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Neural Fold Formation at Newly Created Boundaries between Neural Plate and Epidermis in the Axolotl

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According to a recent model, the cortical tractor model, neural fold and neural crest formation occurs at the boundary between neural plate and epidermis because random cell movements become organized at this site. If this is correct, then a fold should form at any boundary between epidermis and neural plate. To test that proposition, we created new boundaries in axolotl embryos by juxtaposing pieces of neural plate and epidermis that would not normally participate in fold formation. These boundaries were examined superficially and histologically for the presence of folds, permitting the following observations. Folds form at each newly created boundary, and as many folds form as there are boundaries. When two folds meet they fuse into a hollow "tube" of neural tissue covered by epidermis. Sections reveal that these ectopic folds and "tubes" are morphologically similar to their natural counterparts. Transplanting neural plate into epidermis produces nodules of neural tissue with central lumens and peripheral nerve fibers, and transplanting epidermis into neural plate causes the neural tube and the dorsal fin to bifurcate in the region of the graft. Tissue transplanted homotypically as a control integrates into the host tissue without forming folds. When tissue from a pigmented embryo is transplanted into an albino host, the presence of pigment allows the donor cells to be distinguished from those of the host. Mesenchymal cells and melanocytes originating from neural plate transplants indicate that neural crest cells form at these new boundaries. Thus, any boundary between neural plate and epidermis denotes the site of a neural fold, and the behavior of cells at this boundary appears to help fold the epithelium. Since folds can form in ectopic locations on an embryo, local interactions rather than classical neural induction appear to be responsible for the formation of neural folds and neural crest.

Neural folds normally arise along the boundary between the neural plate and the epidermis. A neural fold comprises the uplifted edge of the neural plate and the epidermis that curls over it (Fig. 1). As neurulation proceeds, the folds rise higher and move toward the dorsal midline of the embryo (Fig. 2) where left and right neural folds fuse; epidermis joins epidermis, and the lateral edges of the neural plate join together to form the neural tube. During this process, some cells that were originally in the epithelium of the folds come to lie between the epidermis and the neural tube as a cap (the neural crest) along the roof of the neural tube. Soon cells emigrate from the neural crest, move throughout the embryo, and ultimately contribute to the peripheral nervous system, connective tissues, glandular tissue, and pigment cells (Le Douarin, 1982). This sequence of morphogenetic events leads to some basic questions: (1) What cellular behaviors produce the neural folds? (2) What determines the position at which the folds and crest will form? (3) Do neural crest cells originate in the epidermis, the neural plate, or both?

Several proposals have been made to explain the mechanics of fold formation. The apical surfaces of the neural plate cells may contract, changing the shapes of these cells from cylinders to cones, and rolling the plate into a tube (His, 1874, 1894; Lewis, 1947; Baker and Schroeder, 1967; Burnside, 1971, 1973; Odell *et al.*, 1981). The epidermis may actively spread toward the midline, pushing on the edge of the neural plate so that it buckles upward into a fold (Schroeder, 1970; Jacobson and Jacobson, 1973; Brun and Garson, 1983). An accumulation of mesoderm and/or extracellular matrix beneath the neural plate (or a change in the shape of these underlying materials) may lift the plate edges upward and create a fold (Schroeder, 1970; Morriss and Solursh, 1978). Anterior-posterior elongation in the epithelium, either at the dorsal midline (Jacobson, 1978) or at the edges of the neural plate (Jacobson and Tam, 1982), may help to produce the folds by causing the plate to buckle into a tube.

A new model of epithelial cell behaviors, the cortical tractor model (Jacobson *et al.*, 1985, 1986), proposes that embryonic epithelial cells, like the mesenchymal cells into which they frequently convert, are motile. Studies of actively moving cells have shown that cell movement is accompanied by a fountainoid flow of cortical cytoplasm (for review see Bray and White, 1988). Adhesion molecules that attach to the substrate or to adjacent cells, pass through the membrane, and attach to the cortical cytoskeleton provide anchorage points against which forces produced in the contracting cortical cytoskeleton can act. As the cortical cytoplasm moves, these

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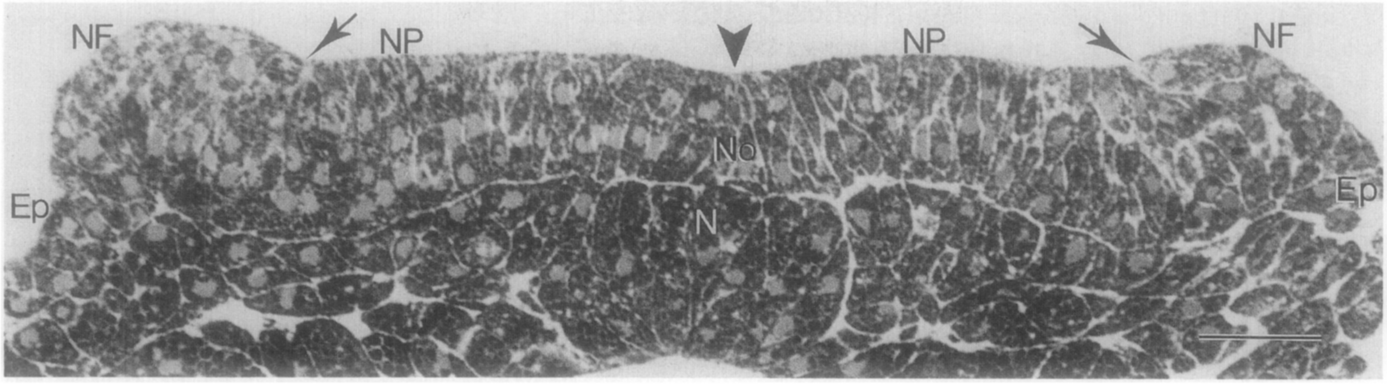

FIG. 1. Plastic cross section through the neural folds (NF) of a normal stage 14 embryo. In the neural folds, the long axes of cells in the neural plate (NP) are oriented toward the boundary (arrows) with the epidermis (Ep). Dorsal to the notochord (N), the long axes of cells in the notoplate (No) are oriented toward the midline (arrowhead). Line scale = 100 μm.

adhesion molecules are brought to the cell surface, move at the surface, and are later internalized. The cortical tractor model proposes that a fountainoid flow of cytoplasm and associated cell movements (called "cortical tractoring" by Jacobson and his co-workers) also occurs in epithelial cells, but that cell movements are partially restricted by the apical junctional complexes that tightly bind epithelial cells to one another. The junctional molecules that compose these complexes, however, move with the flow of the cortical cytoplasm—new molecules are constantly being added to the junctional complexes while others are internalized.

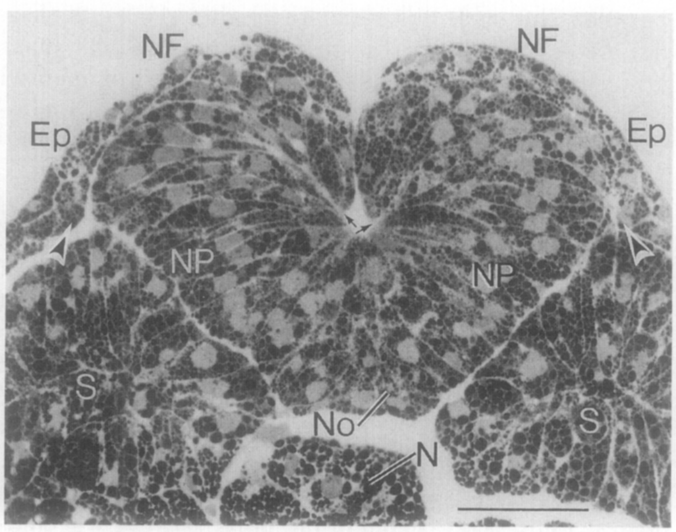

FIG. 2. Plastic cross section through the neural folds (NF) of a normal stage 20 embryo. Closure of the neural folds moves the boundaries (small arrows) between the neural plate (NP) and the epidermis (Ep) toward the midline. Note the tall, columnar cells in the somites (S), and the tabs of epidermis (arrowheads) that extend beneath the neural plate. The separation of the notoplate (No) from notochord (N) is an artifact. Line scale = 100 μm.

Thus the junctional complexes are in a dynamic equilibrium, and the epithelial cells are not frozen in position but can move actively among one another within the epithelial sheet while retaining the integrity of their apical seals. With proper organization, these cellular movements can distort the epithelium and bring about morphogenetic tissue movements such as fold formation.

