridge is not discernible in mammalian embryos, including rats. In amphibians, the anterior portion of the neural ridge is most prominent as a hemicircular rising that delimits the rostral end of the neural plate by connecting the lateral components of the neural ridge. Based on the transplantation studies using albino/wild-type chimeras of *Bufo*, the anterior neural ridge (ANR) is known to be the almost exclusive source of the adenohypophyseal cells (Kawamura and Kikuyama 1992). In birds, a similar experimental approach using quail-chick chimeras has clearly shown that the adenohypophysis originates in the ANR (Couly and Le Douarin 1985; Couly and Le Douarin, 1987). If we compare the gross morphology of the adenohypophyseal primordium at the open neurula stage between amphibian and avian embryos, special attention is drawn to the fact that the anterior portion of the neural ridge in the avian embryo bends towards the ventral side so that the transversal element of the neural ridge becomes less prominent compared with that of the amphibian embryos. Some authors refer to that part of the avian neural ridge as the ventral neural ridge (VNR; Takor and Pearse 1975).

The transversal element of the neural ridge shows a tendency to become less prominent in higher vertebrates. It is generally accepted that the VNR is the avian counterpart of the ANR of the amphibian embryos, with regard to morphology and developmental fate. As there is no morphologically discernible ANR or VNR in mammalian embryos, the present study was undertaken to determine their mammalian counterpart by finding the adenohypophyseal primordium in rat embryos. Similar experimental approach was taken by Osumi-Yamashita et al. (Osumi-Yamashita et al., 1994) in a series of experiments to map the developmental fate of the neural crest. The presumptive pituitary area was specified in their publication as being located in the so-called anterior neural ridge (point 1, Fig. 7); however, RP looked quite faintly labeled, if labeled at all, by labeling of the 'anterior neural ridge' specified in the literature. Our study concentrates on locating the pituitary primordium and revealed that the presumptive adenohypophyseal cells are localized in the extraneural plate ectoderm closely apposed to the rostral end of the neural plate (Fig. 7). These cells developed not only into a component of RP but also into functional secretory cells in the anterior pituitary gland, as revealed by double staining with DiI and anti-ACTH (Fig. 6). In no case did labeling of the neural plate and lateral neural ridge result in the labeling of RP. To our knowledge, the present report is the first experimental demonstration of the localization of presumptive adenohypophyseal cells in early mammalian embryos prior to the formation of RP. In the present study, labeling of the pituitary primordium resulted in the co-labeling of the palatal epithelium, showing that the two entities are closely associated in terms of their topographical arrangement. These results

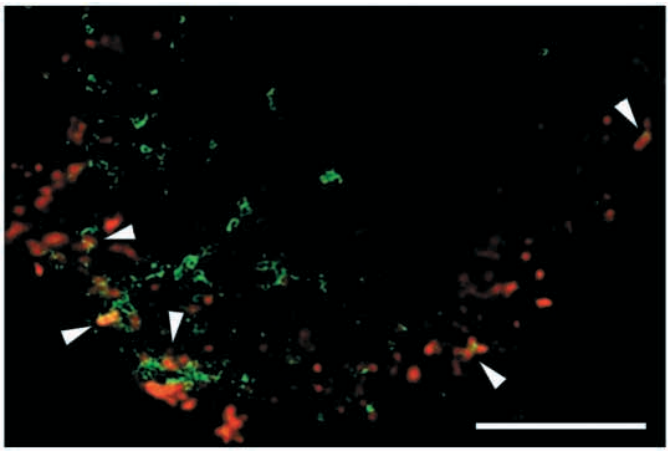

Fig. 6. Co-localization (yellow; arrowheads) of DiI (red) and immunoreactive ACTH (green) in the anterior pituitary gland developed from the explanted Rathke's pouch taken from the embryo shown in Fig. 5C,D. The extraneural area anteriorly apposed to the rostral end of the neural plate developed into functional pituitary cells. Scale bar: 100 μm.

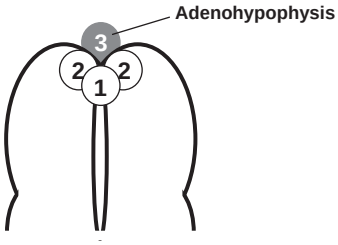

Fig. 7. Localization of the adenohypophyseal primordium on the fetal rat on 9.5 dpc. Of the three points indicated in this figure, only (3) (an extraneural plate ectoderm anteriorly apposed to the rostral end of neural plate) contributes to the formation of adenohypophysis.

reconfirmed the classical idea that, in mammals, the stomodeal primordium gives rise to the adenohypophysis. If we survey the results obtained in this study on rats and in other studies on lower vertebrates, a deformation of the anterior end of the neural plate and associated neural ridge is noted as described at the beginning of the Discussion: i.e. the typical ANR observed in fish and amphibians shifts in avian embryos to a more ventral position, owing to the prominent rising of the lateral components of the neural ridge. The use of the term 'neural ridge' does not seem to be consistent for different classes of vertebrates. According to the recently published fate map of the mouse neural plate (Inoue et al., 2000), the primordia of the telencephalic and diencephalic roof are situated not in the so-called neural ridge but in the 'neural plate'. In the amphibian and avian embryo, these primordia are included in the neural ridge (Kawamura and Kikuyama 1998; Inoue et al., 2000). If we define the neural ridge of mammalian embryo based on the homology of the developmental fate with lower vertebrates, the lateral components of the mammalian neural ridge should be regarded as extending toward the midline and occupying a lateral half of the so-called neural plate, although only the marginal zone of the neuroectoderm has been referred to as the mammalian 'neural ridge' in the classical literature (see Rubenstein et al., 1998). As to a homology of the pituitary primordium among different classes of vertebrates, the mammalian counterpart may be regarded as having vanished from the neuroectoderm in parallel with the disappearance of the transversal element of the neural ridge (ANR and VNR in lower vertebrates). Analysis of the cell lineage is needed to reveal the relationships between the neuro- and stomodeal ectoderm, and the pituitary primordium.

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