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The pituitary adrenocorticotropes originate from neural ridge tissue in *Xenopus laevis*

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SUMMARY

A series of grafting experiments was conducted to determine pituitary origins prior to brain tube closure in *Xenopus laevis*. Extirpation experiments indicated that the ventral neural ridge (VNR) tissue of stage-18+ embryos was essential for pituitary development. Bolton–Hunter reagent was used to label stage-18+ VNR tissue with ^{125}I, and this tissue was then returned to the donor and its subsequent ontogenesis followed. Labelled tissue was ultimately found in the ventral hypothalamus, the ventral retina, and the anterior pituitary. Using immunocytochemical techniques with antisera to adrenocorticotropin (ACTH), it was found that some of the VNR-derived cells were corticotropes. A region of the nucleus infundibularis which was radioactive labelled also gave ACTH-positive immunoreaction. This might indicate that some ACTH-containing neurones of the hypothalamus are VNR in origin. We suggest that stage-18+ VNR is the site of attachment of brain and anterior pituitary ectoderm. Part of this adherence point is eventually incorporated into the anterior pituitary and will form corticotropes. It is concluded that the ventral retina, the preoptic region of the hypothalamus, some hypothalamic ACTH-immunoreactive cells, and the most anterior portion of the adenohypophysis are all ventral neural ridge in origin.

INTRODUCTION

Pituitary origins prior to brain tube closure are largely unknown. Most vertebrate studies assume that the anterior pituitary arises from midline buccal ectoderm located within the anterior region of the buccal cavity just ventral to the neural plate. Soon after brain tube closure, a placode develops into a Rathke's pouch in birds and mammals or a homologous cord of cells in amphibians. The posterior pituitary arises from an evagination of the diencephalon. This evagination, termed the infundibulum, continues to develop in close association with the anterior pituitary. The pars intermedia is thought to be derived from a reorganization of buccal ectodermal tissue closely apposed to this posterior pituitary tissue (Nyholm & Doerr-Schott, 1977).

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Most developmental studies describe a sudden appearance of the epidermal hypophyseal placode. In *Xenopus* embryos, this placode first appears during stages 20 to 21 (Nyholm, 1977). The placode is thought to grow and extend itself caudally between the brain and foregut (Atwell, 1918; Atwell, 1921; Nyholm, 1977). After stage 33/34, the attachment of this extended placode with buccal ectoderm is broken and during stages 35/36 to stage 42, it migrates and situates itself along the border of the infundibular floor (Nyholm, 1977).

There is some controversy concerning the epidermal origins of the anterior pituitary. Takor Takor & Pearse (1975) state that the thickening of ventral ectoderm around the tip of the ventral neuropore stretches the folding neural plate inwards. Flanks of plate tissue are thought to give rise to a 'ventral neural ridge' (VNR) that reaches the stomodeum by the 7-somite stage in the chick embryo. From these studies, Takor Takor & Pearse concluded that the anterior pituitary is completely neuroectodermal in origin. This suggestion is in keeping with their concept that endocrine APUD (Amine Precursor Uptake and Decarboxylation) cells might all arise from either neural or neural crest tissue (Pearse, Polak & Bussolati, 1972; Pearse, 1977).

Due to the increased proliferation of the prosencephalon subsequent to brain tube closure, vital-dye-marking experiments for this rapidly changing area have proven unsuccessful. Most pituitary developmental studies have relied upon histological examination of different embryos as they progress through developmental stages. The disadvantage of this approach is that timing of developmental events could vary from embryo to embryo making unequivocal tissue identifications during these stages of rapid tissue rearrangements very difficult. The present study has used a new approach, namely, labelling of the suspected pituitary anlage with a vital radioactive marker. For this purpose the embryo of the amphibian *Xenopus laevis* was used. The technique involved the radioactive iodination of intracellular and cell surface proteins of an excised tissue using Bolton–Hunter reagent followed by a return of the tissue to its site of excision. This labelling technique was previously used to label whole embryos, and it was shown that labelled cells undergo normal sequences of development (Katz *et al.* 1982). The purpose of our study was to determine if this technique could be used to follow pituitary development during the early embryonic periods of tissue rearrangements and, in particular, to determine if VNR tissue is involved in pituitary development.

MATERIALS AND METHODS

Embryos

Embryos of *Xenopus laevis* were obtained from matings in the aquarium facility of the Department of Zoology, Catholic University, Nijmegen. Spawning was induced by injection of Pregnyl (Organon, Oss, The Netherlands) into the dorsal lymph sac. The male was given a daily injection of 300 i.u. for two days and the female was given a single injection of 600 i.u. The developmental stages of the embryos and tadpoles were determined using the standard table of

Nieuwkoop & Faber (1967). Tadpoles were fed powdered Urticaceae leaves (J. Hooy B.V., Limmen) to which was added 1 % w/w each of powdered milk and yeast.

Grafting and dissections

All experiments were carried out in a modified Holtfreter's solution of the following composition: NaCl, 22·6 mM; $CaCl_2.2H_2O$, 0·6 mM; KCl, 0·47 mM. This solution was made semisterile by bringing it to 70–80°C. The solution was then cooled to room temperature and 50 mg gentamicin (Sigma) l^{-1} was added. The pH was adjusted to 7·5 by addition of Tris (Sigma). Operations were carried out in Petri dishes coated with 2 % agar (Eastman). Embryos were oriented by placing them in depressions cut in the agar. Incisions were made with electrolytically sharpened tungsten needles. Preliminary grafting experiments were conducted to ascertain pituitary origins prior to brain tube closure. Ectodermal or neuroectodermal ridge tissue, taken from embryos at stages 16 to 19, was grafted from donors to the presumptive fin of hosts of the same stage. Donors and hosts were allowed to develop to stage 40, and they were then fixed in Bouin's fluid for histological examination to determine the presence or absence of pituitary tissue.