The various proposals of how folds might form are not mutally exclusive, and although none has been definitely disproved, strong arguments have been presented against some (see Karfunkel, 1974; Gordon, 1985, for reviews). A number of models incorporate the fact that neural folds form at a distinct boundary between the neural plate and the epidermis. One model, the cortical tractor model, predicts that the interactions that organize cell movements at the boundary between the neural plate and the epidermis are responsible for neural fold formation, but the role of this boundary during neurulation has not been examined in detail.

In the present experiments, we test the prediction that epithelial folding results from local interactions between different types of cells at a boundary. To determine whether neural folds will form at a boundary between any portion of the neural plate and epidermis, pieces of neural plate or of epidermis are taken from regions that would not normally participate in fold formation, juxtaposed to create boundaries in ectopic locations, and examined for the presence of folds.

MATERIALS AND METHODS

Embryos of the Mexican axolotl, *Ambystoma mexicanum* (Shaw), were obtained from the Indiana University Axolotl Colony and kept in 10% Holtfreter's solution prior to the experiments. Transplantations were performed in 100% Holtfreter's solution using electrolytically sharpened tungsten needles. When the transplant had healed in place, the embryos were transferred to 50% Holtfreter's solution and maintained at 17°C. In some cases, tissue from pigmented donor embryos was transplanted into albino host embryos, so that the pigmented cells from the graft could be identified as the embryo developed. Embryos between stages 14 and 16 (Schreckenberg and Jacobson, 1975) were used in these experiments because by stage 14, the neural fold was clearly visible; and after stage 16, the narrowing and closure of the neural plate made operating difficult. Since the "neck" between the prospective brain and spinal cord could be easily seen, this area was used for removing tissue from (or implanting tissue into) the neural plate and neural folds. The supranotochordal neural plate (notoplate) appears to have very different properties from the rest of the neural plate (Jacobson, 1981, 1985; Keller *et al.*, 1985a, b; Jacobson *et al.*, 1986), so care was taken to avoid including this region in the transplants. The methods followed in preparing and maintaining embryos have been described in more detail by Jacobson (1967).

Following *in vivo* observation, some experimental embryos were fixed and examined histologically. Plastic or paraffin sections of 69 embryos were examined by light microscopy, and 17 embryos were examined by scanning electron microscopy (SEM). Embryos were fixed for embedding in plastic or for SEM using Karnovsky's fluid diluted to half strength with 0.1 *M* cacodylate buffer (Karnovsky, 1965), postfixed in 1% OsO_4 in 0.1 *M* cacodylate buffer, and dehydrated through an ethanol series. For SEM, specimens were then critical point dried using liquid CO_2 as an exchange medium, coated with 6–7 nm of gold/palladium, and viewed with an ISI-SX-40 scanning electron microscope at 20 kV. For plastic embedding, specimens were cleared in acetone, embedded in Epon by the procedure of Luft (1961), cut at 2–3 μm using glass knives, and stained with 1% methylene blue in 1% sodium tetraborate. For paraffin embedment, embryos were fixed in Kahle's fluid (Jones, 1966), dehydrated and cleared through an ethanol/*n*-butanol series, embedded in Paraplast-plus, and sectioned at 6 μm. Paraffin sections were stained with 0.2% neutral red and (when both the donor and the host were pigmented) counterstained with 0.1% Janus green (Jones, 1966).

RESULTS

Neural Plate Transplanted into Epidermis

When a piece of neural plate is transplanted into lateral or ventral epidermis as illustrated in Fig. 3 (both host and donor pigmented, 61 cases; host albino and donor pigmented, 14 cases), low folds rise along the edges of the graft soon after it heals into the new position. The folds "roll" toward the center of the implant, where they eventually meet and fuse. Initially, the ectopic folds close rapidly, and soon the neural plate graft is visible from the surface only through a small central pore (Fig. 4a), which often remains open after the natural neural folds of the host have fused. Larger implants require a longer period to close. Cells in the mesoderm surrounding the implant, including those subjacent to the folds, appear to be typical mesenchymal cells (Fig. 4b).

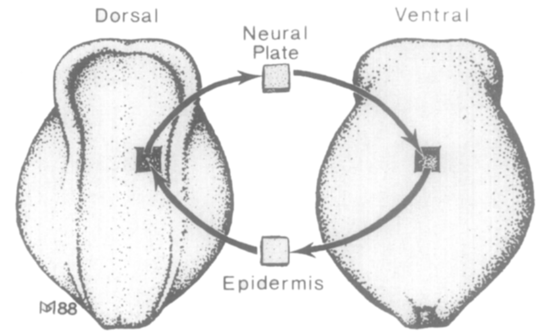

FIG. 3. Diagram of operations in which a piece of neural plate is transplanted into the ventral epidermis, or a piece of ventral epidermis is transplanted into the neural plate.

Pigmented neural plate tissue in an albino host is detectable by superficial examination even when covered by epidermis and allows us to follow the development of the ectopic folds *in vivo*. As the epidermis covers the neural tissue, the folds appear as a gray area between the dark plate and the light epidermis (Fig. 5a). After fold closure, the graft appears as a gray mass lying below the epidermis (Fig. 5b). Later, pigmented cells migrate away from some implants, and often develop the dendritic morphology characteristic of melanocytes (Fig. 5c).

Ectopic folds are histologically similar to normal folds in many respects when viewed in section (Fig. 6). In both normal and ectopic folds, the basal surfaces of neural plate cells are in close apposition with those of the epidermal cells, and a small tab of epidermal cells bulges between the basal surface of the neural plate and the underlying mesoderm (Figs. 2 and 6). Mesodermal cells that occupy the space below the ectopic folds show no evidence of the columnarization (Fig. 6) that is characteristic of cells in the somites beneath natural neural folds (Fig. 2). When implants fail to form folds along one or more sides, sections reveal that the wound has failed to heal and the epidermis and neural plate are not in contact on this side.

Most cells in the transplants attain the wedge-like morphology of those in the natural neural plate, with

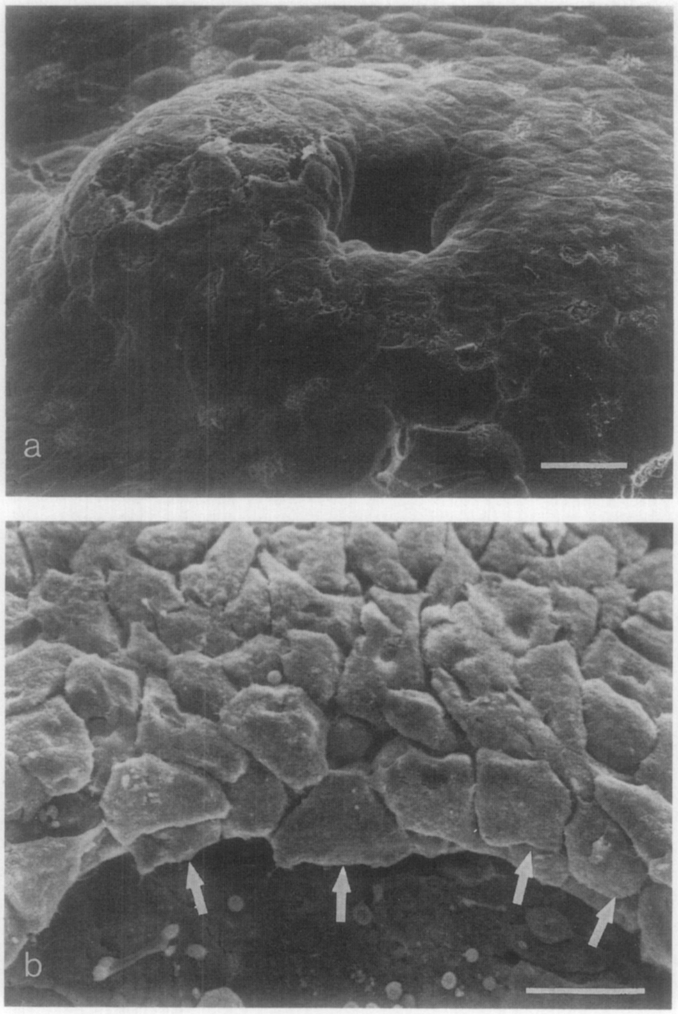

FIG. 4. Results of transplanting a piece of neural plate into the lateral epidermis as viewed by SEM. Time elapsed since operation: 14 hr. Line scales = 50 μm. (a) Folds that formed around the transplant have "rolled" toward its center leaving a pore that will eventually disappear as the folds fuse. (b) Mesoderm subjacent to transplant in (a). Mesodermal cells immediately beneath the folds (arrows) show no evidence of columnarization.

the longest and narrowest cells being located at the periphery of the graft (Fig. 6). The long axes of cells near the periphery of the normal plate are oriented toward the boundary between the epidermis and neural plate, while the long axes of cells near the center of the plate (i.e., cells in the notoplate and adjacent neural plate) are strongly oriented toward the dorsal midline (Figs. 1 and 2). In an implant, the long axes of peripheral neural plate cells are oriented toward the boundary with the epidermis that surrounds the graft. Near the center of an implant, however, the long axes of cells are not as strongly oriented toward the middle as they are in the normal plate (Fig. 6).

