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Raphe of the Posterior Neural Tube in the Chick Embryo: Its Closure and Reopening as Studied in Living Embryos With a High Definition Light Microscope

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ABSTRACT **Chick embryos cultured on a curved substratum show a transient enlargement of the posterior neuropore (PN), mimicking the temporary delay of PN closure as seen in the curly tail (*ct*) mouse mutant (van Straaten et al. [1993] Development 117:1163–1172). In the present study the PN enlargement in the chick embryo was investigated further with a high definition light microscope (HDmic), allowing high resolution viewing of living embryos in vitro. The temporary PN enlargement appeared due to considerable reopening of the raphe of the posterior neural tube, which was followed by reclosure after several hours. The raphe was subsequently studied in detail. It appeared very irregular, with small zones of apposed, open and fused neural folds. During closure, these raphe features shifted posteriorly. A distinct fusion sequence between surface epithelium and neuroepithelium was not seen. During experimental reopening of the raphe in vitro, small bridges temporarily arose, broke and disappeared quickly; they likely represented the first adhesion sites between the neural folds. More prominent adhesion sites partly detached, resulting in bridging filopodia-like connections; they probably represented the first anteroposterior locations of neural fold fusion. Our observations in the living chick embryo in vitro thus show that the caudal neural tube has an irregular raphe with few adhesion sites, which can be readily reopened. As a result of the irregularity, the PN does not close zipper-like, but button-like by forming multiple closure sites.** © 1993 Wiley-Liss, Inc.

Key words: Chick, Embryo, Posterior neuropore, Raphe, Apposition, Fusion

INTRODUCTION

Neurulation, the process of transformation of the flat neural plate into a closed neural tube, has been an intriguing subject for many years. The fact that the underlying mechanisms are scarcely or only partially understood is undoubtedly due to the complexity of the process and to the multitude of suggested genetic and environmental factors which are translated into intrinsic and extrinsic forces in neurulation. An abundance of literature on these subjects is available (for reviews see Karfunkel, 1974; Gordon, 1985; Copp et al., 1990; Schoenwolf and Smith, 1990). Recently, a possible role of axial curvature as an extrinsic force counteracting neurulation was suggested: in the curly tail (*ct/ct*) mouse, mutant for spina bifida, increased ventral curvature of the posterior embryonic axis appeared coincidental with temporary enlargement of the posterior neuropore (PN). The enlargement appeared due to cessation or deceleration of fusion at the proximal site of the PN. It was suggested that, due to increased bending, the neural folds were opposed to converge, prohibiting the fusion point from proceeding in a posterior direction. After a period of delay the fusion point continued to proceed at high speed, parallel to unbending of the PN region (Copp et al., 1990; Brook et al., 1991; van Straaten et al., 1992, 1993). This abnormal process was simulated in chick embryos, which have a flat neural plate posteriorly. Axial curvature was forced by culturing embryos on a concave or convex substratum, which resulted in a temporary enlargement of the PN for several hours, followed by closure (van Straaten et al., 1993). This experiment supported the hypothesis that axial curvature is an important event in neurulation. However, it was possible that the temporal enlargement of the PN was not due to deceleration or cessation of fusion, as seen in the curly tail mouse, but to reopening of the raphe. Such an interpretation was obscured by the caudal shift of the PN as a whole, due to the marked elongation of the neural plate in the PN region (DiVirgilio et al., 1967; Jacobson, 1981; Schoenwolf, 1985). Moreover, the continued progression of closure after several hours of delay (*not* due to unbending of the axis) indicated that a corrective adaptation mechanism must exist.

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Therefore, in the present study the enlargement and reclosure of the PN in concave-cultured chick embryos was studied using a high definition light microscope (HDmic). With this device, the dynamic process of neural tube formation in living avian embryos has been achieved at higher resolution and contrast than was previously possible (Jaskoll et al., 1991). Moreover, morphology of the raphe of the posterior neural tube was studied in detail and the mechanism of reopening and of reclosure of the raphe and PN was experimentally tested. It appeared that PN closure occurs not zipper-like, but button-like at multiple sites.

RESULTS

Neurulation Defined in the Chick Embryo

The present study was performed in stage 10–11$^+$ chick embryos. At these stages, the PN appears diamond-shaped (sinus rhomboidalis). The walls of the PN are in their final stage of elevation; the neural folds converge, appose in the median line and finally fuse (Portch and Barson, 1974; Bancroft and Bellairs, 1975; Schoenwolf, 1979, 1982; Schoenwolf and Smith, 1990). In the antero-posterior direction, from the cervical region onwards, three neurulation zones can be distinguished at these stages: neural tube, neural tube raphe and PN. The neural tube is defined as the zone where fusion of both surface epithelium and neuroepithelium is completed and no medial suture line is visible with HDmic (Jaskoll et al., 1991). The PN regards the most posterior zone where the neural folds are still apart, its distal border defined as the distal side of the sinus rhomboidalis. The raphe zone is between PN and neural tube. The raphe is defined as the medial suture of the closing neural tube. Based on the present results with the HDmic, the raphe appeared quite variable; it included small zones where the neural folds were apposed, where the folds were still apart from each other, or where the folds were partially or completely fused. We will nevertheless use the term "raphe" for this zone throughout this paper.

Interpretation of Neurulation Features as Seen in HDmic

Although the high definition light microscope (HDmic) is especially suited for high resolution views of living embryos, the low power view shown in Figure 1 reveals at least 4 aspects of the neural plate simultaneously, with sufficient depth of field: 1) the PN and neural tube raphe, being delineated by the medial edges of the neural folds; 2) the thickness of the neural walls, at the site where a lateral sulcus is present; 3) the projection of the neural folds onto the horizontal plane, indicating that the folds converge; and 4) the width of lumen of the neural groove. Elevation of the neural walls cannot be deduced from HDmic viewings, although in cases of fast narrowing of the lumen increased elevation is likely.

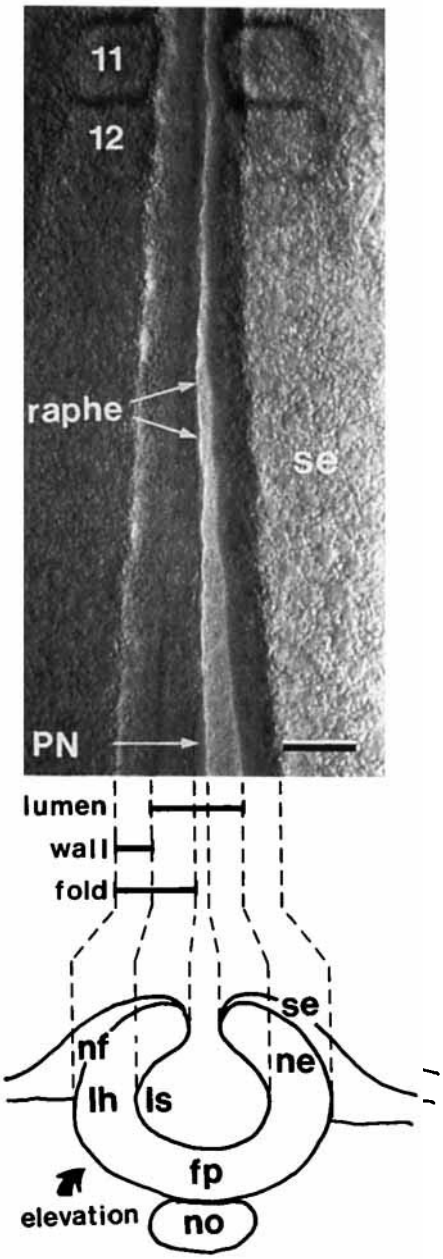


Fig. 1. Features of the posterior region in a 12-somite chick embryo as seen in dorsal view with the high definition light microscope (HDmic) at low power. Somite pairs 11 and 12 are present at the anterior side (**top**). The anterior side of the PN is indicated. The image is focused on the surface epithelium (se). Three pairs of projection lines of the neural groove can be distinguished. Through-focus observations revealed that these lines represent the medial edges of the neural folds, the lateral sulci (ls) and the outlines of the bended neural plate as is explained in the drawing (**bottom**). These lines delineate the projection of the neural folds (nf), the thickness of the neural walls and the width of the lumen. The converging neural fold is composed of surface epithelium (se) and neuroepithelium (ne). The lateral neural plate is elevated, whereby the floor plate (fp) acts as a hinge point. Anterior to the PN, the irregular shape of the neural tube raphe, with both open and apposed parts (arrows), is present. fp, floor plate; lh, lateral hinge point; ls, lateral sulcus; no, notochord. Bar, 100 μm.

