J. Embryol. exp. Morph. 88, 39–54 (1985)
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Relationship between timing of posterior neuropore closure and development of spinal neural tube defects in mutant (*curly tail*) and normal mouse embryos in culture

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SUMMARY

The relationship between timing of closure of the posterior neuropore (PNP) and development of spinal neural tube defects (NTD) has been studied in individual mutant *curly tail* mouse embryos maintained in culture. Moderate delay in PNP closure results in development of tail flexion defects whereas extreme delay of PNP closure is associated with development of open NTD. Experimental enlargement of the PNP at the stage of 25 to 29 somites leads to delayed PNP closure and development of tail flexion defects in 36 % and 38 % respectively of non-mutant A/Strong embryos. In *curly tail* embryos, the effect of experimental enlargement of the PNP summates with the genetic predisposition to produce an increased incidence of spinal NTD among which open defects are proportionately more common. These results indicate that a causal relationship exists between delay in PNP closure and development of spinal NTD in mouse embryos. The method described for distinguishing between prospective normal and abnormal *curly tail* embryos at a stage prior to the appearance of malformations provides an opportunity to study the morphogenetic processes that precede the development of genetically determined spinal NTD.

INTRODUCTION

Spinal neural tube malformations in mutant *curly tail* embryos comprise open lesions that originate as lumbosacral myeloschisis (LSM, the developmental forerunner of spina bifida), and closed lesions that manifest as tail flexion defects (curly tails). Grüneberg (1954) suggested, from morphological observations, that these malformations result from delay in closure of neural folds at the posterior neuropore (PNP). Support for this hypothesis has come from finding that the percentage of *curly tail* embryos which undergo delayed closure is similar to the percentage which later develop tail flexion defects and LSM (Copp, Seller & Polani, 1982). However, before the validity of Grüneberg's hypothesis can be accepted it is necessary to establish, firstly, whether particular *curly tail* embryos

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Key words: mouse embryo, mutant, culture, neuropore, neural tube defects.

that undergo delayed PNP closure are the same as those that later develop spinal neural tube defects (NTD) and, secondly, whether the hypothetical causal nature of the association between delayed PNP closure and development of spinal NTD can be demonstrated experimentally.

An experimental approach to the first of these questions was prompted by the demonstration that *curly tail* embryos may develop spinal NTD during growth in a whole embryo culture system (Copp *et al.* 1982). In the present paper, an experiment is described in which the extent of delay of PNP closure, before culture, has been correlated with the development of spinal NTD, after culture, in individual *curly tail* embryos. The second question posed above has also been investigated using the embryo culture system. In this case, delay of PNP closure has been induced experimentally in normal embryos of a non-mutant mouse strain, with the expectation that, if Grüneberg's hypothesis is correct, spinal NTD should be produced.

MATERIALS AND METHODS

Source and culture of embryos

The *curly tail* mutation is thought to be an autosomal recessive with incomplete penetrance, so that adult *curly tail* mice (all presumed *ct/ct*) exhibit two phenotypes: CT individuals have tail abnormalities, whereas ST individuals are straight tailed. The incidence of malformations has been found to differ depending on whether the parents of a *curly tail* litter are themselves affected (Embury, Seller, Adinolfi & Polani, 1979). In the present experiments, therefore, *curly tail* embryos were obtained from matings of two types: CT × CT and ST × ST. Non-mutant embryos were obtained from random-bred A/Strong mice. Males and females were paired overnight and the day of finding a vaginal plug was designated day 1 of pregnancy.

Conceptuses were dissected on days 10 (A/Strong) and 11 (*curly tail*) of gestation in alpha-modification of Eagle's medium (Stanners, Eliceiri and Green, 1971) containing 10 % foetal calf serum and 4·8 g l^{-1} Hepes. Reichert's membrane and trophoblastic giant cells were removed but the yolk sac and amnion were kept intact. The culture technique was that described by New, Coppola & Terry (1973), with the following modifications. Up to three embryos (five in short-term experiments) were cultured in 5 ml undiluted rat serum, prepared by immediate centrifugation of freshly drawn blood as described by Steele and New (1974), in 60 ml reagent bottles (Scientific Supplies Ltd.). Cultures were gassed, initially, with 5 % CO_2, 95 % air, and the gas atmosphere was replenished after 12 and 24 h of culture, using 5 % CO_2, 95 % O_2. Bottles were rotated at 37 r.p.m. throughout the culture period.

Assessment and manipulation of embryos before culture

Cultures were initiated when embryos had between 25 and 29 somites (see below for assessment of somite number). A small incision was made in the yolk sac, avoiding major blood vessels, over the region of the tail bud and the embryo was inspected to determine PNP category. In some experiments the PNP was enlarged mechanically by the following procedure. The amnion was torn open over the region of the PNP and the tip of a sharp glass needle was inserted under manual control into the PNP, care being taken to avoid penetrating the neural plate. The needle, held perpendicularly to the long axis of the tail bud, was moved in a cranial direction so that the roof plate of the most recently formed portion of neural tube was incised (Fig. 1). In mock-operated controls the amnion was torn open but no incision was made in the neural tube.