Sections through the mass of tissue formed by the closure of the ectopic folds show that the neural tissue has become separated from the epidermis. In section, these neural nodules resemble normal neural tubes in that they each contain a central lumen bordered by cells that possess apical cilia. Nerve fibers resembling those in the marginal layer of the spinal cord occupy the outer portion of the nodule (Fig. 7).

Pigmented cells transplanted into an albino host can be identified in paraffin sections by the presence of pigment granules (especially when the section is stained using only neutral red and then viewed using red light). Most cells containing pigment granules remain in the nodule of neural tissue, but heavily pigmented melanocytes and mesenchymal cells with a few pigment granules occasionally appear.

Homotypic controls in which epidermis is transplanted into epidermis (both host and donor pigmented, four cases; host albino and donor pigmented, eight cases) tested for the possibility of "folds" resulting from wounding. Once the graft heals, cells from the donor epidermis appear to integrate with those of the host. Cells from pigmented donor tissue can be located in albino hosts, both *in vivo* and in sections, revealing that neither folding nor any other discontinuity (other than the difference in pigment) is discernible at the boundary between donor and host tissues (Figs. 8a–8c).

Epidermis Transplanted into Neural Plate

When epidermis is transplanted into the neural plate as illustrated in Fig. 3 (both host and donor pigmented, 66 cases; host albino and donor pigmented, 19 cases), a ridge develops at the site of implantation, and later the neural tube bifurcates in this region. Soon after transplantation, the entire graft is raised into a single broad ridge (Fig. 9). As neurulation progresses, the medial movement of the ipsilateral normal fold stops at the implant, while the contralateral normal fold eventually reaches the midline. When the folds close and the overlying epidermis fuses, the resulting neural tube is bifurcated in the region of the transplant. The larger branch of the tube follows the dorsal midline, and the smaller branch appears as a lateral ridge at the site of the transplant. At later stages, dorsal fins appear along both branches of the neural tube. Often, only discontinuous fragments of a fin form over the more lateral branch of the tube, but occasionally both fins are complete. When the transplant is placed across the midline, the neural tube and dorsal fins branch symmetrically (Fig. 10).

In cross sections, the newly formed ridges contain two areas that morphologically resemble reduced neural folds. At the new boundaries between the neural

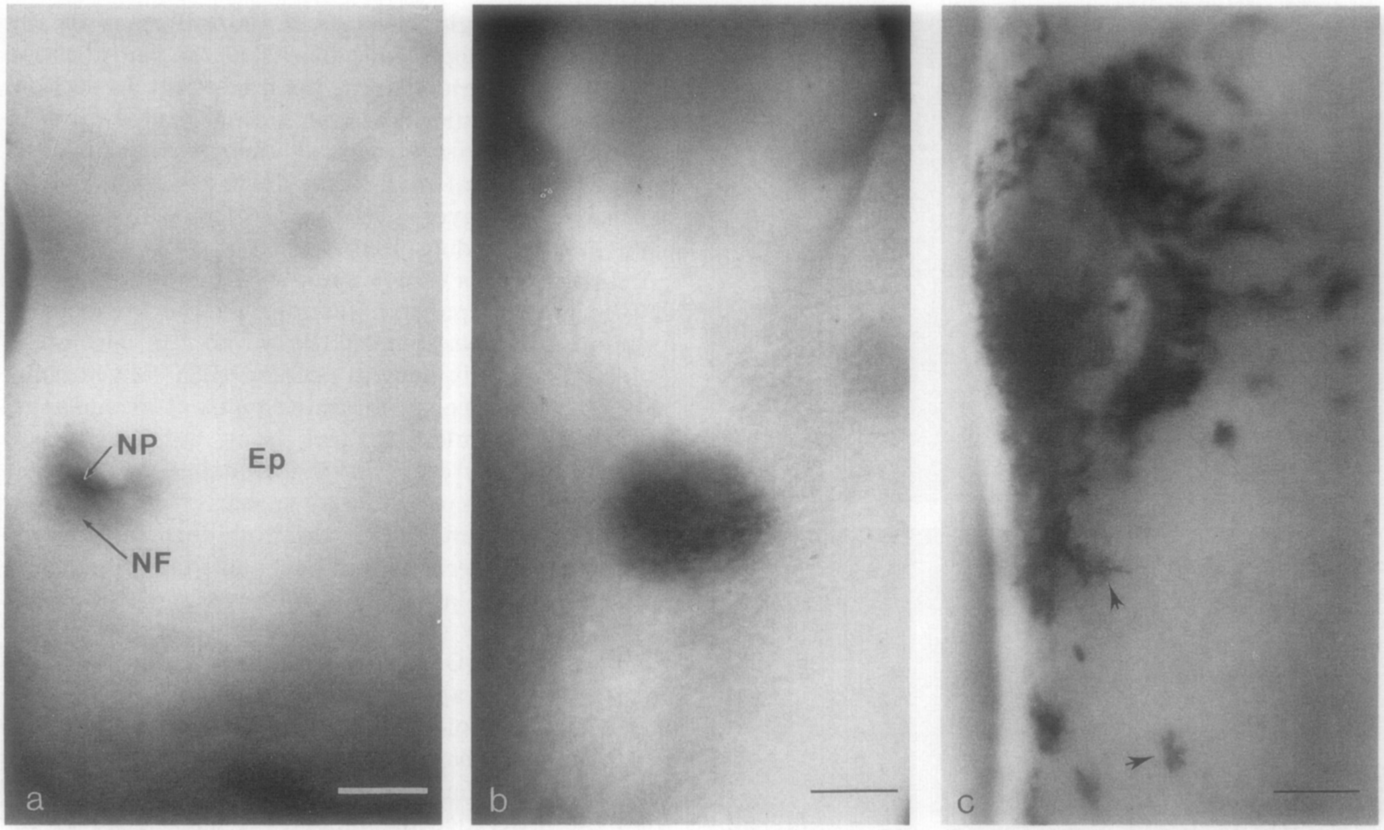

FIG. 5. The formation and closure of ectopic folds around a pigmented neural plate transplant in the epidermis of an albino host. Line scales = 200 μm. (a) At first, the neural plate transplant (NP) appears dark, the neural folds (NF) appear gray, and the surrounding epidermis (Ep) appears white. Time elapsed since operation: 16 hr. (b) Later, in the same embryo, the dark neural tissue is now completely covered by epidermis and appears gray. Time elapsed since operation: 2.5 days. (c) Still later, in a different embryo, darkly pigmented, dendritic cells that appear to be melanocytes (arrows) migrate from transplant. Time elapsed since operation: 10 days.