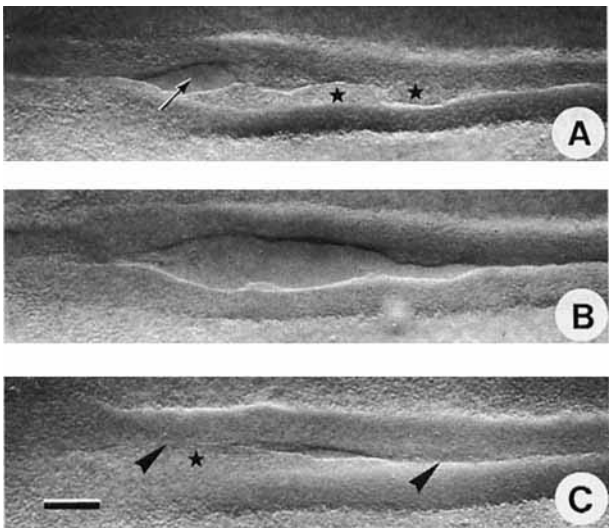


Fig. 2. Series of low power views of the PN and raphe zone in a chick embryo, cultured on a curved substratum. **A:** Immediately after placing the 14-somite embryo in culture. The PN (arrow) is small. In the raphe zone the apposed neural folds (asterisks) are broad, indicating marked convergence. The raphe is very irregular. **B:** 75 min in culture. The PN has enlarged, mainly at the expense of the raphe. The reopening of the raphe is due to some widening of the lumen, indicating de-elevation, but mostly to decrease in extension of the folds, indicating that the folds de-converged to a more upright position. **C:** 8 hr in culture, the embryo has gained 5 pairs of somites. Reclosure of the PN, contributed to by a decreased lumen width (indicating elevation) and extension of the neural folds (indicating convergence). In the distal part the large fold seen (asterisk) is likely due to bending in the lateral hinge point; in the remainder the convergence of the folds is likely due to elevation, not to change in angles at the lateral hinge point. An anterior and posterior neural tube raphe are seen (arrowheads). After 11 hours in culture the folds of the remaining PN were completely apposed in this embryo. Photographs are aligned on the distal end of the neural groove/tube. Anterior is to the right. Bar, 100 μm.

Effect of Axial Curvature on PN Shape and Size

From a previous study it appeared that the PN enlarged in chick embryos, when cultured on a slightly concave or convex substratum. In order to investigate if this enlargement was due to reopening of the raphe, we repeated these experiments and studied the size and relative displacement of the PN during in vitro culture with the HDmic. In 4 out of 22 concave-cultured embryos normal progression of closure was seen, while in 18 embryos (with 9–15 somite pairs) closure delay occurred. In 11 of them (with 11–14 somite pairs) the PN enlarged both in length and width for several hours (Fig. 2). Lengthening appeared due to reopening of the raphe. PN widening was paralleled by widening of the lumen and a marked decrease of the neural fold projection, which probably indicated that elevation was decreased and that the neural folds were in a more upright position. Ultimately the PN came to closure in these 11 embryos; in most cases the lumen remained wide and large neural fold projections were seen, indicating that convergence was increased. (In Fig. 2C, a wide lumen is seen at the posterior side only). Of note in older embryos, and when the amount of reopening reached was substantial, the final closure did not occur at the very end of the neural plate, but more anterior (Fig. 2C).

In 3 of 18 embryos (12, 15, 15 somite pairs), the caudal progression of the proximal PN site occurred slowly, or not at all. Since the distal site of the PN continued to progress (due to elongation of the embryo) the PN enlarged.

In 4 of 18 embryos (9–12 somite pairs), the neural fold projections disappeared and the whole neuroepithelium could be seen in focus at once (Fig. 3), indicating that the folds were bended outwards and, together with the neural walls, were depressed to a flat plate. Even after many hours the walls of the widely opened PN failed to converge. It is likely that the caudal progression of the proximal PN site ultimately halted, as is suggested by the blunt angle between the neural folds in Figure 3 (arrowhead). In the youngest (9-somite pairs) embryo, the flattened neural plate was very wide (330 μm).

Appearance of the Neural Tube Raphe

In order to understand the mechanism of reopening, the appearance and structure of the PN and raphe during normal closure were investigated first. At the stages studied, the neural folds lining the PN were oriented almost parallel to each other as shallow convex crescents, approaching over a considerable length. The edges of these folds appeared not as straight lines, but were rather irregular and displayed medially directed bulges (Figs. 2A, 7A, 9A). More anteriorly, a raphe between both folds was present (Figs. 7A, 9A). Its appearance was irregular: at some sites the neural folds were still separated by a narrow slit, at other sites the folds contacted each other (apposed), leaving a clear groove in between, and in others a medial line was barely or not visible.

The edges of the neural folds were even more irregular after the raphe reopened in concave-cultured embryos. In Figure 4, two successive stages of closure after raphe reopening are presented, whereby a prominent bulge of the upper neural fold overlaps the lower fold considerably. Medial movement of the neural folds continued until other parts of the neural folds apposed each other (Fig. 4B). Such overlaps were also seen during normal closure, although to a lesser extent.

During progressive closure, as followed with the HDmic for several hours, it appeared that the bulging neural folds were the first sites where contact occurred, and that such apposed sites were the first sites where the suture line disappeared. In Figures 5A and 7A, two sites of fusion can be distinguished.