After inspection of the PNP region, with or without manipulation, individual conceptuses were 'tagged' to permit identification following culture. Single strands were teased from a 3–6 mm length of silk suture (Abrasilk), and one or more loops were tied in some strands. A

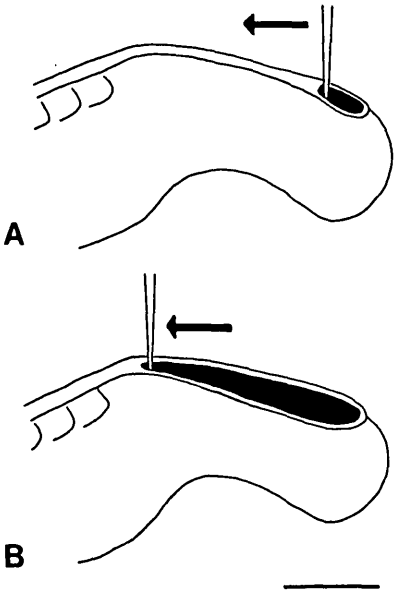

Fig. 1. Diagram to illustrate the method for experimental enlargement of the PNP from the size of (A) Category 1/2 to (B) Category 4/5. A 'window' is prepared in the otherwise intact yolk sac and amnion, over the region of the tail bud. The tip of a sharp glass needle, held perpendicularly to the long axis of the tail bud, is inserted under manual control into the neuropore, and is moved in a cranial direction, incising the roof plate of the most recently formed portion of neural tube. Scale bar equals approximately 0·5 mm.

single strand was inserted through the cut edges of the incised yolk sac so that each conceptus within a particular culture bottle had a unique tag (i.e. silk strand with no loop, single loop or double loop; Fig. 2A). Silk tags did not interfere with embryonic development and were rarely dislodged during culture. Furthermore, in experiments involving mechanical enlargement of the PNP, silk tags served as sutures to prevent herniation of the embryonic tail bud through the torn amnion and yolk sac.

Analysis of embryos following culture

Cultures were terminated after either 8–12 h (short cultures) or 32–36 h (long cultures). Each embryo was dissected from its extraembryonic membranes, inspected for malformations, tested for closure of the PNP by injection of toluidine blue dye into the neural canal (Copp *et al.* 1982), and fixed in Bouin's fluid. Two types of malformations, tail flexion defects and LSM (Fig. 2), were particularly studied, and were defined as described previously (Copp *et al.* 1982). Somites were counted after fixation of embryos, using as landmarks the 11th and 28th somites (Copp *et al.* 1982).

The axial length of PNP was measured for each embryo with an open PNP in short culture experiments using a Wild dissecting microscope and eyepiece graticule at constant magnification. Serial histological sections (thickness 6 μm, stained with haematoxylin and eosin) were prepared from embryos of the following types:

a) *Curly tail* embryos with 25–29 somites, prior to culture, in order to determine the axial lengths of PNPs in different categories;

b) A/Strong embryos fixed immediately, 2 h and 4 h following experimental enlargement of the PNP, in order to study the time course of reconstruction of 'neural folds';

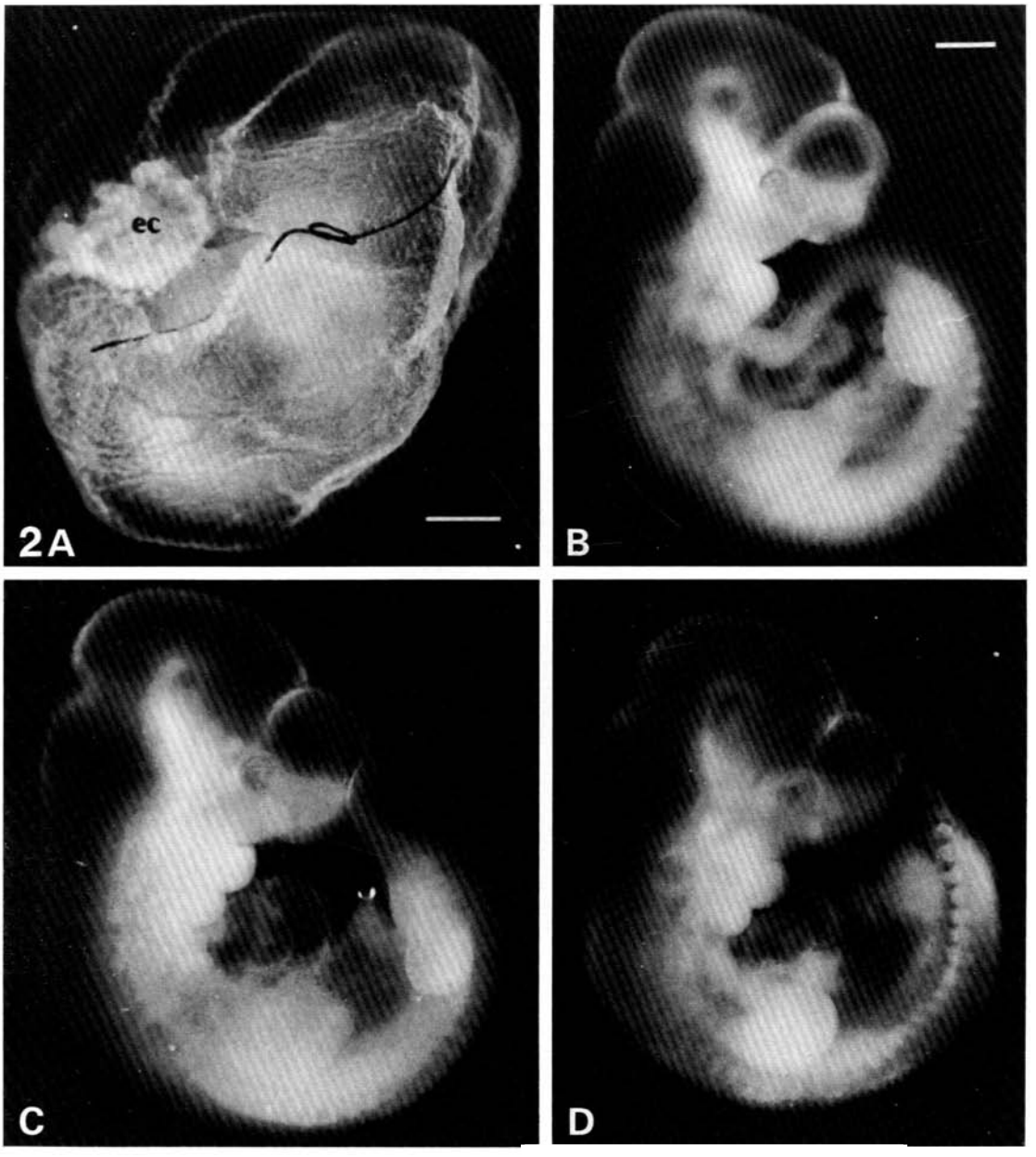

Fig. 2. (A) Conceptus dissected from the uterus on day 11 of gestation, and prepared for culture. A 'window' has been opened in the yolk sac to one side of the ectoplacental cone (ec), so that the tail bud region which lies directly beneath can be inspected to determine PNP category. A silk strand containing a single loop has been inserted through the incised edges of the yolk sac to permit identification of the conceptus following culture. Blood vessels are visible in the yolk sac towards the bottom of the figure. Scale bar equals 1 mm. (B to D) *Curly tail* embryos cultured for 32–36 h, from the stage of 25–29 somites, to illustrate the main 'developmental outcomes' encountered: (B) normal development with straight tail; (C) tail flexion defect; (D) tail flexion defect with LSM. Scale bar equals 1 mm.