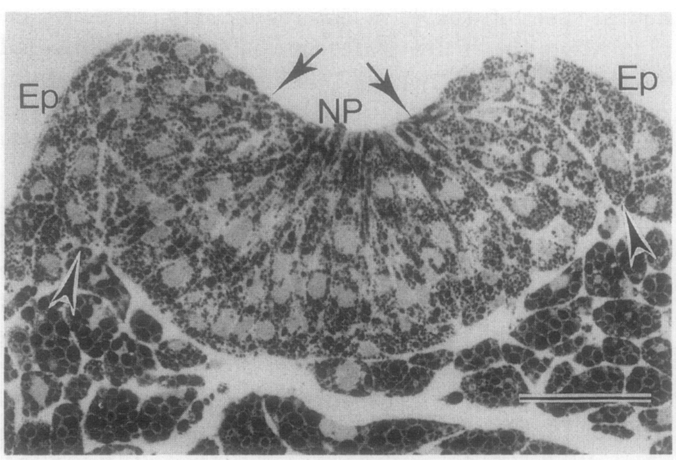

FIG. 6. Plastic cross section through the folds surrounding a piece of neural plate transplanted into the ventral epidermis. Boundaries (arrows) between neural plate (NP) and epidermis (Ep) are incorporated in neural folds, and tabs of epidermis (arrowheads) extend below the neural plate. (Compare to Figs. 1 and 2.) Time elapsed since operation: 17 hr. Line scale = 100 μm.

plate and the epidermis, the long axes of cells in the neural plate are oriented toward the boundary, and the basal surfaces of these cells are in contact with epidermal cells (Fig. 11). Often the basal surfaces of the neural plate cells from either side of the implant touch, but they do not fuse and the tissue on each side remains distinct. Epidermal cells inserting between the neural plate and the mesoderm are prominent on the lateral edge of the implant, but not near the midline. Sections also reveal that folds form around the entire implant. However, as the embryo continues to elongate in the anterior–posterior direction, the folds at the anterior and posterior ends of the implant become smaller. After tube closure, the lumen in one of the two branches may be closed by neural tissue at some points, but each branch is usually hollow for most of its length. An epidermal septum often separates the two branches of the neural tube. When pigmented epidermis is transplanted into the neural plate of an albino host, the central part of this septum contains cells derived from the pig-

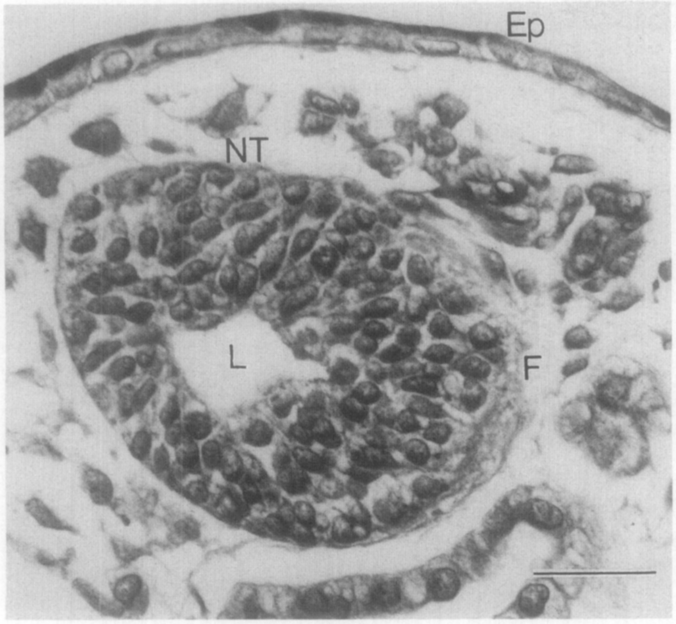

FIG. 7. Paraffin cross section through the "neural tube" formed by a neural plate transplant in the ventral epidermis. The transplant has become a nodule of neural tissue (NT) that has a lumen (L) and nerve fibers (F), and is completely separated from the epidermis (Ep). Time elapsed since operation: 9 days. Line scale = 50 μm.

mented epidermal transplant, while the more peripheral areas contain no pigmented cells. In later development, this septum often extends to the notochord and is separated from the neural tissue by mesenchyme throughout most of its length (Fig. 12).

Homotypic controls in which neural plate is implanted into neural plate (both host and donor pigmented, seven cases; host albino and donor pigmented, five cases) never cause a complete branching of the neural tube. Such operations often retard the closure of the folds, and occasionally produce contortions in the neural tissue, but two distinct branches of the neural tube are never seen. Cross sections through grafts of pigmented plate into an albino host show that the implant is usually incorporated into the host's tube to give a normal morphology (Fig. 13).

Contralateral Exchanges, Rotations, and Multiple Boundaries

Reversing the medio-lateral axis of a strip of tissue containing epidermis, neural fold, and neural plate creates new boundaries by juxtaposing the neural plate and epidermis along the edges of the strip and also reverses the orientation of the existing neural fold in the strip. As illustrated in Fig. 14, this was accomplished in two ways: by rotating the strip in place 180° (thus reversing the anterior-posterior polarity of the strip; three cases) or by transplanting the strip, without rotation, to the contralateral side of a host embryo (retaining the orginal anterior-posterior polarity of the strip; four cases). In either case, folds develop at all of the new boundaries. These embryos develop in a manner similar to those in which epidermis is transplanted into the neural plate (as described above), except that the large original neural fold in the strip now "rolls" laterally rather than toward the midline. (During fold closure, natural and artificially created neural folds always "roll" over the neural plate.) The fold in the strip moving away from the midline does not prevent the contralateral natural fold and the new fold at the medial edge of the strip from fusing into a tube. When the folds fuse, the neural tube bifurcates into two branches, each of which can develop dorsal fins (or fragments of dorsal fins). Rotating tissue in place results in the same course of development despite the reversal of anterior-posterior polarity.

If folds form at all boundaries between neural plate and epidermis, then the number of folds should equal the number of boundaries. Thus, two new boundaries (in cross section) are created when epidermis is transplanted into the neural plate, or the medio-lateral axis of a single strip containing epidermis, neural fold, and neural plate is reversed. Two new boundaries and two original boundaries (a total of four boundaries) should produce four folds if the hypothesis is correct. As described above, four folds are visible in such cases.

Other operations can create more boundaries. Transplanting two pieces of epidermis into the neural plate, one on each side of the midline, by performing the operation illustrated in Fig. 3 twice (four cases), exchanging strips bilaterally (five cases), or rotating the strips in place 180° on both sides of the embryo (three cases), creates a region that has six boundaries in cross section (Fig. 14). In these embryos, folds form at each boundary (Fig. 15). Sections through these embryos show the two large original folds, and four smaller folds at new boundaries. The folds closest to the midline are often extremely small and poorly formed. Fold closure results in the formation of a three-branched neural tube (Fig. 16). The folds that close into the central branch of the neural tube formed entirely at new boundaries.

Control experiments consist of replacing a strip with an ipsilateral one that had not been rotated (four cases) and of rotating a strip 180° and then moving it to the contralateral side (six cases) which reverses the anterior-posterior axis without reversing the medio-lateral axis. Both of these procedures juxtapose epidermis with epidermis and neural plate with neural plate and retain

