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Intrinsic and extrinsic factors in the mechanism of neurulation: effect of curvature of the body axis on closure of the posterior neuropore

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SUMMARY

Neurulation has been suggested to involve both factors intrinsic and extrinsic to the neuroepithelium. In the curly tail (*ct*) mutant mouse embryo, final closure of the posterior neuropore is delayed to varying extents resulting in neural tube defects. Evidence was presented recently (Brook et al., 1991, *Development* 113, 671-678) to suggest that enhanced ventral curvature of the caudal region is responsible for the neurulation defect, which probably originates from an abnormally reduced rate of cell proliferation affecting the hindgut endoderm and notochord, but not the neuroepithelium (Copp et al., 1988, *Development* 104, 285-295). This axial curvature probably generates a mechanical stress on the posterior neuropore, opposing normal closure. We predicted, therefore, that the *ct/ct* posterior neuropore should be capable of normal closure if the neuroepithelium is isolated from its adjacent tissues. This prediction was tested by in vitro culture of *ct/ct* posterior neuropore regions, isolated by a cut caudal to the 5th from last somite. In experimental explants, the neuroepithelium of the posterior neuropore, together with the contiguous portion of the neural tube, were separated mechanically from all adjacent non-neural tissues. The posterior neuropore closed in these explants at a similar rate to isolated posterior neuropore regions of non-mutant embryos. By contrast, control *ct/ct* explants, in which the caudal region was isolated but the neuroepithelium was left attached to adjacent tissues, showed delayed neurulation. To examine further the idea that axial curvature may be a general mechanism regulating neurulation, we cultured chick embryos on curved substrata in vitro. Slight curvature of the body axis (maximally 1° per mm axial length), of either concave or convex nature, resulted in delay of posterior neuropore closure in the chick embryo. Both incidence and extent of closure delay correlated with the degree of curvature that was imposed. We propose that during normal embryogenesis the rate of neurulation is related to the angle of axial curvature, such that experimental alterations in curvature will have differing effects (either enhancement or delay of closure) depending on the angle of curvature at which neurulation normally occurs in a given species, or at a given level of the body axis.

Key words: embryo, mouse, chick, neurulation, posterior neuropore, axial curvature

INTRODUCTION

Neurulation is the complex process by which the neural tube develops from the neural plate. It involves elevation and subsequent inward bending of the neural folds and medial fusion of their tips. Several factors have been suggested to regulate these events, both processes intrinsic to the neural plate as well as those originating in the surrounding tissues (for reviews see Gordon, 1985; Copp et al., 1990; Schoenwolf and Smith, 1990).

One possible mechanism that has so far received little attention is the role of curvature of the body axis in regulation of neurulation. In the mouse embryo, Jacobson and Tam (1982) found a coincidence between closure of the neural plate in the mesencephalic region and a temporary cessation of axial unbending in this region. More recently, evidence was presented for a role of axial curvature in determining the rate of closure of the posterior neuropore (PNP) in the mouse mutant curly tail (*ct*; Brook et al., 1991). Embryos homozygous for the *ct* mutation develop lumbosacral spina bifida in 20% of cases and tail flexion defects in a further 40% of offspring (Grüneberg, 1954; Embury et al., 1979; Copp et al., 1982). At mid-gestation, *ct/ct* embryos have an enlarged PNP as a result of delayed neural tube closure; the magnitude and duration of this delay is known to correlate with the severity of the neural tube defects that result (Copp, 1985; van Straaten et al., 1992).

Brook et al. (1991) showed that enhanced ventral curvature of the PNP region is correlated with delay of PNP closure in *ct/ct* embryos and demonstrated, moreover, that experimental prevention of the enhanced ventral curvature

abolished the delay in closure. These results suggested that enhanced ventral curvature is the primary cause of the PNP closure defect in the *ct* mutant. It was suggested that ventral curvature imposes a mechanical stress on the caudal region which opposes neurulation. According to this model, the neural plate is regarded as playing a passive role in the generation of closure delay in *ct/ct* embryos, since the curvature probably arises from a cell proliferation defect affecting ventral tissues, the notochord and endoderm, but not the neuroepithelium (Copp et al., 1988).

A prediction of this model of *ct* developmental defects is that the neural plate of *ct/ct* embryos is unaffected in its ability to undergo closure at the PNP, but is prevented from doing so by mechanical stress imposed by adjacent structures. To test this prediction, we have studied the ability of explanted caudal regions (neural folds and contiguous neural tube, mechanically isolated from the surrounding tissues in order to release mechanical stress) of *ct/ct* embryos to undergo PNP closure in vitro. As controls, we explanted intact caudal regions of *ct/ct* embryos of the same body length but additionally contained the non-neuroepithelial tissues. The result shows that, as predicted, the *ct* PNP region is capable of apparently normal PNP closure in the absence of most adjacent structures.

To examine further whether axial curvature may be able to modify the rate of PNP closure in species other than the mouse, we have cultured chick embryos on weakly curved substrata. PNP closure is delayed in a manner that is related directly to the angle of curvature imposed, suggesting that axial curvature may be a general mechanism regulating neurulation in the vertebrate embryo.

MATERIALS AND METHODS

Mouse strains

The curly tail (*ct*) mutation arose in a female of the inbred GFF strain that was subsequently mated with a CBA/Gr male. The F_1 offspring were kept as a closed, random-bred colony thereafter (Grüneberg, 1954). Outbred Swiss CD-1 (Sw) mice were commercially obtained (Charles River Wiga GmbH, Germany). Both strains were maintained on a 12 hours light:12 hours dark regime. Sw mice were paired overnight, and *ct/ct* mice between 8 and 12 a.m. to produce timed pregnancies. The day of finding a plug was designated day 0 of pregnancy.