Radiolabelling

Bolton–Hunter reagent (*N*-succinimidyl 3-(4-hydroxy, 5-(I^{125})-iodophenyl) proprionate) was used to label embryonic tissue. The reagent was prepared as described by Bolton (1976) using carrier-free ^{125}I (1 mCi 5 μl^{-1}, Amersham). Following iodination the radioiodinated reagent was extracted in 250 μl benzene and 20 μl samples were placed in 1·5 ml Eppendorf vials and dried over nitrogen gas. The vials, each containing 28 μCi dried labelling reagent (specific activity 1·8 μCi ng^{-1}) were stored at −20°C. The reagent was redissolved in 20 μl Holtfreter's solution just before addition of the tissue sample. The VNR of a stage-18+ embryo was removed using tungsten needles. All adhering epidermis was cut or teased away from the thickened neural ridge tissue. The VNR was then transferred to the Bolton–Hunter reagent vial and incubated for 5 min. In order to stop the labelling process, excess Holtfreter's solution containing 1 % BSA was added to the Bolton–Hunter reagent vial (Katz *et al.* 1982). The labelled tissue was then returned to its site of origin paying special attention to orientation. All tissue manipulations were conducted with fine hair loops (Rugh, 1962).

Autoradiographic techniques

Labelled embryos were allowed to develop to different stages and then were fixed in Bouin's fluid for 3–4 h. After fixation, embryos were processed and embedded in paraffin paying careful attention to embryo orientation. They were then sectioned at 5–7 μm, and sections were attached to gelatinized slides. Liquid emulsion autoradiography was carried out according to the procedure of Bogoroch (1972). Slides were coated with Ilford K5 emulsion, they were then air-dried and placed in a light-tight box containing desiccant. Following a 5- to 6-day exposure period, they were developed with D-19 developer (Eastman Kodak) and stained with haemotoxylin–eosin.

Immunocytochemical techniques

Sections adjacent to autoradiographic sections were processed for immunocytochemistry. Results of previous studies (Verburg-van Kemenade, Willems, Jenks & van Overbeeke, 1984) indicated that the labelled region might correspond to the area of the pituitary gland containing cells involved in adrenocorticotropin (ACTH) production. Immunocytochemical identification was achieved by the peroxidase–anti-peroxidase method of Steinberger (1979) using 4-Cl-napthol as an oxygen acceptor. Anti-ACTH antisera was raised against synthetic $ACTH_{1-24}$ (Organon). The antigen was conjugated with glutaraldehyde to Bovine serum albumin (BSA). The antiserum shows a weak cross-reaction with melanotropins which allows simultaneous identification of melanotropes (Verburg-van Kemenade *et al.* 1984).

RESULTS

Grafting and dissection studies

Early pituitary origins were determined by grafting experiments. The anterior areas of open neurula (stages 16 to 19) *Xenopus laevis* embryos were grafted from a donor on to a host embryo. The loss of pituitary function in donor animals was initially assessed by the donor's inability to exhibit melanophore expansion on a black background. It has been established (Terlou & van Straaten, 1973; Verburg-van Kemenade *et al.* 1984) that during ontogenesis, *Xenopus* tadpoles develop a functional and regulated pars intermedia by stage 40. At this stage animals can adapt to background colour. Animals on black background have expanded pigment in dermal melanophores due to the release of melanotropins from the functional pars intermedia. Animals lacking a functional pars intermedia are incapable of melanotropin release and the melanophores remain punctuate. Removal of the ventral neural ridge (VNR) tissue (Fig. 1) was particularly effective in disrupting normal pituitary function. Removal of this VNR region from stage-17 embryos resulted in donor stage-40 larvae displaying punctuate melanophores in 75 % of the cases (27 of 36 embryos). Removal of the VNR tissue from stage-18+ embryos resulted in 90 % (27 of 30 operations) of the stage-40