The irregular structure of the raphe was further studied by processing embryos for light microscopy (LM) immediately after they had been photographed with the HDmic. If no suture line was present in HDmic viewings, the LM sections showed complete fusion of the surface epithelium (Fig 5B). A thin line in HD-

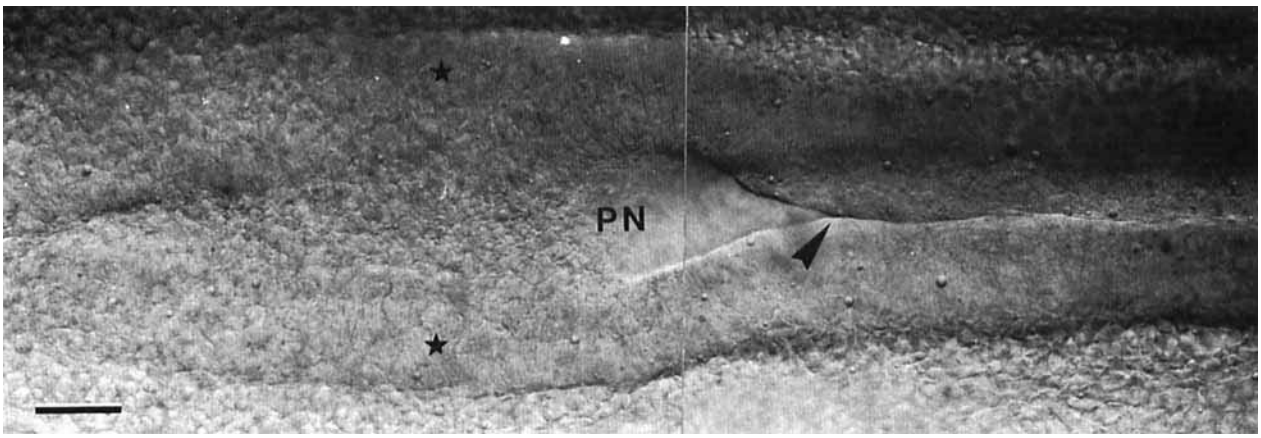


Fig. 3. High power view of diverged posterior neural folds (asterisks) of the PN in a 12-somite chick embryo after culture for 2.5 hr on a curved substratum. Anterior is to the right. Arrowhead, distal end of the raphe. Bar, 50 μm.

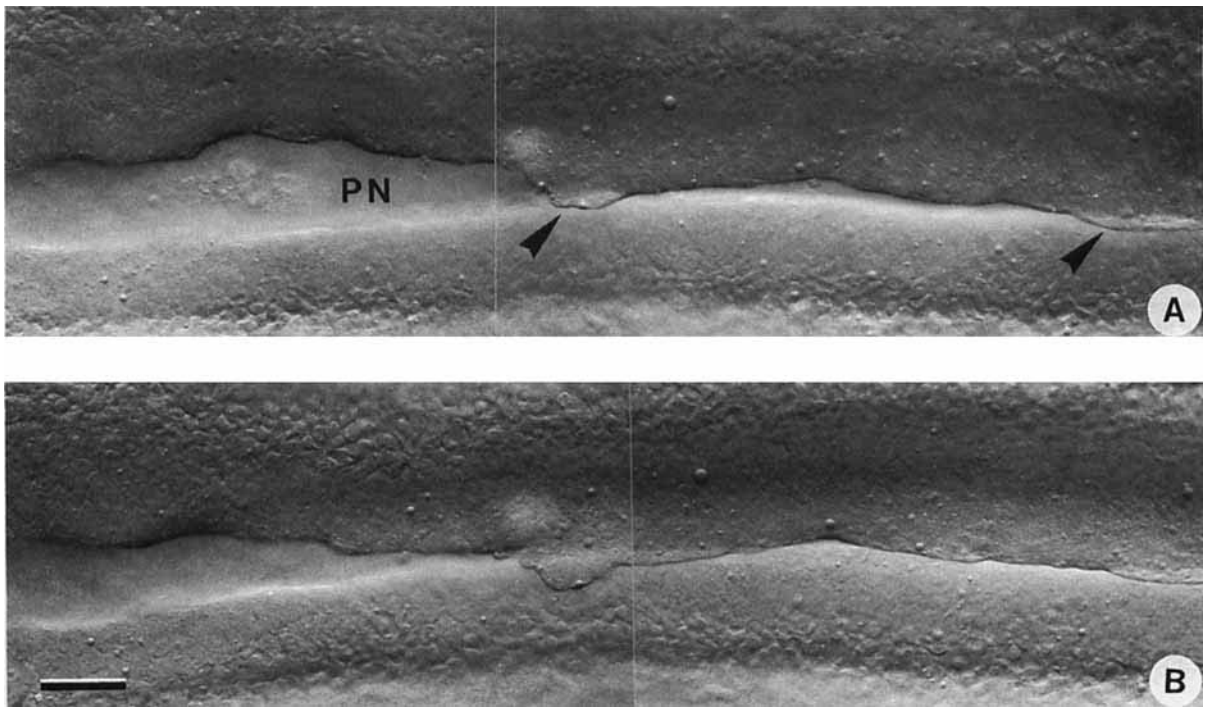


Fig. 4. High power view of PN reclosure in a 12-somite chick embryo during culture on a curved substratum. At the beginning of the culture, the PN was almost closed. After initial reopening the PN commenced reclosure. **A:** One hour in culture. In the raphe zone, fold overlap during reclosure is seen at two sites (arrowheads). **B:** 2.5 hr in culture. Continued closure of PN and raphe, whereby one fold is overlapping considerably. The seemingly thin appearance of the fold is due to the specific way of illumination; in reality, the thickness of the folds amounts approximately 50 μm. Photographs are aligned on large folds. Bar, 50 μm.

mic corresponded with fusion or apposition of the surface epithelium, with a small dorsal gutter left in between (Fig. 5C). A clear line in HDmic appeared mostly as apposed (Fig. 5D), sometimes open neural folds. Non-apposed neural folds in HDmic appeared mostly separated (Fig. 5E), sometimes apposed in LM sections.

During development, a posterior shift of the main sites of apposition and fusion occurred, but the pattern of open, apposed and fused neural folds for both surface epithelium and neurepithelium appeared irregular, especially at the younger stages. An LM inventory of the raphe, performed in serial LM sections at three developmental stages (Fig. 6), confirms this posterior shift and the irregular pattern. Initial fusion was seen ei-

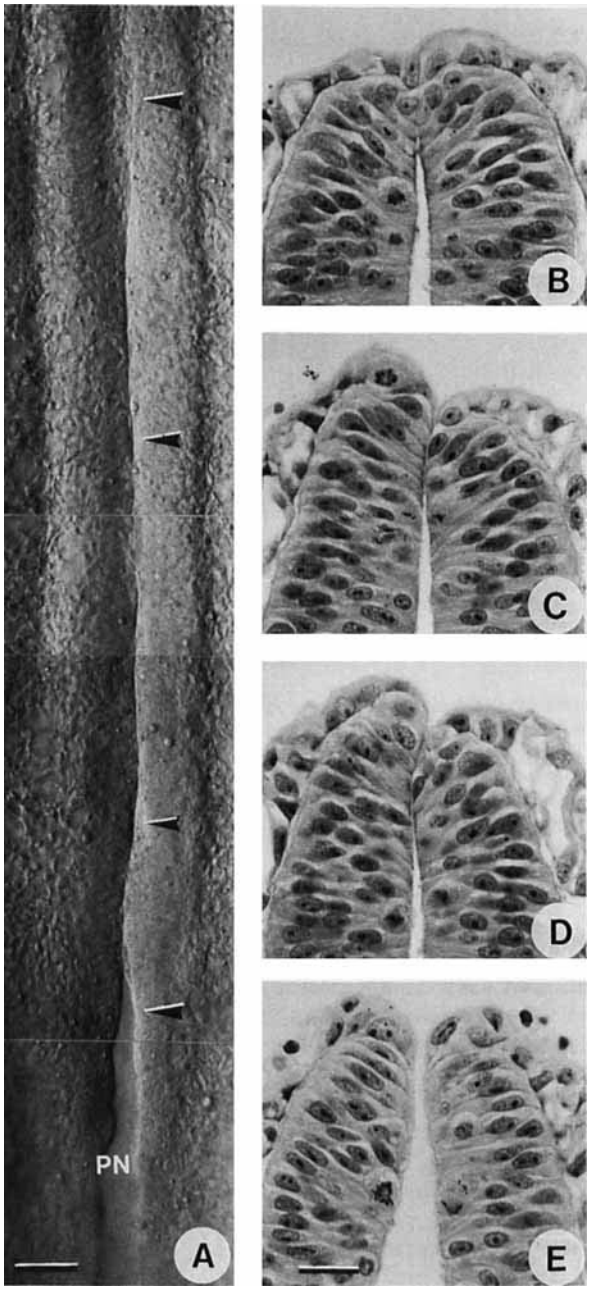


Fig. 5. Appearance of the PN and raphe with HDmic and LM in a 14-somite chick embryo. **A:** High power view of the posterior region of the living embryo. The PN is almost closed. The raphe is irregular, with apposed and fused sites. Anterior is up. Bar, 50 μm. **B–E:** Histology of the PN and raphe from the same embryo. The approximate levels of the sections are indicated by arrowheads (in A). B: Fusion of surface epithelium and neuroepithelium. C: Both epithelia apposed. D: The neuroepithelium is apposed while the surface epithelium is open. E: Level of the open PN. Bar, 25 μm.