Culture of caudal regions and neural tubes of mouse embryos

Female mice were killed on day 9 (Sw) or day 10 (*ct*) of pregnancy and the uterus was explanted into a modification of Eagle's minimal essential medium (Gibco), buffered with 20 mM Hepes and containing 10% fetal calf serum. Embryos were dissected free of their extraembryonic membranes. The number of somites was determined; only embryos with 20-29 somites were processed further. The hypothesis was tested that PNP closure should occur normally in *ct/ct* neural plates after removal of the surrounding non-neural tissues. The caudal region was isolated from some embryos by transsecting the entire body axis at the level of the 5th from last somite, using a tungsten needle. In preliminary experiments, we determined the extent to which isolated mouse neuroepithelium will undergo neural tube closure in vitro. Closure did not occur in the neural plate/neural folds of the PNP when they were cultured in isolation from the contiguous closed neural tube. On the other hand, closure occurred in an apparently normal rostrocaudal sequence when the neural plate/neural folds were left attached to a portion of the already closed neural tube. Thus in subsequent experiments, all explants consisted of the neural plate/neural folds of the PNP plus an attached portion of neural tube. In experimental explants, the neuroepithelium was cultured in isolation from non-neural tissues. Separation of tissues following digestion with cold 0.1% trypsin in PBS for 10 minutes, was found to result in failure of closure of the PNP in culture. Therefore, tissue separation was accomplished using tungsten needles without the use of enzymes. This resulted in a portion of the surface ectoderm remaining attached to the neural folds and a few mesodermal cells remaining adherent to the ventrolateral part of the neural plate (Fig. 5A,B). The notochord was always removed. In control explants, the neuroepithelium was left attached to the non-neural tissues.

For culture, isolated caudal regions or isolated neuroepithelium of PNP regions were placed in microwells (Nunclon, Intermed, Denmark) containing 1 ml of medium 199 (Gibco), supplemented with 10% heat-inactivated calf serum and 1% penicillin/streptomycin (Gibco), for a maximum of 8 hours. The length and width of the PNP was measured every hour, using an eyepiece graticule fitted on a stereo microscope, with the PNP held upright. The number of explants studied is presented in Table 1.

PNP closure rate was expressed in μm/hour (calculated from the slope of the regression line as shown in Fig. 3 for the chick embryo). Statistical comparisons were made using the Student's *t*-test.

Scanning electron microscopy (SEM) and light microscopy

Mouse embryos were fixed in Bodian's fluid (90 ml ethanol (80%), 5 ml acetic acid (99%) and 5 ml formaldehyde (37%)) for 1 hour and dehydrated in ethanol. This fixative has been found, from past experience, to give excellent results with both SEM and light microscopy. For SEM, embryos were critical point dried in liquid CO_2, attached to aluminium stubs with silver paint, coated with gold/palladium and observed on a Cambridge SE 180 scanning electron microscope operating at 15 kV. For light microscopy, cultured caudal parts of mouse embryos were embedded in Historesin (Kulzer and Co. GmbH, Philipp-Reis-Strasse 8, D-6393 Wehrheim (Taunus), Germany), sectioned at 5 μm and stained with toluidine blue.

PNP closure in the chick embryo

Fertilized White Leghorn eggs were obtained from the animal care facility of the University of Maastricht. A large group of chick

Table 1. Rate of closure of the PNP in caudal regions and isolated neuroepithelium of Sw and *ct/ct* embryos

	stage* (somites)	no. explants	PNP closure rate (μm/hour) mean	s.d.
Sw caudal region	20 - 24	66	50	13
Sw caudal region	25 - 29	38	49	18
ct/ct caudal region	20 - 24	35	13	16
ct/ct caudal region	25 - 29	67	21	24
Sw neuroepithelium	20 - 29	17	72	20
ct/ct neuroepithelium	20 - 29	33	89	43

*indicates the number of somites present at the beginning of the culture period. *ct/ct* caudal regions have a significantly slower closure rate than Sw ($P < 0.005$). PNPs close at a significantly higher rate in isolated neuroepithelium than in control caudal regions for both Sw ($P < 0.005$) and *ct/ct* ($P < 0.005$).

embryos (n=379) was used to determine the normal closure pattern of the PNP (Fig. 8). Embryos were excised at various times of incubation between 1.5 and 2 days and explanted in a flat Petri dish. Subsequently, the PNP length was measured and the number of somites was counted. Between the stages of 11 and 16 somites, regression analysis was performed to test whether there was a significant decrease in PNP length with advancing developmental stage (i.e. increasing number of somites). The rate of closure of the PNP was found from the slope of the regression line.

Explantation of chick embryos

At 1.5 days incubation, embryos were excised 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 in order not to hinder growth of the embryo, but was subsequently left in situ in order to prevent evaporation. The culture substratum consisted of equal portions of agar 1% (Difco, Detroit, MI) in 0.9% NaCl and egg white. Only embryos with 11-16 somites, and with an open PNP, were used. Bending of the embryonic axis was provoked by 5 differently shaped substrata: concave with angles of −1° and −0.5°, flat, and convex with angles of +0.5°, and +1° per mm. The culture arrangement is illustrated in Fig. 1. The curved substrata were made using watchglasses with defined curvatures. For convex substrata, molten material was allowed to solidify in the watchglass. For concave substrata, the watchglass was pressed into the substratum material before it had fully solidified. Petri dishes containing embryos on these substrata were maintained in a moist chamber at 37°C for up to 10 hours. Under a stereo-microscope, the PNP was readily visible through the transparent vitelline membrane (Fig. 2). The length of the PNP and the number of somites were measured immediately after explantation and at intervals of about 100 minutes throughout the culture period.

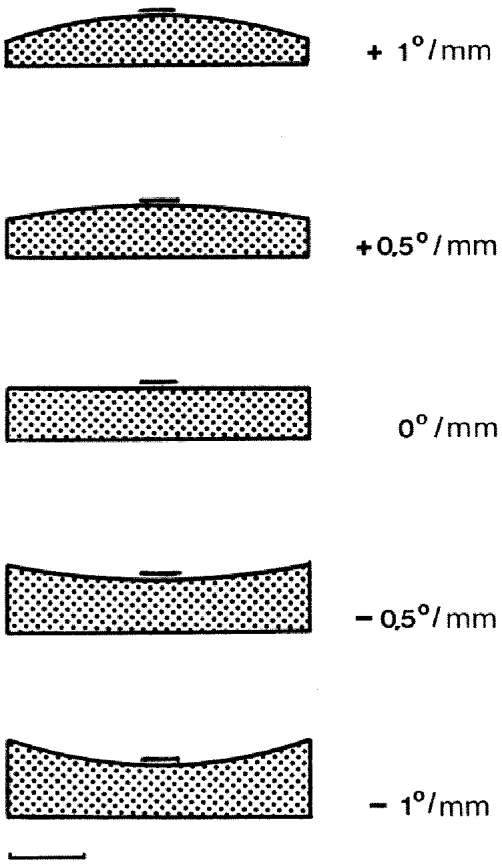

Fig. 1. Drawings of the curved substrata used for chick embryo cultures, with the embryo drawn on top of the substratum. The angles of curvature used were very small. Since the length of the PNP of a 12-somite embryo amounts to about 1 mm (see Fig. 2), the curvature numbers given roughly indicate the bending angle of the PNP region. Drawings are at actual size (scale bar, 10 mm).