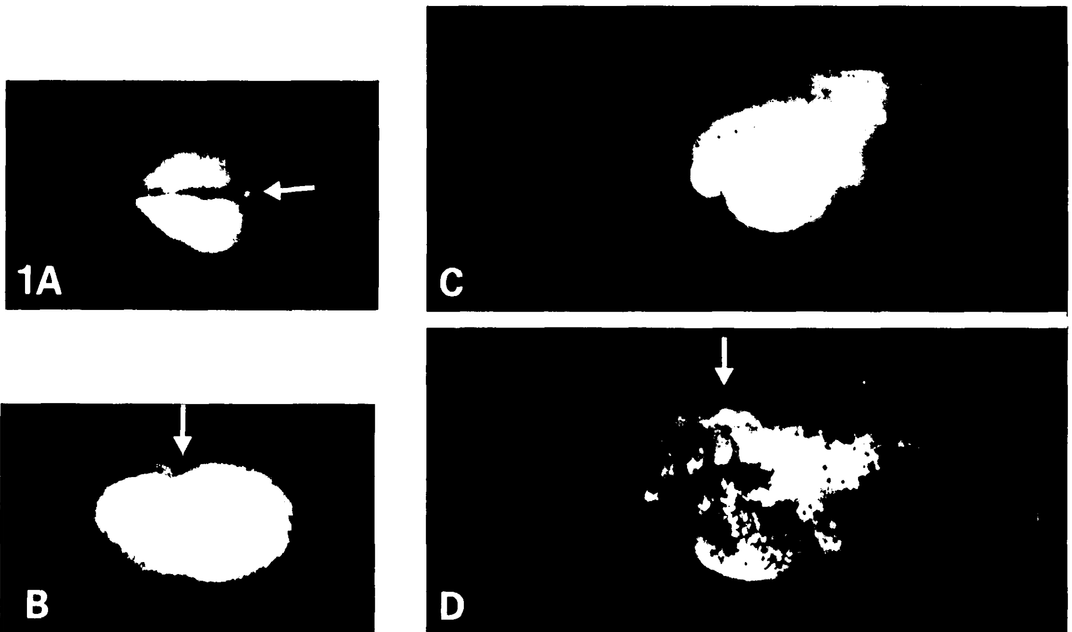

Fig. 1. (A,B) Appearance of donor and host embryos shortly after removing ventral ridge tissue from donor embryo and grafting it in host fin. (A) Epidermal tissue quickly grows over the excised region of donor (arrow). (B) The tissue graft survives in host fin (arrow). (C,D) Appearance of stage-42 larvae of donor and host embryo. The melanin pigment of dermal melanophores of donor larvae remains in a punctuate condition indicating a lack of pituitary melanotropin (C) while the host larvae display hyperpigmentation (D). Arrow indicates position of graft in host larvae.

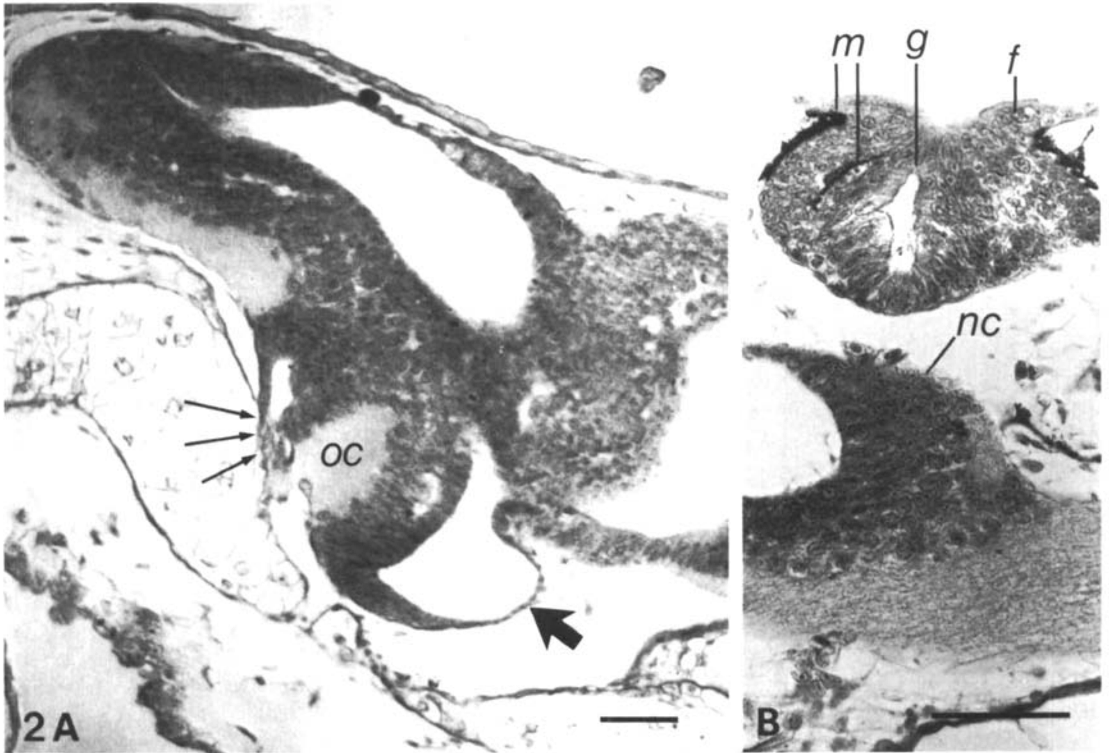

Fig. 2. (A) *Xenopus larvae* which had their stage-18+ VNR extirpated, completely lacked a hypophysis (single arrow). The infundibulum was unaffected by this operation. The preoptic recess usually exhibited minor breaks and tears (small arrows). *oc*, optic chiasma. Bar, 0·1 mm. (B) The grafted stage-18+ VNR was allowed to develop in hosts. This grafted tissue rolled up and formed a spinal-cord-like structure. It often induced vacuolation within host spinal tissue. *g*, graft; *m*, melanophores; *nc*, host neural cord; *f*, fin epidermis of host. Bar, 0·1 mm.

larvae having punctuate melanophores. An example of one of these donor animals that failed to adapt to black background is shown in Fig. 1.

The complete absence of pituitary tissue was verified histologically (Fig. 2A). If VNR tissue from a stage-18+ embryo was removed, at stage 40 these larvae lacked not only the pars intermedia, but the entire pituitary gland (Fig. 2A). The preoptic recess area of these 'hypophysectomized' stage-40 larvae also exhibited distortions and tears, but the infundibulum seemed unaffected (Fig. 2A, arrows).

Host animals assessed at stage 40 were found to have expanded pigment in the dermal melanophores regardless of background colour (Fig. 1D). The tissue graft exhibited a number of characteristics common to neural tissue. It rolled up and formed a spinal cord-like structure in the host animals (Fig. 2B). The possibility that these explants could be producing and releasing pro-opiomelanocortin-related peptides was confirmed in immunocytochemical studies which showed a positive reaction with antisera to ACTH and α-MSH. This will be the subject of a later study.