c) *Curly tail* and A/Strong embryos following long cultures, in order to compare the areas and perimeters of neural tube and neural canal, and area of neurectodermal tissue, by the method described previously (Copp *et al.* 1982).

Criteria for exclusion of embryos from experiments or analysis

Only embryos with 25–29 somites were used in culture experiments. The intact extra-embryonic membranes precluded accurate counting of somites, so that an indirect criterion, the presence of well-defined forelimb buds, was taken as evidence of more than 25 somites; prior to this stage the forelimb bud is present only as an ill-defined swelling of the lateral body wall. The finding of somites caudal to the hindlimb bud rudiment, during direct inspection of the tail bud region through the yolk sac incision, indicated the presence of 30 or more somites.

Criteria for exclusion of embryos from analysis after culture were as follows:

a) Embryos could not be identified unequivocally due to dislodgement of silk tags during culture (two embryos).

b) Unexpected developmental abnormalities arose which precluded meaningful analysis, i.e. lateral bends in embryonic tails (two embryos) and adhesion beween amnion and incised neural folds (one embryo).

c) Embryos that had fewer than 35 somites after long cultures or fewer than 30 somites after short cultures. This criterion was adopted since tail flexion defects cannot be identified with certainty in embryos with fewer than 35 somites and PNP closure is not complete in the majority of normal embryos with fewer than 30 somites (Copp *et al.* 1982). This criterion led to the exclusion of 33 out of a total of 204 embryos in long cultures and 12 out of 63 embryos in short cultures. The possible significance of these excluded embryos is considered later.

RESULTS

Preliminary observations showed that *curly tail* embryos with 25–29 somites can be classified into five categories according to the cranial extent of the PNP (Fig. 3). Although all intermediate conditions occur, the distinction between categories 2, 3 and 4 is usually straightforward. Embryos in categories 1 and 2 are less easily distinguished, and therefore the categories have been combined for purposes of analysis. Category 5 embryos are encountered infrequently, and so categories 4 and 5 have also been combined. Approximately equal numbers of embryos occur in the combined categories 1/2, 3 and 4/5.

A total of 126 tail bud regions from *curly tail* embryos with 25–29 somites were assessed for PNP category, and then prepared as serial transverse histological sections. The mean (± standard deviation) number of 6 μm sections comprising the PNP of embryos in the three categories was as follows: category 1/2, $38{\cdot}6 \pm 11{\cdot}3$; category 3, $66{\cdot}6 \pm 7{\cdot}6$; category 4/5, $89{\cdot}3 \pm 14{\cdot}4$. The difference in size of PNP between categories 1/2 and 3, and between 3 and 4/5, are both highly significant ($P < 0{\cdot}001$), indicating minimal overlap between the categories as defined above.

Culture of curly tail *embryos after assessment of PNP category*

Table 1 shows the outcome of long cultures (i.e. 32–36 h) in which PNP category was determined before culture. A significantly smaller proportion of embryos in