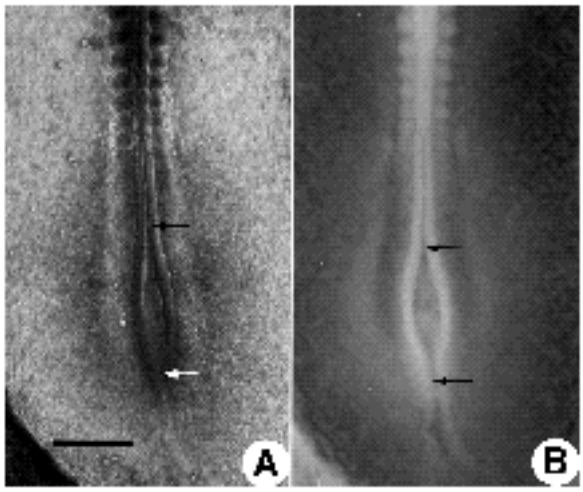

Fig. 2. The PNP region of a 12-somite chick embryo (dorsal view), as observed in the experiments with curved substrata. (A) Embryo in culture, located on a substratum and covered with the vitelline membrane. The neural folds are clearly visible. The length of the PNP, as indicated by the arrows, was measured using an eyepiece graticule fitted onto a stereomicroscope. (B) The same embryo without the vitelline membrane and briefly fixed with Bodian's fluid in order to demonstrate the effect of fixation. The PNP is indicated by arrows. Fixation has widened the PNP slightly, but the length of the PNP is hardly altered. Scale bar, 500 μm.

Analysis of PNP closure pattern in the explanted chick embryo

Changes in length of the PNP during the culture period were graphically depicted as a closure pattern for each embryo. Examples of the two most commonly observed closure patterns are presented in Fig. 3. Some embryos underwent progressive closure of the PNP from the beginning of culture ('non-delayed' pattern in Fig. 3) whereas others exhibited an initial enlargement of the PNP followed, after a variable period, by progressive PNP closure ('delayed' pattern in Fig. 3). In both cases, PNP closure proceeded in a linear fashion and the rate of PNP closure was found from the slope of the regression line (see Fig. 3). The length of the period of delay was measured as described in Fig. 3. The numbers of embryos analysed in each group are listed in Table 2. Testing for correlation between the incidence of closure delay and axial angle for the groups from 0° to +1° and for the groups from 0° to −1° was done using the chi-squared test for trends with a continuity correction. Testing for correlation between the extent of closure delay and substratum angle for the groups from 0° to +1° and for the groups from 0° to −1° was done using the Jonckheere test for ordered alternatives with correction for tied observations; this is a modification of the Kruskal-Wallis one way analysis of variance by ranks (Siegel and Castellan, 1988).

For every embryo in culture, the rate of increase in somite number was found from the regression line of increasing number of somites against hours in culture. Statistical comparison between

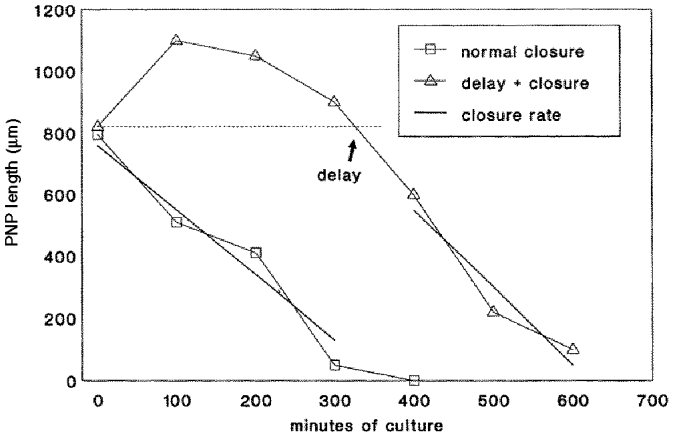

Fig. 3. Examples of the two most commonly observed PNP closure patterns in the chick embryo in vitro as illustrated by data from 2 representative embryos. The duration of closure delay was the time taken for the PNP length to fall to its original value at the start of the culture (as shown by the dashed line). In the example given, the closure delay was 325 minutes. The closure rate was found from the slope of the regression line through at least 3 data points (provided none were zero). In the case of embryos with closure delay, only data points below the dashed line were used for calculation of closure rate.

the five groups of embryos was made using Student's *t*-test (Table 2).

Additional embryos were used for illustration. After 6 hours in culture the covering vitelline membrane was gently removed, the embryos were briefly fixed in Bodian's fluid and immediately photographed (Fig. 9). The brief fixation hardly altered the actual length of the PNP. Some increase in width was seen (compare Figs 2A,B).

RESULTS

PNP closure in cultured caudal regions of the mouse embryo

To test the ability of the isolated mouse PNP to close in vitro, caudal regions were mechanically separated from the rest of the embryo and cultured. In Sw explants, the length of the PNP decreased steadily and, in the most advanced embryos, closure of the PNP was completed during the 8 hour culture period. PNP closure involved a gradual decrease in the length and width of the rhomboidal area of open neural folds, primary due to rostrocaudal movement of the cranial end of the neuropore. Abnormalities of closure pattern, such as de novo fusion of the neural walls within the neuropore, were not seen, and the SEM appearance of the closed neural tube was normal (Fig. 4A). Light microscopy of histological sections of the explants also showed a normal appearance except that some extruded cells were present within the neural tube (Fig. 4B). No difference was observed in the rate of PNP closure, expressed as decrease of PNP length per hour, between embryos explanted at the 20-24 or 25-29 somite stages (Table 1).