ther between the surface epithelium or between the neurepithelium; a spatiotemporal preference of fusion by one epithelium was not seen.

It thus appeared that closure of the posterior neural tube occurred locally, and simultaneously at several sites, and did not proceed antero-posteriorly like a linear zipper, although an overall trend from anterior to posterior is clearly present.

In order to see the impact of embryo processing on the structural integrity of PN and raphe, embryos were viewed in ovo after the least distortion possible. It appeared that the irregular shape of the neural tube raphe already exists in the relatively undisturbed embryo.

Experimental Manipulation To Produce Reopening of the Raphe

The reopening of the raphe in concave-cultured chick embryos suggested that the raphe easily reopens. We performed two additional experiments in order to study the features and expected ease of reopening of the raphe in more detail.

In the first experiment, the posterior region of six embryos was mechanically widened with a forceps. The raphe could be opened up to a fusion site (Fig. 7). The projection of the neural folds was reduced, and the lumen became very wide. The same experiment performed at older stages resulted in less widening; several relatively tight adhesive bridges prevented further separation of the neural folds (Fig. 8). After removal of the forceps the walls approached each other and regained their original position within a few minutes in all cases studied.

In the second experiment, 5 embryos were immersed in a minimal amount of fluid. When embryos were left in an uncovered Petri dish on the warm stage of the HDmic, both the PN and raphe widened, resembling the results in concave-cultured embryos, although marked effects were observed now within 15 min (Fig. 9). Reopening in the anterior direction occurred up to a point of fusion. During widening of the raphe, small bridges frequently arose between the separating folds. These adhesions subsequently broke and both halves retracted to the neural folds within 1–2 min (Fig. 10), much like viscous droplets which melt with their substratum. In this experiment, dehydration is the likely cause of reopening.

DISCUSSION

Are Irregularities of PN Neural Folds and of Raphe an Artifact?

The present study indicates that the edges of the neural folds of the PN are irregular in 9–15 somite stage chick embryos, and that the folds contact each other rather locally in the neural tube raphe. This appeared the natural state of the PN and raphe. However, it became evident that the raphe can easily be disrupted. When the embryos were viewed for the first time with the HDmic, they had undergone several procedural steps which could have deformed the PN and raphe: 1) cracking the egg shell; 2) dropping the egg contents in a bowl with saline; 3) cutting out the embryo; 4) dragging the embryo onto a carrier; 5) allowing

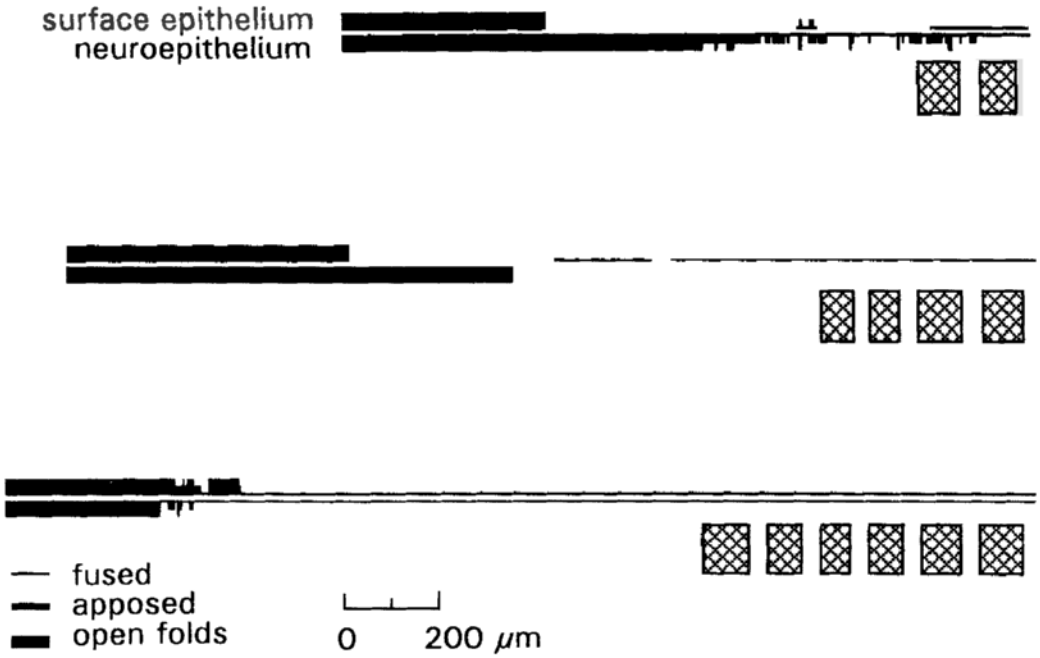


Fig. 6. Structure of the raphe zone as seen in LM serial sections in three embryos of 10, 12, and 14 somite pairs. The neural fold edges were judged as open, apposed or fused for its components: surface epithelium and neuroepithelium. Somites are indicated on the right. The PN (thick, continuous bar) was open in all three embryos, although to a different extent. In the 10-somite embryo the raphe exists as open, apposed and fused sites. The pattern is irregular. Also in the cervical region (beyond the figure) no continuous fusion was seen. In the 12-somite embryo the neural folds of the raphe have open, apposed and fused parts, but more anteriorly continuous fusion was seen, exept for some small open or apposed sites. In the 14-somite embryo the larger part of the raphe has disappeared, due to fusion. Only posteriorly some open and apposed sites are seen. In general, surface epithelium and neuroepithelium did not fuse simultaneously. Drawings are aligned at somite pairs 10. The left end of the figures corresponds with the distal site of the PN.

the embryo to slide from the carrier onto the substratum; 6) release of the vitelline membrane; 7) adaptation of the embryo to the shape of the substratum during removal of most of its immersion fluid. Some or all of these procedural steps might result in apposition of initially open regions, or in reopening of already apposed or even fused regions; the irregularities of the neural fold edges might be the result of reopening of the folds or of wrinkling of the folds during embryo processing. However, chick embryos in ovo, after the minimum disturbance possible, also had irregular neural fold edges, and an irregular raphe did occur over a considerable length. Although distortion of the raphe during embryo processing is not excluded by this observation, it does indicate that the in vitro HDmic figures show features which resemble the in ovo state of the PN and raphe of the chick embryo.

Apposition of neural folds occurs simultaneously at several sites along the raphe. This multilocal feature can be explained by at least two aspects of neurulation: 1) bulging of the neural folds, and 2) fast elevation of the neural walls.

