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Establishment of rostrocaudal polarity in tectal primordium: *engrailed* expression and subsequent tectal polarity

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Summary

In the E4 (embryonic day 4) chick tectal primordium, *engrailed* expression is strong at the caudal end and gradually weakens toward the rostral end. We used quail-chick chimeric tecta to investigate how the caudorostral gradient of *engrailed* expression is established and whether it is correlated with the subsequent rostrocaudal polarity of tectal development.

To examine the positional value of the tectal primordium, we produced ectopic tecta in the diencephalon by transplanting a part of the mesencephalic alar plate heterotopically. In the ectopic tectum, the gradient of the *engrailed* expression reversed and the strength of the expression was dependent on the distance from the mes-diencephalon junction; the nearer the ectopic tectum was to the junction, the weaker the expression was. Consequently, the pattern of the *engrailed* expression in the host and ectopic tecta was nearly a mirror image, suggesting the existence of a repressive influence around the mes-diencephalon junction on the *engrailed* expression.

We examined cytoarchitectonic development in the ectopic tecta, which normally proceeds in a gradient along the rostrocaudal axis; the rostral shows more advanced lamination than the caudal. In contrast, the caudal part of the ectopic tecta (near to the mes-diencephalon junction) showed more advanced lamination than the rostral. In both the host and ectopic tecta, advanced lamination was observed where the *engrailed* expression was repressed, and *vice versa*.

Next we studied the correlation between *engrailed* expression and retinotectal projection from a view of plasticity and rigidity of rostrocaudal polarity in the tectum. We produced ectopic tecta by anisochronal transplantations between E3 host and E2 donor, and showed that there is little repressive influence at E3 around the mes-diencephalon junction. We then made chimeric double-rostral tectum (caudal half of it was replaced by rostral half of the donor tectum) or double-caudal tectum at E3. The transplants kept their original staining pattern in hosts. Consequently, the chimeric tecta showed wholly negative or positive staining of *engrailed* protein on the grafted side. In such tecta retinotectal projection pattern was disturbed as if the transplants retained their original position-specific characters.

We propose from these heterotopic and anisochronal experiments that the *engrailed* expression can be a marker for subsequent rostrocaudal polarity in the tectum, both as regards cytoarchitectonic development and retinotectal projection.

Key words: *engrailed*, rostrocaudal polarity, tectal development, retinotectal projection, quail-chick chimera.

Introduction

The optic tectum receives axons from the retina in a topographically ordered manner. For the precise retinotectal connections, it has been suggested that tectal cells are positionally specified prior to receiving retinal axons (Cowan and Hunt, 1985). Several developmental events occur sequentially in the tectum with regard to rostrocaudal polarity. One of homeobox-containing genes, *engrailed*, is reported to be expressed in chick tectal primordia (Gardner *et al.* 1988; Patel *et al.* 1989) with caudorostral gradient. The expression is strong at the mes-metencephalon junction, and gradually weakened toward the rostral. The immunoreactivity of *engrailed* protein appears at E2 (embryonic day 2, 8-9 somite stage) in the chick. The *engrailed* expression is the first detectable character concerning rostrocaudal polarity, so far reported. In subsequent

tectal development, from E4 in the chick, there is a rostrocaudal gradient in cytoarchitectonic differentiation (LaVail and Cowan, 1971) with laminar organization more advanced at the rostral than the caudal. Moreover, just prior to receiving retinal fibres, tectal cell membranes are shown to be regionally specified (Walter *et al.* 1987*a*; Walter *et al.* 1987*b*). A glycoprotein that has been fractionated from posterior tectal cell membranes by Stahl *et al.* (1990) is regarded as a candidate for directional cues for ingrowing retinal axons.

Plasticity of rostrocaudal polarity in the tectal primordium at an early stage, E2 in the chick, has been revealed by rotation experiments of the mesencephalic alar plate. After rotation of the tectal primordium, tectal polarity with regard to the *engrailed* expression (Martinez and Alvarado-Mallart, 1990), laminar organization (Matsuno *et al.* 1991) and retinotectal projection (Ichijo *et al.* 1990) was regulated and adjusted to that of the host. These results revealed that (1) rostrocaudal polarity of tectum is plastic at E2; (2) environmental influence is important for the establishment of rostrocaudal polarity in tectum.

Here we investigated how the rostrocaudal polarity of the *engrailed* expression is influenced by environmental cues, and whether the subsequent rostrocaudal polarity of tectal development is associated with gradient of the *engrailed* expression. We analysed *engrailed* expression patterns and subsequent development in quail–chick chimeric tecta, in which rostrocaudal polarity of the *engrailed* expression was altered by heterotopic transplantations of tectal primordium.

Materials and methods

Transplantations

(1) *Ectopic tectum by isochronal transplantation at E2*

Fertilized chick and quail eggs obtained from local farms were incubated for 36–40 h in a humidified atmosphere at 38°C. In all cases, grafts were from quail embryos, and hosts were chick embryos. Transplantations were performed unilaterally at the 10-somite stage (stage 10 of Hamburger and Hamilton, 1951). A part of the diencephalic alar plate was excised from the host chick embryos and a quail mesencephalic alar plate was transplanted as shown in Figs 1A and 2. The rostrocaudal orientation of the graft was preserved in most cases, but in some cases grafts were prepared from the contralateral side and rotated along the rostrocaudal axis. The chimeras were re-incubated for further 1 to 5 days (till E3 to E7).

As a control, we transplanted a part of the diencephalon homotopically at the 10-somite stage.

(2) *Ectopic tectum by anisochronal transplantations between E2 and E3*

To investigate whether there is an environmental influence on

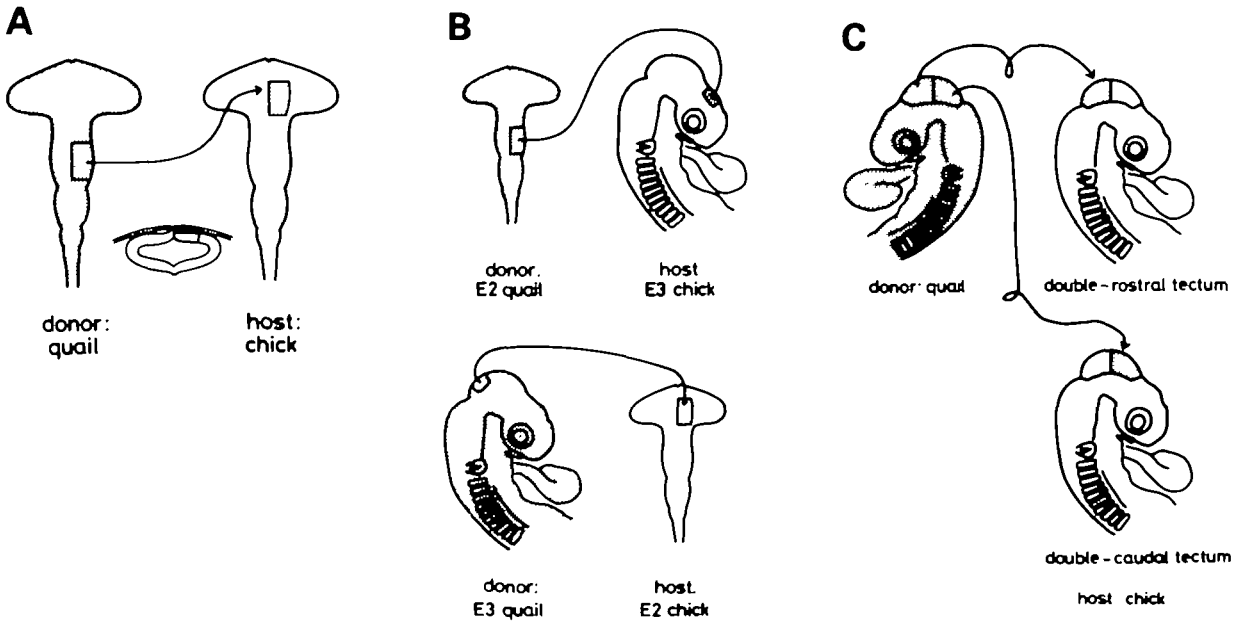


Fig. 1. Schematic drawings of heterotopic transplantations. In all cases, donor is quail (dotted) and host is chick. The grafts contain neuroepithelium, neural crest, mesenchyme and ectoderm. (A) Producing ectopic tectum by an isochronal transplantation at E2. The mesencephalic alar plate was transplanted into a part of the diencephalon unilaterally, with the rostrocaudal orientation preserved in main experiment. The caudal limit of the graft is a little rostral to the mes–metencephalic constriction. (B) Producing ectopic tectum by the anisochronal transplantations between E2 and E3. (B, upper) The mesencephalic alar plate was excised from E2 quail embryo and transplanted into a part of the diencephalon of E3 chick unilaterally, with the rostrocaudal orientation preserved. (B, lower) The tectal primordium was excised out from E3 quail embryo and transplanted into a part of the diencephalon of E2 chick unilaterally, with the rostrocaudal orientation preserved. (C) Producing double-rostral or double-caudal tectum at late E2 or E3. (C, upper) A rostral half of tectal primordium on the left side of quail embryo was transplanted into the caudal half of chick tectal primordium on the right side (double-rostral tectum). (C, lower) A caudal half of tectal primordium on the left side of quail was transplanted into the rostral half of chick tectal primordium on the right side (double-caudal tectum). This series of transplantations were performed between the 12- and 39-somite stages (stages 11–19 of Hamburger and Hamilton).