In *ct/ct* caudal regions, PNP length also decreased during culture, but at a significantly lower rate and with more variability between embryos than in Sw (Table 1). In several explants the PNP length actually increased during the culture period, although a clear delay pattern such as was seen in the chick embryo (see Fig. 3), was not found.

Table 2. Incidence and duration of closure delay and rate of somite increase in chick embryos of 11-16 somites during in vitro incubation on curved substrata

angle of axial curvature (°/mm)		−1°	−0.5°	0°	+0.5°	+1°
no. embryos used	*n*	23	13	69	23	30
% of embryos with delay of PNP closure		96	100	9	30	90
duration of closure delay (minutes)	mean	279	181	10	53	176
	s.d.	102	85	37	85	106
increase in somite number (per hour)	mean	0.63	0.60	0.67	0.68	0.62
	s.d.	0.10	0.11	0.14	0.11	0.17

With increased curvature, the incidence of closure delay ($P < 0.001$) as well as its duration ($P < 0.001$) increased significantly in both convex and concave directions. No significant differences in rate of somite gain were seen for the five groups of embryos ($P > 0.05$).

PNP closure in the isolated neuroepithelium of the PNP-region

The neuroepithelium of the PNP-region was isolated and cultured in order to test whether PNP closure would occur

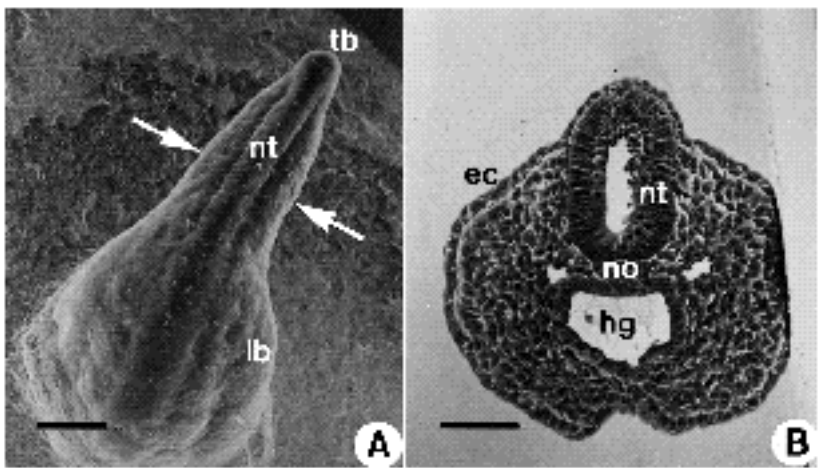

Fig. 4. Control explants of the PNP region. (A) Scanning electron micrograph of the dorsal surface of the explant of a Sw mouse embryo with 27 somites after 6 hours in culture. The closed neural tube (nt) is visible through the dorsal surface ectoderm. tb, tail bud; lb, limb bud. Scale bar, 200 µm. (B) Histological section through an explant of a Sw mouse embryo with 24 somites. The explant has been cultured for 4 hours after which the PNP was closed. The surface ectoderm (ec) covers the closed neural tube (nt). Some extruded cells are present within the neural tube. hg, hind gut; no, notochord. Scale bar, 100 µm.

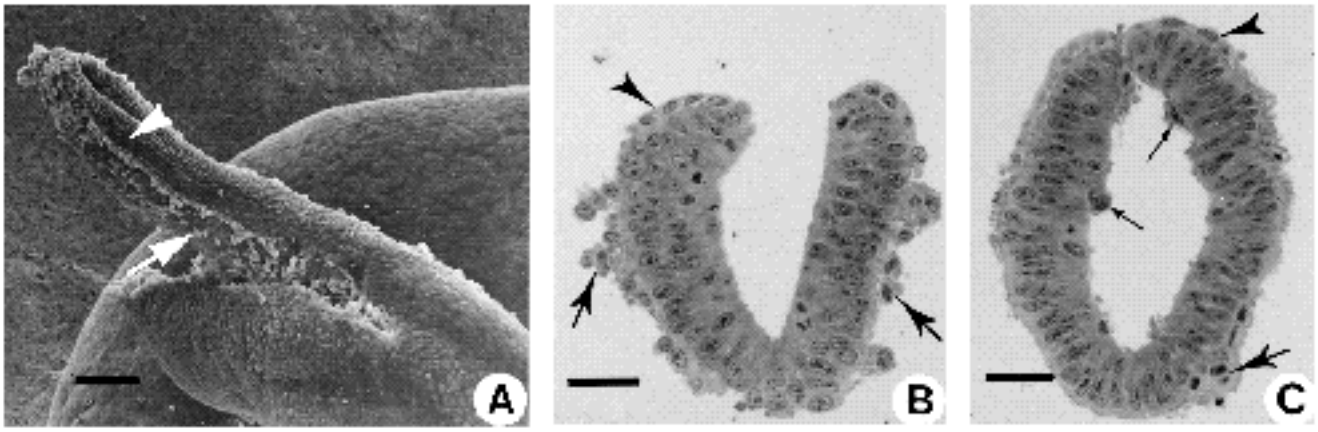

Fig. 5. Neuroepithelial explants. (A) Scanning electron micrograph of the caudal region of an Sw embryo with 27 somites. The caudal neural tube including the PNP region has been separated from the underlying mesoderm by tungsten needles. Ectodermal cells (arrowhead) are still present on the neural folds and a few mesodermal cells (arrow) are adherent to the neural tube. Subsequently the neural tube was transsected from the remainder of the embryo and brought into culture. Scale bar, 100 μm. (B) Histological section through an explant from a Sw mouse embryo of 22 somites, immediately after isolation. As in A, ectodermal cells (arrowhead) are adherent to the neural folds and some mesodermal cells (arrows) are adherent at the ventrolateral side of the neuroepithelium. Scale bar, 50 μm. (C) Neuroepithelial explant from a Sw mouse embryo of 23 somites after 3 hours in culture, when the PNP was closed. In this section the neural folds are apposed and the ectoderm is fused. The neuroepithelial cells resemble those in B, with some degenerating cells present on their apical surface (small arrows). At the basal surface, cells are no longer attached to the neuroepithelium, except for some pycnotic mesodermal cells (arrow). The surface ectoderm is still present (arrowhead). Scale bar, 50 μm.

