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# Tissue Boundaries and Cell Behavior during Neurulation

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**We have analyzed the dynamics of the boundaries between the neural plate and the epidermis and between the neural plate and the notoplate. Our experiments confirm that these two boundaries have important roles in neurulation. Measurements of the lengths of neural fold (the boundary between epidermis and neural plate) in embryos of axolotls and newts reveal that neural folds abutting the prospective brain decrease in length while neural folds abutting the prospective spinal cord increase in length during neurulation. We tested the proposition that boundaries of the neural plate with epidermis and with notoplate are essential for proper neurulation. Cuts made along the boundaries with epidermis or with notoplate stop, or greatly diminish, neural plate elongation and tube formation. Explanting the axolotl neural plate without any bordering epidermis stops plate elongation and prevents neural tube closure, but neural plates explanted with a rim of epidermis elongate and close into tubes. Cutting the notoplate boundary stops midline elongation in the newt embryo or diminishes it in the axolotl embryo. We conclude that the notoplate boundary and part of the boundary of the epidermis that abuts the prospective spinal cord organize cell behavior to elongate the neural plate and help close the neural tube. The boundary of the neural plate with the epidermis is essential for tube closure both because it organizes plate elongation in the spinal cord region and because cell behavior becomes organized at the boundary such that neural folds are raised and a rolling moment is produced that helps form the neural tube.** © 1995 Academic Press, Inc.

## INTRODUCTION

Cells behave in a variety of ways that together accomplish neurulation. Cells of the neural plate change their shapes, becoming taller and reducing their overall diameters and apical surfaces, thus helping reduce the width of the neural plate, but also tending to reduce its length (Burnside and Jacobson, 1968). Some bending of the neural plate may occur as a result of these changes in cell shape. The length of the neural plate actually increases considerably during neurulation (Jacobson and Gordon, 1976), and this is brought about by the orderly changing of positions of neuroepithelial cells. There is a strict correlation between plate elongation and neural tube closure, and tube closure requires plate elongation (Jacobson, 1978, 1981, 1984).

Neuroepithelial cells intercalate and remain trapped at the boundary between the neural plate and the notoplate, increasing the length of this boundary and thus of the neural plate (Jacobson *et al.*, 1985, 1986). These intercalations of cells reduce the width of the plate as they increase its length. The lengthening of the boundary produces orthogonal buckling forces that may help form the neural plate into a tube (Jacobson, 1978), but computer simulations suggest that these Poisson buckling forces cannot form a tube by themselves (Jacobson *et al.*, 1986). Neural plate cells also attempt to crawl beneath the bordering epidermis, producing a powerful rolling moment that helps roll the plate into a tube (Jacobson *et al.*, 1986; Moury and Jacobson, 1989; reviewed in Jacobson, 1994).

The experiments we present here test the proposition that neurulation depends upon the changes in cell behavior that occur when different cell types encounter one another at the boundaries between tissue domains within and around the neural plate. These experiments are done on amphibian embryos that, in contrast to amniote embryos, do not grow during neurulation (Jacobson, 1978).

The neural plate of the salamander embryo begins with two tissue boundaries defined. One boundary is between prospective epidermis and neural plate (Fig. 1A). The second boundary is near the midline within the neural plate between the domain of plate cells that overlies the prospective

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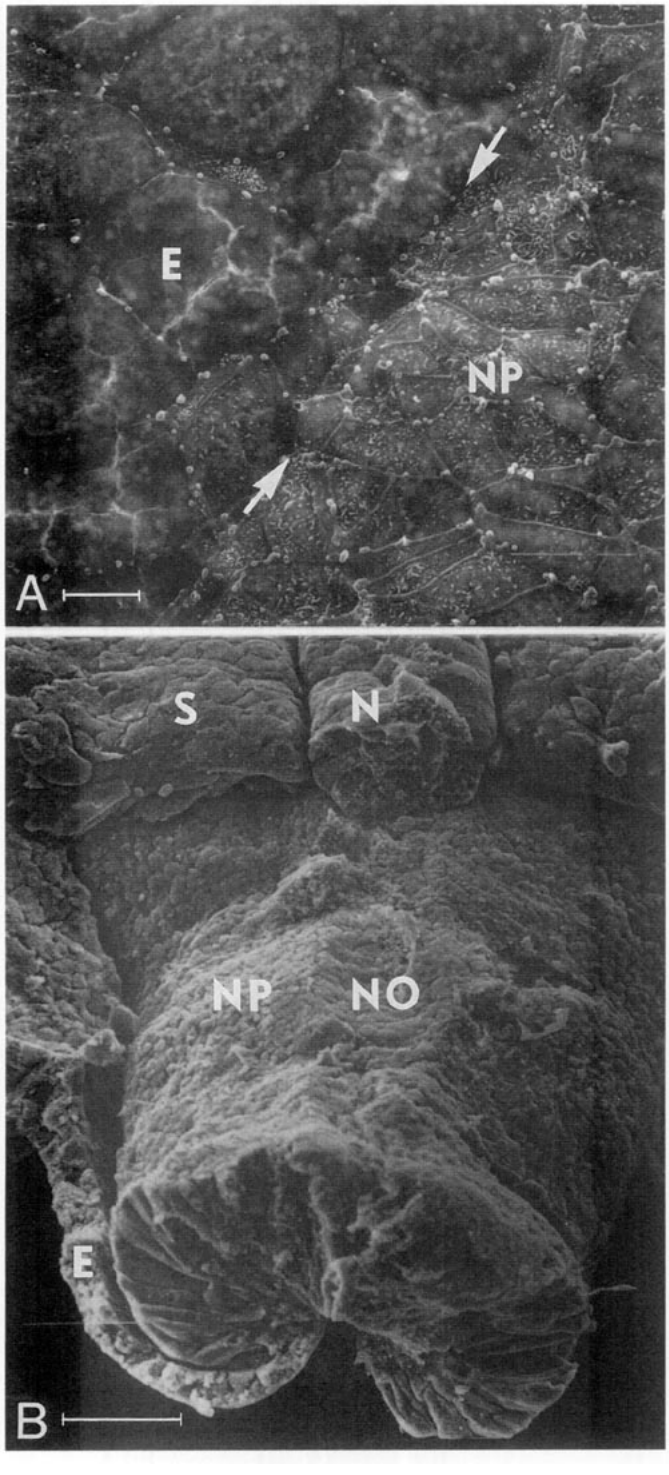


**FIG. 1.** (A) SEM of the dorsal surface of a newt embryo (*T. torosa*) at neurula stage 17. The arrows mark the boundary between the neural plate (NP) and the epidermis (E). Bar, 1 $\mu$m. (B) SEM of the underside of a newt embryo (*T. torosa*) at neurula stage 17. Where the notochord (N) is removed, the underside of the notoplate (NO) is visible in the midline of the neural plate (NP). S, somite. Bar, 100 $\mu$m.

notochord and the rest of the neural plate. The midline domain of the neural plate is named the "notoplate" (Jacobson, 1981). The notoplate was first described in studies by Jacobson and Gordon (1976), who noticed that the cells of the notoplate area behave differently from cells in the rest of the neural plate. In that paper, the area was called the "supranotochordal region." When the neural tube forms, the notoplate composes the initial floor plate. The notoplate is at the position of the "median hinge-point" of the chick neural plate (Schoenwolf, 1994). The edges of the notoplate can be seen in surface views of some embryos because of pigmentation differences and in section and scanning electron microscopy (SEM) views (Fig. 1B) by different appearances of the cells. The basal surfaces of notoplate cells adhere to the underlying prospective notochord.