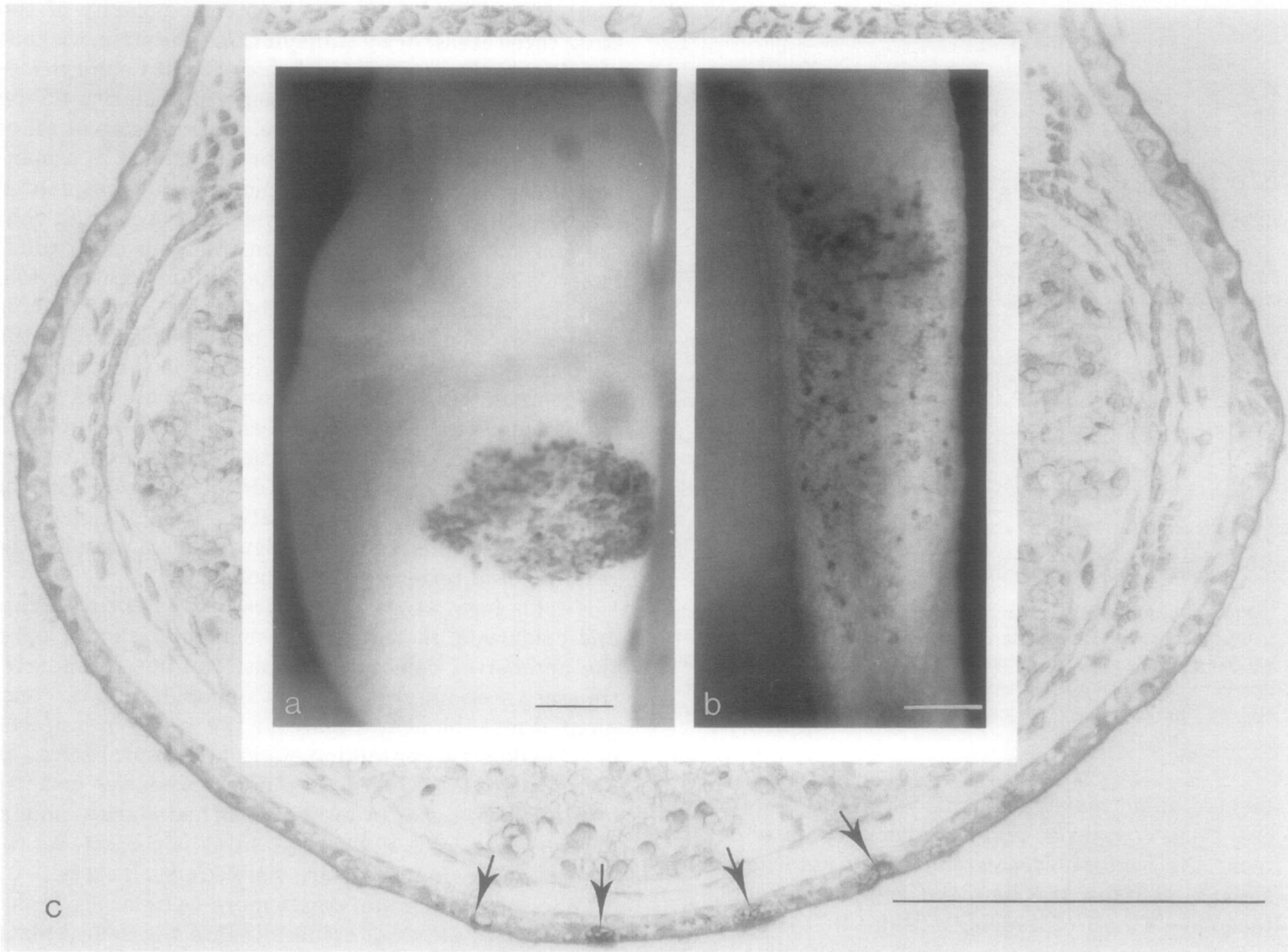

FIG. 8. Views of a single control embryo: pigmented epidermis transplanted into the epidermis of an albino host. Note the absence of folds at any point in development. Line scales = 300 μm. (a) Time elapsed since operation: 17 hr. (b) Time elapsed since operation: 5 days. (c) Paraffin cross section through larva. Although the epidermis of the donor and the host have integrated, and the embryo appears normal, the transplant can be identified by the presence of pigmented cells (arrows). Time elapsed since operation: 10 days.

the original orientation of the original neural fold. Fold formation and closure is normal in the controls.

DISCUSSION

By demonstrating that folds appear at any site where epidermis encounters neural plate, these experiments test and confirm the proposition that neural folds result from conditions at this boundary. Folds appear at all of the boundaries created by transplanting pieces of neural plate into epidermis, by transplanting pieces of epidermis into neural plate, or by reversing the mediolateral orientation of strips consisting of neural plate, neural fold, and epidermis so that the epidermis is in the neural plate and vice versa. Furthermore, in the few cases in which a neural fold did not appear after these operations, sections showed that healing had failed between epidermis and neural plate so that no boundary existed in the regions lacking folds. Controls in which epidermis was transplanted into epidermis or neural plate into neural plate produced no folds.

When a piece of neural plate is transplanted into the epidermis, the resulting folds closely resemble their natural counterparts. However, when a piece of epidermis is transplanted into the neural plate, the folds that form tend to be small. Several factors may limit the size of the folds in these cases. (1) Neural folds roll up over the neural plate, and a rather small epidermal implant surrounded by neural plate must contribute its limited number of cells to folds that are moving away

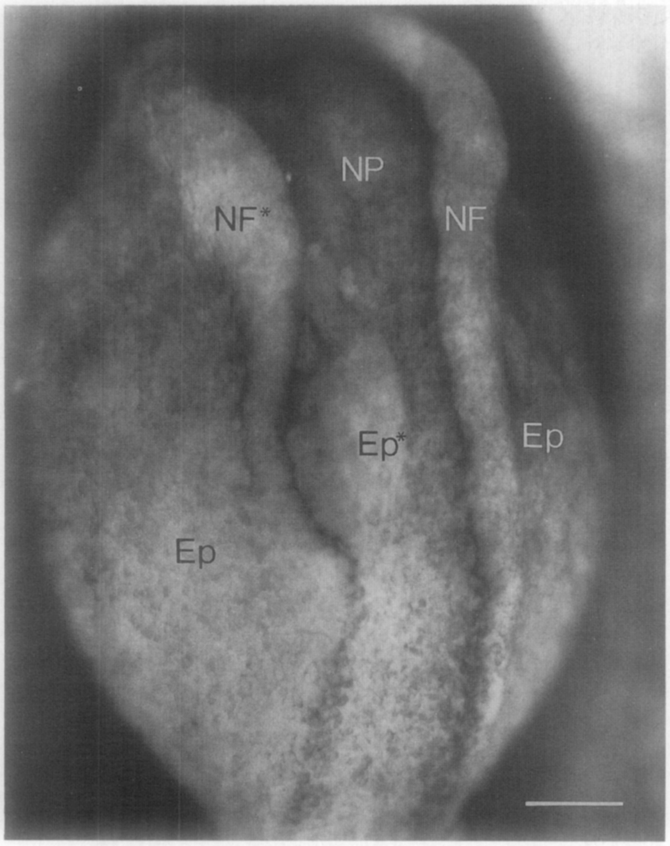

FIG. 9. A piece of epidermis (Ep*) transplanted into the neural plate (NP). Note that the movement toward the midline of the neural fold on the side of the transplant (NF*) is retarded relative to that of the contralateral neural fold (NF). Epidermis of the host (Ep). Time elapsed since operation: 9 hr. Line scale = 300 μm.

from it in every direction. (2) The tight adhesion between cells of the notochord and notoplate anchors the epithelium to the notochord and may prohibit the epithelium from folding when only a few ranks of neural plate cells separate an epidermal implant from the notoplate. (3) Although the epidermal implant "stops" the adjacent natural fold from rolling toward the midline, the natural folds anterior and posterior to the implant continue to roll toward the midline and distort the epithelium in the region where new folds are forming. (4) As the plate rolls up, the basal ends of the tall columnar cells in the neural plate surrounding the implant often touch one another beneath the implant, and these unusual interactions may produce distortions that affect the shaping of the region.

Upon closure, folds that form when a piece of neural plate is transplanted into the ventral epidermis become hollow vesicles. When sectioned in any direction, such vesicles look much like neural tubes. Rather than elongating into a tube, however, these vesicles are nearly spherical. Because these implants are no longer near the dorsal midline of the embryo, they lack the convergent extension machinery that is normally provided by the notoplate and the underlying notochord, both of which normally contribute to proper shaping of the neural tube.

The questions of whether the created neural folds produce neural crest cells, and whether epidermis, neural plate, or both contribute to the neural crest will be addressed in detail in a paper to follow. We find that the neural folds at the created boundaries do form neural crest cells and that both neural plate and epidermis contribute to them.

How Is the Boundary between the Neural Plate and the Epidermis Established?

The boundary between the neural plate and the epidermis appears to be defined as the neural plate is induced by the underlying chordamesoderm. Nieuwkoop *et al.* (1952) found that neuralization of gastrula ectoderm transplanted into the early neural plate depended upon the graft's distance from the midline. They concluded that the components of the neural plate and the neural crest were defined by their position relative to

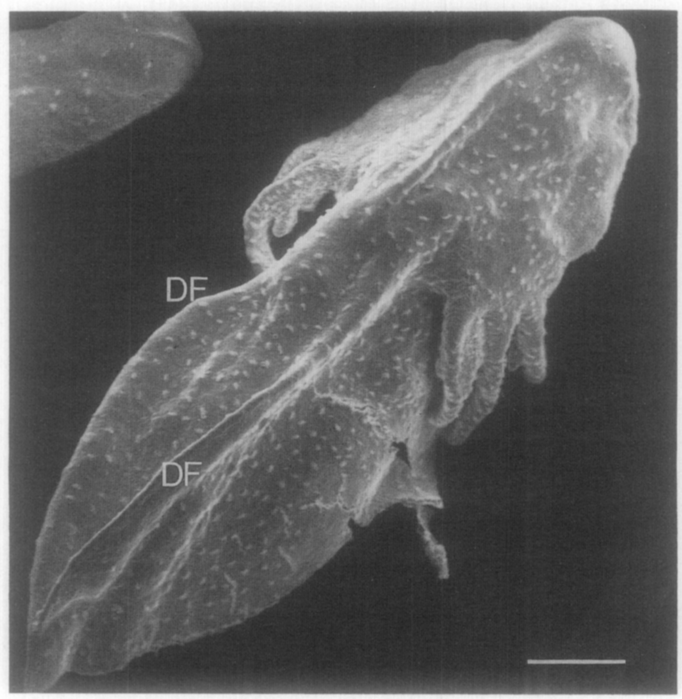

FIG. 10. When a piece of epidermis is transplanted into the neural plate across the dorsal midline, SEM shows that the larva develops a symmetrically bifurcated dorsal fin (DF). (Posterior portion of larva has been removed during processing.) Time elapsed since operation: 8 days. Line scale = 500 μm.