Tracer studies with radiolabelled neural tissue

The principle steps for autoradiographic assessment of the fate of radiolabelled tissue is illustrated in Fig. 3. The most difficult step proved to be the recovery of the tissue from the reaction vial for reimplantation into the embryo. Orientation of the labelled tissue was easily accomplished due to the fact that the VNR tissue maintained its folded wedge shape subsequent to excision, labelling and re-insertion. Shown in Fig. 4 is the autoradiographic analysis of a stage-42 larvae that had received a radiolabelled VNR at stage-18+. Labelled tissue was found within areas of the retina, the anterior hypothalamic area and the anterior pituitary. The labelled hypothalamic area was usually observed as banded stripes between unlabelled areas, an appearance which possibly reflects cell proliferation within the labelled region leading to areas of less-intense radioactivity. The anterior pituitary area that possessed label was always located within the ventral, most anterior portion of the adenohypophysis just adjacent to the median eminence. Further studies, carefully noting precise areas of incision, indicated that the midventral portion of the stage-18+ VNR tissue gave rise to ventral retina tissue. The bulk of the stage-18+ VNR tissue (mid-dorsal portions) eventually gave rise

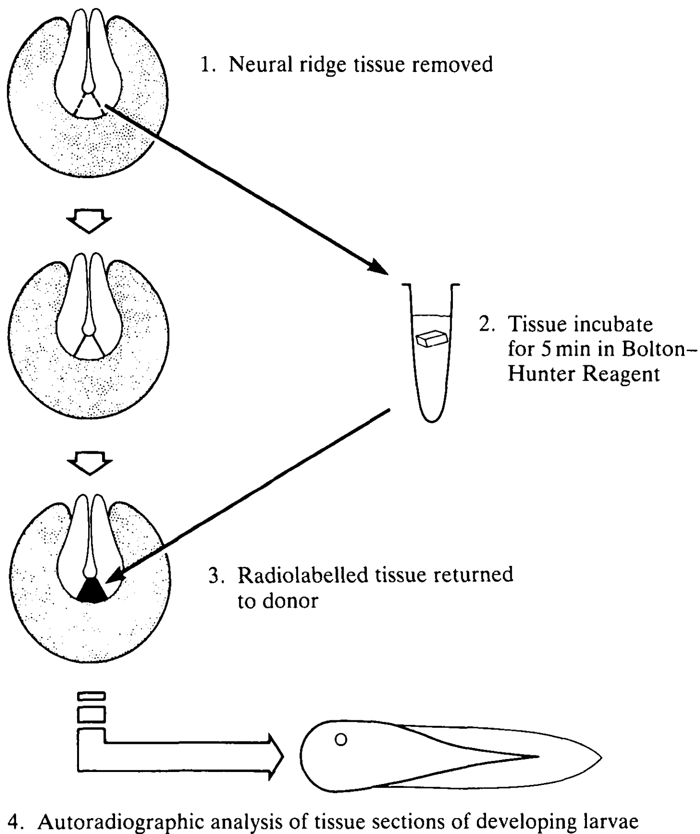

Fig. 3. Schematic diagram of procedure to radiolabel ventral neural ridge tissue with ^{125}I.