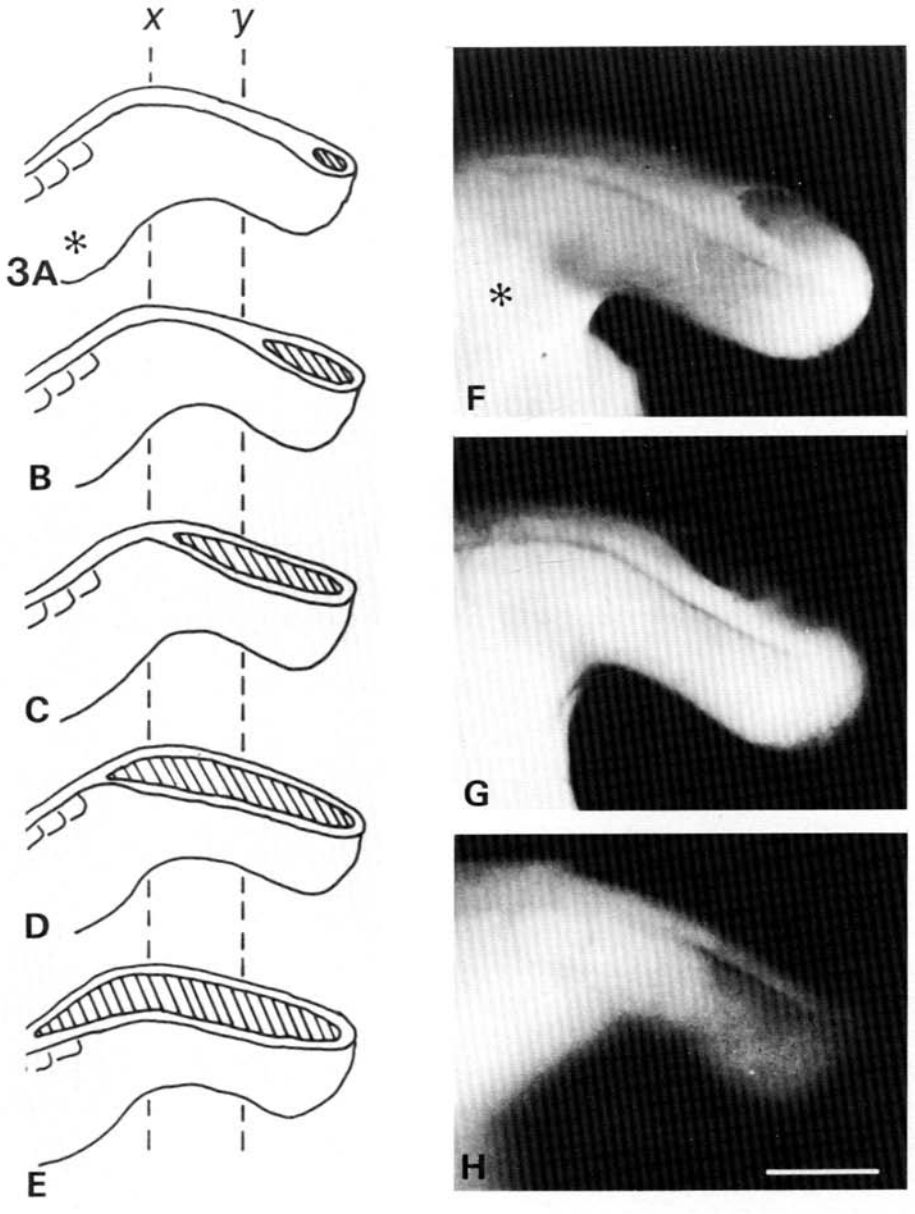

Fig. 3. Diagrams and photographs of the tail bud regions of *curly tail* embryos with 25–29 somites, to illustrate the five PNP categories. In categories 1 (A) and 2 (B and F), the PNP is restricted to the expanded distal region of the tail bud and does not extend beyond level Y. In category 3 (C and G), the PNP extends beyond the distal expansion, but does not reach the level of the hindlimb bud rudiment (i.e. does not extend beyond level X). In category 4 (D and H), the PNP extends into the region of the hindlimb bud (i.e. beyond level X) but does not encroach upon the rows of somites. In category 5 (E), the PNP overlaps, in its cranial extent, with the somite rows. Asterisks indicate hind limb bud rudiment. Scale bar on photographs equals 0·5 mm.

category 1/2 developed spinal NTD than in categories 3 ($\chi^2 = 5{\cdot}8$, d.f. = 1, $P < 0{\cdot}025$), and 4/5 ($\chi^2 = 15{\cdot}3$, d.f. = 1, $P < 0{\cdot}001$). There was no significant difference in the proportion of embryos which developed spinal NTD in categories 3 and 4/5. However, when embryos with tail flexion defects alone are considered separately from those with LSM in the statistical test (2 × 3 contingency table) categories 3 and 4/5 also differ significantly ($\chi^2 = 7{\cdot}15$, d.f. = 2, $P < 0{\cdot}05$). Thus, although total spinal NTD and tail flexion defects occurred with approximately equal frequency in category 3 and 4/5 embryos, LSM was found predominantly among category 4/5 embryos. The overall incidence of spinal NTD (66·2 %) was not significantly different from that found in a recent study of *curly tail* embryos developing entirely *in vivo* (60·7 %; $\chi^2 = 0{\cdot}58$, d.f. = 1, $P > 0{\cdot}05$; Copp *et al.* 1982). Another similarity between the two studies is the finding that the overall incidence of spinal NTD does not differ significantly ($\chi^2 = 0{\cdot}53$, d.f. = 1, $P > 0{\cdot}05$) between embryos derived from CT × CT and ST × ST matings.

Almost half of category 1/2 embryos had completed closure of the PNP after 8–12 h of culture, and in the remaining embryos the PNP was very small, indicating that closure was imminent (Table 2). Only a single embryo in category 3, and no category 4/5 embryos, underwent PNP closure during a similar culture period. Moreover, the axial length of the PNP was significantly greater in embryos of categories 3 ($t = 3{\cdot}56$, d.f. = 11, $P < 0{\cdot}01$), and 4/5 ($t = 6{\cdot}28$, d.f. = 16, $P < 0{\cdot}001$), when compared with those category 1/2 embryos in which the PNP was open.

Table 1. *Outcome of long cultures (32–36 h) of* curly tail *and A/Strong embryos, with or without experimental enlargement of the PNP before culture*

Embryo type	PNP category		Outcome of culture		
	Before operation	After operation	Straight tail	Tail flexion defect	LSM ± tail flexion defect
1. *Curly tail*					
Non-operated	1/2	–	17 (7, 10)*	10 (4, 6)	0
	3	–	7 (3, 4)	15 (8, 7)	2 (1, 1)
	4/5	–	2 (2, 0)	15 (8, 7)	9 (7, 2)
Operated	1/2	4/5	4	4	2
	4/5	beyond 4/5	0	0	9
Mock-operated	3	–	3	8	0
2. *A/Strong*					
Non-operated	1/2	–	19	1	0
Operated	1/2	4/5	16	10	0
Mock-operated	1/2	–	14	0	0

* Parentheses indicate the number of embryos derived, respectively, from CT × CT and ST × ST matings.

Culture of A/Strong and curly tail *embryos after experimental enlargement of the PNP*

All A/Strong embryos with 25–29 somites so far examined have been found to fall into category 1/2. Experimental enlargement of the PNP to the size of category 4/5 resulted in development of tail flexion defects, but not LSM, in 38 % of A/Strong embryos (Table 1). Mock-operated embryos did not develop spinal NTD. A single non-operated A/Strong embryo developed a tail flexion defect, but the malformation in this embryo involved the most distal part of the tail, beyond the somitic region, which is not characteristic of tail flexion defects in *curly tail* and operated A/Strong embryos.

Although a similar proportion of operated and mock-operated A/Strong embryos underwent closure of the PNP during the short culture period (Table 2), open neuropores were very small in mock-operated embryos, indicating that closure was about to occur, whereas open neuropores in the operated group were significantly larger ($t = 9{\cdot}60$, d.f. $= 4$, $P < 0{\cdot}001$). The proportion of operated A/Strong embryos with large neuropores (36 %) did not differ significantly ($\chi^2 = 0{\cdot}01$, d.f. $= 1$, $P > 0{\cdot}05$) from the proportion that later developed tail flexion defects (38 %).