We have previously proposed that cell behaviors are organized along these boundaries during neurulation (Jacobson *et al.*, 1985, 1986; Moury and Jacobson, 1989). Jacobson *et al.* (1985, 1986) described how cells in an epithelium could change neighbors while dynamically retaining apical seals with surrounding cells. Direct observations of cell behavior during newt neurulation indicated that such neighbor changes were random within each tissue domain, but when a cell reached the notoplate/neural plate boundary it remained there and elongated the boundary. A similar "trapping" of cells may also occur at the neural plate/epidermis boundary, but the elevation and rolling of the neural folds make direct observation of cell behaviors at this boundary impossible during most of neurulation. It is possible, however, to determine whether cell behaviors are organized at boundaries between tissue types by disrupting these boundaries and then examining the resulting changes in tissue movements.

We present measurements that demonstrate that the portion of the epidermis/neural plate boundary along the prospective spinal cord elongates throughout neurulation in salamander embryos, but that the portion of the boundary abutting the prospective brain plate shortens during most of neurulation.

Our experimental evidence shows that the epidermis/neural plate boundary is essential for closure of the neural tube and for some of the elongation of the neural plate. We also present experiments that indicate that the notoplate/neural plate boundary may be necessary for elongation of the midline of the neural plate. Preliminary results from such experiments done on *Taricha torosa* have been published (Jacobson, 1981, 1985, 1991). We summarize those experiments and give new and somewhat different results from replications of these experiments on the axolotl embryo, using better measuring techniques with computer-assisted morphometrics.

## MATERIALS AND METHODS

### *Specimens*

Axolotl embryos (*Ambystoma mexicanum*) were shipped from the Indiana University Axolotl Colony; embryos or

gravid females of the West Coast newt (*Taricha torosa*) were shipped from the San Francisco area. All were kept in the laboratory at 17°C in 10% Holtfreter's balanced salt solution (Holtfreter, 1931), prepared according to the formula in Jacobson (1967). Axolotl embryos were staged according to the staged series of Schreckenberg and Jacobson (1975); newt embryos were staged according to the staged series of Twitty and Bodenstein (Rugh, 1948).

### *Measurements and Statistical Methods*

Lengths in living embryos and explants were measured either directly, using a calibrated ocular micrometer, or from video images analyzed with a computer-assisted morphometrics program (BioQuant IV, R & M Biometrics, Nashville, TN). During measurement, the embryos were tilted to maintain a tangential field of focus to minimize optical foreshortening.

Comparisons of means were performed using two-tailed Student's $t$ tests. Student's $t$ tests for paired data were used when appropriate. Whether the data was paired or unpaired has been noted when reporting statistics.

### *Locating the Border between the Prospective Brain and the Prospective Spinal Cord*

The position of the brain–spinal cord border was judged for embryos at stage 15 using the fate map constructed by Jacobson (1959) for the axolotl. This position is easily seen at stage 15 since it coincides with the point where the neural plate flares outward. At later stages, the position of the "flare" moves craniad, but the level of the posterior brain boundary can be found by the positions of the emerging somites. At stage 17, the "first" somite, which later becomes somite 3, has condensed. Its cranial edge marks the boundary between brain and spinal cord (see Figs. 8 and 10 in Jacobson and Meier, 1984). Since we followed and measured individual living embryos through neurulation, we could follow the progress of somite formation and find the brain–spinal border between somites 2 and 3. Somites 1 and 2, the two occipital somites of the salamander (Jacobson and Meier, 1984), condense later than the 3rd somite.

In the newt, changes in the lengths of neural folds during neurulation were measured by a frame-by-frame analysis of two time-lapse movies. Measurements were made on projections of individual frames. The movies were made at one frame per minute, so the time interval that had elapsed between one measured frame and the next could be read directly from the frame counter on the projector. The border between brain and spinal cord was located at stage 17 at the anterior edge of the first (later third) somite. Variegations in egg pigment within cells at the border between the neural plate and the spinal cord allowed us to follow the positions of the border both forward and backward through time (by running the movie forward or backward).

### *Operations*

Surgery was performed in 100% Holtfreter's solution using sharpened iridectomy scissors or electrolytically sharpened tungsten needles. Neural plates were explanted into Corning 35-mm plastic tissue culture dishes. During more delicate operations (e.g., separation of neural plate and notoplate), embryos were held in hemispherical indentations in the paraffin/plasticine lining of glass operating dishes (Jacobson, 1967). After healing (about 1 hr), embryos were transferred to fresh Holtfreter's solution in 35-mm plastic tissue culture dishes and reared at 17°C. All experimental embryos (both newts and axolotls) were examined when normal, intact control embryos (cultured in parallel with the experimental embryos) had reached stages 20 to 21 (neural tubes completely closed, about 17–24 hr). Because several different types of operation were used, the details of individual operations have been presented in the appropriate parts of Results.

### *Scanning Electron Microscopy, Plastic Sections, Paraffin Sections*

Specimens for SEM were fixed in Karnovsky's fixative (Karnovsky, 1965) diluted to half strength with 0.1 *M* cacodylate buffer (pH 7.4), then rinsed, and dissected in the same buffer. Following secondary fixation in 1% $OsO_4$ in 0.1 *M* cacodylate buffer, specimens were washed in buffer, dehydrated through an ethanol series (30 to 100%), and critical-point-dried using $CO_2$ as the exchange fluid. Specimens were then mounted on SEM stubs with double-stick tape, sputter-coated with 8–10 nm of gold–palladium alloy, and examined at 15 kV with an ISI Super III-A scanning electron microscope.

For plastic sections, embryos were processed as for SEM through dehydration with ethanol, then cleared in acetone, embedded in Epon by the procedure of Luft (1961), cut at 2–3 $\mu$m with glass knives, and then stained with 1% methylene blue in 1% sodium tetraborate.

For paraffin sections, embryos were fixed in Kahle's fixative (Jones, 1966), dehydrated and cleared through an ethanol/butanol series, embedded in Paraplast-plus, sectioned at 6 $\mu$m, stained with 0.2% neutral red, and counterstained with 0.1% Janus green (Jones, 1966).