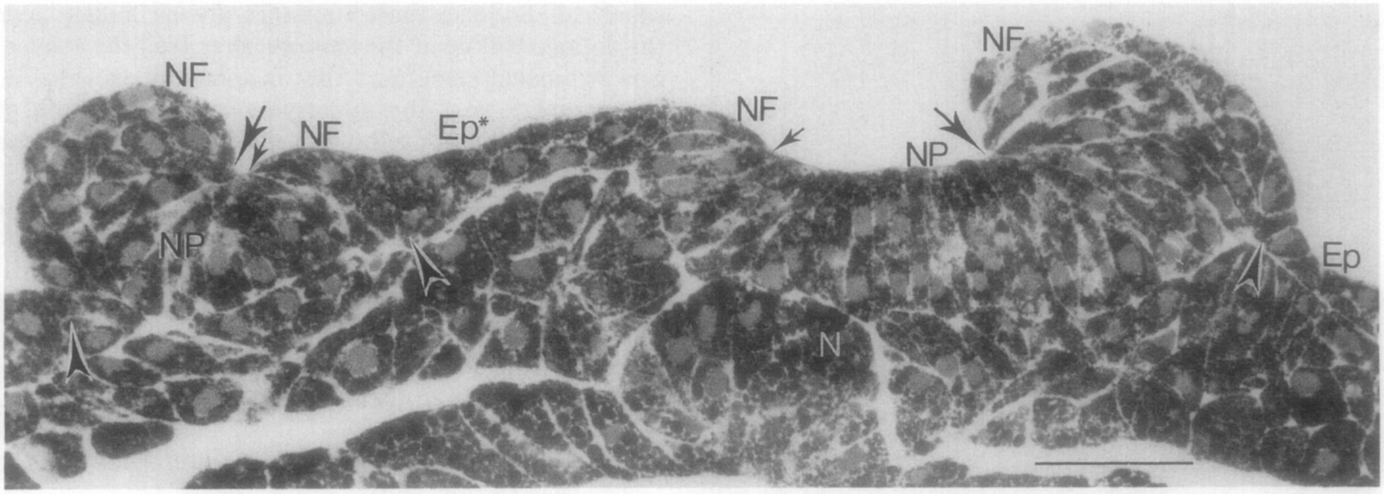

FIG. 11. Plastic cross section through folds formed when an epidermal transplant (Ep*) is placed in the neural plate (NP). Neural folds (NF) are visible at the original boundaries (large arrows) between the epidermis (Ep) and the neural plate, and at the new boundaries (small arrows) created around the transplant. Note the tabs of epidermis (arrowheads) that extend below the neural plate. The notochord (N) is labeled to aid in orientation. Time elapsed since operation: 33 hr. Line scale = 100 μm.

the strongest inducer, normally the chordamesoderm of the midline, and they suggested that the induction largely traveled through the plane of the responding tissue. In addition to spatial factors, Nieuwkoop (1985) has recently suggested that a temporal decrease in the competence of tissues to respond to neural induction is instrumental in defining the border of the neural plate.

If cells become competent to begin folding or to become crest cells because their position in space and/or time exposes them to a low level of neural inducer (Nieuwkoop *et al.*, 1952; Nieuwkoop, 1985) or to a combination of "neuralizer" and other factors (Rollhäuser-ter Horst, 1977), then cells that are exposed to different concentrations or combinations of these factors should not form folds or become crest cells. Some experiments seem to indicate that neural folds and neural crest cells will form only in their natural location. DuShane (1935) reported that embryos in which the neural folds were

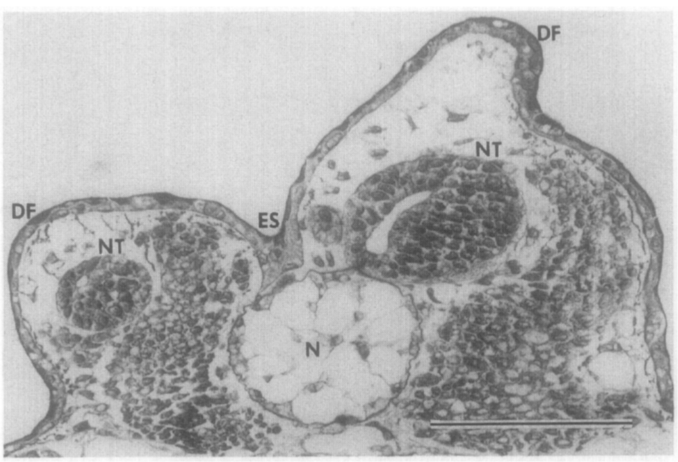

FIG. 12. Paraffin cross section through an embryo with a bifurcated neural tube and an epidermal septum that were formed by transplanting a piece of epidermis into the neural plate. The epidermal septum (ES) separates the two branches of the neural tube (NT) and extends to the notochord (N). Dorsal fins (DF) form above each branch of the neural tube. Time elapsed since operation: 9 days. Line scale = 200 μm.

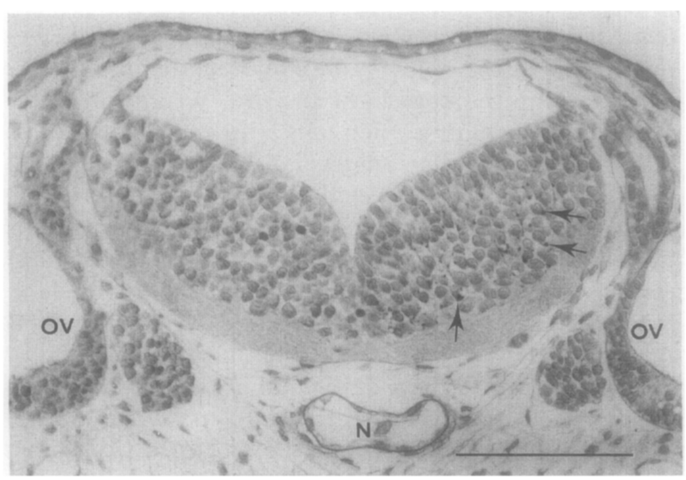

FIG. 13. Paraffin cross section through a control embryo: pigmented neural plate tranplanted into the neural plate of an albino host. Although no folding occurs and the embryo appears normal, the transplant can be identified by the presence of pigmented cells (arrows). The notochord (N) and otic vesicles (OV) are labeled to aid in orientation. Time elapsed since operation: 11 days. Line scale = 200 μm.

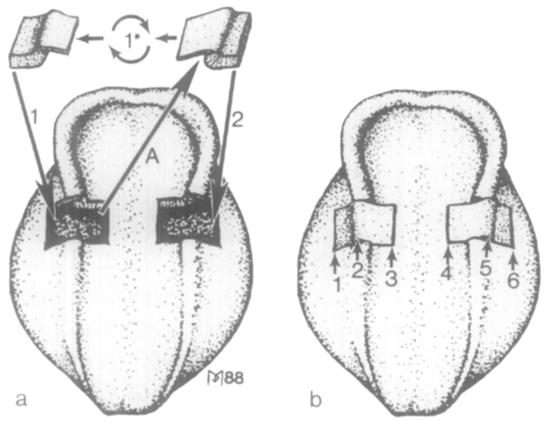

FIG. 14. Diagrams illustrating the creation of new boundaries by reversing the medial-lateral axis of a strip of tissue containing epidermis, neural fold, and neural plate. (a) First, the strip is removed (A). Either this strip can be rotated 180° (1*) and then transplanted ipsilaterally into the same or a different embryo (1) or it can be transplanted contralaterally into a different embryo without rotation (2). The operations depicted here would not be performed simultaneously on a single embryo. (b) If either of these operations is performed bilaterally, six boundaries between epidermis and neural plate appear (in cross section). The two original boundaries remain (2, 5), and four new boundaries are created (1, 3, 4, 6).