The medial bulgings of the folds are likely the first sites which contact the opposing fold during convergence, resulting in local rather than linear apposition. By through-focus observation with the HDmic it was seen that they do not only bulge in a horizontal, but also in a vertical direction. This might explain misalignments during apposition, resulting in overlap of the folds (Fig. 4), which was particularly evident during closure of reopened raphes where folds were relatively large and thus more easily subject to mis-alignment.

Elevation of the neural walls and convergence of the neural folds are important shaping events in the formation of the posterior neural tube. This has been described for the chick embryo in detail (Portch and Barson, 1974; Bancroft and Bellairs, 1975; Santander and Cuadrato, 1976; Silver and Kerns, 1978; Jacobson, 1981; Schoenwolf, 1979, 1982, 1985, 1991; Schoenwolf and Smith, 1990). In short, during elevation of the PN region the lateral neural walls are lifted upwards, thereby bending around a medial hinge point, the floor plate. Preceeding elevation, the dorsolateral parts of the neural walls, the neural folds, start to bend at the lateral hinge points, whereby a lateral sulcus becomes visible. The total medial movement of the neural folds—convergence—is thus the result of both elevation of the neural wall and bending of the folds at the lateral hinge points. In the chick embryo, elevation of the PN walls and thus convergence, occurs relatively fast, and both Silver and Kerns (1978) and Schoenwolf

(1979) reported that the neural folds in the posterior region came into contact along their entire length. We therefore suggest that the appearance of multilocal apposition sites along the raphe is the result of a combination of both the bulging anatomy and the simultaneous convergence of the folds over a considerable distance.

Raphe Subject to Reopening

Due to the fast apposition of the neural folds in the antero-posterior direction, the permanence of contacts between the apposed folds is likely to be tenuous. Indicative for this, the apposed neural folds in the newly formed raphe can easily be separated. The only signs of adhesiveness between the folds are cellular bridges arising between splaying folds (Jaskoll et al., 1991). The present study further substantiated that such bridges were likely features of initial closure, being exposed only because of (artifactual) separation of neural folds. The neural cell adhesion molecule (N-CAM) is thought to play a role in fusion; it was observed especially on the neural fold edges before and during apposition (Jaskoll et al., 1991). The artifactual exposure of cellular bridges in the present experiments suggest N-CAM mediated adhesion was present, but that more permanent cell contacts were not yet established.

At later stages, mechanical reopening resulted in tight, difficult to break thread-like connections between the folds. This suggests that the raphe adhesions were more permanent. Such filopodia-like connections probably indicate persistent cellular connections, i.e., sites where fusion had taken place. They are probably identical to those seen with scanning electron microscopy (SEM) (Bancroft and Bellairs, 1975; Schoenwolf, 1979, his Fig. 19). Schoenwolf (1979) suggested that these structures might be artifacts, produced during embryo processing. Our experiments strongly support the conclusion that these bridges *are* artifactual, and are created by forced reopening of tightly adhesive sites.

Areas with some adhesion showed a clear raphe in HDmic. Subsequent thinning of the line, and its final disappearance likely indicated progressive fusion of cell membranes. Experimental reopening never proceeded beyond such sites of fusion. In LM, at such sites continuity of the neuroepithelium and/or surface epithelium were seen. These areas are likely the same as described in SEM studies as local cellular junctions (Santander and Cuadrato, 1976; Schoenwolf, 1979).

PN Closure: No Zipper-Like Action

The present study has shown that the PN in the living chick embryo does not close in a linear manner, but rather locally. The raphe thus appears as more than a simple "suture." This was already noticed by Bancroft and Bellairs (1975) from SEM observations; they explained the irregular pattern of the raphe "as if a few teeth of a zip fail to close in usual order, but are able to do so subsequently." It is also consistent with the SEM observations of Silver and Kerns (1978), who described the fusion as discontinuous, both superficial to deep, and anterior to posterior. Both surface epithelium and neuroepithelial cells are involved in fusion. The surface epithelium was reported to fuse first in the PN region (Bancroft and Bellairs, 1975; Schoenwolf, 1979, 1982), but our results do not confirm such an ordered sequence; they better fit with the abovementioned SEM observations of Silver and Kerns (1978). Desmond and Field (1992) defined the point of fusion by tearing apart the raphe with a tungsten needle. It is possible that they reopened beyond the posterior-most fusion site, thereby damaging several sites of fusion. In fact, our results indicate that it is difficult to define one single fusion point; rather a series of fusion sites exist, with increasing tension in the anterior direction. We found that sites converging first (bulges) are the same sites to appose and fuse first; from such sites closure probably occurs in both the anterior and posterior direction.

We conclude that no linear zipper-like closure occurs in the posterior raphe zone. Fusion starts at several sites. In general, however, an antero-posterior gradient of closure does exist.

Neural Tube Closure in Other Regions of the Chick Embryo

Initial apposition of the chick neural folds occur in the mesencephalic region at stage 8, as studied by SEM (Bancroft and Bellairs, 1975) and HDmic (Jaskoll et al., 1991). Although a bidirectional zipper mechanism is suggested, Jaskoll et al. (1991) described "zipping up" as imperfect, with small regions remaining temporarily open, although not as distinct as presently reported for the raphe. The same authors clearly demonstrated de novo closure in the cervical region; initial adhesion was seen at the level of somites, whereas the intersomitic areas closed last. It was suggested that the somites assisted in elevation of the neural plate during their formation. Since from this stage (9) onwards the raphe progresses in posterior direction faster than somites are formed (see Fig. 6), most irregularities seen in the present study are not associated with somite development.

Mechanisms of Reopening and Reclosure of the Raphe

From HDmic observations it was found that in mechanically reopened raphes the elevated walls became depressed and the convergent folds were tilted upright. As can be deduced from Figure 1, the tension was likely exerted at the level of the lateral hinge points. In the dehydration experiments, reduction of convergence and elevation also occurred. We suggest that dehydration resulted in shrinkage of the blastoderm, pulling the neural folds laterally, again with the point of maximum tension exerted at the level of the lateral hinge points. In both experiments, the raphe reclosed quickly after initial disruption. It is possible that neurulation forces (resulting in elevation and convergence) per-