## RESULTS

### *Changes in Length of the Boundary between Epidermis and Neural Plate in Normal Embryos*

To quantify the changes in lengths of the neural folds during neurulation, we measured the epidermis/neural plate boundary directly from two sets of living axolotl embryos. All embryos in a set were from the same clutch of eggs, which helps minimize variations in embryo size within a set. The data from the two sets were not pooled since the two sets were from different mothers. The lengths of the

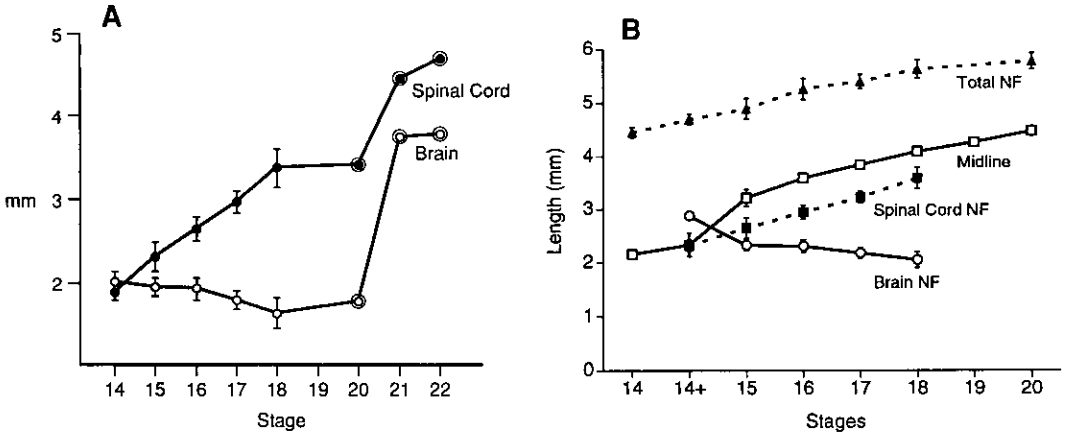


**FIG. 2.** (A) Mean lengths of the edges of the brain plate (open circles) and of the spinal plate (solid circles) were measured in a set of 10 living axolotl embryos through the course of neurulation (stages 15 to 20) (lines and bars indicate standard deviation). The points for stages 20 to 22 (postneurula stages) were measured from photographs of our staged series for axolotls (Schreckenberg and Jacobson, 1975). The edge of the spinal cord, but not of the brain, lengthens during neurulation; both lengthen after neurulation. (B) Mean lengths of the neural fold of the spinal cord, the brain neural fold, total neural fold, and of the midline measured through neurulation (stages 14 to 20) from another set of 10 living axolotl embryos.

neural folds abutting the prospective brain and spinal cord were measured (separately) for each embryo at each stage of neurulation using computer-assisted morphometrics.

The results from the first set of 10 embryos show that the lengths of the neural folds abutting the prospective spinal cord increase during neurulation, while those abutting the prospective brain decrease (Fig. 2A). Measurements taken from photographs in our staged series (Schreckenberg and Jacobson, 1975) indicate that the lengths of both brain and spinal cord increase rapidly when the neural plate has completely rolled into a tube (after stage 20). Similar results were obtained from the second set of 10 embryos (Fig. 2B). In this set, however, we also measured the total lengths of the neural folds and the lengths of the midlines. In the axolotl embryo, the total length of the neural fold increases steadily during neurulation.

We also measured the lengths of the neural folds in two *Taricha torosa* embryos whose neurulation had been recorded as time-lapse movies. The results of frame-by-frame analyses of these two movies were similar; Fig. 3 shows the results of the analysis of one such film. In *T. torosa*, the total length of neural fold remains about the same or decreases slightly during neurulation (stages 13–20), but increases after the closure of the neural tube. As in the axolotl, the neural folds abutting the prospective spinal cord increase in length during neurulation, while the neural folds bordering the prospective brain decrease in length. Folds in both the brain and spinal cord regions increase in length after closure of the neural tube.

### *Disrupting the Boundary between Epidermis and Neural Plate*

To test whether the boundary between the epidermis and the neural plate is essential for neural tube formation, we explanted the neural plate of axolotl embryos either with (Fig. 4A) or without (Fig. 4C) neural folds and thus with or without a rim of epidermis. We made the cuts sufficiently deep to enter the archenteron, so that the mesoderm and

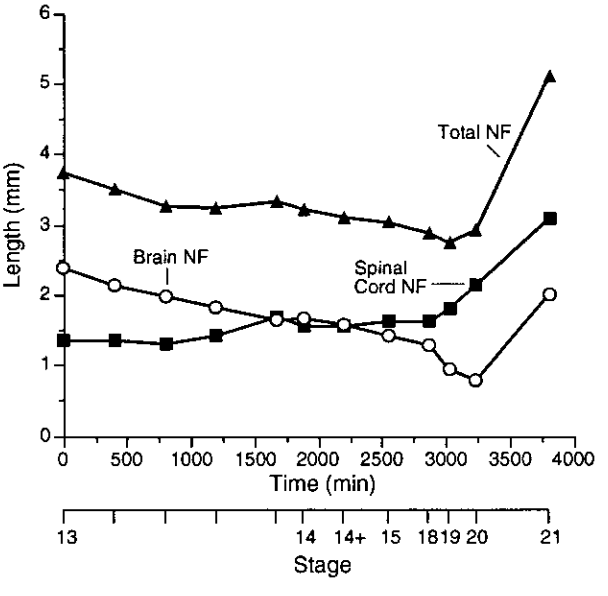


**FIG. 3.** Lengths of the neural folds of a newt, measured from a time-lapse movie of neurulation taken at one frame per minute. The total length of the neural fold and the length of the brain neural fold decrease during neurulation (stages 13 to 20) while the length of the spinal cord neural fold increases. After neurulation (stages 20–21) all increase. Note that stages do not occupy equal amounts of time.