in *ct/ct* embryos in vitro, after abolition of mechanical stress forces arising from the surrounding non-neural tissues. In such explants, the process of PNP closure (Fig. 5) morphologically resembled that seen in control explants, in which the neuroepithelium was left attached to non-neural tissues. The PNP closed at a similar rate in Sw and *ct/ct* explants, and the rates were significantly higher than in explanted caudal regions of both strains (Table 1). The strains varied, however, in the magnitude of the difference in closure rate. Isolated PNP-regions exhibited a 4- to 7-fold increase in closure rate compared with caudal regions in *ct/ct* embryos, whereas the increase in PNP closure rate in isolated PNP-regions of Sw embryos was less than 2-fold.

Morphology of PNP closure in *ct/ct* embryos

Scanning electron microscopy of the caudal region of *ct/ct* embryos demonstrated the marked ventral curvature of the PNP region at the 27 somite stage (Fig. 6A), which has been observed previously (Brook et al., 1991). Dorsolateral views of the PNP at this stage show that the apices of the neural folds are splayed apart, indicating that ventral curvature opposes neural folding at this stage (Fig. 6B). However, 7 hours later in development, at the 31 somite stage, the angle of curvature has reduced and the apices of the neural folds can be seen to be approaching each other prior to completion of PNP closure (Fig. 6C).

PNP closure in the chick embryo

In the chick embryo, de novo closure of the neural folds in the cervical region was seen between the 5 and 6 somite stages. From this stage onwards, a wave of caudally directed neural tube closure leads to a decrease in length of the region of open neural folds (referred to in the present study as the PNP) until the PNP finally closes between the 16 and 18 somite stages (Figs 7,8). The rate of PNP closure between the stages of 11 and 16 somites amounted to 174 μm per somite stage.

Effect of axial bending on PNP closure in the chick

Groups of chick embryos were cultured on 5 differently curved substrata, with angles of −1°,−0.5°, 0°, +0.5° and +1° per mm respectively (Fig. 1). The effect on culture of such slight curvatures was a temporary increase in PNP length (indicated as delay in PNP closure) followed by closure of the PNP (Fig. 3). Delay in PNP closure occurred most frequently in embryos cultured at −1°, −0.5° and +1° (maximal at −1°), and was least common in embryos cultured at 0° and +0.5° (Figs 9,10). Both the incidence and the extent of closure delay correlated with the degree of curvature in both concave and convex directions.

In preliminary experiments using diagnostic ultrasound techniques we examined chick embryos developing in ovo and found an angle of curvature of +0.4° per mm (van Straaten and Hoogland, unpublished data). This value fell between the two substratum angles that caused minimal delay of PNP closure: 0° and +0.5°.

Effect of 'dorsal side up' culture and axial bending on chick embryo development

Several parameters of embryonic development were studied to determine whether there may have been non-specific adverse effects of the culture methods. Embryos of all groups showed no marked morphological differences after 6 hours in culture (Fig. 9), and most were still viable after 24 hours. The rate of somite increase, averaged over all groups, was 0.65 per hour, a value that is very similar to

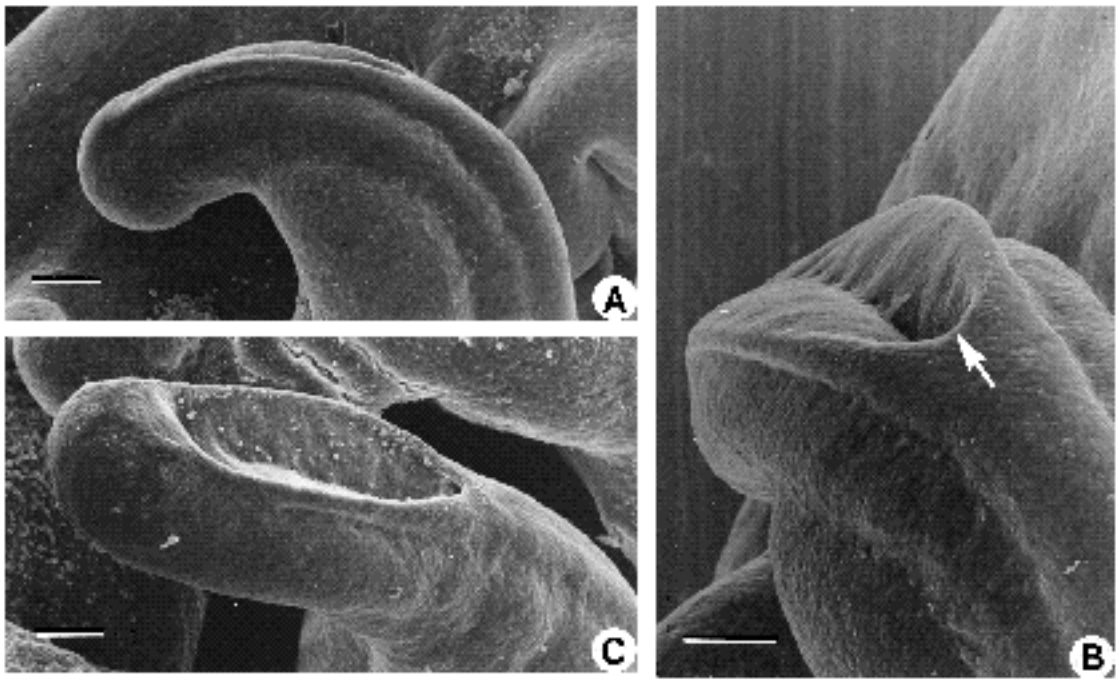

Fig. 6. Caudal region of two *ct/ct* mouse embryos with 27 (A,B) and 31 somites (C), exhibiting a delay in PNP closure. (A) Ventral bending of the embryonic axis is enhanced and PNP length is abnormally large. (B) The dorsolateral walls of the PNP are splayed outwards, probably as a result of the enhanced bending. Rostrocaudal closure of the PNP (arrowhead) is inhibited at this stage. (C) Unbending has occurred in the PNP region of the 31-somite embryo, probably allowing the neural walls to elevate and move in a medial direction. Neurulation accelerates at this stage. Scale bar, 100 μm.