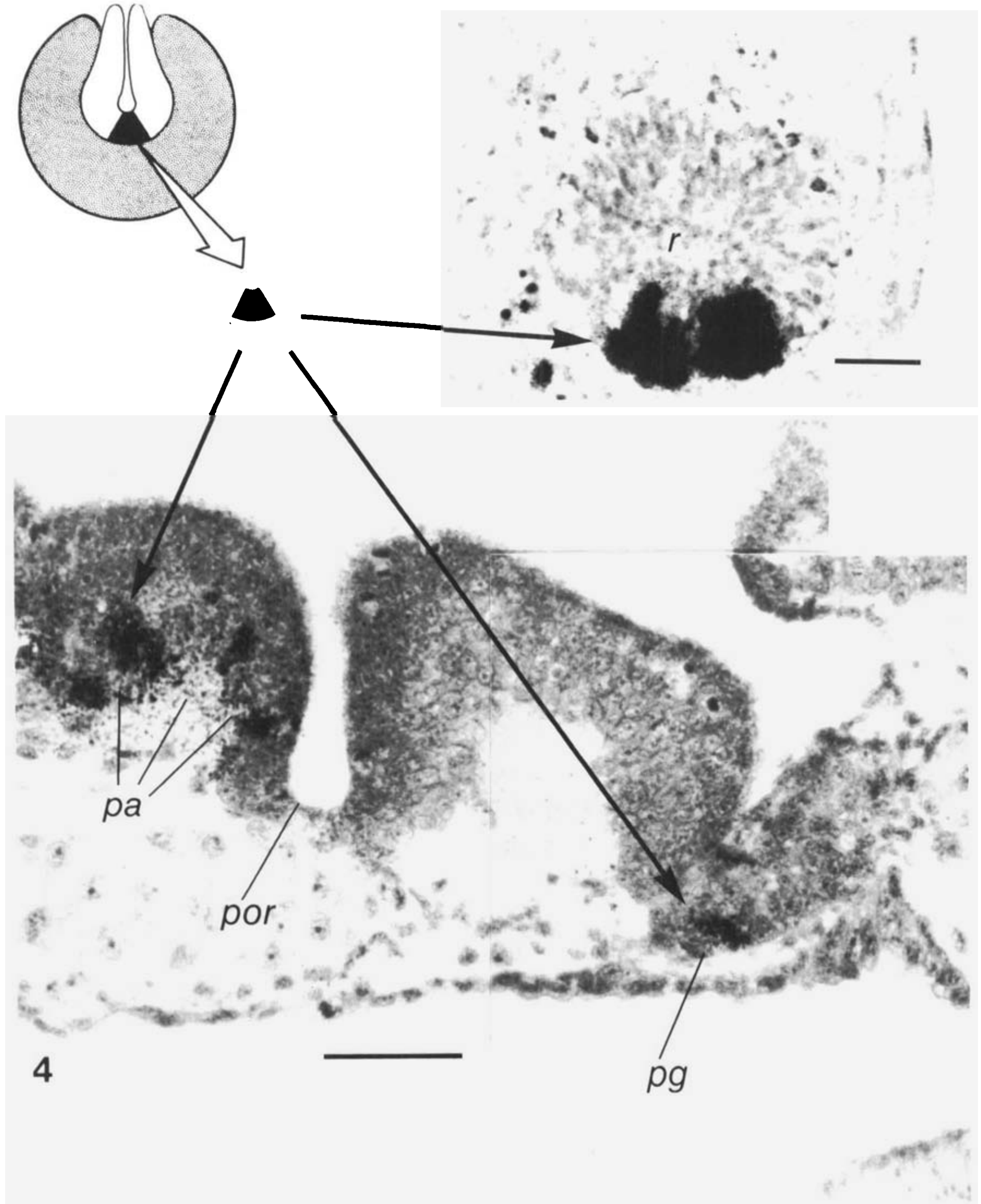

Fig. 4. Autoradiographic analysis of stage-42 embryo, the VNR of which was radiolabelled at stage 18+. Radiolabelled stage-18+ VNR tissue consistently contributed to the formation of three distinct organs. The edges of the VNR eventually became localized within ventral retinal tissue. The ventral portion of the VNR was localized within the anterior pituitary. The bulk of the VNR tissue was found within the anterior hypothalamus just anterior to the preoptic recess (*por*). *pa*, preoptic area; *pg*, pituitary gland; *r*, retina. Bars, 0·1 mm.

to hypothalamic tissue anterior to the preoptic recess. In five of twenty operations (usually using stage-19 embryos) pineal tissue was also labelled.

To follow the morphogenesis of the VNR stage-18+ tissue, autoradiographic analysis was conducted on animals at various stages between receiving the radiolabelled tissue (stage-18+) and the stage at which the pituitary is fully developed (stages 40–42). At stage 30, anterior regions of the prosencephalon were labelled (Fig. 5). At stage 33/34, label was observed within the dorsal diencephalon, the ventral optic cup, the preoptic region of the hypothalamus and the most anterior portion of the pituitary anlage. At stage 42, label is found within the anterior hypothalamus, the ventral area of the retina and the ventral, anterior area of the adenohypophysis (Fig. 5). A few cells in the caudal hypothalamus were observed to be labelled.

Immunocytochemical analysis

Autoradiographic and immunocytochemical analysis of adjacent pituitary sections of tadpoles with VNR tissue labelled at stage 18+ shows a strong correspondence between ACTH-immunoreactive cells and radiolabelled cells of VNR origin (Fig. 6). Even the few caudal hypothalamic areas that exhibited radioactive label also showed ACTH-immunoreactivity (Fig. 6A,B, arrow). The ACTH antiserum used displays weak cross-reaction with α-MSH (Verburg-van Kemenade *et al.* 1984), thus accounting for the weak positive reaction in the pars intermedia (Fig. 6B). The pars intermedia is not radiolabelled (Fig. 6A).

DISCUSSION

Katz *et al.* (1982) found that the Bolton–Hunter reagent can be used to label living cells with ^{125}I using techniques similar to the methods used to label isolated macromolecules *in vitro*. They noted that labelled cells underwent normal developmental movements and changes. The ^{125}I is covalently bound, not reutilized and does not appear to alter the normal physiology and development of embryos. Katz *et al.* (1982) used this labelling technique to distinguish long-lived proteins in *Xenopus* embryos. They suggested that it could be used to radioactively label discrete populations of cells. The extreme hydrophobicity of the Bolton–Hunter reagent permits the rapid labelling of intracellular as well as cell surface proteins with a high specific activity (Katz *et al.* 1982). The present study reports the application of this method to follow tissue movements of prelabelled grafts.

The ventral neural ridge (or anterior neural fold) of a stage-18+ embryo consists of three parts: the neural crest, prospective neural tube tissue and epidermis (Brun, 1985). The descending epidermal fold tissue gives rise to epidermal components of neural fold (Brun, 1985). For our radiolabelling studies, care was taken to first separate and remove the thin, epidermal portion of the VNR. The remaining portion of the VNR was labelled in our studies. The observation that epidermal-derived areas such as the buccal ectoderm were not found to be labelled after these operations indicates that removal of the epidermal component prior to