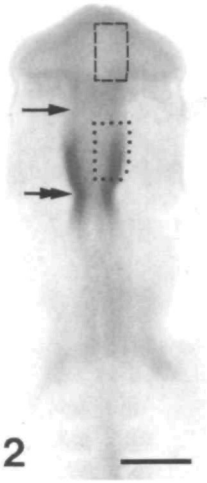


Fig. 2. A normal chick embryo at stage 10, immunostained with anti-*engrailed* protein antibody. Quail embryos have a similar staining pattern (not shown here). Dashed line shows the area receiving the graft. Dotted line shows corresponding area for preparing the graft from quail, to produce ectopic tectum isochronally. The staining is strong at the mes–metencephalon junction (double arrow) and weakened at the mes–diencephalon junction (arrow). Bar, 200 μm.

engrailed expression at the area of the E3 mes–diencephalon junction, we transplanted the mesencephalic alar plate of E2 quail (the 10-somite stage, corresponds to the stage 10 of Hamburger and Hamilton), which is competent to respond to induction, into the E3 (stage 16–17 of Hamburger and Hamilton) chick diencephalon (Fig. 1B, upper).

To investigate whether the E3 mesencephalic alar plate is sensitive to environmental influence or not, we transplanted a caudal part of the tectal primordium of E3 quail (the 25- to 30-somite stage, corresponds to the stage 16–17 of Hamburger and Hamilton) into the E2 chick (the stage 10 of Hamburger and Hamilton) diencephalon (Fig. 1B, lower), which is known to influence *engrailed* expression around the mes–diencephalon junction.

(3) *Double-rostral or double-caudal tectum by transplantations at late E2 or E3*

These types of transplantations were performed isochronally on late E2 or E3. From the host chick embryo, the rostral or caudal half of the right tectal primordium was excised with a microscalpel. The caudal or rostral half of the left tectal primordium was prepared from a quail embryo as a graft. A rostral half of the quail tectal primordium was transplanted into the caudal half of a host chick tectum (double rostral tectum), or a caudal half of the tectal primordium was transplanted into a rostral half of a chick tectum (double caudal tectum) (Fig. 1C). In these transplantations, the rostrocaudal direction of the transplants was inverted while the mediolateral direction was preserved.

Staining engrailed *protein*

For immunostaining of *engrailed* protein, chimeras were fixed at E4 with 3.7% formaldehyde in Pipes buffer for 1 to 2 h at room temperature. E4 was chosen for analysis because at this stage the diencephalon and tectal primordium are well developed in size and show clear immunoreactivity of the *engrailed* protein in host. Concerning the incubation time after transplantations, our isochronal transplantations at E2 and those by Martinez and Alvarado-Mallart (1990) confirmed that 1 day is enough for environmental influences to change the pattern of the *engrailed* expression. After fixation, eyes and epidermal and mesenchymal tissues were removed with fine forceps, and only neural tissues were processed for the immunoreaction. The tissues were soaked in cold methanol for 30 min, and 3% H_2O_2 dissolved in methanol for another 30 min to kill endogenous peroxidase. After washing, they were reacted with anti-*engrailed* protein monoclonal antibody, 4D9, obtained from American Type Culture Collection, and stained by the DAB (3,3′-diaminobenzidine) method, according to Patel *et al.* (1989). Whole mounts of the stained embryos were observed and photographed.

Investitation of cytoarchitectonic development in tectum

Some chimeras with ectopic tecta produced by isochronal transplantation were fixed at E7 in Carnoy's solution, and brains were taken out and embedded in paraffin. They were sagittally sectioned and stained according to Feulgen-Rossenbeck method.

Investigation of retinotectal projection map

Retinotectal projection patterns were examined in chimeric embryos with double-rostral or double-caudal tecta by labelling a small population of retinal ganglion cells anterogradely. A fluorescent dye, DiI (1,1′dioctadecyl-3,3,3′,3′ tetramethylindocarbocyanine perchlorate; Molecular Probes), was applied at the nasal or temporal margin of the retina with a sharp tungsten needle at E14 *in ovo*. The embryos were decapitated at E16, and their retinae and brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. After fixation, cuts were made in the retinae and optic tecta to aid flattening and they were whole-mounted with solution of 9 parts of glycerol and 1 part of 0.1 M phosphate buffer (pH 7.4) containing 5% *n*-propyl gallate as described by Nakamura and O'Leary (1989), and observed under an epifluorescence microscope.

Identification of grafts in hosts

After observations of *engrailed* protein staining or of retinotectal projection on whole mounts, specimens were embedded in paraffin, and serially sectioned. The *engrailed*-stained specimens were deparaffinised and coverslipped with glycerol, to ensure nuclear-specific staining of *engrailed*. Then

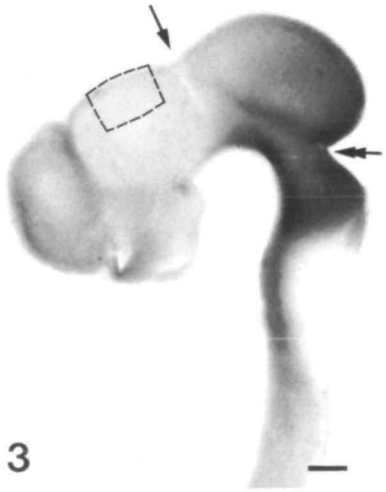


Fig. 3. A stage 22 chimera that received homotopic transplantation in the diencephalon at the 10-somite stage, immunostained with anti-*engrailed* protein antibody. Eyes, epidermal and mesenchymal tissues are removed. Dashed line shows the transplanted area. The staining is strongest at the mes–metencephalon junction (double arrows) and gradually weakened toward the rostral end of tectal primordium (arrows). Bar, 200 μm.