Regular observations of A/Strong embryos in the first 4 h after experimental enlargement of the PNP showed that re-closure of the neuropore occurs in a craniocaudal direction, as in the undisturbed embryo. Histological examination of embryos immediately following neural tube incision revealed a discontinuity between neurectoderm and surface ectoderm in the region of the newly created 'neural folds' (data not shown). By 2 h, neurectoderm and surface ectoderm had begun to re-establish continuity, and the process was completed by 4 h after

Table 2. *Incidence of PNP closure and size of open PNP after short cultures (8–12 h) of (1) non-operated* curly tail *embryos, and (2) A/Strong embryos in which the PNP was enlarged experimentally*

	PNP after culture		
Embryo type	Closed PNP	Open PNP	Mean axial length of open PNP in mm. (± S.D.)
1. *Curly tail*			
Category 1/2	4	6	0·28 (0·07)
Category 3	1	7	0·53 (0·17)
Category 4/5	0	12	0·67 (0·19)
2. *A/Strong*			
Operated (PNP enlarged from category 1/2 to 4/5)	7	4	0·76 (0·07)
Mock-operated	8	2	0·22 (0·06)

operation. Cells destroyed during the enlargement procedure persisted as collections of necrotic debris at the apices of the newly created neural folds.

Experimental enlargement of the PNP in *curly tail* embryos from category 1/2 to 4/5 produced an overall increase in the incidence of spinal NTD that developed during the long culture period (Table 1). Moreover, LSM developed in two out of ten of these embryos although it was never seen in cultures of non-operated category 1/2 embryos (Table 1). Enlargement of the PNP to the level of the midpoint of the hindlimb bud in category 4/5 embryos (i.e. considerably beyond the usual cranial extent of PNP in category 4/5) resulted in development of LSM in all cases. Category 3 embryos, treated as mock-operated controls, developed spinal NTD in the same proportions as in non-operated embryos.

Significance of embryos excluded from analysis

The exclusion criteria outlined in Materials and Methods resulted in the exclusion from analysis of a number of embryos following culture. The approximately even distribution of excluded embryos among PNP categories in long cultures of *curly tail* embryos (three in category 1/2, six in category 3, seven in category 4/5) suggests that they constitute a random sample of embryos cultured. Nevertheless, it is important to consider the possibility that, on the contrary, excluded embryos comprise a special group in which the trend following culture would have been markedly different from that of embryos included in the analysis. To take an extreme example, suppose that all excluded category 1/2 embryos had developed spinal NTD and all excluded category 4/5 embryos developed straight tails. In this case the overall trend of the data, combined from excluded and included embryos, would not be affected: the developmental outcomes of embryos in categories 1/2 and 4/5 remain significantly different ($\chi^2 = 5{\cdot}6$, d.f. $= 1$, $P < 0{\cdot}05$).

Morphology of tail flexion defects

Tail flexion defects that developed in non-operated *curly tail* embryos, and in operated A/Strong and *curly tail* embryos resembled morphologically those of *curly tail* embryos that developed entirely *in vivo* (Copp *et al.* 1982). The only consistent difference was that in operated embryos the tails were often less sharply flexed (Fig. 4) than in non-operated *curly tail* embryos.

Histological examination revealed further similarities between *curly tail* flexion defects developing *in vitro* and *in vivo*: in both cases the neural tube in flexed regions was over-expanded, occupying a significantly larger area of transverse sections than in straight tails (cultured embryos, $t = 3{\cdot}18$, d.f. $= 4$, $P < 0{\cdot}05$; uncultured embryos, see Table 2 of Copp *et al.* 1982), and was positioned on top of the mesodermal elements rather than embedded within them (data not shown).

Examination of serial histological sections of operated A/Strong embryos (Fig. 5) showed that in more than 50 % of cases the distal portion of the developing tail contained one or more regions in which the neural tube was duplicated laterally (i.e. diplomyelia), or, in a few embryos, represented by three or more neural tube

elements (Fig. 5C). Somites, notochord and gut appeared normal in these tails. The number of embryos affected by diplomyelia was the same whether the tail had