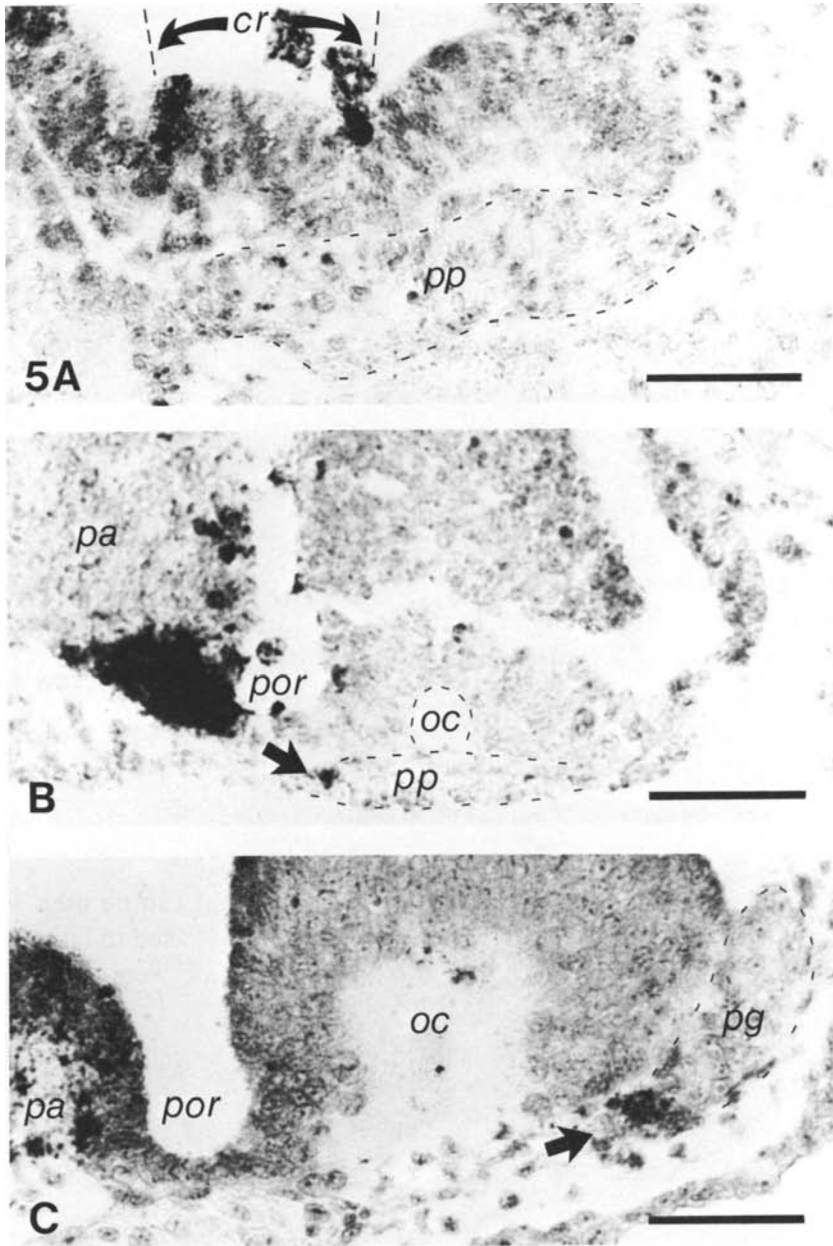

Fig. 5. Morphogenetic movements of the labelled stage-18+ VNR. (A) At stage 30 most of the label is observed within the anterior forebrain in the region of the chiasmatic ridge (*cr*). The outline of the pituitary primordium (*pp*) is indicated with a dotted line. No label was found within the primordium. (B) At stage 33/34 the pituitary primordium has detached or is torn free from the preoptic area (*pa*). Thus the most anterior portion of this pituitary anlage is labelled (arrow). Also indicated is the preoptic recess (*por*) and the optic chiasma (*oc*). (C) By stage 42, the pituitary gland (*pg*) is under the infundibulum and label becomes incorporated within the adenohypophysis (arrow). Bars, 0·1 mm.

labelling was successful. Therefore, we conclude that the labelled regions of the developing embryo are of neural or neural-crest origin.

In Fig. 7 we have summarized our conclusions concerning the fate of radiolabelled ventral neural ridge tissue. Using the vital radioactive marker, we observed a number of interesting features concerning hypothalamic and pituitary development. Soon after the reinsertion of the VNR tissue along the suture area of the closing brain tube it adheres to and is overgrown by unlabelled epidermis. This attachment to anterior epidermis is probably necessary to internalize the pituitary primordium, since our preliminary extirpation experiments (Figs 1, 2) showed that removal of the VNR leads to the complete absence of a pituitary gland, rather than