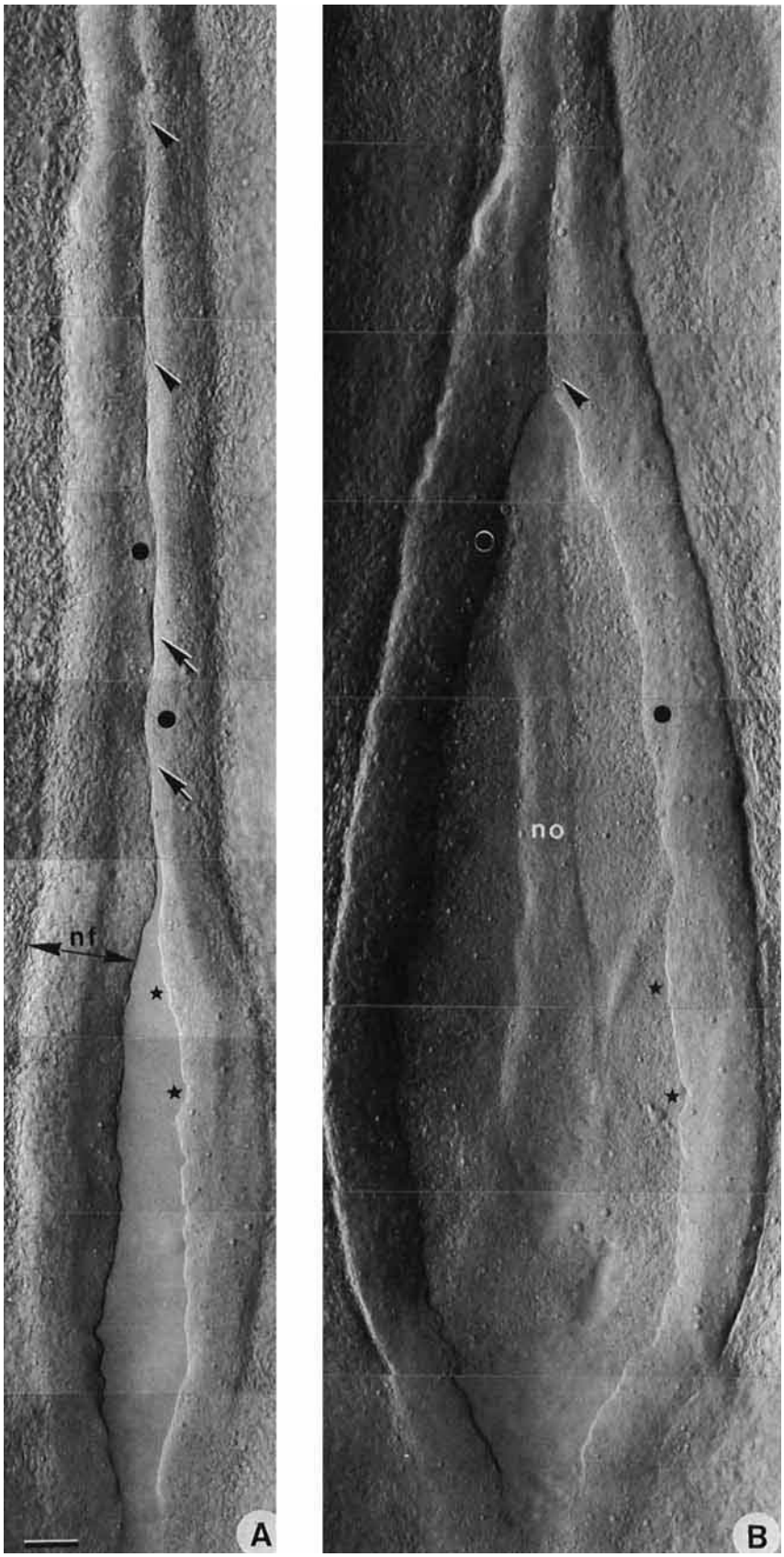


Fig. 7.

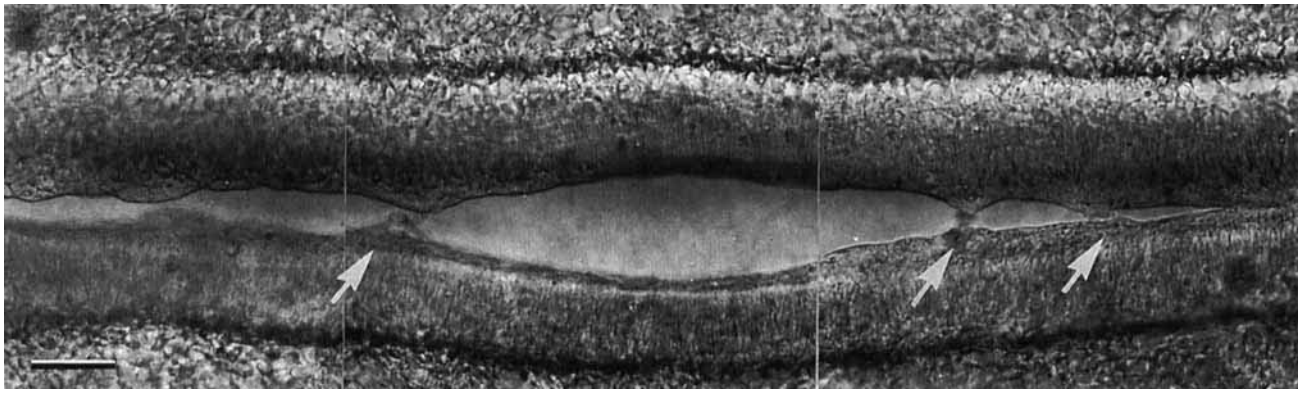


Fig. 8. Mechanical reopening of the raphe. In this 13-somite embryo the PN was closed initially. The raphe was reopened by forceps as in Figure 7. Several hard-to-break, tight connections (arrows) resisted the forceps tension and kept the folds together. Anterior is to the right. Bar, 50 μm.

sisted during the experimental procedure and subsequently restored the shape of the neural groove, or that compensatory forces, unrelated to normal neurulation, occurred.

The mechanism of the slow reopening of the raphe in concave-cultured embryos might be compared to a straight U-shaped gutter, which is forced to a concave shape. Even the slightest bending will immediately result in eversion of the gutter edges. Apparently, the neural folds could not adapt to the new situation adequately. However, the opened raphe did reclose after several hours, likely caused by increased convergence. Such adaptation to changes in axial angles indicates that during neurulation a mechanism of corrective adaptation can be mobilized with respect to fold convergence.

Convergence is the result of elevation (bending of the neural plate at the medial hinge point) but especially of medial bending of the neural folds at the lateral hinge points. Forces for medial bending are thought to be generated both intrinsic and extrinsic to the neural plate. As intrinsic forces, differential longitudinal stretching of the neural plate and cell wedging at the lateral hinge points (due to apical constriction) is suggested; as extrinsic forces, the surface ectoderm could play a role (reviewed by Schoenwolf and Smith, 1990). In amphibians, active crawling of neuroepithelial cells underneath the surface epithelium is suggested, which would result in bending of the fold (Jacobson, 1991). In the chick embryo, the surface epithelium and neuroepithelium of the cephalic neural fold were described to be attached at the top and the lateral hinge point only. Differential growth of both layers, therefore, could cause bending (Caso et al., 1992). Surface epithelium was suggested to provide the major extrinsic force for bending (elevation and convergence) of the neural plate, due to medially directed extension (Schoenwolf and Alvarez, 1991; Alvarez and Schoenwolf, 1992). Further studies of fold convergence and its corrective adaptation mechanisms are clearly needed.