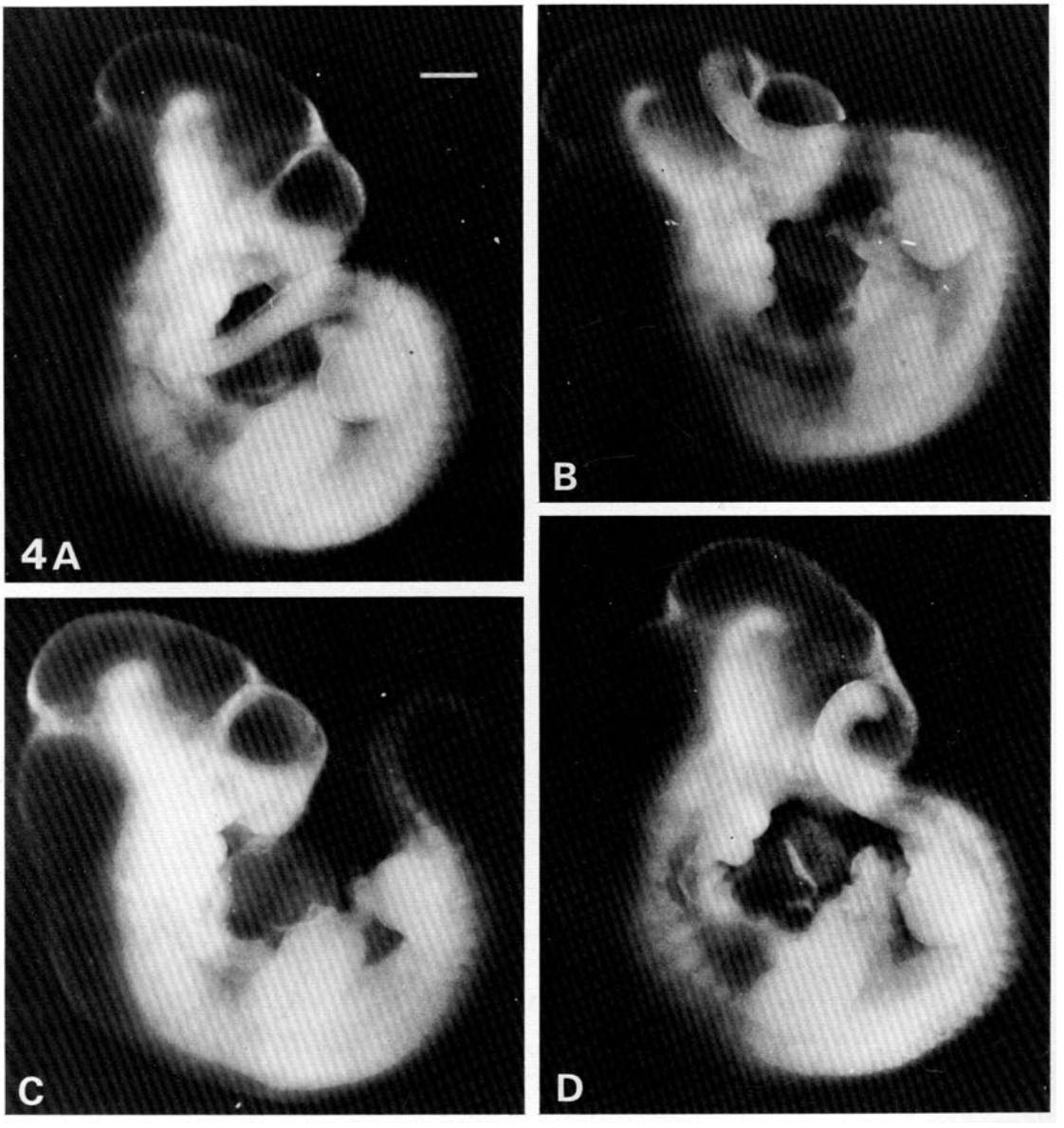

Fig. 4. Non-mutant A/Strong embryos cultured for 32–36 h from the stage of 25–29 somites. (A) Mock-operated control embryo; (B to D) embryos in which the PNP was enlarged prior to culture from category 1/2 to 4/5. In (B) the tail exhibits a dorsal bend that is less than 90°, with respect to the long axis of the tail, and the embryo is classed as 'straight tailed'. In (C) and (D), the tails have flexions in excess of 90°, and are classed as tail flexion defects. The tail in (C) is less sharply flexed than is usually found in *curly tail* embryos (compare with Fig. 2C), whereas the tail in (D) is more closely comparable to the *curly tail* malformation. Scale bar equals 1 mm.