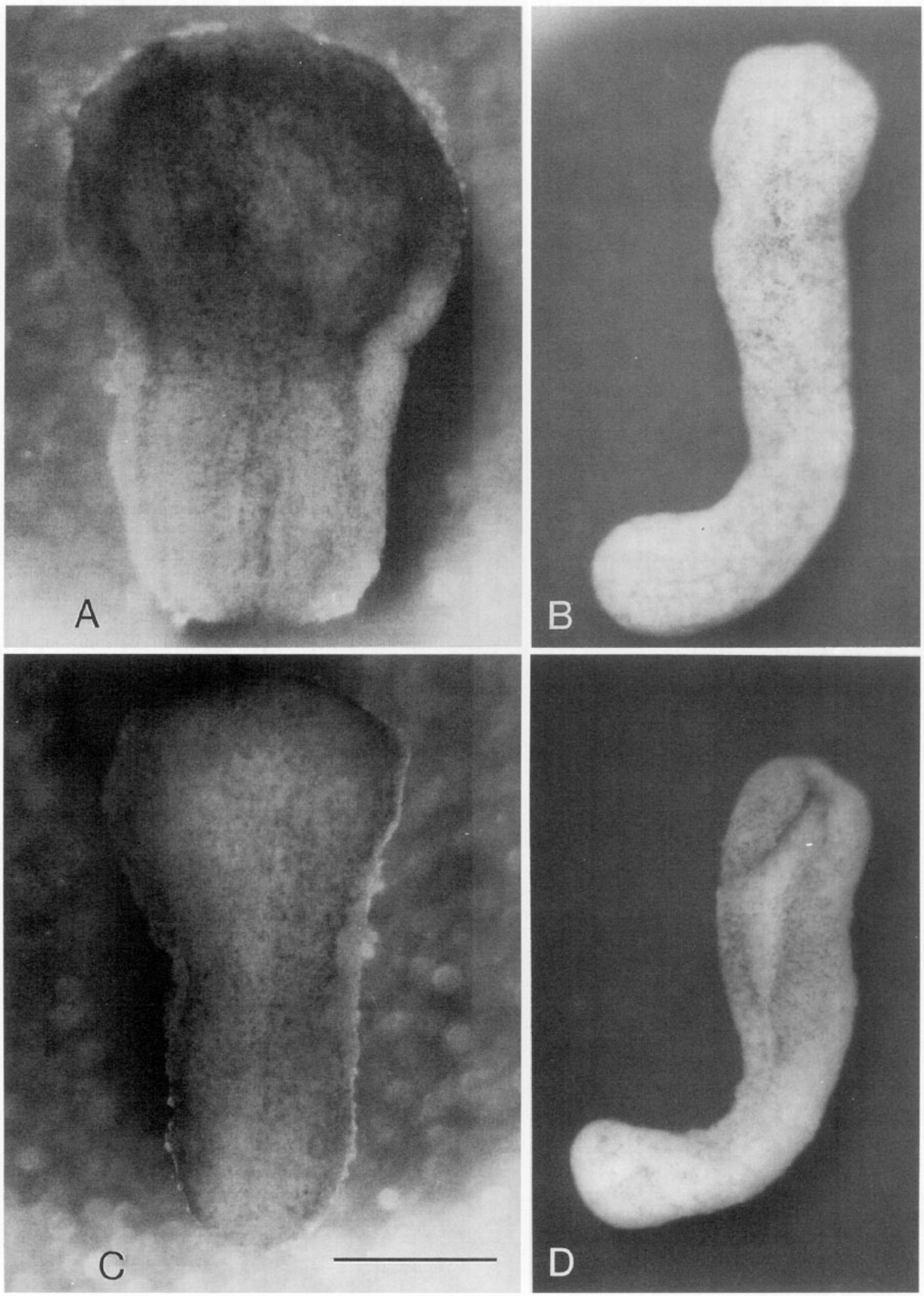
A
B
C
D

**TABLE 1**

Changes in the Length of the Midline after Operations That Disrupt the Epidermis/Neural Plate Boundary in the Axolotl, *Ambystoma mexicanum*

| Description | Mean length (mm) ± SD | | Mean % Change[a] (%) ± SD |
|---|---|---|---|
| | Start (stage 15) | End (stage 20/21) | |
| Set 1 | | | |
| Without neural folds (8 cases) | 2.9 ± 0.25 | 3.0 ± 0.44 | +2 ± 12.0 |
| With neural folds (8 cases) | 3.0 ± 0.20 | 3.5 ± 0.23 | +17 ± 11.5 |
| Set 2 | | | |
| Without neural folds (6 cases) | 2.1 ± 0.21 | 1.8 ± 0.33 | −12 ± 11.6 |
| With neural folds (6 cases) | 2.3 ± 0.16 | 3.0 ± 0.14 | +29 ± 11.3 |
| Total percentage elongation (pooled from Sets 1 and 2) | | | |
| Without neural folds (14 cases) | | | −4 ± 13.6 |
| With neural folds (14 cases) | | | +22 ± 12.5 |

[a] Percentage change (the difference between the starting length of the midline and the midline length at the end of the experiment, divided by the starting midline length) was calculated for each explant. Start was at stage 15; end was at stage 20–21.

endoderm that directly underlie the neural plate were included with the explants. These experiments thus test the effects of deprivation of different amounts of lateral tissues without the confusion of effects that could result from leaving bare the basal surfaces of the neural plate cells. All operations were done at stage 15 when the neural plate is still open and flat.

In the 14 explants that included a fringe of epidermis, 12 of the neural plates (86%) rolled completely into a closed tube during the culture period (Fig. 4B), and the remaining two explants closed their tubes leaving but minor closure defects. In each of the 14 explants that lacked epidermis, the neural plates remained open in the cranial region, but closed in the more caudal region (Fig. 4D).

We measured the lengths of the midlines of the neural plates before operating, and the lengths of the plates or tubes after culture (Table 1). Although all embryos were stage 15 at the time of operation, we used two sets of embryos from different egg clutches with different egg sizes. The mean midline lengths (either with or without folds) of explants from Set 1 were greater than their counterparts from Set 2 (a highly significant difference; unpaired data, $P < 0.001$). Therefore, we treated embryos from the different clutches separately.

For explants of neural plate with neural folds (both sets), the mean midline length was significantly greater following culture (paired data: Set 1, $P = 0.003$; Set 2, $P = 0.001$).

For explants without neural folds (both sets), the mean midline lengths before and after culture were not significantly different (paired data: Set 1, $P = 0.528$; Set 2, $P = 0.060$). In each set, when the mean lengths after culture were compared to one another, the explants with folds were significantly longer than those without folds (unpaired data, $P \leq 0.004$ in each case). However, we should also note that, like the mean midline lengths before culture, the mean midline lengths (with or without folds) from Set 1 after culture remained significantly greater than their counterparts in Set 2 (unpaired data, $P < 0.001$ in both cases).

From the paired data, we calculated the individual percentage change (i.e., the difference in starting and ending lengths, divided by the starting length) for each explant. This procedure allowed us (1) to calculate a mean percentage change and its standard deviation (Table 1) and (2) to pool the percentage of changes from both sets of data because it helped to normalize differences in the sizes of embryos from different clutches. The mean elongation of explants with folds was 22%, compared to a mean decrease in length of 4% in those explants without folds, a highly significant difference (unpaired data; $P < 0.001$).