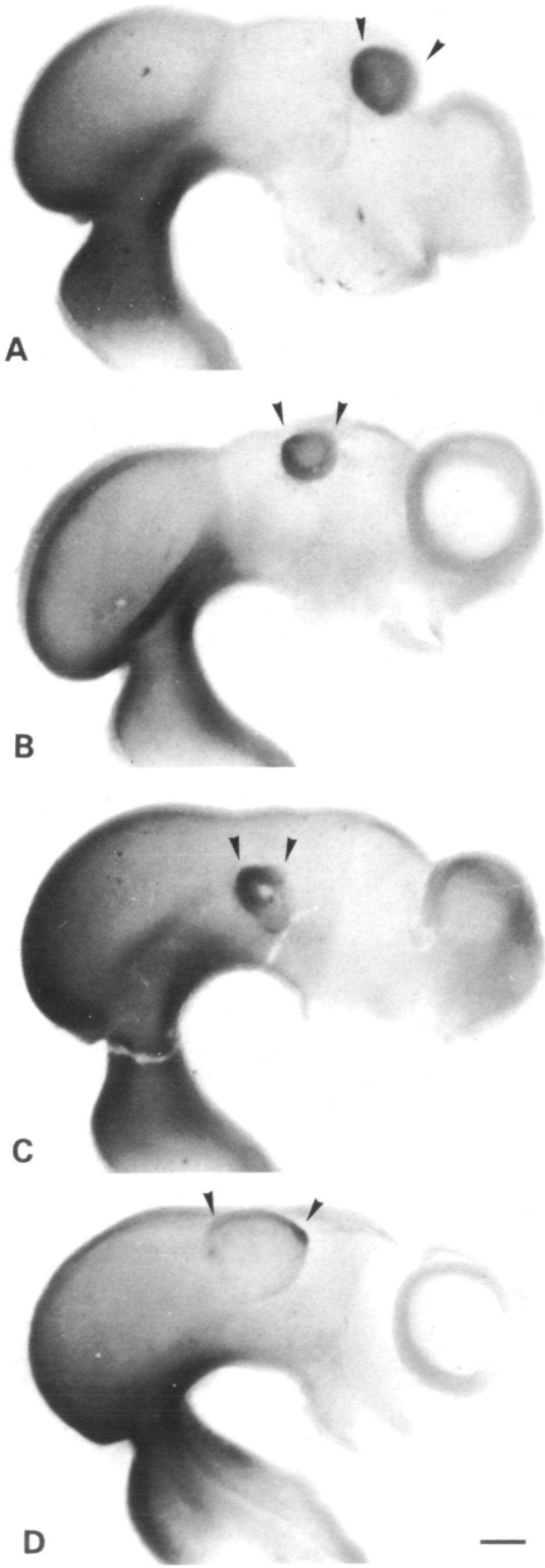


Fig. 6. (A–C) Chimeras after transplantation of the E2 mesencephalic alar plate into the E3 diencephalon, immunostained with anti-*engrailed* protein antibody. Ectopic tecta are produced at various sites (arrowheads); rostral (A), middle (B) and caudal (C) portion of the diencephalon. In all cases, staining of *engrailed* protein in the ectopic tecta is strongest at the caudal end and weakest at the rostral. This pattern is the same as the original fate of the graft. Even when the ectopic tectum was located near the mes–diencephalon junction, the staining was not repressed (C). (D) A chimera after transplantation of the E3 tectal primordium into the E2 diencephalon. Staining of *engrailed* protein in the ectopic tectum (arrow) is strong at the rostral end and weakened toward the caudal end. Bar, 200 μm.

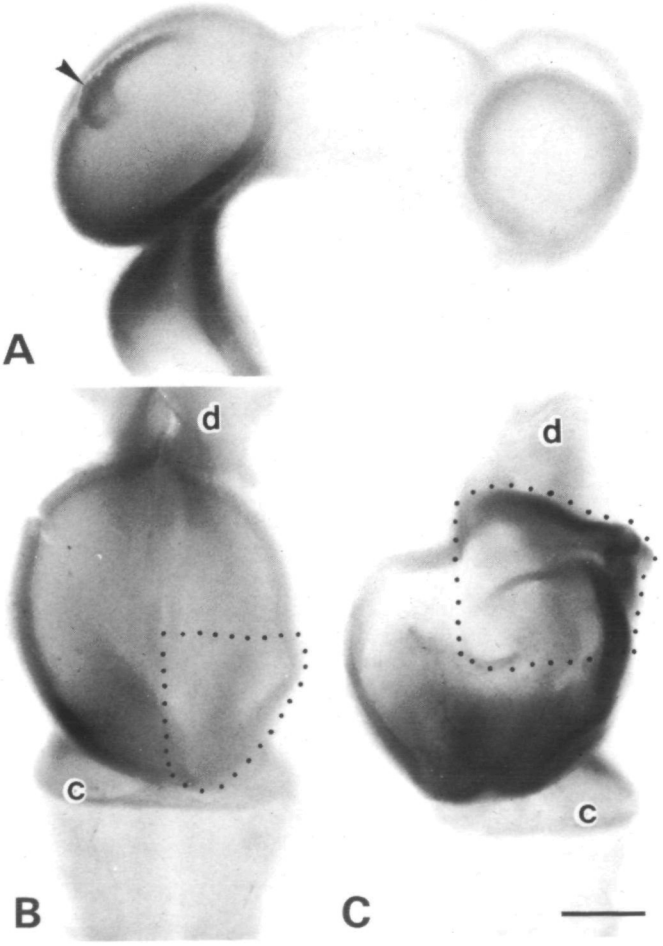


Fig. 7. Chimeras with double-caudal (A, C) or double-rostral (B) tectum, immunostained with anti-*engrailed* protein antibody. (A) A chimera transplanted at the 14-somite stage. The transplant (rostral to the arrowhead) shows weak staining although its original fate is to express *engrailed* strongly. (B) A chimera transplanted around the 25-somite stage, a dorsal view. The transplant (encircled by dotted line) does not show strong staining at the caudal portion of the host tectum. (C) Chimera transplanted around the 25-somite stage. The transplant shows strong staining at the rostral portion of the host tectum. d, diencephalon; c, primordium of cerebellum. Bar, 500 μm.