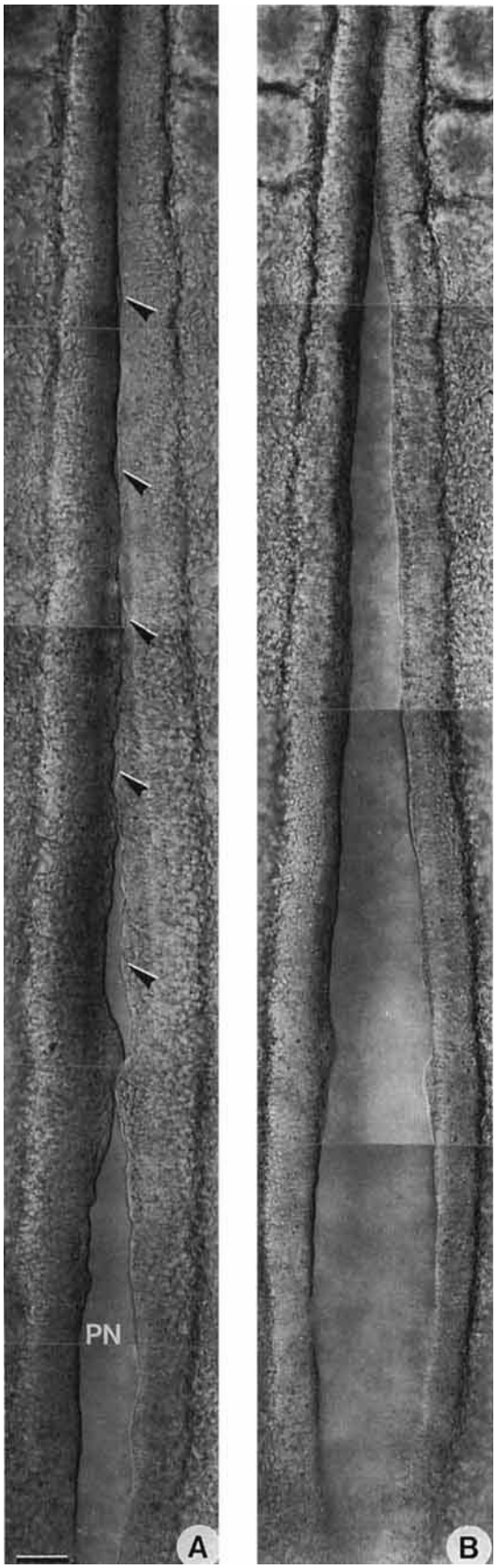
The present study has shown that neurulation in the posterior chick embryo is a delicate process which can be easily disrupted, but which also shows adaptive correction mechanisms. It is important to study these details of neurulation in living mammals in order to find out if multilocal adhesion, ease of reopening and corrective adaptation does occur there, too. Such data may elucidate causes and mechanisms by which closure defects of the NP arise.

Fig. 7. Mechanical reopening of the PN and raphe in a 12-somite chick embryo. **A:** Features of the PN and raphe zone immediately after placing the embryo in culture. The lower part of the figure shows the PN zone, where the folds are still separated from each other. The upper part shows the raphe zone, where contact between the folds occurs. The neural walls are elevated over the whole trajectory, as appears from their close position, although the least in the PN zone. In longitudinal aspect, the walls are slightly curved in the PN zone, and approximately mirror-imaged. In this dorsal viewing, extension of the neural folds (nf) is indicative of their rate of convergence. The edges of the neural folds, both in the PN and raphe zone, are very irregular. In the latter, open or apposed (arrows) neural fold sites are seen, and at least two fused sites (arrowheads) can be recognized. **B:** Photographs made during separation of the walls by forceps, placed laterally to the PN. The PN is increased in width considerably and the extension of its neural folds is decreased, but they do not diverge. The medial edges of the folds are still irregular (compare, e.g., at asterisks). The raphe did reopen anteriorly up to the first fusion site (arrowhead). The neural fold edges of the previous raphe are still irregular, and some of the bulges can be traced back (compare, e.g., at dots). The apposition site in between both fusion sites in A has not changed shape. The visibility of the notochord (no) indicates that the neural walls and folds are de-elevated considerably. Figures are aligned on the surface blebs near the asterisks. Anterior is up. Bar, 50 μm.

EXPERIMENTAL PROCEDURES

Examining Chick Embryos In Ovo

White Leghorn eggs, obtained from K and R (Westminster, CA) were incubated in an humidified incubator at 37°C for 42–48 hr. For in ovo studies, two pinholes were made in the air chamber, the chamber was subsequently filled with saline through one hole and both holes sealed. Then a hole of 5 × 8 mm was made



in the shell, above the embryo. The shell membranes were carefully excised, whereby the embryo did not sink down because the air chamber was saline-filled. The area of interest of the exposed embryo was immediately viewed with a WILD stereo-microscope.

Examining Embryos With High Definition Light Microscopy

The procedure to culture chick embryos on specified substrata has been described elsewhere (van Straaten et al., 1993). In brief, eggs were carefully broken and slowly opened in a bowl with warm saline. The embryo was cut out outside their area vasculosa, and placed in a petri dish on a substratum with their dorsal side up. The covering vitelline membrane was released from the blastoderm at its peripheral attachment but was subsequently left in situ in order to prevent dehydration. Excess fluid was removed to allow the embryo to adapt to the shape of the substratum. The culture substratum consisted of equal portions of agar 1% (Difco, Detroit, MI) in 0.9% NaCl and egg white. The culture substratum was flat, or concave with an angle of $-1°$ per mm. The Petri dishes were covered and maintained in a moist chamber at 37°C. Only embryos with 9 to 15 somite pairs (stages 10–11$^+$, Hamburger and Hamilton, 1951) were used. Embryos were examined with the EDGE high definition light microscope (HDmic), (Scientific Instrument Corporation, Los Angeles, CA), equipped with a 37°C temperature controlled stage and long working distance objectives. The embryo was trans-illuminated from three directions from below by obliquely installed light sources with different light intensities. This resulted in a high contrast. Kodak TMax100 was used for photography. To study neurulation in flat and concave-cultured embryos, once every hour the Petri dish was placed on the microscope stage, the lid removed and the area of interest photographed within 30 seconds. This short period was introduced in order to minimize evaporation, although exposure even up to 5 min did not result in dehydration effects (see below). The process of neurulation was followed during 3–12 hr. The vitelline membrane was optically clear, but would sometimes interfere with clear vision, as can be seen in Figure 10.

Histology and Reconstruction of the Raphe Zone

For light microscopy, 3 chick embryos (10, 12, and 14 somite pairs) were photographed with the HDmic and

Fig. 9. Reopening of the PN and raphe after dehydration in a 12-somite chick embryo. **A:** The PN and raphe zone immediately after placing the embryo in culture. Approximately identical features as described for Figure 8A can be seen. In the raphe zone both open (arrowheads) and apposed folds are present; clear fusion sites were not seen. **B:** Reopening due to 10 min of evaporation on the microscope stage. A decrease in extension of the neural folds is seen both in the PN and previous raphe zone. This decrease is at least partly explained by de-elevation, as indicated by the widening of the lumen. The raphe zone has reopened over a considerable length, although probably not up to a fusion site. Anterior is up. Bar, 50 μm.