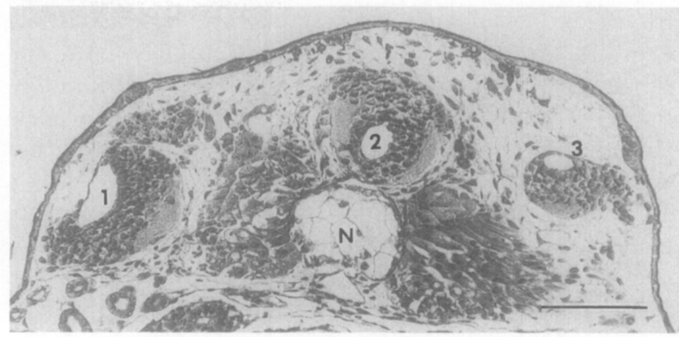

FIG. 16. Paraffin cross section through the three branches of the neural tube (1, 2, 3) formed when two contralateral strips are exchanged without rotation (see No. 2 on Fig. 14a). The branch of the neural tube (2) that lies dorsal to the notochord (N) was formed entirely from folding at newly created boundaries. Time elapsed since operation: 15 days. Line scale = 200 μm.

bilaterally extirpated developed normally, but contained no neural crest derivatives. In similar experiments, Jacobson and Jacobson (1973) reported the absence of folds when they removed the neural fold and also strips of adjacent epidermis and neural plate, and concluded that competence to raise a fold is limited to cells near the natural boundary between the neural plate and the epidermis. However, other explanations could account for the absence of folds and neural crest cells in these experiments. Small folds may not have been visible on superficial examination, and neither study describes the appearance of sections through these neurulae. Removing the natural neural fold temporarily destroys the boundary, and such a wound may delay the reformation of the boundary to such an extent that fold and crest production is inhibited. Alternatively, a large wound may have cut into the notoplate, forcing the epidermis to join with this tissue rather than with the neural plate. Other studies (Jacobson, 1981, 1985; Keller *et al.*, 1985a, b; Jacobson *et al.*, 1986) indicate that the notoplate has different properties and functions from those of the neural plate, perhaps including an inability to form folds.

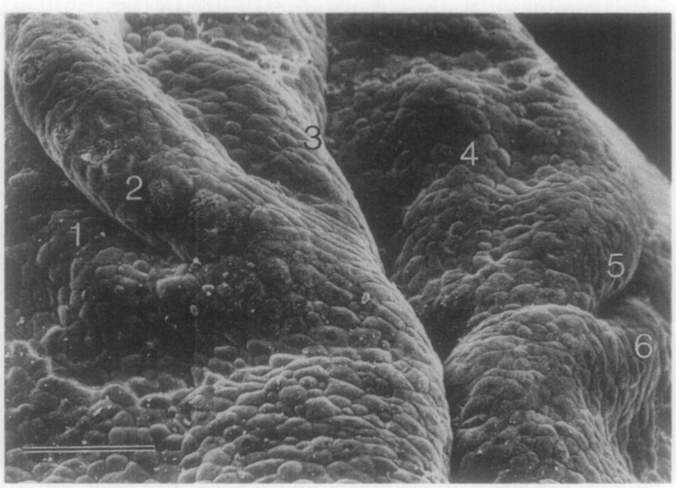

FIG. 15. View by SEM of folds formed after bilaterally exchanging strips without rotating them. This procedure (see No. 2 in Fig. 14a) retains the two large original folds (2, 5) and forms new folds (1, 3, 4, 6) at four newly created boundaries. Time elapsed since operation: 6 hr. Line scale = 100 μm.

In our experiments, when a piece of neural plate containing neither cells from the edge of the plate nor cells from the notoplate was transplanted into the ventral epidermis, and when epidermis was transplanted into the neural plate, folds formed at the edges of the graft. In such ectopic sites, the correct concentrations of factors or inductive influences imputed to occur in the dorsal regions to produce folds and crest should not occur. Therefore, the induction responsible for fold and crest formation must occur across any boundary created between neural plate and epidermis, and it must originate locally in the cells at the boundary.

Neural cell adhesion molecule (N-CAM) and its RNA appear in the presumptive neural plate during or shortly after neural induction (Jacobson and Rutishauser, 1986; Kintner and Melton, 1987). The differences in cell behavior and morphology that result from neural induction may be related to emerging differences in the presence and distribution of N-CAM and other cell adhesion molecules (Crossin *et al.*, 1985; Edelman, 1985; Balak *et al.*, 1987). Since the adhesive properties of cells in adjacent tissues can determine whether such cell populations will mix or sort out (Steinberg, 1978), differential adhesion may play a role in morphogenetic movements—including the buckling of an epithelial sheet (see Jacobson, 1981, 1985; Mittenthal and Mazo, 1983).

Must the Boundary between the Neural Plate and the Epidermis Be Involved in Neurulation?

In a normal embryo, fold formation always occurs at the boundary between the epidermis and the neural plate, yet the folding phenomenon could be independent of the boundary. Increasing the volume of material beneath a region of the epithelium could push the epithelium into a fold regardless of the presence of a boundary. Such an increase has been attributed to columnarization of mesodermal cells (Schroeder, 1970; Karfunkel, 1971) and to swelling of extracellular matrix (Solursh and Morriss, 1977; Morriss and Solursh, 1978; Morriss-Kay and Crutch, 1982). Boerema (1929) observed that folds formed when neural plate was transplanted into ventral epidermis, and concluded that mesoderm was not involved in fold formation. Karfunkel (1974) justly criticizes Boerema's study for failing to examine the underlying mesodermal cells for changes in shape. Our observations reaffirm Boerema's conclusion, since folds form at ectopic boundaries without a corresponding columnarization of underlying mesodermal cells (Figs. 4 and 6).

Poisson (or Eulerian) buckling (Jacobson and Gordon, 1976; Jacobson, 1978; Gordon, 1985; Jacobson *et al.*, 1986) could also produce folds in the absence of a boundary between the epidermis and the neural plate. Since a homogeneous sheet is usually used to illustrate this phenomenon (Jacobson, 1978), boundaries within the sheet are obviously not necessary for folding to occur. Intercalations of cells at boundaries between the neural plate and the notoplate (Jacobson, 1978) and between the neural plate and the epidermis (Jacobson and Tam, 1982) have been suggested to produce the linear elongation needed to stretch the epithelium and form a fold. Elongation of the underlying notochord may also contribute synergistically to midline elongation of the neural plate (Jacobson, 1985), but it is not essential for neurulation (Malacinski and Youn, 1981; Jacobson *et al.*, 1985). Neural plate transplants placed in the ventral epidermis lacked notoplate and were removed from the influence of the extending chordamesoderm, yet folds still formed around the graft. Although intercalation of cells at the newly created boundary between the neural plate and the epidermis was possible, no elongation of these transplants occurred, and the boundary contracted as the folds came together and fused. In light of these observations, elongation of the neural plate does not appear to be essential for fold formation, but when elongation of the neural plate does occur, the resultant buckling may assist fold formation.

Karfunkel (1974) concluded that the forces necessary for neurulation were not produced in the fold itself because the neural fold stopped moving toward the midline when cuts were made on either side of it. However, when we repeated this experiment, cross sections revealed that the fold did not relax—if anything it became slightly larger than its counterpart (Figs. 17a and 17b). Adhesions in the fold, then, are strong enough to resist tensions created by cells moving away from the fold in opposite directions to close wounds, and therefore are probably involved in maintaining and stabilizing the fold. Furthermore, the observation that folds form whenever neural plate and epidermis are juxtaposed suggests that the relationship between the position of the boundary and the site of fold formation is more than a coincidence—conditions at the boundary actually elicit fold formation.

How Might the Boundary between the Neural Plate and the Epidermis Organize the Morphogenetic Movements of Neurulation?