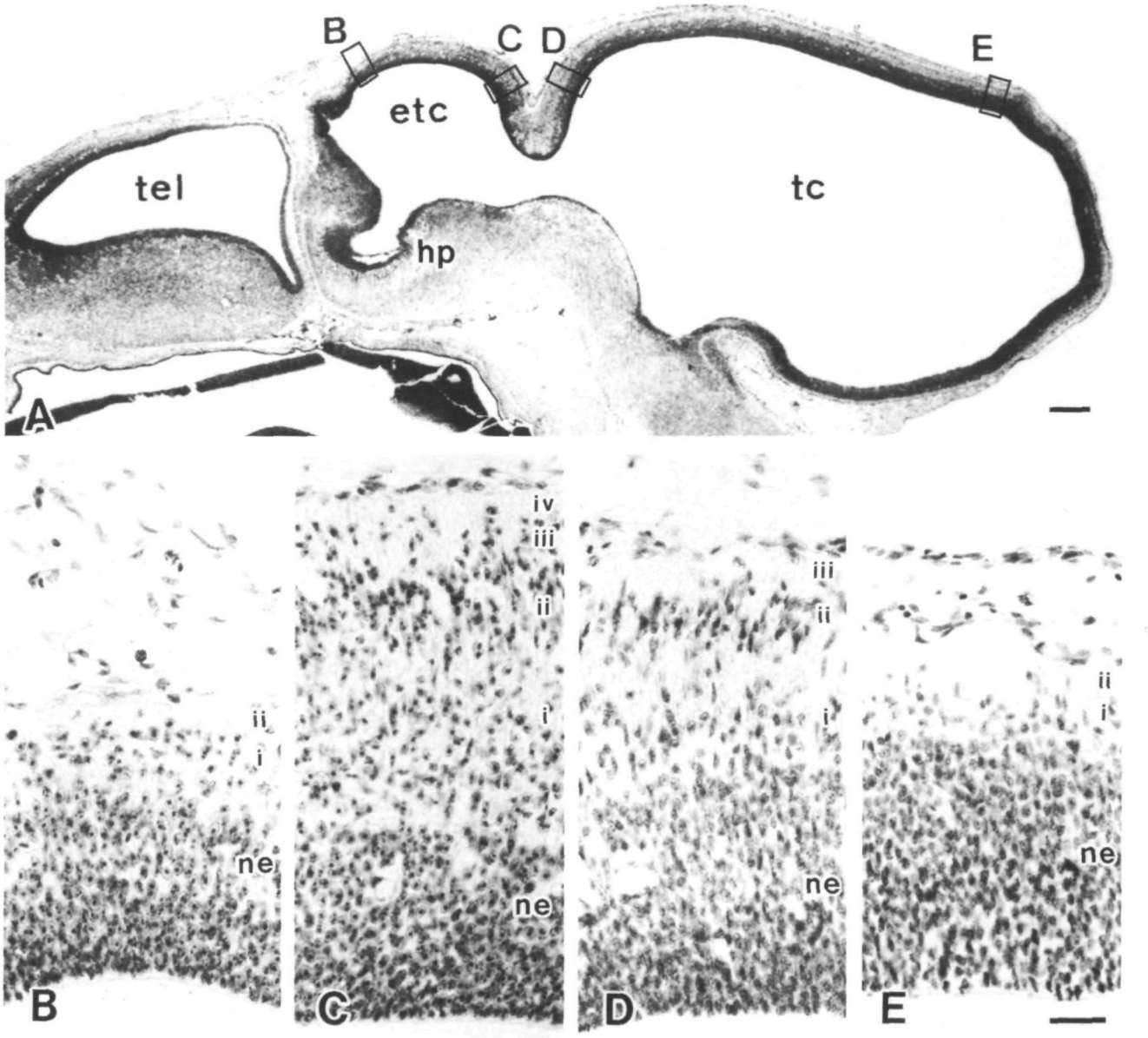


Fig. 8. A chimera with an ectopic tectum in the diencephalon (transplantation was performed between E2 chick and quail), fixed at E7, sagittally sectioned and stained by Feulgen-Rossenbeck method. (A) Low magnification. There is an ectopic tectum (etc) between the telencephalon (tel) and the host tectum (tc). hp, hypophysis primordium. (B–E) High magnification of the ectopic (B,C) and host (D,E) tectum. The ectopic tectum consists of quail cells with condensed heterochromatin (B,C). A rostral part of the ectopic tectum (B) shows immature laminar structure than the caudal part of the ectopic tectum (C). The caudal part shows a thicker wall than the rostral and more advanced lamination (i–iv). A rostral part of the host tectum (D) has the laminar structure comparable to a caudal part of the ectopic tectum. In D, the laminar organization is slightly delayed compared with C, because of the different schedule of development between chick and quail. A caudal part of the host tectum (E) shows thin and immature laninar structure as B. ne, neural epithelium. Bar, (A) 200 μm, (B–E) 20 μm.

all sections were stained according to the Feulgen-Rossenbeck method to distinguish quail and chick cells (Le Douarin, 1973). In the study of retinotectal projection, we analysed only those chimeras that did not show severe malformations and those where approximately half of the tectum was replaced by quail tissue.

Results

engrailed *protein staining in isochronally produced ectopic tecta*

First, we confirmed that chimeric embryos that received a part of the quail diencephalic alar plate homotopically

had normal brain vesicles apparently (Fig. 3). The graft occupied almost a dorsolateral quarter of the diencephalon in frontal sections, and about one to two thirds of the rostrocaudal length of the diencephalon in sagittal sections. The chimeric embryos showed the normal pattern of the *engrailed* protein staining in the tectum (Fig. 3); strong at the caudal end and weakened toward the rostral.

Chimeras after transplantation of the mesencephalic alar plate into the diencephalon were fixed mainly at stages 19–22 of Hamburger and Hamilton (the 4th day of incubation). At the dorsolateral diencephalon, the chimera had an extravesicle which has been shown to be an ectopic tectum (Alvarado-Mallart and Sotelo, 1984; Alvarado-Mallart *et al.* 1990; Nakamura, 1990) (Figs 4, 5). Feulgen-Rossenbeck staining revealed that ectopic tecta consisted of quail cells (Fig. 4). The diameter of the ectopic tecta varied from a quarter to a half of the longitudinal length of the diencephalon. Position of the ectopic tecta also varied from immediately adjacent to the telencephalon to adjacent to the host tectum.

The pattern of *engrailed* protein staining in the ectopic tecta varied according to their positions. In ectopic tecta that were located at the rostral third of the diencephalon, the majority (20 out of 24) showed a rostrocaudal gradient of staining, strong at the rostral and weakened toward the caudal end. As we did not change the rostrocaudal orientation of the graft at the transplantation, the gradient was consequently inverted fron the original fate of the graft (Fig. 4). The gradient of staining was a reflection of strength of each nuclear staining (Fig. 4). The other 4 ectopic tecta were rather small and did not show a clear gradient. Their staining was always as strong as at the caudal side of the mesencephalon (Fig. 5A). When the ectopic tecta were located at the middle third of the diencephalon, the inverted gradient was also observed in many cases (19 out of 23), but the staining was not so strong as in those that occupied the more rostral side (Fig. 5B). The caudal part of the graft, near to the host tectum, was stained very faintly. When the ectopic tecta were located at the caudal third of the diencephalon, almost all (11 out of 12) ectopic tecta showed negative staining (Fig. 5C). Only one chimera showed weak staining at the most rostral end of the ectopic tectum. These position-dependent staining patterns in ectopic tecta were also observed in chimeras fixed at E3. Chimeras that received grafts with the rostrocaudal orientation inverted at the transplantation also showed the position-dependent staining patterns.

engrailed *protein staining in ectopic tecta produced by transplantations between E2 and E3*

In chimeras that received the E2 mesencephalic alar plate into the diencephalon at E3, most (7 out of 10) of the ectopic tecta kept their original pattern of the *engrailed* expression. That is, the staining in the ectopic tecta was in a caudorostral gradient; strongest at the caudal and weakened toward the rostral (Fig. 6A–C). The gradient was independent of the position of the ectopic tectum in the diencephalon. Even when the ectopic tectum was located close to the mes–diencephalon junction, the expression was not repressed. In the other 3 chimeras, the ectopic tecta were small and strongly stained.

In most of the ectopic tecta (24 out of 28) produced after the transplantation of the E3 tectal primordium into the E2 diencephalon, *engrailed* protein staining was strong at the rostral and weakened toward the caudal end (Fig. 6D), as after isochronal transplantation at E2. Similarly, the strength of the gradient was dependent on the distance from the mes–diencephalon junction. The other 4 chimeric tecta did not show the clear gradient, but the relation between strength of staining and distance from the mes–diencephalon junction was observed.

In both experiments, the ectopic tecta consisted of quail cells and the neuroepithelial wall was continuous between the host and the graft even after the transplantations between the different stages of embryos.

engrailed *protein staining in double-rostral and double-caudal tecta*

In the series of transplantations before the 20-somite stage (late E2), 7 double-caudal and 5 double-rostral tecta were processed for the immunohistochemical staining. In the chimeric tecta, the staining pattern of *engrailed* protein followed the host program in both double-rostral and double-caudal tecta (Fig. 7A).

In the series of transplantations performed at the 23- to 29-somite stages (E3), 6 double-rostral and 3 double-caudal tecta were processed for imunohistochemical staining. In double-rostral tecta, the transplant did not show strong staining at the caudal part of the host tectum, which resulted in little-stained tecta from the rostral to the caudal end (Fig. 7B). In double-caudal tecta, the transplant showed strong staining at the rostral part, which resulted in strongly stained tecta from the rostral to caudal end (Fig. 7C).