the rate of 0.66 per hour in ovo, for embryos with 11-16 somites (Hamburger and Hamilton, 1951). Moreover, culture on a curved substratum did not affect the rate of somite formation (Table 2), suggesting that the observed delay in onset of PNP closure was not due to generalised developmental delay. The average rate of PNP closure in embryos on a flat substratum amounted 113±53 μm/hour (n=52); by calculation, the average rate of closure in these embryos was 113/0.66=171 μm/somite stage, which compares favourably with the value of 174 μm/somite stage reported above. Ultimately, the PNP closed in all embryos studied.

We conclude that embryos in this study developed in a manner closely similar to embryos in ovo, so that it is likely that the observed delay in PNP closure in embryos cultured on curved substrata was due to a specific effect of axial curvature and not to non-specific effects of the culture conditions.

DISCUSSION

Closure of the mouse neural tube in vitro

We have found that, in Sw embryos, the PNP closes in vitro both in isolated caudal regions and in the neuroepithelium of the PNP-region which has been separated from most of its adjacent non-neural tissues. This result indicates that mouse PNP closure can proceed in a neural plate that is almost devoid of surrounding tissues. An alternative expla-

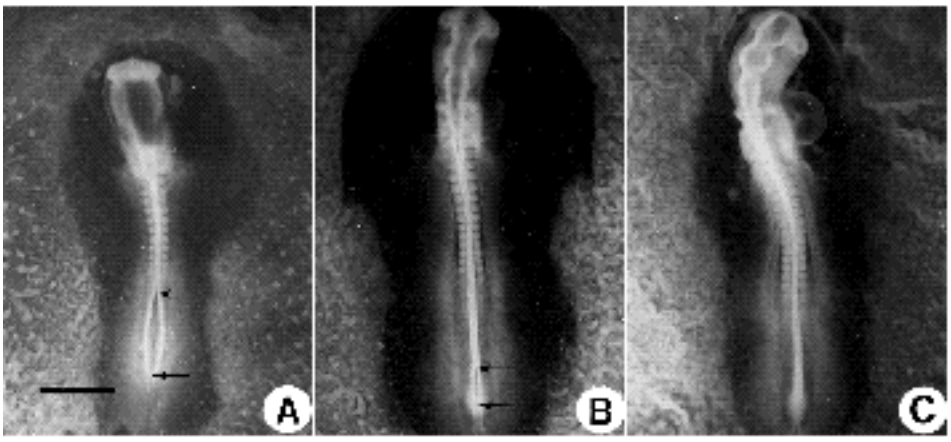

Fig. 7. A series of chick embryos of 11, 15 and 17 somites to illustrate the decrease in PNP length (arrows) during development. Embryos were placed in a flat Petri dish, briefly fixed in Bodian's fluid and photographed. Scale bar, 1 mm.

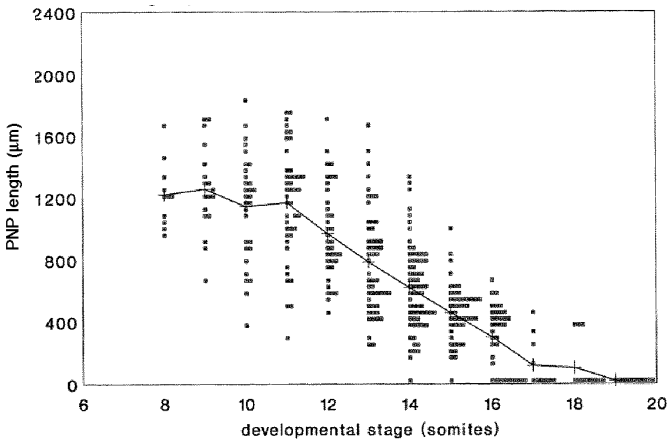

Fig. 8. Changes in length of the PNP during development of the chick embryo. Individual data are plotted as dots. A line is drawn through the average values per somite stage. Regression analysis showed a significant decrease between somite stages 11 and 16 ($P < 0.001$). The slope of the regression line was 174 μm/somite stage. Most PNPs close at the stages of 16 and 17 somites. Data are not presented for the 6 and 7 somite stages, since the caudal end of the PNP could not be seen in these embryos.

nation for our observation, however, is that the rolling up of an isolated neuroepithelium to form a tube, in vitro, may represent an artifactual response of the epithelium to separation from its mesenchyme. This interpretation may apply particularly to the behaviour of small pieces of neuroepithelium that have been found, in other studies, to roll up in vitro with their apical surface on the outside rather than on the inside of the tube, as discussed by Schoenwolf and Smith (1990). In the present study, however, we explanted large regions of neuroepithelium, which contained both the PNP and a portion of closed neural tube. Indeed, we found that the region of neural plate/neural folds would only undergo closure in vitro if left attached to a portion of neural tube. Closure of the PNP proceeded in these explants in a rostrocaudal direction, with a similar morphology to PNP closure in vivo. It seems possible, therefore, that continuation of rostrocaudal neural tube closure throughout the PNP in the mouse embryo may be a morphogenetic process that is solely dependent on changes intrinsic to the neuroepithelium, although the ectoderm may also be involved (see below). It is important to bear in mind, however, that rostrocaudal progression of the PNP fusion point may differ, mechanistically, from the process of de novo closure of the neural tube, which has been suggested to involve forces originating extrinsic to the neuroepithelium (Schoenwolf and Smith, 1990).