**FIG. 4.** The axolotl neural plate is explanted with underlying tissues and a rim of adjacent neural fold epidermis (A) or without adjacent neural fold epidermis (C). The photographs were made within 1 min of the operation. When intact axolotl control embryos reached closed-tube stage 20–21, the same explants of neural plate and other tissues were photographed again in dorsal view. (B) An explant of neural plate with a rim of adjacent neural fold epidermis has closed into a tube. (D) An explant of neural plate without adjacent epidermis remains an open plate except in the caudal spinal cord. Bar, 1 mm.

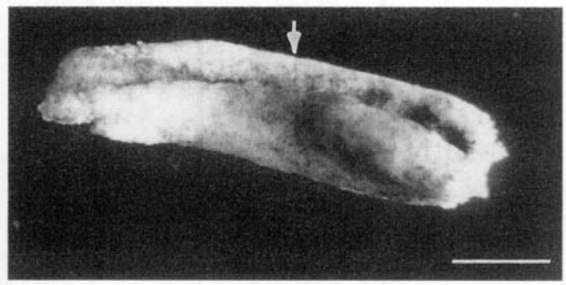

FIG. 5. Explant of an axolotl neural plate from which all epidermis was removed from one side and a rim of epidermis was left on the other side, photographed 8 hr after explantation. The side with epidermis (bottom in photo) began rolling at the border of brain plate and future spinal cord (arrow) and has rolled there to the opposite edge of the plate. The rolling progressed cranially and caudally some distance, but the anterior and posterior neuropores remain open at this time. The side with no epidermis (top in photograph) has not rolled. Bar, 0.1 mm.

### *Neural Plate Explants with Epidermis on One Side but Not on the Other*

In these experiments, one side of the neural plate was cut with a rim of epidermis remaining as in Fig. 4A, and the other side of the neural plate was cut without any epidermis as shown in Fig. 4C.

The side of the explant that retains a rim of epidermis rolls toward the midline, starting at the brain–spinal cord border and progressing both cranially and caudally as in a normal embryo. The opposite side, without epidermis, does not fold, nor does it roll toward the midline. When the edge of the plate with the rim of epidermis reaches the midline, it would normally meet the neural fold from the other side and tube formation would be complete in that region. In the absence of a neural fold from the other side, the side with epidermis continues to roll past the midline until it reaches the opposite side of the neural plate, where it stops. The two sides do not appear to fuse. This unilaterally formed "tube" is first complete at the brain–spinal border and then progresses some distance both cranially and caudally, but ceases without closing either the anterior or the posterior neuropores in any of the five examples (Fig. 5).

The lengths of the borders of the neural plates were measured with the morphometrics unit at 5 and 8 hr after the operation. The lengths of the neural plate borders that had a rim of epidermis were compared to the lengths of the neural plate borders that had no epidermis at each of the times. Five hr after the operation, the neural plate edges on the sides with epidermis had a mean length of 3.22 ± 0.64 mm; the sides with no epidermis had a mean length of 3.08 ± 0.63 mm. The lengths of the two sides were significantly different ($P = 0.005$, paired data). At 8 hr after the operation, the neural plate edges on the sides with epidermis had a mean length of 3.06 ± 0.16 mm; the sides with no epidermis had a mean length of 2.78 ± 0.13 mm. The lengths of the two sides were still significantly different ($P = 0.04$, paired data).

Sections show (Fig. 6) that on the side of the explant that has some epidermis, the epidermal rim is rolled over the plate as tube formation proceeds on this side of the explant. The side of the explant that has no epidermis shows no signs of rolling to form a tube.

### *Disrupting the Boundary between Notoplate and Neural Plate*

The object of these experiments was to determine whether the boundary between the notoplate and the neural plate affects the behavior of the cells on each side such that the cells intercalate and elongate the boundary. The boundary between notoplate and neural plate was severed (unilaterally or bilaterally) so that elongations of portions with and without the notoplate could be compared. All cuts entered the archenteron, but did not extend below the archenteron. The cut embryos immediately gaped widely and remained gaping through the period of culture. At the end of the culture period, the lengths of cut edges of neural plates of newt or axolotl embryos were highly significantly smaller compared with those of normal, intact embryos that had been cultured for the same period of time (unpaired data, $P < 0.001$ in each case).

Three types of operation were performed in axolotls and newts: In Operation A, the left boundary between the notoplate and neural plate was cut, creating a left part that contained neural plate, but no notoplate/neural plate boundary, and a right part that contained some neural plate

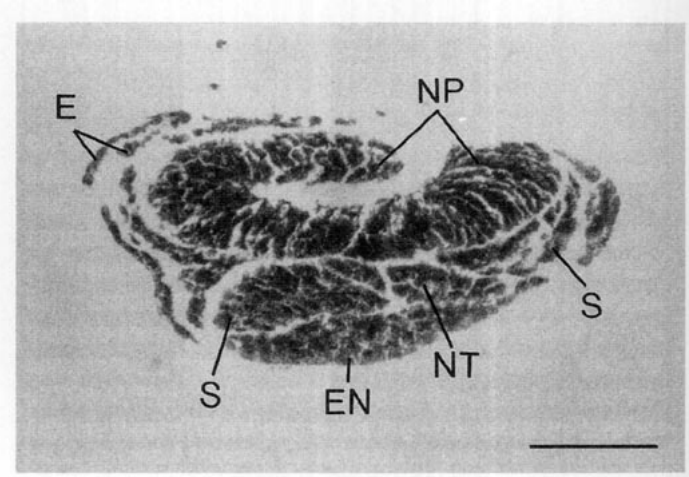


FIG. 6. Paraffin transverse section of an explant as described in Fig. 5, through a region slightly cranial to the position indicated by the arrow in Fig. 5. On the side with contiguous epidermis (left in photo), the neural plate has rolled nearly to the opposite edge of the plate. The side with no epidermis (to the right in the photo) has not rolled up. NP, neural plate; E, epidermis; EN, endoderm; NT, notochord; S, somitic mesoderm. Bar, 0.1 mm.

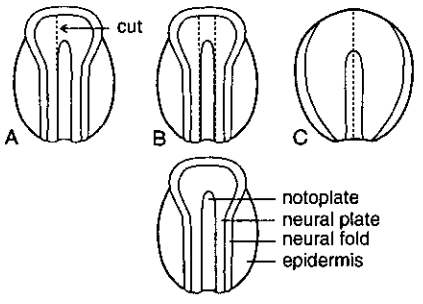


**FIG. 7.** Operation schemes to test the role of the notoplate boundary. (A) A cut is made along the left boundary of the notoplate so the left neural plate contains no notoplate and the right part has all the notoplate. (B) Cuts are made along both the left and right boundaries of the notoplate. Neither the left nor the right parts of the neural plate have any notoplate boundary, and the center part has all the notoplate, but no boundary with the neural plate. (C) In this control experiment, a cut is made down the midline, splitting the notoplate. Both the left and the right parts of the neural plate have some notoplate boundary.

and the entire notoplate including its intact boundary with the right portion of the neural plate (Fig. 7A). In Operation B, both the left and right sides of the notoplate were severed, separating the embryo into three parts, none of which had an intact boundary between the notoplate and neural plate (Fig. 7B). As a control, in Operation C, the midline of the embryo was cut so that the left and right halves each had some neural plate and some notoplate and thus contained an intact boundary between neural plate and notoplate (Fig. 7C).