Cytoarchitectonic development in ectopic tecta

Ectopic tecta fixed at E7 showed a laninar structure

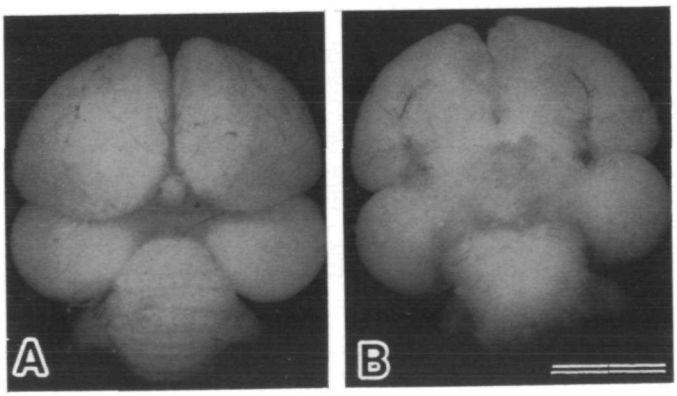
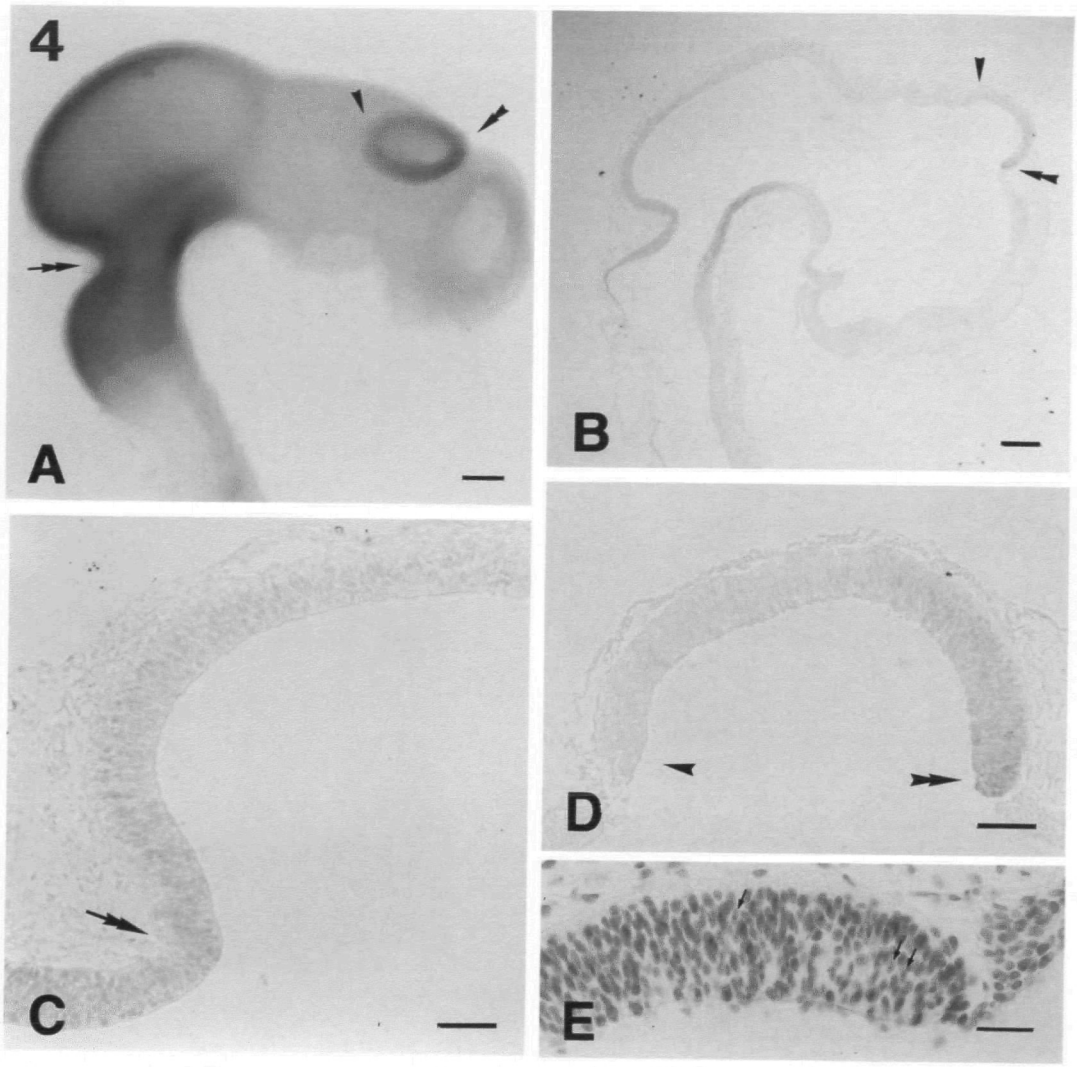


Fig. 9. A chimera with double-caudal tectum (No. 417) fixed at E16, dorsal (A) and ventral (B) views. The right tectum is chimeric and the rostral half consists of the quail cells. The brain does not show malformations. Bar, 500 μm.



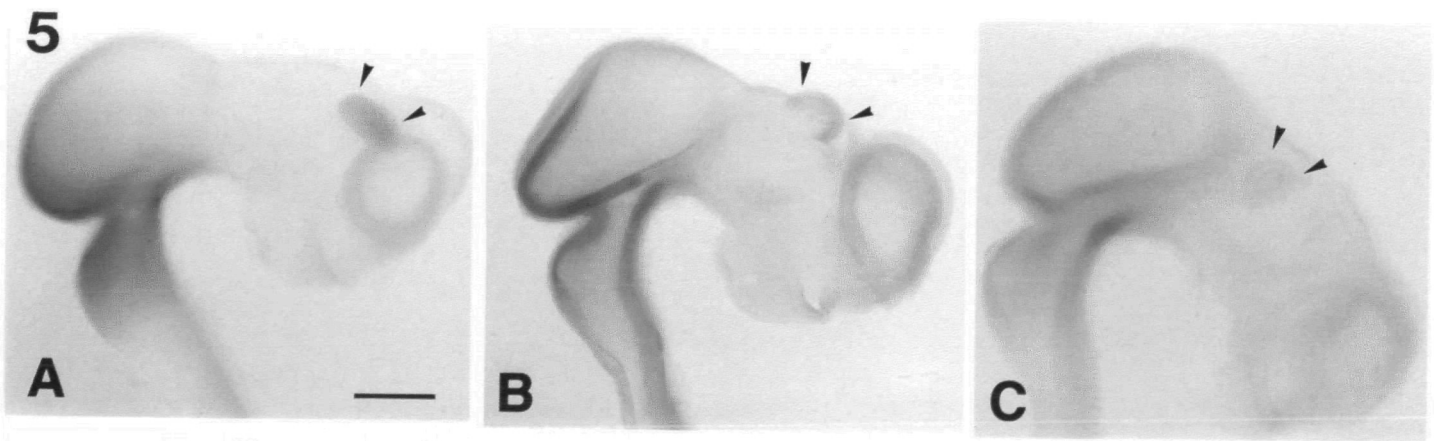


Fig. 4. Chimeras with an ectopic tectum in the rostral diencephalon, immunostained with anti-*engrailed* protein antibody. (A) Whole-mounted chimera, a lateral view. In the ectopic tectum, the staining at the rostral end (double arrowhead) is as strong as the host mes–metencephalon junction (double arrow), and gradually weakened toward the caudal end (arrowhead). (B) A sagittal section with an ectopic tectum at the rostral diencephalon. The rostral end of the ectopic tectum (double arrowhead) shows stronger staining than the caudal end (arrowhead). (C) A part of B at the host caudal mesencephalon. The staining is strong at the mes–metencephalon junction (double arrow) and gradually weakened toward the rostral (right-hand side). The gradient of staining observed in whole mounts reflects the strength of each nuclear staining. (D) A part of B at the ectopic tectum. The staining is strongest at the rostral end (double arrowhead) and gradually weakened toward the caudal end (arrowhead). (E) High magnification of the ectopic tectum at the rostral end, Feulgen-Rossenbeck staining. The ectopic tectum consists of quail cells, which have condensed heterochromatin (arrows). Bar, (A,B) 200 μm, (C,D) 50 μm, (E) 20 μm.

Fig. 5. Chimeras with ectopic tecta (arrowheads) at various positions in the diencephalon, immunostained with anti-*engrailed* protein antibody. (A) Chimera with a small ectopic tectum at the rostral end of the diencephalon. Whole ectopic tectum is stained strongly. (B) Chimera with an ectopic tectum at the middle third of the diencephalon. The rostral end of the ectopic tectum shows the positive staining, but not so strong as the host mes–metencephalon junction. The caudal end of the ectopic tectum is almost negative. (C) Chimera with an ectopic tectum at the caudal third of the diencephalon. The ectopic tectum shows almost negative staining. Bar, 500 μm.