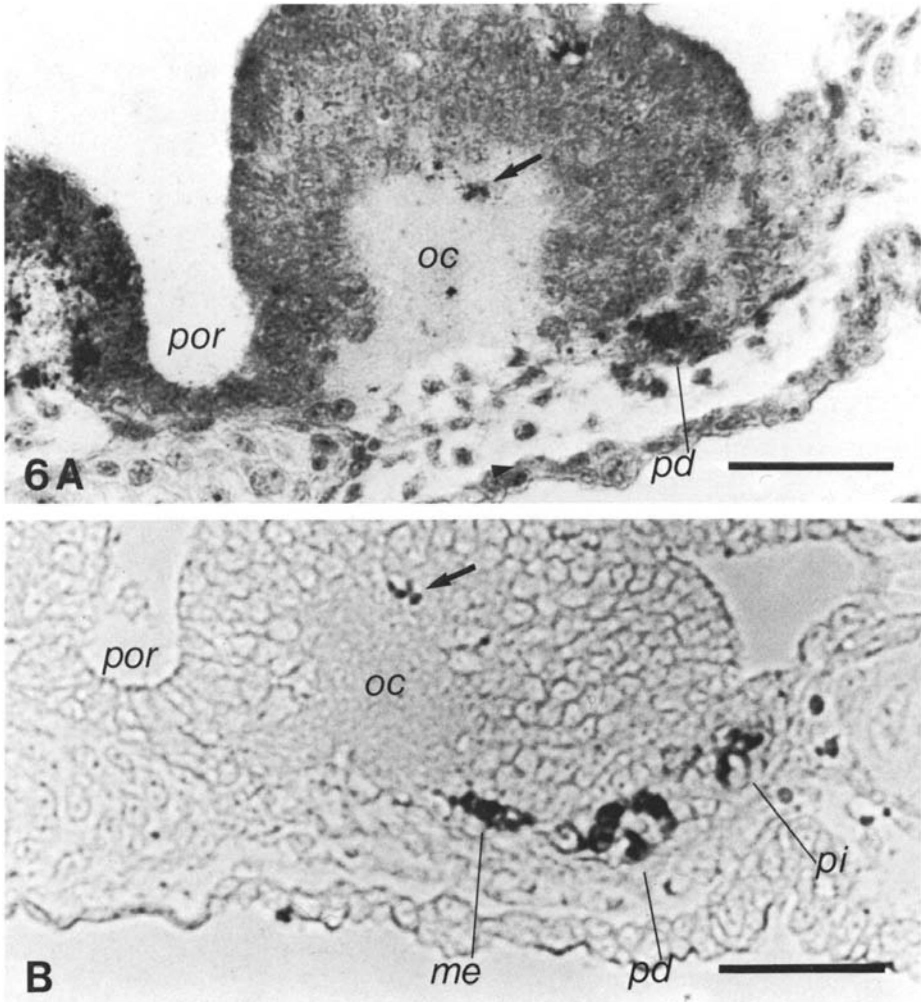

Fig. 6. The radiolabelled VNR (A) shows a strong correlation with the ACTH-immunoreactive cells (B). Even the hypothalamic area (nucleus infundibularis ventralis) that exhibits label corresponded to an ACTH-immunoreactive area (indicated by arrow). *por*, preoptic recess; *oc*, optic chiasma; *pd*, pars distalis; *me*, median eminence; *pi*, pars intermedia. Bar, 0·1 mm.

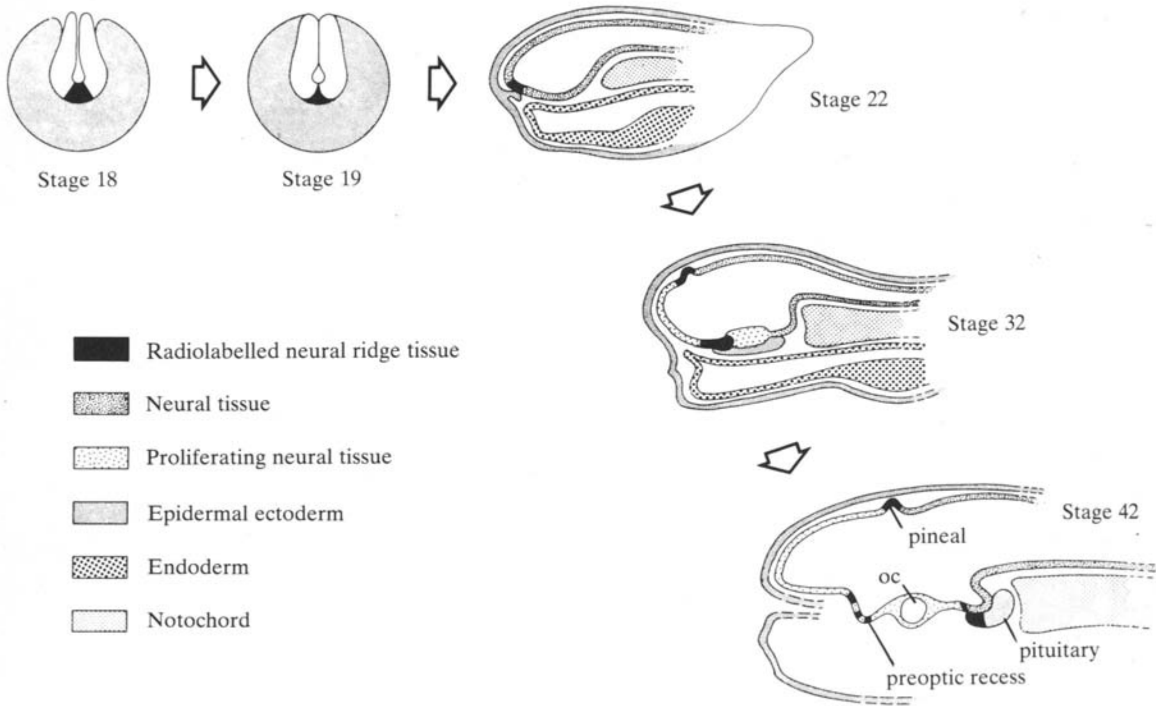

Fig. 7. Schematic illustration of the most probable morphogenetic events leading to the labelling of three distinct brain regions. Stage 18: introduction of the radiolabelled VNR tissue. Stage 19: internalization of the radiolabelled tissue during epidermal overgrowth and brain tube fusion. Stage 22: attachment of overlying epidermis occurs along suture line of involuted VNR tissue. Stage 32: proliferation of prosencephalon within the radioactive VNR tissue subdivides the label into two areas. Stage 42: further proliferation results in the final distribution of radiolabelled areas.