We have previously used this technique to examine the role of the notoplate/neural plate boundary in the newt (Jacobson, 1981, 1985, 1991). A brief review and quantification of these experiments is given in Table 2. Originally we merely wished to determine the difference in the lengths of the various parts of an embryo after culture. These values were recorded as a separate data set for each individual, but the data in these sets were not paired with their own starting lengths. In the present analysis we have pooled midline lengths of normal embryos from several clutches to generate sample mean lengths for the starting stages. We have also pooled all available experimental cases for each sort of experiment. Although the present results differ in some respects from the previous reports which were based on subsets of the data analyzed here, they all illustrate similar trends.

The cut in Operation A (Fig. 7A) produced embryos whose right parts (with boundary) formed closed tubes, while their left parts (without boundary) did not (illustrated in Jacobson, 1981). The mean length of the left parts decreased by 4% compared to the mean length of the stage 15 embryos, but the difference in the means is not significant (unpaired data, $P > 0.500$). The mean length of the right parts increased by 13% compared to stage 15 embryos; this difference is not quite significant (unpaired data, $P = 0.058$). There is a highly significant difference between the lengths of the left and right parts ($P < 0.001$; paired data).

The cut in Operation B (Fig. 7B) produced embryos with three parts, none of which have a boundary between notoplate and neural plate. The slight changes in mean lengths of the left and right pieces compared to the stage 15 controls was not significant (unpaired data, $P > 0.500$). The center portion, which contained all of the notoplate, decreased length during incubation by nearly 36%, which is highly significantly less than the length of the stage 15 embryos (unpaired data, $P < 0.001$).

Operation C (Fig. 7C) is a control experiment. These operations were performed at stage 14 when the notoplate is broader and can be more accurately bisected than would be possible at stage 15. After culture, the mean length (left or right sides) was significantly longer than the mean length of the stage 14 controls (unpaired data, $P < 0.01$). The lengths of the left and right sides after culture were not significantly different from one another (paired data, $P = 0.157$).

Repetitions of these experiments using axolotl embryos gave somewhat different results. The length of the dorsal midline of each axolotl neural plate was measured at the start of the experiment and the cut edges of the severed plate parts were measured at its termination (Table 3). The dorsal midlines of normal embryos cultured in parallel were also measured at stage 20–21. The data were collected and quantified from projected video images using the computer-assisted morphometrics program.

We made the cut in Operation A (Fig. 7A) on two sets of embryos from different clutches (16 embryos at stage 15– to 15 for Set 1 and 12 embryos at stage 14 for Set 2). Both sets yielded similar results. At the end of the experiment, the mean lengths of both the (left) parts with no notoplate/neural plate boundary and the (right) parts with a single, intact notoplate/neural plate boundary showed a highly significant increase compared to the mean starting lengths (paired data, $P < 0.001$ for both sets). The mean lengths of the (left) parts with no boundary after culture were also highly significantly less than the mean lengths of the (right) parts with a single intact boundary (paired data; $P < 0.001$ for both sets).

For Operation B, we used 24 embryos (stages 14 to 15–) from a single clutch (Fig. 7B). The mean length of the lateral parts (neural plate only, left or right) at the end of the experiment was highly significantly greater than the mean starting length (paired data; $P < 0.001$ for each side). The difference in mean lengths of the left and right portions at the end of the experiment was barely significant (paired data, $P = 0.045$). The mean length of the central parts (notoplate only) at the end of the experiment showed a highly significant decrease compared to their own mean starting length or to the mean lengths of the left and right pieces following culture (paired data; $P < 0.001$).

Two sets of control operations were performed as shown in Fig. 7C (16 embryos at stage 14 for Set 1 and 20 embryos

**TABLE 2**
Summary of Lengths of Midlines and of Cut Edges, *Taricha torosa*

| Operation[a] | Stage | Mean length (mm) ± SD | Change[b] | No. of cases |
|---|---|---|---|---|
| A | | | | |
| Midline at start | 15 | 3.38 ± 0.64 | | 27 |
| Left (neural plate only) | 20 to 21 | 3.25 ± 0.86 | −4% | 18 |
| Right (notoplate and neural plate) | 20 to 21 | 3.83 ± 0.89 | +13% | 18 |
| B | | | | |
| Midline at start | 15 | 3.38 ± 0.64 | | 27 |
| Left (neural plate only) | 20 to 21 | 3.31 ± 0.44 | −2% | 12 |
| Center (notoplate only) | 20 to 21 | 2.17 ± 0.49 | −36% | 9 |
| Right (neural plate only) | 20 to 21 | 3.39 ± 0.50 | 0% | 12 |
| C | | | | |
| Midline at start | 14 | 2.18 ± 0.00[c] | | 7 |
| Left (neural plate and one-half notoplate) | 20 to 21 | 2.53 ± 0.24 | +16% | 9 |
| Right (neural plate and one-half notoplate) | 20 to 21 | 2.56 ± 0.19 | +18% | 9 |
| Normal control embryos | | | | |
| Midline of intact controls | 20 to 21 | 5.80 ± 0.75 | | 25 |
| % Change from stage 14 (Operation C) to stage 20–21 | | | +166% | |
| % Change from stage 15 (Operation A, B) to stage 20–21 | | | +72% | |

[a] The operations (A, B, C) are illustrated in Fig. 7.
[b] The percentage change is the difference between the starting midline length and the length of the cut edge at the end of the experiment, divided by the starting midline length.
[c] All these cases were the same length.

at stage 14− to 14 for Set 2). In both sets, both sides showed a highly significant increase over starting length (paired data, $P < 0.001$). In Set 1, the difference in the left and right lengths after culture was barely significant (paired data, $P = 0.042$), while in Set 2, the difference in the left and right lengths was nonsignificant (paired data, $P = 0.477$).

The results of Operation A, Set 2 were compared to the results in control Operation C, Set 1. In both experiments, the starting stage was 14 and the mean starting sizes were not significantly different from one another (unpaired data, $P = 0.061$). The left part that had no notoplate boundary in Operation A elongated significantly less than either the left or the right parts in control Operation C that do have a notoplate boundary (unpaired data, $P < 0.01$ in both cases). The elongation of the right part of Operation A (that does have a notoplate boundary) was not significantly different from the elongation of the right part of Operation C (unpaired data, $P = 0.075$) nor of the left side of Operation C (unpaired data, $P = 0.145$), both of which have notoplate boundary. We also compared the results of Operation B to the control embryos in Operation C, Set 1. Although the starting stages were slightly different, the starting values were not significantly different (unpaired data, $P = 0.330$). After culture, the mean lengths of the left, center, or right pieces from Operation B were highly significantly smaller (unpaired data, $P < 0.001$ in all cases) than the mean lengths of the control Operation C (either left or right).