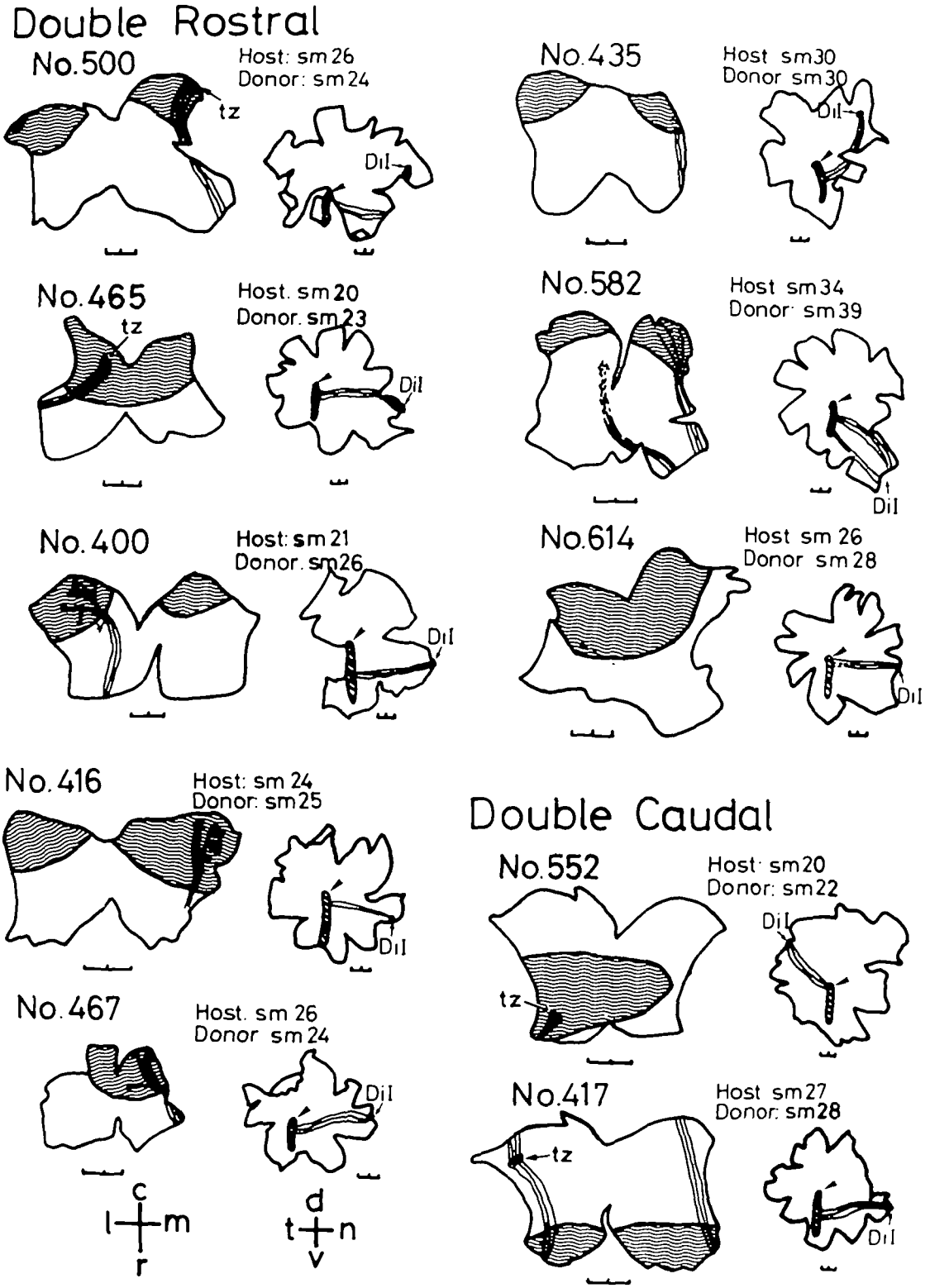


Fig. 10. *Camera lucida* drawings of retinal fibre trajectories on the retinae and the tecta, showing all cases analysed in this study. Stages of the embryo at the transplantation is indicated. DiI, the place where DiI crystal was applied; tz, terminal zone of the labelled fibres; l, lateral; m, medial; r, rostral; c, caudal of tectum; t, temporal; n, nasal; d, dorsal; v, ventral of retina. Arrowhead: optic nerve head. Bar, 2 mm.

characteristic of the developing tectum (LaVail and Cowan, 1971; Senut and Alvarado-Mallart, 1986). The wall of the caudal side of the ectopic tectum was thicker and showed more advanced lamination than that of the rostral side. This gradient of cytoarchitecture was inverted from the original fate of the graft, and resulted

in the mirror image to the host tectum. The ectopic tecta generally showed a little more advanced lamination than the host tectum; probably because quail embryos develop faster than chicks, and the graft follows its own cytodifferentiation schedule (Senut and Alvarado-Mallart, 1987) (Fig. 8).

Retinotectal projection to the double-rostral or double-caudal tecta

Retinotectal projection was analysed in 10 chimeric tecta that showed almost normal appearance (Fig. 9). In chimeras in which transplantations were carried out around the 20-somite stage (2 out of 10 chimeras), the retinotectal projection map was regulated as to that of the host. Nasal retinal fibres (normally project on the caudal) entered the double-rostral tectum from the rostral, extended to the caudal, and projected on the transplant with a tight focus (No. 465 in Fig. 10). In the double-caudal tectum, temporal retinal fibres (normally project to the rostral) entered the tectum and projected on the transplant at the rostral part with a tight focus (Fig. 11, No. 552 in Fig. 10). Both patterns were as expected from the normal retinotectal projection map.

In 4 double-rostral tecta in which transplantation was carried out around the 25-somite stage, fibres from the nasal part of the retina reached the caudal part of the tectum and sent arbors to the transplant. However, in 3 cases (No. 400, 416, 467 in Fig. 10) out of 4, arbours were wandering and dispersed, and did not converge into a tight focus around the geometrical terminal zone (Fig. 12). Some fibres were degenerative. In one case (No. 500) nasal fibres made tight terminal arborizations on the transplant.

In the other 3 double-rostral tecta in which transplantation was carried out around or after the 30-somite stage (Nos. 435, 582, 614 in Fig. 10), almost all nasal retinal fibres reached the caudal, but did not send arbours or were completely degenerative.

In one double-caudal tectum in which transplantation was carried out after the 25-somite stage (No. 417 in Fig. 10), nasal retinal fibres reached the caudal part and made tight terminal arborizations in a topographically normal manner. However, axons sent many small side-branches to the transplant at the rostral part (Fig. 13), suggesting that the transplant was partially characterised as caudal tectum which attracts nasal retinal fibres. Each retinotectal projection on the chimeric tecta is summarized schematically in Fig. 14.

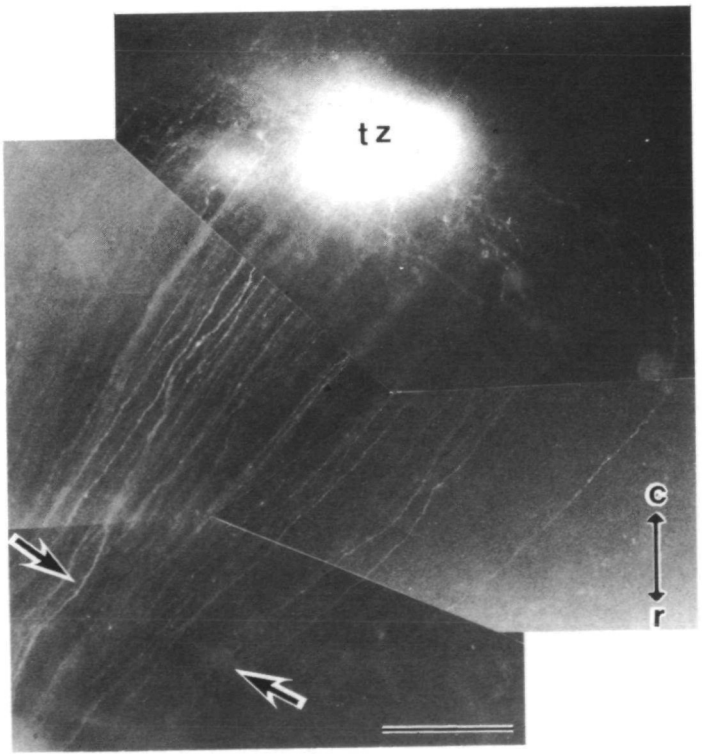


Fig. 11. Projection of the temporal retinal fibres to the rostral part of the double-caudal tectum (No. 552). In this case, transplantation was performed at the 22- (quail) and 20- (chick) somite stages. Temporal retinal fibres (normally project to the rostral) project on the transplant which had originally been the caudal. Rostrocaudal polarity of this chimeric tectum was regulated according to the host program. Rostrocaudal directions are indicated by r and c with arrows. Large arrows indicate the rostral limit of the tectum. tz: target zone. Bar, 250 μm.

Discussion

In this study, we produced ectopic tecta in the diencephalon by transplanting a part of the mesencephalic alar plate heterotopically, and examined its rostrocaudal polarity with regard to the *engrailed* expression and laninar organization. Most isochronally produced ectopic tecta expressed *engrailed* in a rostrocaudal gradient; strongest at the rostral, and gradually weakened toward the caudal. The gradient was inverted from the original fate of the graft, and the strength of the *engrailed* expression was dependent on the distance from the mes–diencephalon junction; the nearer it was to the mes–diencephalon junction, the weaker the expression was.