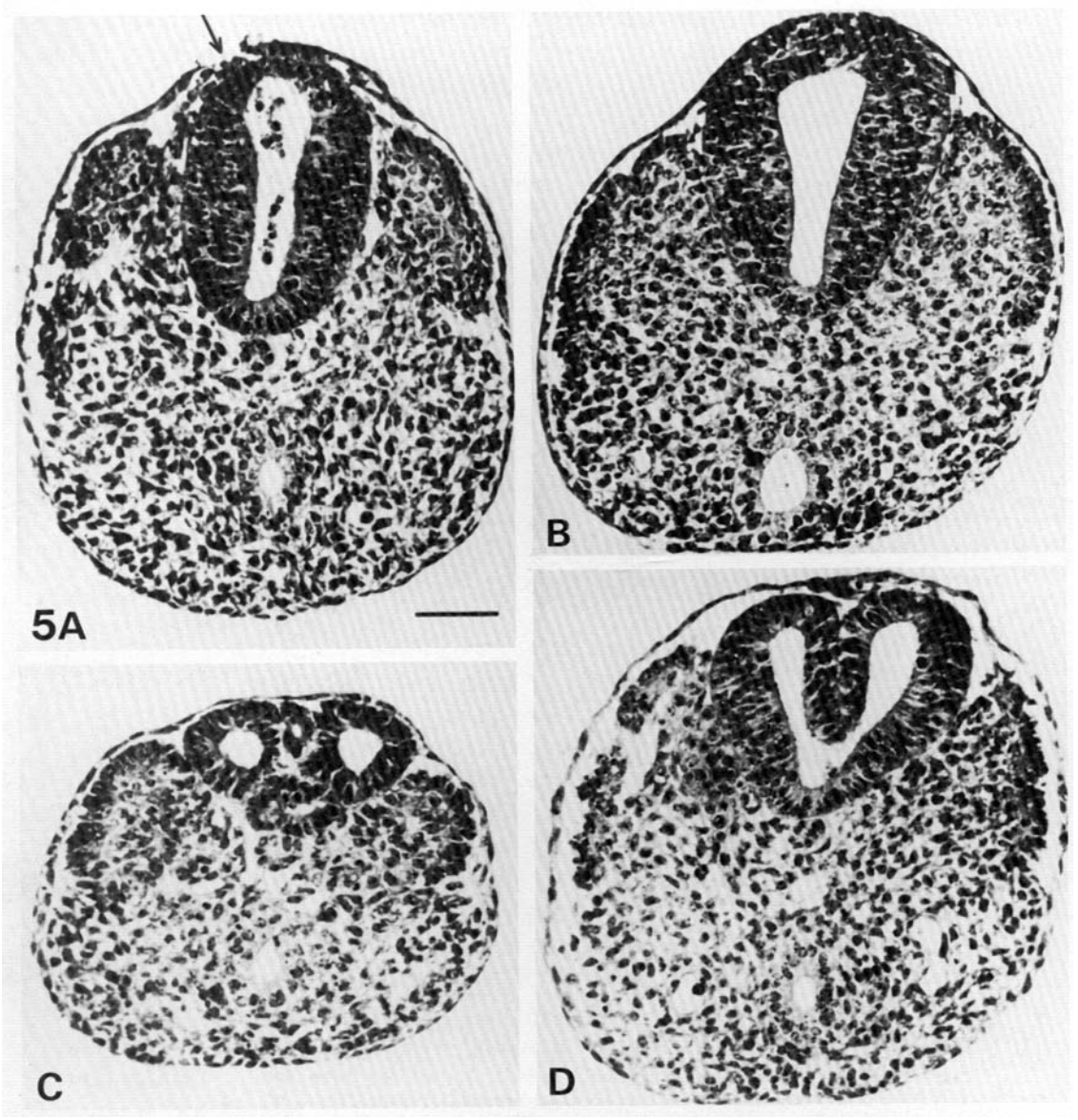

Fig. 5. Transverse histological sections through tails of non-mutant A/Strong embryos cultured for 32–36 h, from the stage of 25–29 somites. (A) Mock-operated control embryo; (B to D) embryos in which the PNP was enlarged prior to culture from category 1/2 to 4/5. Embryos in (A), (B) and (C) developed straight tails, whereas that in (D) developed a tail flexion defect. In (A) the neural tube is deeply embedded in the mesodermal elements, whereas in (B) it is less deeply embedded. In (D) the neural tube has undergone forking, so that four separate neural canals are visible. In (D), which shows a section taken from the proximal limb of the flexion defect, an infolding of the roof plate of the neural tube has developed so that, although the cross-sectional area of neural canal is not enlarged, the perimeter of the canal is considerably increased. In both (C) and (D) the neural tube is positioned abnormally on top of the mesodermal elements. Arrow: the discontinuity of surface ectoderm is probably a fixation artefact. Scale bar equals 0·1 mm.

a flexion defect or was straight (six out of ten tails examined in each case). However, flexed tails were, in no case, affected by diplomyelia throughout their length (32 to 81 % of distal tail length affected), whereas five of the affected straight tails exhibited duplication or multiple splitting of the neural tube throughout the entire distal tail region. Mock-operated A/Strong embryos (Fig. 5A) and non-operated embryos were not affected by diplomyelia (10 embryos examined in each case).

Measurements of areas occupied by neural tube, neural canal and neurectodermal tissue showed no differences between flexed and straight tails in operated A/Strong embryos (paired t-tests, $P > 0{\cdot}05$), providing support for the observation that, unlike non-operated *curly tail* flexion defects, the neural tube was not over-expanded in operated A/Strong malformations. On the other hand, perimeters of both neural tube ($t = 3{\cdot}59$, d.f. $= 9$, $P < 0{\cdot}01$) and neural canal ($t = 4{\cdot}06$, d.f. $= 9$, $P < 0{\cdot}01$) were significantly greater in flexed than in straight tails of A/Strong embryos. Thus, the caudal neural tube in operated A/Strong embryos with tail flexion defects was enlarged but not over-expanded. This discrepancy can be attributed to infolding of the dorsal roof plate of the neural tube, which was a regular feature of these embryos (Fig. 5D).

DISCUSSION

The main findings of this paper can be summarised as follows.

1) An abnormal cranial extent of the PNP is found in 64 % of *curly tail* embryos with 25–29 somites and is associated, in individual embryos developing *in vitro*, with delayed closure of the PNP and development of spinal NTD.

2) Experimental re-opening of the most recently closed portion of neural tube in embryos of a non-mutant mouse strain, in which spontaneous NTD are extremely rare, leads to development of spinal NTD (tail flexion defects) in 38 % of cases. Examination of these embryos during the first 12 h following operation reveals that a) continuity is re-established between neurectoderm and surface ectoderm within 4 h of re-opening, b) closure of the newly created 'neural folds' in the region of re-opening proceeds in a craniocaudal direction as in the undisturbed embryo and c) the PNP is significantly larger after 12 h of culture in operated than in non-operated embryos indicating that closure of the PNP is delayed following its mechanical enlargement.

3) Experimental enlargement of the PNP in *curly tail* embryos leads to an increase in the overall incidence of spinal NTD and in the relative incidence of LSM, the most severe form of spinal NTD seen in *curly tail* mice.

These results demonstrate that delay in closure of neural folds at the PNP can be produced by manipulations of both genome (in this case by the *curly tail* mutation) and environment (i.e. by experimental enlargement of the PNP) and that in both cases the subsequent developmental outcome includes appearance of spinal NTD. Moreover, the effects of genetic and environmental manipulations have been shown to summate in operated *curly tail* embryos. Taken together,

these observations suggest strongly that there is a causal relationship between delay of PNP closure and development of spinal NTD in the mouse embryo.

The developmental mechanisms that underlie the appearance of spinal NTD in these experiments are clearly heterogeneous: delay of PNP closure in non-operated *curly tail* embryos probably results from primary failure of neural fold elevation due to an, as yet, unknown cellular abnormality (Copp, 1983) whereas delay of PNP re-closure in operated A/Strong embryos is the direct result of experimental manipulation. On the other hand, the mechanisms whereby delay of PNP closure (or re-closure) leads to development of spinal NTD in non-operated *curly tail* and operated A/Strong embryos can be less clearly differentiated. The similarities in gross morphology and the finding of neural canal enlargement in tail flexion defects of both groups of embryos suggests that a common developmental mechanism may exist. Although the occurrence of diplomyelia in tail flexion defects of operated A/Strong, but not of non-operated *curly tail*, embryos could indicate a difference in developmental mechanism it may, alternatively, represent a secondary effect of the re-opening procedure that is not directly connected with the development of spinal NTD. For instance, imperfect apposition of the apices of the newly created neural folds may occur during reclosure of the PNP leaving a residual dorsal infolding of the neurectoderm within the neural tube. It is possible, therefore, that the sequence of developmental events intervening between delay of PNP closure and development of spinal NTD is similar whether the PNP closure delay has a genetic or environmental origin.