## DISCUSSION

Boundaries between tissue domains within and around the neural plate appear to restrict the behavior of cells, with the result that the boundaries elongate, the neural plate reshapes, and the plate rolls into a tube. Jacobson *et al.* (1985, 1986) gave evidence for this proposition and used computer simulations to confirm that the physical forces invoked were realistic.

The present studies give additional evidence that cell behavior is organized at tissue boundaries and better define what is occurring at the boundary between neural plate and epidermis.

### *The Role of the Boundary between Notoplate and Neural Plate*

We had already seen in previous studies that the boundary of the neural plate with the notoplate organizes the activi-

**TABLE 3**
Summary of Lengths of Midlines and of Cut Edges in the Axolotl, *Ambystoma mexicanum*

| Operation[a] | Stage | Mean length (mm) ± SD | Change[b] | No. of cases |
|---|---|---|---|---|
| A (set 1) | | | | |
| Midline at start | 15-/15 | 3.05 ± 0.55 | | 16 |
| Left (neural plate only) | 20/21 | 3.62 ± 0.27 | +19% | 16 |
| Right (notoplate and neural plate) | 20/21 | 3.93 ± 0.28 | +29% | 16 |
| A (set 2) | | | | |
| Midline at start | 14 | 2.16 ± 0.08 | | 12 |
| Left (neural plate only) | 20/21 | 3.30 ± 0.34 | +53% | 12 |
| Right (notoplate and neural plate) | 20/21 | 3.67 ± 0.31 | +70% | 12 |
| B | | | | |
| Midline at start | 14/15- | 2.34 ± 0.22 | | 24 |
| Left (neural plate only) | 20/21 | 3.03 ± 0.26 | +30% | 24 |
| Center (notoplate only) | 20/21 | 2.04 ± 0.24 | −13% | 24 |
| Right (neural plate only) | 20/21 | 3.21 ± 0.31 | +37% | 24 |
| C (set 1) | | | | |
| Midline at start | 14 | 2.27 ± 0.19 | | 16 |
| Left (neural plate and one-half notoplate) | 20/21 | 3.84 ± 0.27 | +69% | 16 |
| Right (neural plate and one-half notoplate) | 20/21 | 3.71 ± 0.28 | +63% | 16 |
| C (set 2) | | | | |
| Midline at start | 14-/14 | 2.08 ± 0.15 | | 20 |
| Left (neural plate and one-half notoplate) | 20/21 | 3.46 ± 0.33 | +67% | 20 |
| Right (neural plate and one-half notoplate) | 20/21 | 3.40 ± 0.33 | +63% | 20 |
| Normal control embryos | | | | |
| Midline of intact controls | 20/21 | 4.96 ± 0.46 | | 15 |
| % Change from stage 14-/14 (Operation C, 2) to stage 20/21 | | | +138% | |
| % Change from stage 14 (Operation A, 2) to stage 20/21 | | | +123% | |
| % Change from stage 14 (Operation C, 1) to stage 20/21 | | | +119% | |
| % Change from stage 14/15- (Operation B) to stage 20/21 | | | +112% | |
| % Change from stage 15-/15 (Operation A, 1) to stage 20/21 | | | +63% | |

[a] The operations (A, B, C) are illustrated in Fig. 7.
[b] The percentage change is the difference between the starting midline length and the length of the cut edge at the end of the experiment, divided by the starting midline length.

ties of intercalating cells from both sides by trapping at the boundary any cells that touch it. Time-lapse cinematography has allowed direct observation of cells at the notoplate/neural plate boundary. Cells move randomly when approaching this boundary, but remain fixed when they reach it (Jacobson *et al.*, 1986).

Our new boundary-cutting experiments (Figs. 7A, 7B, and 7C; Tables 2 and 3) provide additional evidence that this boundary has a role in the elongation of the midline of the neural plate. In both the newt and the axolotl, elongation of the cut edges of the halves of the neural plates that contain notoplate (and its boundary with the neural plate) is highly significantly greater than the starting lengths or than the lengths of cut edges of neural plates without notoplate boundary.

### *The Boundary between Epidermis and Neural Plate Is Necessary for Proper Neurulation*

Two sets of experiments demonstrate that a boundary between neural plate and epidermis is necessary for the completion of neurulation and support our hypotheses that the boundary between the neural plate and the epidermis is necessary both to help roll the plate into a tube and to elongate the edge of the plate. The first set consists of explants of neural plates with and without neural folds (Figs. 4A–4D; Table 1). Explants made without neural folds lack the neural plate/epidermis boundary and they neither rolled into tubes nor elongated during neurulation. Explants with intact neural folds elongate significantly (though less than in unoperated embryos), and they close their neural tubes completely or, in a couple of cases, nearly completely.

In the second set of experiments, each explant of neural plate had a rim of epidermis on one side and none on the other. After culture, the side with epidermis was significantly longer than the bare side, and the side with epidermis rolled to the opposite edge while the side with no epidermis did not roll up (Figs. 5 and 6). It seems clear that contact with epidermis alters the behavior of neural plate cells.

Any explanation of the role of the neural plate/epidermis boundary in neurulation must address regional differences in tissue movements along this boundary (Figs. 2A, 2B, and 3). The total length of the neural fold remains about constant or decreases during neurulation in the newt embryo, but increases steadily during neurulation in the axolotl embryo. However, when we examine the two main subdivisions (i.e., brain and spinal cord) of this boundary individually, our measurements show that, during neurulation in both the axolotl and the newt, the neural folds surrounding the brain plate decrease in length while the neural folds abutting the prospective spinal cord increase in length. In both species, after the neural plate closes into a tube (stage 20), the total length of neural fold (now the dorsal midline) and the lengths of the brain and spinal cord regions increase.

### *Cell Behaviors at the Boundary between Epidermis and Neural Plate*

In the absence of cell rearrangements, shrinkage of the apical surface areas of neural plate cells would shorten the epidermis/neural plate boundary everywhere. During neurulation, cells in the neural plate become taller and thinner, and their apical surfaces decrease dramatically (Burnside, 1971, 1973; Ferreira and Hilfer, 1993). Neural plate cells next to the neural folds are the first to exhibit these changes. Later, cells progressively nearer the midline undergo similar changes (Jacobson *et al.*, 1986). Neural plate cells near to the epidermis/neural plate boundary in the prospective brain area show the greatest reduction in their apical surface areas (Jacobson and Gordon, 1976). As these cells reduce their surface areas, the length of the border of the brain plate that they compose should also decrease.