By a rotation experiment of the mesencephalic alar plate along the rostrocaudal axis at E2, Martinez and Alvarado-Mallart (1990) showed that the pattern of the *engrailed* expression in the rotated mesencephalon was regulated and adjusted to the host pattern. That is, the caudal part of the graft (originally rostral) expressed *engrailed* strongly, and the rostral (originally caudal) showed the weak expression at E3. They supposed from their result that environmental cues are important to establish the gradient. Martinez *et al.* (1991) also showed that metencephalon, when transplanted to prosencephalon, can induce *engrailed* expression in the contiguous host prosencephalon, suggesting a positive influence of metencephalon for *engrailed* expression. Their proposition might be appropriate because *wnt-1*, homologous to *wingless* (Rijsewijk *et al.* 1987) which is a positive signal for *engrailed* in *Drosophila* (DiNardo *et al.* 1988; Martinez Arias *et al.* 1988), is relatively well expressed at the mes–metencephalon junction in mice (Wilkinson *et al.* 1987). The homologous gene might be

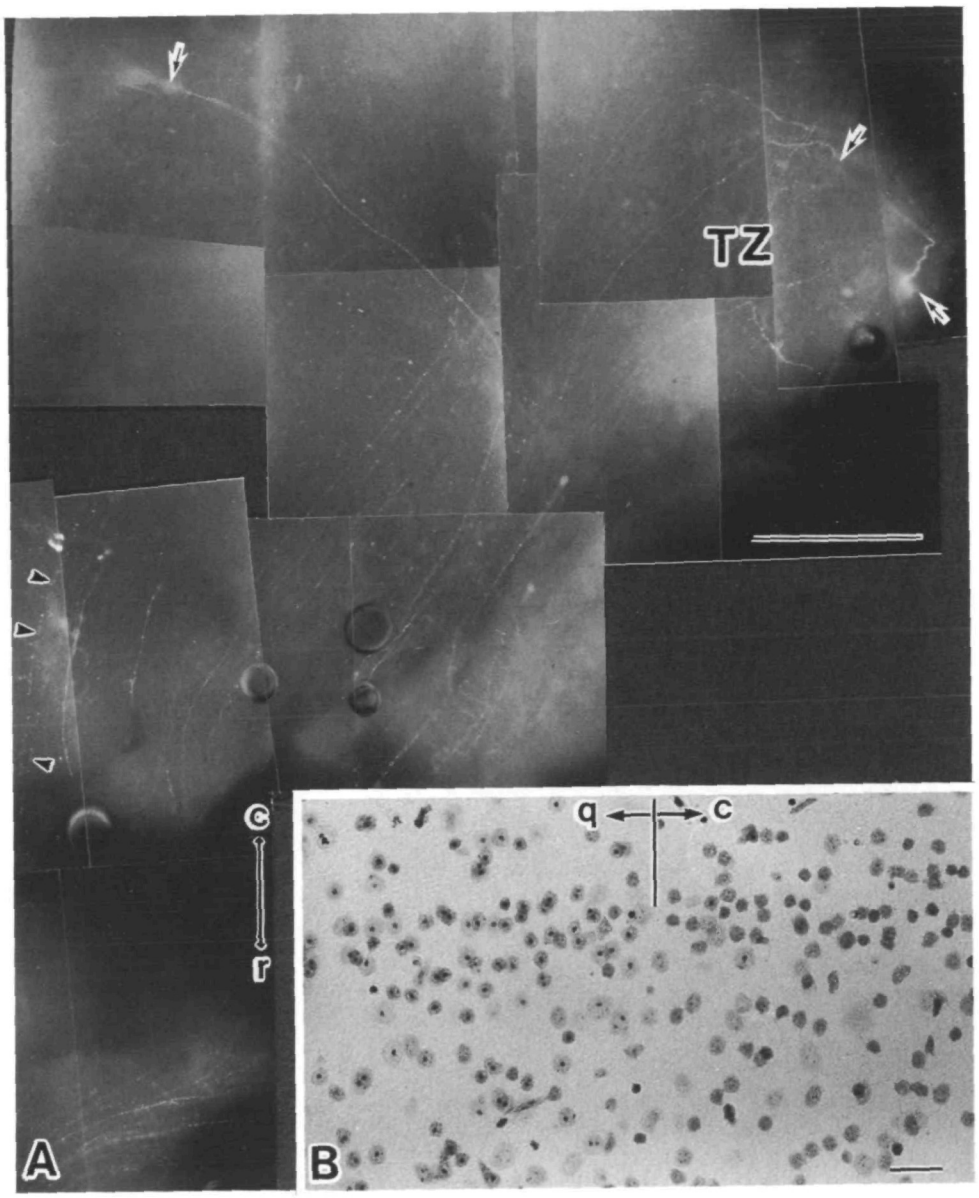


Fig. 12. (A) Trajectory of the nasal retinal fibres on the caudal part of the double-rostral tectum (No. 467). In this case, transplantation was performed at the 24- (quail) and 26- (chick) somite stages. Nasal retinal fibres (normally project to the caudal) reach the caudal part of the chimeric tectum, but does not make a tight focus of terminal arborizations. Fibres make small arborizations independently (arrows). Some fibres are degenerative (arrowheads). (B) Micrograph near the boundary of chick (c) and quail (q) tissues. Quail cells are easily distinguished because of the condensed heterochromatin. Bar, (A) 500 μm, (B) 20 μm.

expressed in chick in a similar pattern and regulate the expression of *engrailed*. As *Wnt-1* is supposed to be a secreted protein and involved in cell–cell signalling (Papkoff and Schryver, 1990), it is plausible that *wnt-1* contribute to the establishment of the caudorostral gradient of *engrailed* expression. In *wnt-1(−)* homozygous mice, produced by McMahon and Bradley (1990) and Thomas and Capecchi (1990), most of the mesencephalon and some rostral metencephalon were absent suggesting importance of *wnt-1* for brain development. Expression pattern of *engrailed* in this mutant would be of interest.

The present study confirms that the ectopic tecta changed their patterns of the *engrailed* expression according to their new environment, and suggests the existence of a repressive influence on *engrailed* expression around the mes–diencephalon junction. The influence is supposed to be in a form of a gradient, peak at the junction and decreased toward the rostral and caudal directions. It may thus be responsible for the field.

The existence of a signal that is responsible for the establishment of rostrocaudal polarity of the tectum at the mes–diencephalon junction is consistent with the study of Chung and Cooke (1975). They rotated the tectal primordium alone or with diencephalic tissues along the rostrocaudal axis in *Xenopus* embryos, and showed that the retinotectal projection map was reversed only when the ectopic diencephalon developed caudally. In agreement with their results, we showed that the rostrocaudal polarity detected by retinotectal projection associates with that of the *engrailed* expression, and that the relative position to the diencephalon is important for the tectum to establish rostrocaudal polarity.

We have shown in the heterotopic and anisochronal transplantations of the mesencephalic alar plate into the diencephalon that E3 chick host has little repressing