Relationship between tail flexion defects and LSM

In the absence of PNP enlargement, LSM was almost confined to embryos that belonged to category 4/5 prior to culture, whereas tail flexion defects without LSM developed in embryos of all categories, and were commonest in category 3. This finding indicates that not only is delayed closure of the PNP the main defect underlying development of spinal NTD, but also that the degree of delay in PNP closure at the stage of 25–29 somites may determine the type of malformation that results. Support for this idea comes from the results of experimental enlargement of the PNP in *curly tail* embryos. A shift in the pattern of malformations developed by operated embryos was seen: enlargement from category 1/2 to 4/5 led to the appearance of LSM in two out of ten embryos, and enlargement beyond category 4/5 resulted in all embryos developing LSM.

The relationship between degree of delay in PNP closure and type of malformation that results could involve simple thresholds in the size of PNP at the stage of 25–29 somites. Below the lower threshold (minimal delay in PNP closure), normal development results; between upper and lower thresholds (moderate delay), tail flexion defects develop, whereas above the upper threshold (extreme delay), LSM develops. However, the lack of an absolute correlation between PNP size and developmental outcome (Table 1) is not predicted by this hypothesis, and is not readily explained on the basis of inaccuracy in the method of assigning embryos to PNP categories: accurate measurements of the craniocaudal extent of

the PNP demonstrated minimal overlap between categories. An alternative explanation is that individual embryos differ in their rate of neural fold closure subsequent to the stage of 25–29 somites. Although the overall rate of closure is greater, on average, in embryos of category 1/2 than 3, and is least in category 4/5, the actual rate of closure may vary during different phases of neural tube development. For instance, category 1/2 embryos with a 'slow' final phase may lag behind their littermates sufficiently to develop tail flexion defects, whereas category 3 embryos with a 'fast' final phase may 'catch up' and develop normally. According to this hypothesis, most, but not all, embryos would be expected to develop 'true to category', as was verified by the experiments. The finding that experimental enlargement to category 4/5 of the PNP in A/Strong embryos failed to produce a single case of LSM could be interpreted as evidence that non-mutant embryos undergo reclosure of the enlarged PNP at a rate that is too rapid to permit development of LSM, although insufficient to avoid development of tail flexion defects in all cases.

It may be concluded that tail flexion defects and open spinal NTD share a common pathogenetic mechanism, differing only in the severity of the underlying defect. Since tail flexion defects occur with high frequency in *curly tail* litters, these malformations provide a useful model system for studying the pathogenesis of open spinal NTD. Moreover, the method for distinguishing between PNP categories permits comparisons to be made between prospective normal and abnormal *curly tail* embryos at a stage prior to the appearance of malformations. Differences that may be identified early in development are more likely to reflect primary pathogenetic mechanisms than are abnormalities identified after the appearance of NTD when secondary degenerative processes would be expected to operate.

Pathogenetic mechanism of tail flexion defects

The developmental mechanism whereby delay of PNP closure results in appearance of a tail flexion defect is unknown. It is possible that delay of PNP closure imposes mechanical stresses on the developing tail bud due to an imbalance between the 'developmental schedules' of the neural tube and of non-neural structures. Such mechanical stresses are expected to be particularly severe in the zone of transition between formation of the neural tube by closure of neural folds and by canalization of the medullary cord which occurs at a level immediately caudal to the PNP (Dryden, 1980). In the present experiments, a large proportion of operated A/Strong embryos that failed to develop tail flexion defects exhibited duplication or multiple splitting of the neural tube throughout the region of the tail bud. All embryos that developed tail flexion defects had major regions of non-duplicated neural tube. Perhaps complete schisis of the neural tube serves to dissipate the stresses imposed on the developing tail bud by delay of PNP closure and results in development of a straight tail.

A number of mouse strains other than *curly tail* exhibit tail malformations in association with NTD, e.g. *Bent tail* (Johnson, 1976) *Danforth's short tail*

(Gluecksohn-Schoenheimer, 1945), and *Splotch* (Auerbach, 1954). These mutants differ considerably in the final form of their tail defects, presumably reflecting the complexity of interactions between the various structural elements during morphogenesis of the tail bud. Nevertheless, in the few mutants for which observations on early embryos have been reported, the initial abnormality of tail development often comprises a dorsal flexion that resembles closely the defect as it first appears in *curly tail* (e.g. *Splotch*; Auerbach, 1954). It remains to be determined whether disturbance of PNP closure may be of fundamental importance in the development of tail abnormalities in these mutants.

Pathogenesis of open NTD: re-opening versus *failure of closure*

According to the re-opening hypothesis of Gardner (1973), open NTD result from rupture of a previously closed neural tube due to high intraluminal pressure. The finding of an over-expanded neural canal in the region of tail flexion defects in *curly tail* embryos could be interpreted as supporting this hypothesis. For instance, lesser degrees of overexpansion, although insufficient to cause rupture of the neural tube, could lead via some unknown mechanism to development of tail flexion defects (Copp *et al.* 1982). The present paper has shown, however, that tail flexion defects can occur in A/Strong embryos in the absence of an over-expanded neural canal. Although neural tube and canal perimeters were enlarged, the neurectodermal layer was folded inwards, and the lumen was consequently not increased in size. It remains a possibility that over-expansion of the neural canal is a prerequisite for development of open NTD, and that tail flexion defects arise by a quite different mechanism. On the other hand, the findings of this paper indicate that the two malformations arise by a common pathogenetic mechanism which operates via delay of PNP closure. It seems more likely, therefore, that over-expansion of the neural canal represents a secondary effect of delay in PNP closure in the absence of experimental intervention: that is, in *curly tail* embryos but not in operated A/Strong embryos.

I thank Professor P. E. Polani and Mr. J. A. Crolla for reading the manuscript and Mrs. H. M. Roberts for excellent technical assistance. This work was supported by grants from the Mental Health Foundation, the Association for Spina Bifida and Hydrocephalus, and Action Research for the Crippled Child.

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(Accepted 5 February 1985)