Other possible mechanisms for shortening the edge of the brain plate include disintercalation of cells along that border, or, more likely, ingression of the cells at the border into the forming folds where they may be stored to migrate later as neural crest cells (Fig. 8A).

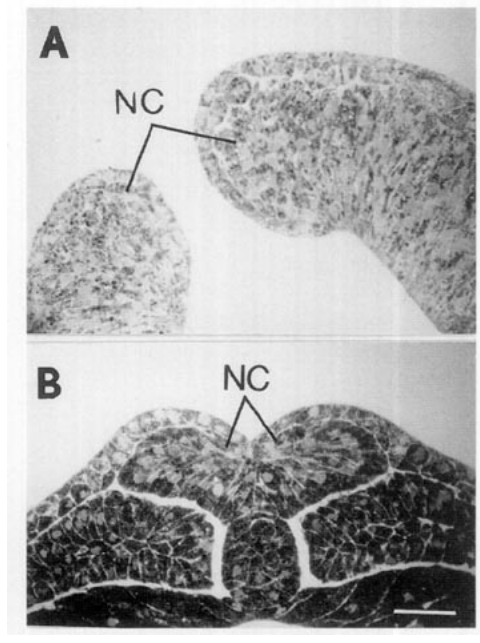


**FIG. 8.** Plastic transverse sections of an embryo of *T. torosa* at stage 17 show many putative stored neural crest cells (NC) in the neural folds of the brain plate (A) and few in the spinal cord region (B). Bar, 0.1 mm.

The same mechanisms that shorten the edge of the brain plate should tend to shorten the edge of the neural plate in the prospective spinal cord region, but our measurements show that this border elongates rather than shortening. One is forced to conclude that cells intercalate along that portion of the boundary and then remain stuck there as we observed at the notoplate/neural plate boundary (Jacobson *et al.*, 1986). The cells at the border of the spinal cord region of the neural plate reduce their apical surfaces considerably less than do similar cells along the brain plate border (Jacobson and Gordon, 1976) and the neural folds in the spinal cord region do not appear to contain many stored crest cells (Fig. 8B), so mechanisms that would shorten this portion of the boundary of the neural plate are expressed much less than in the region of the brain plate. In the axolotl embryo, the elongation of the spinal cord region of the plate edge is sufficient to increase the total length of the neural folds during neurulation, the total elongation in the spinal region being greater than the shrinkage of the edge of the brain plate.

We had previously proposed that crawling of neural plate cells beneath the epidermis creates a rolling moment that lifts the epidermis into a fold up over the neural plate (Jacobson *et al.*, 1985, 1986). Thus, in the absence of a boundary with the epidermis, the neural plate will lack such a rolling

moment and fail to form a tube, as our experiments demonstrated. Our earlier studies (Moury and Jacobson, 1989, 1990) also demonstrated that the ability to form a fold is not limited to the natural boundary between neural plate and epidermis; any piece of neural plate apposed to any piece of epidermis will form a neural fold (and crest) at the newly created boundary.

Bending the neural plate into a fold and ingression of prospective neural crest cells may represent different manifestations of a single basic type of cell behavior. Jacobson *et al.* (1985, 1986) proposed that the basal and lateral surfaces of cells in an epithelium can actively crawl or "tractor" on adjacent cells. The extensive apical–basal elongation of neural plate cells that begins at the boundary with epidermis and then progresses toward the midline is initiated by interactions with adjacent epidermal cells.

Neural plate cells crawling beneath the epidermis create an upward rolling moment directed toward the midline that helps raise the neural folds and close the neural tube. The cells of the neural plate that contact the epidermis elongate themselves and reduce their apical surfaces (reducing plate width) as they crawl beneath the epidermis. Continued tractoring produces such severe cell elongation that apical surfaces are reduced to points which eventually break, releasing the cells (now prospective neural crest cells) from the epithelium (Jacobson *et al.*, 1986). These processes progress medially.

### *The Notoplate/Neural Plate Boundary and the Epidermis/Neural Plate Boundary Can Act Synergistically*

The entire spinal cord region of the neural plate undergoes convergence-extension movements as shown by mapping of cell trajectories (Burnside and Jacobson, 1968 (newt); Jacobson, 1959 (axolotl); Keller *et al.*, 1992 (*Xenopus*)). Intercalation and trapping of cells at the epidermal boundary in the spinal cord region and along the entire notoplate boundary elongates these boundaries, and thus the neural plate, accounting for "extension." "Convergence" toward the midline as a result of the same trapping of cells at the boundaries is less easily visualized. The contiguous epidermal and neural ectoderm of the embryo is most firmly attached to the underlying mesoderm where the notoplate contacts the underlying notochord. When notoplate cells become trapped at their boundary with the neural plate, they lengthen this boundary and reduce the width of the notoplate by a corresponding amount. This reduction in width creates an apparent movement of cells and tissues toward the midline. Likewise, when neural plate cells intercalate and become trapped at the epidermal boundary in the spinal cord region, they elongate the boundary and reduce the width of the plate. Again, the apparent motion will be toward the anchored midline. Thus, interactions at the neural plate/notoplate boundary act synergistically with similar interactions at the neural plate/epidermis boundary to lengthen the spinal cord region and narrow the plate. If either of these boundaries is cut, elongation of the midline of the embryo is reduced. In the absence of one boundary, the other may allow some elongation.

One can compare the relative contributions of the two boundaries from the data in Tables 1 and 3. In the axolotl embryo, if one removes the epidermis, plate elongation is −4% (Table 1) despite the presence of an intact notoplate boundary. If one removes the notoplate boundary, leaving the epidermal boundary intact, the plate elongates by 19% (Table 3A, Set 1). Thus, in the axolotl embryo, the boundary between epidermis and neural plate has the greatest effect on plate elongation.

The two boundaries act synergistically to elongate the midline, but the most important boundary for closing the neural plate is that between the neural plate and the epidermis. Interactions at this boundary initiate cell behavior in which neural plate cells attempt to crawl beneath the epidermis, elongate themselves in the process, and raise a neural fold (Jacobson *et al.*, 1985, 1986).

Finally, although axolotl and newt embryos behaved similarly in most of our observations and experiments, we did observe two differences that appear to be related to the neural plate/epidermis boundary. Prior to neural tube closure in the newt, the total length of this boundary stays nearly constant or decreases slightly; in the axolotl it increases steadily (cf. Figs. 2A, 2B, and 3). Furthermore, the lengths of portions of the neural plate without a notoplate boundary decrease in the newt, but increase in the axolotl—although much less than their counterparts with a notoplate boundary (cf. Tables 2 and 3). These may represent true species differences—perhaps indicating that the neural plate/epidermis boundary plays a greater role in the elongation of the axis of the axolotl than it does in the newt.

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