Mechanisms by which driving forces for neurulation could be generated within the neuroepithelium have not been conclusively determined. Apical constriction of neuroepithelial cells is generally believed to be a likely driving force for neurulation (Karfunkel, 1974; Gordon, 1985; Nagele et al., 1989; Jacobson, 1991), but constriction of apical microfilaments, alone, cannot account for all the changes that occur during closure of the chick neural tube (Schoenwolf et al., 1988). The neural plate rapidly elongates and simultaneously narrows (convergent extension) during caudal neurulation, a process involving local proliferation and/or cell rearrangement (Jacobson and Gordon, 1976; Jacobson, 1981, 1991; Schoenwolf and Alvarez, 1989; Schoenwolf and Sheard, 1989, 1990; Smith and Schoenwolf, 1988), and this could also generate forces

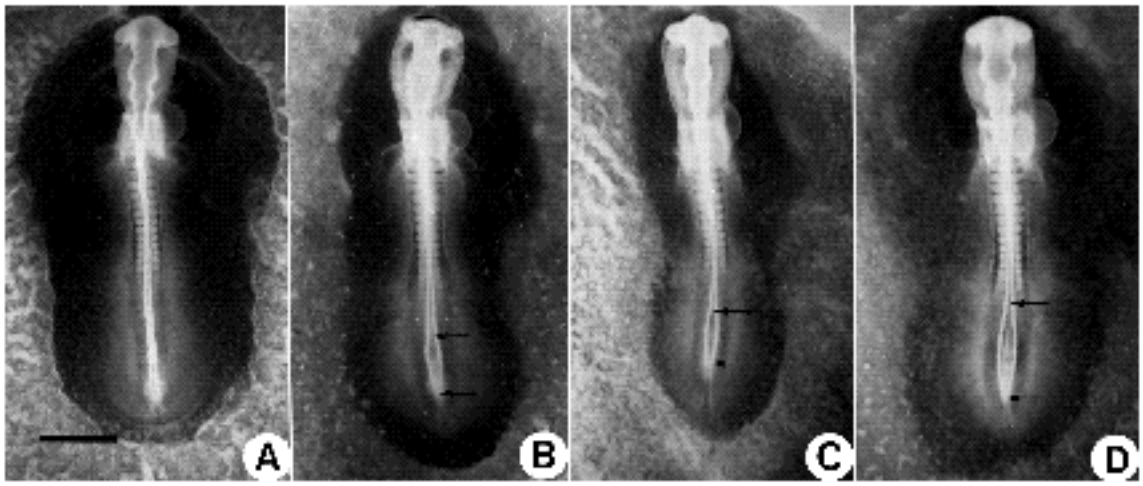

Fig. 9. Chick embryos explanted on different substrata and cultured for 6 hours. (A) Non-cultured control embryo with 15 somites; its PNP is closed (exceptional at this stage, see Fig. 8). (B) Embryo cultured on a convex substratum of +1°/mm. At the start of culture the embryo had 12 somites and a PNP length of 1050 μm. During culture, the PNP initially increased in length, resulting in closure delay of 280 minutes. The figure shows the embryo after 6 hours of culture; it has 15 somites and a PNP length of 820 μm. (C) Embryo cultured on a flat substratum. At the start of culture this embryo had 10 somites and a PNP length of 1620 μm. During culture, the PNP length decreased at an average closure rate of 133 μm/hour. After culture, the embryo shown has 14 somites and a PNP length of 800 μm. (D) Embryo cultured on a concave substratum of −1°/mm. At the start of culture the embryo had 11 somites and a PNP length of 1410 μm. The PNP length increased markedly during culture and resulted in a delay of 310 minutes; the closure pattern resembled that shown in Fig. 3, upper graph. The figure shows an embryo with 15 somites and a still large-sized PNP (1330 μm) after culture. Apart from the variation in PNP length no marked morphological differences are evident between the embryos. Scale bar, 1 mm.

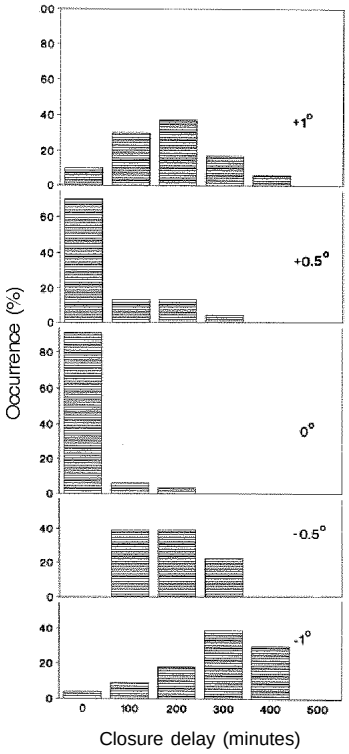

Fig. 10. Correlation between PNP closure delay and angle of axial curvature in the chick embryo. From top to bottom, the axial curvature changes from convex (+1° per mm) to concave (−1° per mm). A marked delay of PNP closure is seen with increasing curvature. Mid-value classes of closure delay are plotted on the x-axis.

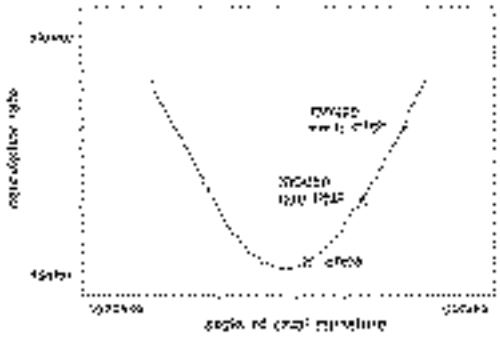

Fig. 11. Hypothetical relationship between the angle of axial curvature and the rate of neurulation. Experimental alteration of the angle of curvature causes the rate of neurulation to change as predicted by the curve. Response to alteration of curvature will depend, for a given species or level of the body axis, on the angle of curvature present during undisturbed neurulation. 'X' indicates the angle of curvature present in undisturbed development in the chick and mouse caudal regions.

assisting closure. With respect to convergence of the neural folds, Jacobson (1991) has suggested that the neuroepithelium of the neural folds may crawl actively on the inside of the surface ectoderm, while Alvarez and Schoenwolf (1992) have identified expansion of the surface ectoderm as a cause of bending of the neural plate. In the present study, a portion of the surface ectoderm was left attached to the dorsolateral aspects of the neuroepithelium, so that the neural folds were intact in our explants and closure could have occurred by either of these mechanisms.