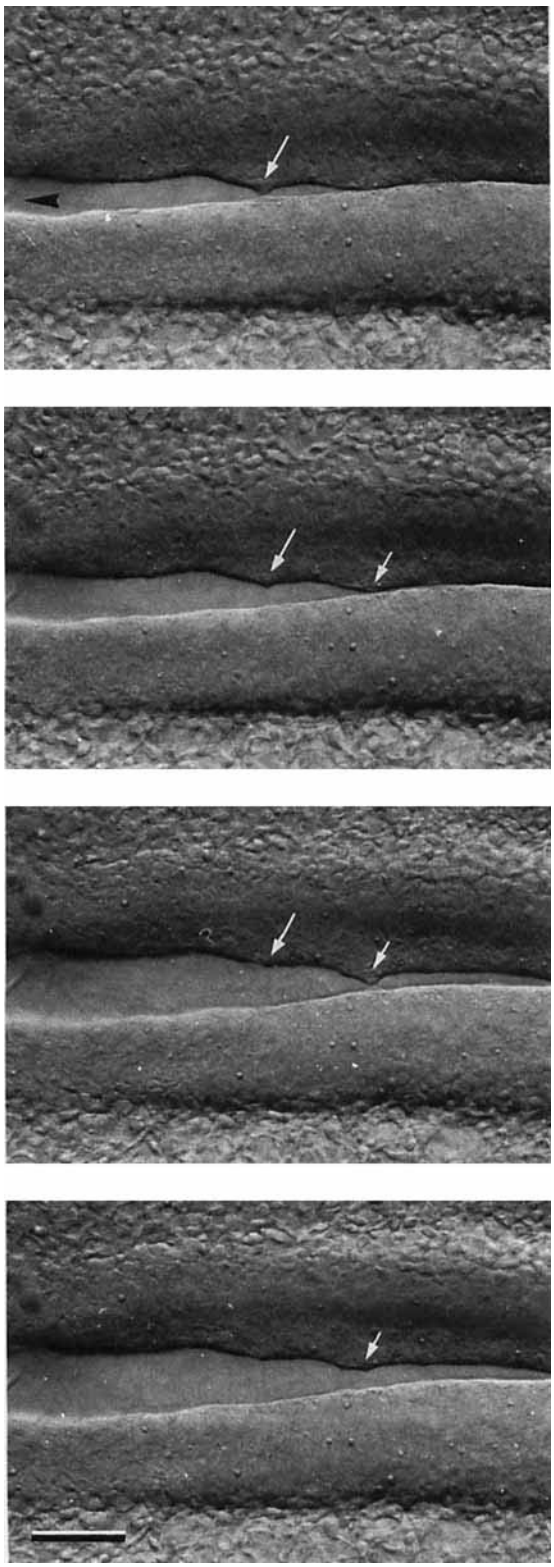


Fig. 10. Reopening of the raphe by dehydration in a 12-somite chick embryo. At the beginning of culture, no openings were seen. Five minutes after the start of dehydration experiment these four figures were made with intervals of one minute. Two adhesive bridges can be seen arising, breaking and retracting (arrows). Some wrinkles of the covering vitelline membrane are seen (arrowhead). Anterior is to the right. Bar, 50 μm.

immediately placed in Bodian's fixative [90 ml ethanol (80%), 5 ml acetic acid (99%) and 5 ml formaldehyde (37%)]. They were subsequently dehydrated in ethanol, embedded in Historesin (Kulzer and Co. GmbH, Philipp-Reis-Strasse 8, D-6393 Wehrheim (Taunus), Germany), and again photographed. By comparing both photographs, the embryonic length appeared 89% of its original value. The embryos were sectioned at 5 μm, stained with toluidine blue. Based on several surface markers and the calculated shrinkage, the sections could be related to the initial photograph (Fig. 5). Every section was screened for open, apposed or fused surface epithelium and neuroepithelium; these data are depicted in Figure 6.

Experimental Procedures To Reopen the Raphe

Embryos were removed from the egg as described above and placed in a Petri dish without both agar substratum and vitelline-membrane. Reopening of the raphe was established as follows: in 5 cases, the embryo was immersed in some fluid, and photographed with the HDmic; the legs of a watchmakers forceps were placed bilaterally to the closing region, about 200 μm lateral to the neural folds, and released in order to mechanically reopen the raphe, which was immediately photographed; subsequently the forceps was removed, the Petri dish was closed and after several minutes the closing region was examined again. In 6 embryos only a minimum amount of fluid was left onto the embryo; during examination of the embryo with the HDmic the Petri dish was left uncovered for several minutes, allowing evaporation.

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REFERENCES

Alvarez, I.S. and Schoenwolf, G.C. (1992) Expansion of surface epithelium provides the major extrinsic force for bending of the neural plate. J. Exp. Zool. 261:340–348.

Bancroft, M. and Bellairs, R. (1975) Differentiation of the neural plate and neural tube in the young chick embryo. Anat. Embryol. 147: 309–335.

Brook, F.A., Shum, A.S.W., van Straaten, H.W.M., and Copp, A.J. (1991) Curvature of the caudal region is responsible for failure of neural tube closure in the curly tail (ct) mouse embryo. Development 113:671–678.

Caso, M.F., De Paz, P., Fernandez Alvarez, J.G. Chamorro, C., and Villar, J.M. (1992) Delamination of neuroepithelium and nonneural ectoderm and its relation to the convergence step in chick neurulation. J. Anat. 180:143–153.

Copp, A.J., Brook, F.A., Estibeiro, J.P., Shum, A.S.W., and Cockroft, D.L. (1990) The embryonic development of mammalian neural tube defects. Prog. Neurobiol. 35:363–403.

Desmond, M.E. and Field, M.C. (1992) Evaluation of neural fold fusion and coincident initiation of spinal cord occlusion in the chick embryo. J. Comp. Neurol. 319:246–260.

DiVirgilio, G.N., Lavenda, N., and Worden, J.L. (1967) Sequence of events in neural tube closure and the formation of neural crest in the chick embryo. Acta Anat. 68:127–146.

Geelen, J.A.G. and Langman J. (1977) Closure of the neural tube in the cephalic region of the mouse embryo. Anat. Rec. 189:625–640.

Gordon, R. (1985) A review of the theories of vertebrate neurulation and their relationship to the mechanics of neural tube birth defect. J. Embryol. Exp. Morphol. 89 (suppl):229–255.

Hamburger, V. and Hamilton, H.G. (1951) A series of normal stages in the development of the chick embryo. J. Morphol. 88:49–92.

Jacobson, A.G. (1981) Morphogenesis of the neural plate and tube. In: Connelly, T.G., Brinkley, L.L., and Carlson, B.M. (eds). "Morphogenesis and Pattern Formation," New York: Raven Press, pp 233–263.

Jacobson, A.G. (1991) Experimental analyses of the shaping of the neural plate and tube. Am. Zool. 31:628–643.

Jaskoll, T., Greenberg, G., and Melnick, M. (1991) Neural tube and neural crest—a new view with time-lapse High-Definition photomicroscopy. Am. J. Med. Genet. 41:333–345.

Karfunkel, P. (1974) The mechanism of neural tube formation. Int. Rev. Cytol. 38:245–271.

Portch, P.A. and Barson, A.J. (1974) Scanning electron microscopy of neurulation in the chick. J. Anat. 117:341–350.

Santander, R.G. and Cuadrato, G.M. (1976) Ultrastructure of the neural canal closure in the chicken embryo. Acta. Anat. 95:368–383.

Schoenwolf, G.C. (1979) Observations on closure of the neuropores in the chick embryo. Am. J. Anat. 155:445–466.

Schoenwolf, G.C. (1982) On the morphogenesis of the early rudiments of the developing central nervous system. Scanning Electron Microsc. I:289–308.

Schoenwolf, G.C. (1985) Shaping and bending of the avian neuroepithelium: morphometric analysis. Dev. Biol. 109:127–139.

Schoenwolf, G.C. (1991) Cell movements driving neurulation in avian embryos Development (Suppl):157–168.

Schoenwolf, G.C. and Alvarez, I.S. (1991) Specification of neuroepithelium and surface epithelium in avian transplantation chimeras. Development 112:713–722.

Schoenwolf, G.C. and Smith, J.L. (1990) Mechanisms of neurulation—traditional viewpoint and recent advances. Development 109:243–270.

Silver, M.H. and Kerns, J.M. (1978) Ultrastructure of neural fold fusion in chick embryos. Scanning Electron Microsc. II:209–215.

van Straaten, H.W.M., Hekking, J.W.M., Copp, A.J., and Bernfield, M. (1992) Deceleration and acceleration in the rate of posterior neuropore closure during neurulation in the curly tail (ct) mouse embryo. Anat. Embryol. 185:169–174.

van Straaten, H.W.M., Hekking, J.W.M., Consten, C., and Copp, A. (1993) Intrinsic and extrinsic factors in the mechanism of neurulation: effect of curvature of the body axis on closure of the posterior neuropore. Development 117:1163–1172.