Forces resulting from shrinkage of the apical surfaces of cells of the neural plate might cause folding at all boundaries with a more ductile tissue like the epidermis (Jacobson and Gordon, 1976). The "pursestring" (Baker and Schroeder, 1967; Burnside and Jacobson, 1968; Burnside, 1971, 1973; Schroeder, 1973) or "network" (Nagele and Lee, 1978, 1980; Lee and Nagele, 1985) arrangement of actin filaments observed in the apices of these cells is consistent with apical constriction. From our observations and experiments, we have no reason to doubt that apical constriction of neural plate cells could generate forces that contribute to neurulation. The abrupt change in physical properties at the boundary between the neural plate (whose cells are becoming more columnar) and the more ductile epidermis (whose cells are becoming more squamous) could cause neural folds to rise along this line. Thus, even if apical constriction were the only mechanism used during neurulation, it is likely that the boundary between the neural plate and the epidermis defines the position of folding in response to tensions created by the contraction of the apical surfaces of the cells in the

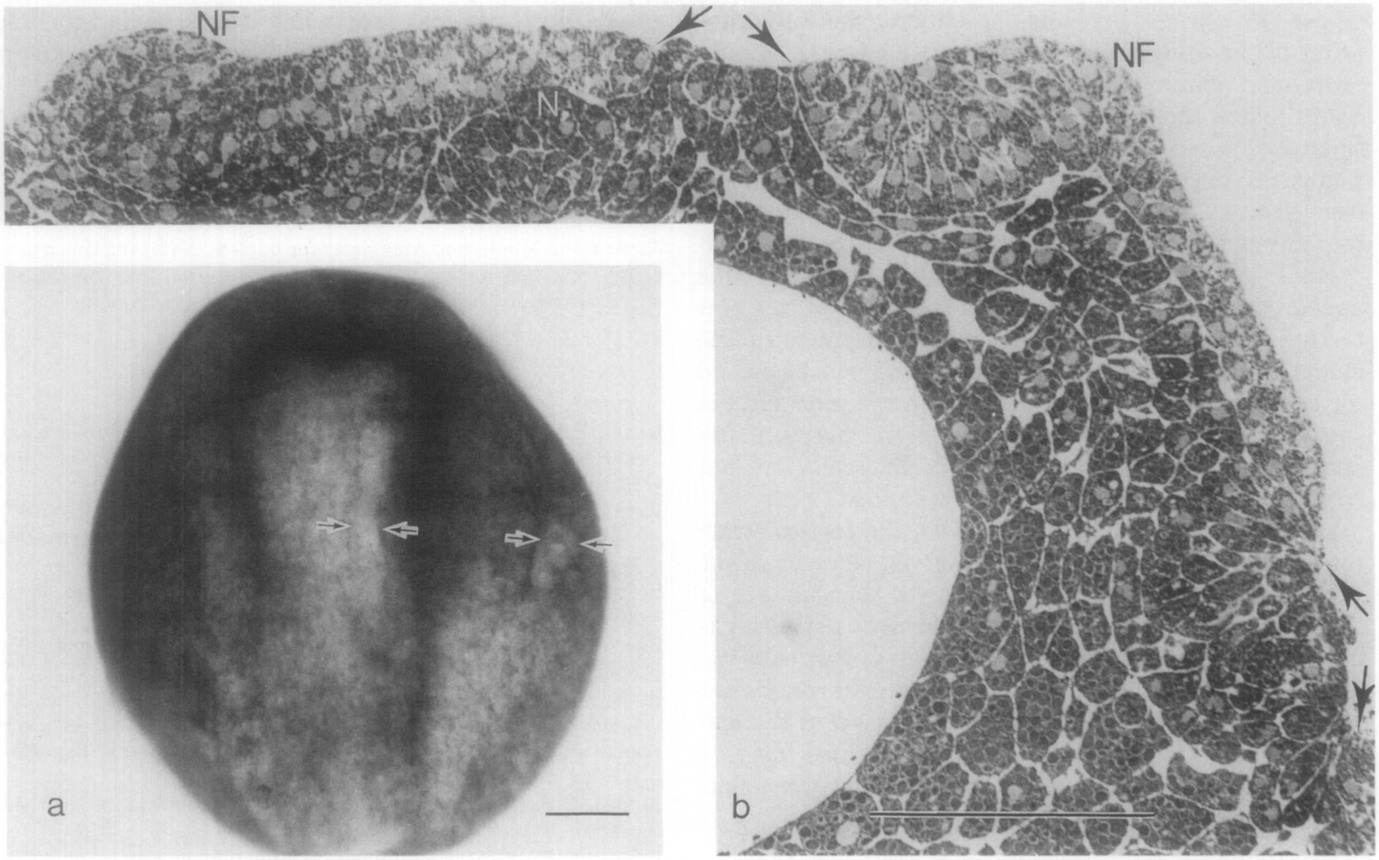

FIG. 17. Removing a piece of neural plate and a piece of epidermis does not cause fold between the lesions to relax, even though cells must move away from the fold to close the wounds. Time elapsed since operation: 30 min. Line scales = 300 μm. (a) Surface view showing the lighter colored lesions. The lateral edges of the lesions are marked (arrows). The arrows also indicate the approximate plane of section for Fig. 17b. (b) Plastic cross section near level indicated by the arrows in Fig. 17a. Despite lesions in epidermis and neural plate, both of the neural folds (NF) appear to be approximately equal in size. The lateral edges of the lesions are marked (arrows).

neural plate. Certain aspects of fold morphology, however, are difficult to reconcile with the expected morphology of a fold produced solely by the contraction of the apical surfaces of cells in the neural plate, but can be explained if the cells of the neural plate and the cells of the epidermis crawl on one another in the fold.

During neurulation, the folds roll up and over the central portions of the neural plate and cells near the edge of normal and implanted neural plates (Figs. 1, 2, 6, and 11), and (to a lesser extent) cells near the midline in normal neural plates (Figs. 1 and 2) become very elongated and wedge-shaped. These observations indicate that cells must be changing shape in a spatially and/or temporally regulated sequence, rather than simultaneously throughout the neural plate (Lewis, 1947; Jacobson and Jacobson, 1973; Odell *et al.*, 1981). Even then, the rolling of the epidermis up over the neural plate is difficult to explain by apical constriction alone. Both a "rolling" and a progression of shape changes are implicit in the cortical tractor model (Jacobson *et al.*, 1985, 1986) which suggests that the tractoring of cortical cytoplasm in embryonic epithelial cells can exert forces on neighboring cells.

Different activity in the apical and basal ends of epithelial cells—junctional complexes restrict movement in the apical areas, and protrusive activity occurs mainly on the basal and lateral surfaces—can explain the epidermal tabs that always extend beneath the edges of the forming neural tube. (Note that cells in the neural plate may tractor on epidermal cells, and/or epidermal cells may tractor on the cells of the neural plate.) The epidermis is under tension (Lewis, 1947; Karfunkel, 1974; Jacobson and Gordon, 1976) and should be taut over the neural folds, but along the line where the basal surfaces of epidermal cells first come into contact with the basal surfaces of cells in the neural plate, the "tabs" of epidermal cells extend beneath the forming neural tube (Figs. 1, 2, 6, and 11). In chicks and mice, the cells in these tabs appear to differ from other epidermal cells (Martins-Green, 1988). Moreover, when epidermis was implanted into the neural plate, the final result was a pair of neural tubes separated by an epidermal septum that sometimes extended to the notochord. This septum appears to be derived from the tabs that formed when the epidermal implant encountered the host's neural plate. If the epidermal implant was pigmented, then pigmented cells appeared in the ventral part of the septum, but the rest of the septum was of host origin. Thus, much of the epidermal tissue in the septum must have been pulled (or crawled) to its final position. Both the tabs and the septum can be explained by tractoring between the basal ends of neural plate and epidermal cells, but not by apical constriction in neural plate cells.

Like the apical constriction model, the cortical tractor model can also explain the observed changes in the shape of the neural plate cells. As a consequence of tractoring, the cytoplasm of each neural plate cell is displaced basolaterally. Because their apical ends remain attached to one another by junctional complexes, these cells elongate instead of crawling out of the epithelium. This movement away from the apex may be responsible (at least in part) for reducing the apical area of each neural plate cell.

CONCLUSIONS

This work suggests that local interactions of cells at the boundary between the neural plate and the epidermis are sufficient to raise the neural folds. Neural folds form after neural induction establishes this boundary, but they are not directly induced themselves. Rather, the folds form as a consequence of the apposition of neural plate cells to epidermal cells. Once the boundary between the neural plate and the epidermis is established, the behaviors and shapes of cells on the two sides of the boundary become different and then interact with one another in such a manner that they raise neural folds. We have shown that the folds resulting from these interactions form whenever a piece of neural plate is placed in apposition to a piece of epidermis.

Observations of fold formation at newly created, ectopically located boundaries between the epidermis and the neural plate indicate that Poisson buckling and shape changes in the mesoderm are not essential for folding, but they are not antagonistic to this process and probably help to raise and stabilize the fold during normal neurulation. Our findings are consistent with predictions made by the cortical tractor model and the apical constriction model, but some phenomena that we observed cannot be explained by apical constriction alone. Both cortical tractoring at the boundary and apical constriction of cells in the neural plate probably are required for neurulation.

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