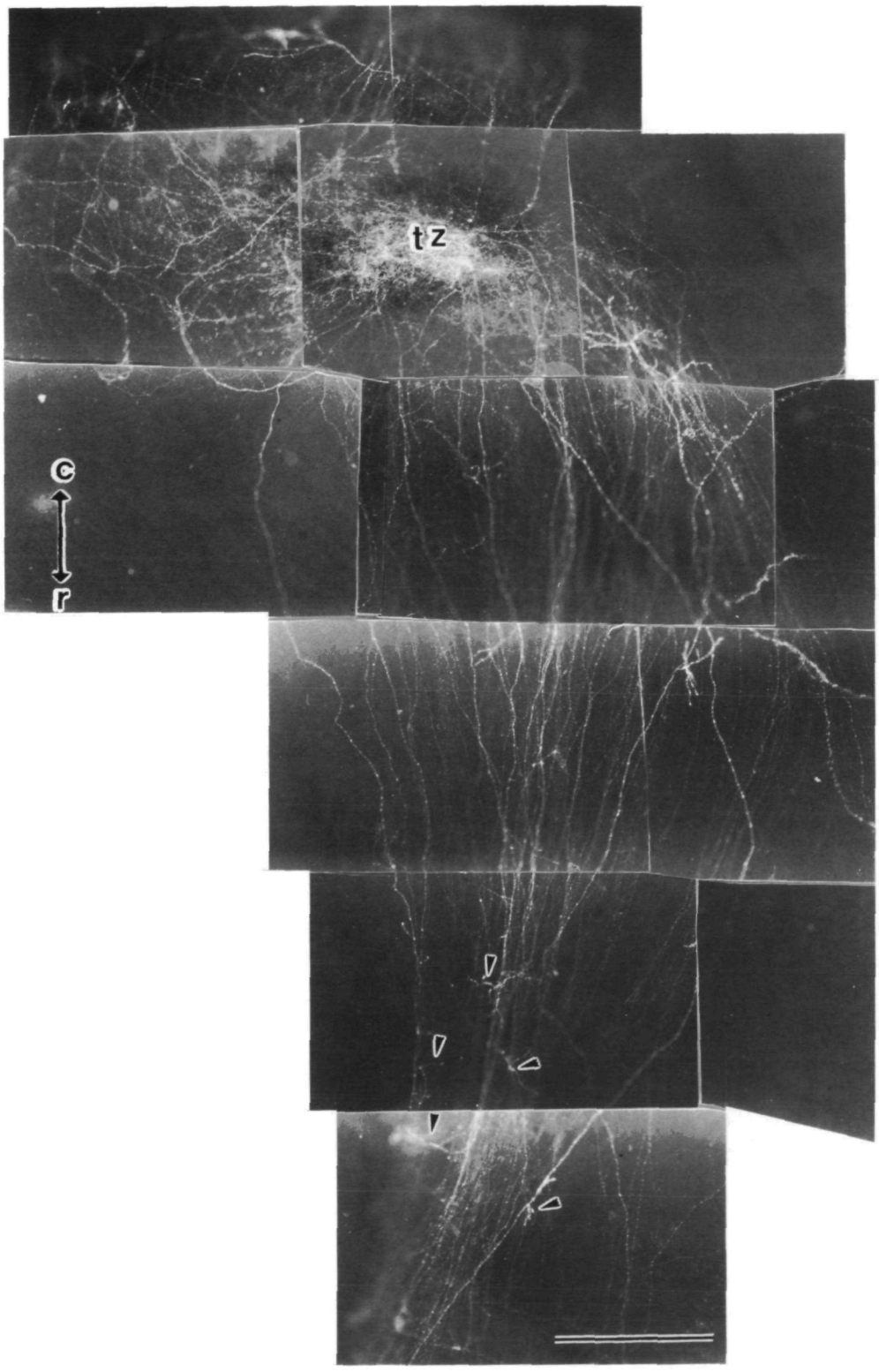


Fig. 13. Trajectory of the nasal retinal fibres on the double-caudal tectum (No. 417). In this case, transplantation was performed at the 28- (quail) and 27- (chick) somite stages. Nasal retinal fibres enter the chimeric tectum from the rostral side, extend to the caudal and make a tight focus at target zone (tz). The fibres, however, send many side-branches at the rostral part which consists of transplant. Rostrocaudal directions are indicated by r and c with arrows. Bar, 500 μm.

effect on *engrailed* expression around the mes–diencephalon junction but that the E3 graft is still competent to respond to the repressing influence of younger host. This result is confirmed in double-caudal tecta produced by transplanting the caudal half of the quail tectal primordium into the rostral half of the chick tectum on the contralateral side at E3. The graft continued to express *engrailed* at the rostral part of the host tectum instead of the expected caudorostral gradient, suggesting that the repressive influence was diminished in E3 chick host, although the graft was competent to respond to changes in expression pattern.

In normal chick (LaVail and Cowan, 1971) and quail (Senut and Alvarado-Mallart, 1986) tecta, there is a

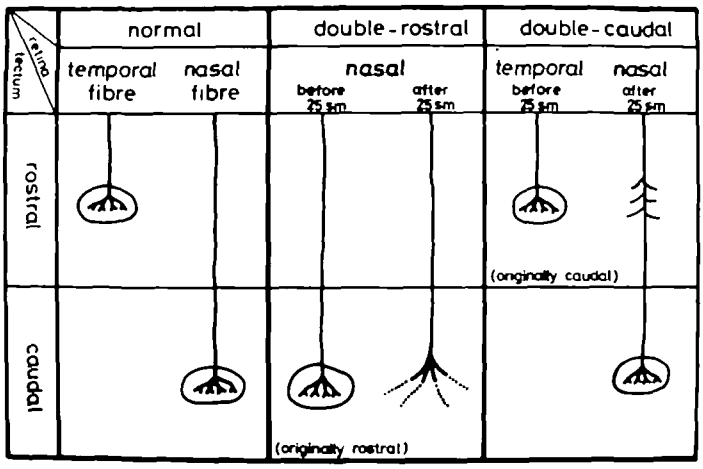


Fig. 14. A schematic diagram showing ordered or disturbed retinotectal projections in chimeric double-rostral or double-caudal tecta. Temporal retinal fibres normally project to the rostral part of tectum, and nasal retinal fibres project to the caudal part of tectum with a tight focus. In double-rostral tectum, in which a caudal half of tectum has been replaced by transplantation of a rostral half of donor tectum, nasal retinal fibres reach the graft. When the transplantation was carried out before the 25-somite stage, nasal fibres normally make a tight focus on the graft. However, when the transplantation was carried out after 25-somite stage, the nasal fibres cannot make a tight focus and fibres degenerate. It is as if the nasal fibres do not recognize the transplant as their target. In double-caudal tectum, a rostral half has been replaced by transplantation of a caudal half of donor tectum. When the transplantation was carried out before the 25 somite-stage, temporal fibres make a tight focus on the graft. When the transplantation was carried out after the 25-somite stage, nasal retinal fibres make a tight focus on the caudal part, with many side-branches at the rostral part. It is as if the transplant attracts nasal fibres at the rostral part.

gradient in cytoarchitectonic differentiation from E4; the rostral side shows more advanced lamination than the caudal side. Recently, Matsuno *et al.* (1991) reported that, even after rotation of the tectal primordium at E2, the rostral (originally caudal) side showed more advanced lamination than the caudal (originally rostral) at E6 to E8. As chimeric tectum produced by rotation of mesencephalic alar plate at E2 showed the adjusted pattern of the *engrailed* expression to the host at E3 (Martinez and Alvarado-Mallart, 1990), it was suggested that the gradient of cytoarchitectonic development is correlated with the pattern of the *engrailed* expression. This was confirmed in our study since in E7 chimeras with ectopic tecta, the caudal side of the ectopic tectum and the rostral side of the host tectum differentiated faster than the opposite sides; that is, cytoarchitectonic development was well advanced in regions where the *engrailed* expression was repressed, and *vice versa*. It is suggested that the pattern of the *engrailed* expression could be a marker for subsequent rostrocaudal polarity of cytoarchitectonic development in the tectum.

It has been reported that patterns of *engrailed* expression (Martinez and Alvarado-Mallart, 1990) and retinotectal projection (Ichijo *et al.* 1990) are regulated and adjusted to those of the host after rotation of the tectal primordium at E2. However, we found that transplantations of the rostral half of the tectal primordium into the caudal half or *vice versa* at late E3 showed that the *engrailed* expression was not adjusted to the host pattern and that ordered retinotectal projection was also disturbed as if the transplants preserved their original characters concerning the positional information. This suggests that the tectum loses its plasticity with regard to the *engrailed* expression pattern and retinotectal projection map at the same stage, and that the pattern of the *engrailed* expression could also be a marker of rostrocaudal polarity of the retinotectal projection map.

We conclude that (1) the pattern of *engrailed* expression is under the control of environmental cues which include a repressive influence around the mes–diencephalon junction; (2) patterns of *engrailed* expression can be an excellent marker representing rostrocaudal polarity of the tectum with regard to subsequent cytoarchitectonic development and retinotectal projection, (3) the ability of the host to influence *engrailed* expression of the graft is lost at around E3 but the graft is still competent to respond. It is possible that *engrailed* may be responsible for the rostrocaudal polarity of tectum. Recent study (Joyner *et al.* 1991) concerning targeted mutagenesis of *En-2* gene by homologous recombination in mouse embryonic stem cell is one of several works to elucidate the functional role of *engrailed* gene in vertebrate. It was reported that the *En-2*(−) homozygous mutant mice did not show severe malformations except that the cerebellum was smaller than normal, and it was concluded that *En-2* is functionally redundant in embryonic development. Considering the possibility of rescue mechanisms by other genes and that the report did not investigate the tectal polarity, we feel that further studies are required to assess the biological function of *engrailed* in tectum.

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