Axial curvature as the mechanism of defective neurulation in the ct mutant

We found that explanted caudal regions of *ct/ct* embryos undergo much slower closure of the PNP in vitro than the caudal regions of Sw embryos. On the other hand, the isolated neuroepithelium of the PNP-region closes at a much faster rate in *ct/ct* embryos, and at a similar rate to the isolated neuroepithelium of Sw PNP-region. The implication of these findings is that the intrinsic neuroepithelial mechanism of PNP closure appears not to be affected by the *ct* mutation. Rather, the cause of the delay in PNP closure in *ct/ct* embryos originates outside the neuroepithelium.

This conclusion is in agreement with the findings of Brook et al. (1991) who showed that *ct/ct* embryos with delayed PNP closure have an increased angle of ventral curvature in the caudal region. Experimental prevention of curvature was found to normalize neurulation, suggesting that ventral curvature is the primary cause of the defect in PNP closure. The increased ventral curvature of *ct/ct* embryos probably originates from an abnormally reduced rate of cell proliferation, which affects ventral tissues, the hindgut endoderm and notochord, but not the neuroepithelium (Copp et al., 1988). Regional differences in proliferative activity might indeed be important. Takamatsu and Fujita (1987) studied cell proliferation rates in the cranial region of the chick embryo and concluded that such differences between the notochord, neural tube and endoderm play a causal role in formation of the cephalic flexure.

In the present study, scanning electron microscopy showed that, in 27-28 somite ct/ct embryos with enhanced ventral curvature of the caudal region, the neural folds are bent outwards, presumably inhibiting the rostrocaudal sequence of PNP closure. As development proceeds, however, the caudal region unbends progressively (Brook et al., 1991) and this would seemingly allow the neural folds to resume their convergent movements, allowing subsequent apposition and fusion. Neurulation accelerates and PNP closure is completed, albeit in a delayed fashion, in the least affected ct/ct embryos (van Straaten et al., 1992). In the most severely affected embryos, however, PNP closure is not completed, resulting in spina bifida.

Other findings indicate a relationship between axial curvature and both caudal and cranial neurulation. Early in spinal neurulation, the caudal region is markedly convex and the rate of PNP closure is 19 μm/hour. However, during the final stages of closure, the caudal region unbends and the rate of closure rises to 70 μm/hour (Brook et al., 1991; van Straaten et al., 1992). In the present study, the PNP closure rate in isolated neuroepithelial explants was also 70 μm/hour, consistent with the idea that the isolated neuroepithelium is freed from inhibitory effects of adjacent tissues, such as curvature of the body axis. With respect to cranial neurulation, Jacobson and Tam (1982) found that fusion of the plate edges in the prosencephalon (de novo neurulation) occurred only after the dorsal bulge is reversed at that site. They also found that closure of the mesencephalic neural plate in mouse embryos occurred following a marked diminution in the angle of the cephalic flexure, suggesting that closure of the neural tube in the cranial region, like the spine, is inhibited by axial curvature.

Axial curvature and PNP closure in the chick embryo

In order to investigate further the idea that altering the angle of axial curvature can affect the rate of neurulation, we cultured neurulation-stage chick embryos on curved substrata. PNP closure was delayed by imposing small angles of curvature, of either a convex or concave nature, and the degree of closure delay correlated with the angle of curvature. These experiments utilised an unusual culture method: embryos were explanted 'dorsal side up', in order to obtain a good view of the PNP, in contrast to the more commonly used method in which embryos are cultured upside down on the vitelline membrane (New, 1955). Embryonic development appeared undisturbed by this variation in technique, as determined by the rate of somite number increase, the ultimate closure of all PNPs and a high survival rate after 24 hours. It seems likely, therefore, that the delay in PNP closure that occurred in embryos cultured on curved substrata reflects a specific effect of axial curvature on spinal neurulation in the chick embryo, as in the *ct* mouse. It can also explain the occurrence of neural tube defects in chick embryos after an air bubble is introduced between the blastoderm and the vitelline membrane (Fineman et al., 1986). Axial curvature is enhanced by this technique, probably leading to persistent delay of PNP closure.

Since neural fold elevation was well advanced when chick embryos were explanted in the present study, it seems likely that convergence of the neural folds, and subsequently, apposition and fusion, are the main processes affected by curvature, as discussed earlier for the mouse embryo.

Relationship between axial curvature and neurulation: a general hypothesis

The evidence from studies of the mouse and chick embryo (Jacobson and Tam, 1982; Brook et al., 1991; van Straaten et al., 1992; the present study) suggest that the angle of axial curvature is an important factor affecting the rate and timing of neurulation. Differences in PNP closure rates as seen in chick (120 μm/hour) and mouse (70 μm/hour) embryos might reflect species differences. However, since their somite gain during this period is very similar (chick: 0.66 somites/hour, Hamburger and Hamilton, 1951; mouse: 0.58 somites/hour, van Straaten et al., 1992), these differences may reflect variations in the angle of curvature at which neurulation normally occurs in the mouse and chick embryo.

We propose that the relationship between angle of curvature and rate of progression of neurulation can be described by a U-shaped curve (Fig. 11). Embryos of different species, and at different levels of the body axis within a single species, have different angles of curvature during undisturbed neurulation, and so respond differently to experimental alteration of the angle. It should be possible to test this hypothesis by altering the angle of curvature experimentally at other levels of the body axis, to determine whether neurulation is accelerated or delayed according to the prediction of the model. For instance, neurulation in the cervical and upper thoracic region of the mouse occurs before 'turning', when the body axis exhibits concave curvature (Copp et al., 1990). According to the hypothesis, straightening of the axis should enhance neurulation, whereas a further increase in angle of curvature should retard neurulation. It is interesting to note that mouse embryos homozygous for the loop-tail (*Lp*) mutation fail to initiate neurulation in the cervical region, and that an early abnormality has been reported to be a shortening of the notochord (Smith and Stein, 1962). It remains to be determined whether *Lp/Lp* embryos exhibit increased axial curvature at the time of onset of cervical neurulation.

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