the development of a gland simply missing its VNR contribution. Since only a small portion of the anterior pituitary was ultimately found to be radioactive when the VNR was radiolabelled, presumably unlabelled epidermal ectoderm, and possibly other unlabelled regions of neural ridge tissue, could make a major contribution to the development of the pituitary gland. During stages 22 to 32, VNR tissue and the pituitary placode are progressively brought to a more interior position due to both forebrain overgrowth (Eagleson & Malacinski, 1986; Jacobson, Miyamoto & Mai, 1979) and to pituitary growth during its contact with the basal diencephalon (Daikoku *et al.* 1982). After being anchored interiorly during forebrain closure and growth, the pituitary placode grows in a caudal direction along the diencephalon floor (stages 22 to 32, Fig. 7). During this growth, secondary adherences are made with the floor of the diencephalon, and the placode grows past the optic chiasma to eventually adhere caudally with the infundibulum. Our results indicate that at stage 33/34, the most anterior portion of the pituitary placode is loosened or torn free from its initial point of adherence to tissue originating from VNR. This VNR tissue is by this time situated slightly

anterior to the preoptic recess. A small portion of radiolabelled tissue torn from this preoptic region is eventually incorporated into the anterior pituitary (stage 42, Fig. 7). The rapid diencephalon growth during this period (Daikoku *et al.* 1982) may be responsible for the loosening of the anterior contact of the pituitary anlage. During stages 39/40 to 42 displacement and reorganization of the pituitary beneath the infundibulum may be due to active migration as suggested by Nyholm (1977). Such movements could account for the final positioning of our labelled portion of the anterior pituitary tissue.

Labelling stage-18+ VNR also resulted in the appearance of label in stage-42 retinal tissue. Careful dissection studies (with radiolabelling) determined that the edges of the stage-18+ VNR ultimately form retinal tissue. This verifies the studies of Brun (1981) that indicated that the prospective eye vesicles migrate into the neural folds during neurulation. Altogether, our results show that extensive movement of the primordia of the hypothalamus and optic vesicles occurs during neurulation. When development has reached the stage where neural folds are forming, the primordia for optic vesicles and hypothalamus are found to be located within these folds rather than in neural plate as previous fate maps indicated (Lewis, 1907; Jacobson, 1959). The results of our experiments compare favourably with those of Jacobson (1982) who used horse radish peroxidase as label: our radioactive labelled area corresponds with the most-forward part of the anterior–ventral compartment labelled by Jacobson (1982), and he too ultimately observed label in the anterior hypothalamus and ventral retina.

The immunohistochemical studies indicated that the adenohypophyseal areas of VNR origin ultimately differentiate into corticotropes. Interestingly, a few immunoreactive brain ACTH cells in the caudal hypothalamus also exhibited radiolabel. This suggests a VNR origin for some of these hypothalamic ACTH cells. Immunoreactive and bioactive ACTH material is present in the brains of all mammalian and amphibian species tested thus far (Liotta & Krieger, 1983). Our present study localized ACTH immunoreactivity within cells of the nucleus infundibularis ventricalis (NIV) area of the caudal hypothalamus. Previous studies indicate that neurones from the NIV area terminate in the median eminence (Liotta & Krieger, 1983), and we also localized ACTH-like material within the median eminence. As proposed by Pearse (1977), at least a portion of the anterior pituitary is of neural origin. Our study, indicating a neural rather than an epidermal origin for the anterior pituitary corticotropes, supports this proposal. This is the first case where a specific pituitary endocrine cell type has been traced back to its early embryonic origin. As discussed earlier, only a discrete region of the anterior pituitary was of VNR origin and thus further studies will be necessary to trace the embryonic origins of the other cell types within this tissue. In this regard, it will be of particular interest to determine whether the pars intermedia melanotropes are also of neural origin, as the secretory peptides of these cells are derived from the same precursor protein as that utilized in the corticotropes to produce ACTH (Martens, Biermans, Jenks & van Overbeeke, 1982; Verburg-van Kemenade *et al.* 1984). It is also interesting to note that MSH immunoreactivity

has been reported in the pineal gland (see O'Donohue & Dorsa, 1982), a gland in which we occasionally observed label of VNR origin.

The present findings have strong implications concerning previous studies of hypothalamo-pituitary interrelations during development. A number of these studies have examined the influence of the diencephalon upon pituitary corticotrope differentiation (Watanabe, 1982) and the influence of the hypothalamus upon ACTH-immunoreactive neuronal differentiation (Daikoku, Chikamori, Adachi & Maki, 1983). Our observation that the corticotropes in the pituitary and some ACTH-immunoreactive cells in the hypothalamus are both of VNR origin might lend new insight to the interpretation of the results of these studies. Watanabe (1982) found that removal of the rat diencephalon from Rathke's pouch contact at day 12·5 resulted in a marked decrease in adenohypophyseal ACTH appearance. Separation of these areas at day 13·5 had only a slight effect upon ACTH appearance (Watanabe, 1982). In view of our results we would suggest that, possibly, at day 12·5 in the rat the VNR area may not yet be subdivided into hypothalamic and pituitary areas. With separation of the tissue at this stage, the presumptive corticotropes remained attached to hypothalamic tissue and are thus removed. At day 13·5, tension due to forebrain elongation may have divided the VNR area into hypothalamus and adenohypophysis and, therefore, the pituitary primordium now contains VNR tissue which ultimately develops into corticotropes.

In conclusion, radiolabelling of small grafts of embryonic tissue using Bolton–Hunter reagent allows for mapping much more precise areas than was previously possible. Applying this technique to the stage-18+ VNR of *Xenopus laevis* embryos has shown that this region ultimately forms a number of brain structures and a portion of the adenohypophysis.

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