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REVIEW ARTICLE

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Curly tail: a 50-year history of the mouse spina bifida model

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Abstract This paper reviews 50 years of progress towards understanding the aetiology and pathogenesis of neural tube defects (NTD) in the *curly tail* (*ct*) mutant mouse. More than 45 papers have been published on various aspects of *curly tail* with the result that it is now the best understood mouse model of NTD pathogenesis. The failure of closure of the spinal neural tube, which leads to spina bifida in this mouse, has been traced back to a tissue-specific defect of cell proliferation in the tail bud of the E9.5 embryo. This cell proliferation defect results in a growth imbalance in the caudal region that generates ventral curvature of the body axis. Neurulation movements are opposed, leading to delayed neuropore closure and spina bifida, or tail defects. It is interesting to reflect that these advances have been achieved in the absence of information on the nature of the *ct* gene product, which remains unidentified. In addition to the principal *ct* gene, which maps to distal Chromosome 4, the *curly tail* phenotype is influenced by several modifier genes and by environmental factors. NTD in *curly tail* are resistant to folic acid, as is thought to be the case in 30% of human NTD, whereas they can be prevented by *myo*-inositol. These and other features of NTD in this system bear striking similarities to the situation in humans, making *curly tail* a model for understanding a sub-type folic acid-resistant human NTD.

Keywords NTD mutant · Axial curvature · Proliferation disturbance · Inositol · RARs

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Introduction

The *curly tail (ct)* mutant mouse provides a model system for the analysis of neural tube defects (NTD). The *ct* gene has variable expression and is incompletely penetrant, with homozygotes developing exencephaly (around 3% of embryos), spina bifida aperta (10%) and a curled tail (50%). The *ct* mutation was discovered in 1950, and first described in a detailed paper by Hans Grüneberg 4 years later (Grüneberg 1954). Twenty five years then elapsed before the mutant was studied further, as a possible model for human NTD. From the late 1970s onwards, a stream of papers began to emerge, resulting in the partial unraveling of the pathogenetic mechanism of spinal NTD in *curly tail* mice. Our understanding of the mode of development of spinal defects in *curly tail* now exceeds that of any other mouse NTD mutant, despite the fact that the *ct* gene has not yet been identified.

Human NTD are considered to exhibit a multifactorial aetiology involving multiple interacting genes and environmental factors (Copp 1998). Hence, analysis of a single mouse mutant may be considered of limited value as a model for human NTD, particularly in view of the large number of other mouse NTD models, based on mutation in a wide range of genes, that are available (Juriloff and Harris 2000). On the other hand, the striking resemblance of the *curly tail* mouse phenotype to NTD in humans (detailed below), has long made it a favourite model for analysis. More recently, the interest in folic acid in the prevention of human NTD has further pin-pointed *curly tail* as a key model system: whereas several mouse mutants exhibit folic acid-preventable NTD, *curly tail* is folic acid-resistant, possibly providing a paradigm for the sub-category of human NTD (estimated at around 30%) that do not respond to folic acid. For these reasons, the *curly tail* mouse continues to be a valuable model for at least certain forms of human NTD.

Origin of the *curly tail* mouse

The *curly tail* mutation arose spontaneously in a litter of inbred GFF mice at the Glaxo Laboratories (Greenford, Middlesex, UK) in 1950. The litter contained one male and three females with various tail deformities. Only one female produced offspring, after mating with a CBA/Gr male, and the *curly tail* stock was derived from the offspring of this female. The stock has been maintained, ever since, as a random-bred closed colony, in which all individuals are considered to be of genotype *ct/ct*. Since mice of the GFF strain are no longer available, there has been no suitable wild type control strain available for comparison with *curly tail*, until the recent development of partially congenic strains.

In the original study by Grüneberg (1954), the *curly tail* mutation appeared incompletely penetrant, with variable expressivity. The expression with the highest incidence, curled tail (denoted CT), consists of a narrow spiral twist in the middle or proximal half of the tail. Some mice exhibit twists more proximally, at the root of the tail and, in these cases, the distal portion of the tail is very short. This 'proximally curled' form of the tail phenotype is often associated with lumbosacral spina bifida (SB) in which the spinal cord and vertebral canal are both open to the outside. While pups with the more extreme forms of SB die at or shortly after birth, milder forms, showing a lanceolate opening in the sacral region only, survive and the SB is overgrown by skin in the first few days of life (Fig. 1). The third phenotypic expression of the *curly tail* mutation is exencephaly (EX), which was rare in Grüneberg's study (Fig. 2c). The remaining mice (40–50%; denoted ST) appear unaffected, with straight tails and normally closed spinal cord and brain.

Following Grüneberg's study, the *curly tail* mice were not used until 1975, when breeding pairs were transferred to Mary Seller and Paul Polani at Guy's Hospital, London.

Fig. 1 Adult *curly tail* mouse (*ct/ct*) with a curl in the proximal region of the tail and a healed sacral spina bifida. Such mice appear otherwise normal, are fertile, and live to an old age

These workers recognized the value of *curly tail* as a mouse model for human NTD, a view stimulated by their discovery of increased alphafetoprotein (AFP) in the amniotic fluid of affected fetuses (Adinolfi et al. 1976). This finding was subsequently extended by Jensen et al. (1991), who reported elevated AFP in maternal serum during *curly tail* pregnancy. Hence, a close parallel exists between NTD in *curly tail* and in humans. The work by Seller, Polani and colleagues acted as a stimulus for a large number of further studies of *curly tail*, over the next two decades. In the remainder of this article, we review the main findings of these studies, emphasising the light they have shed on the genetics, interaction with environmental factors and pathogenesis of NTD in the *curly tail* mouse.

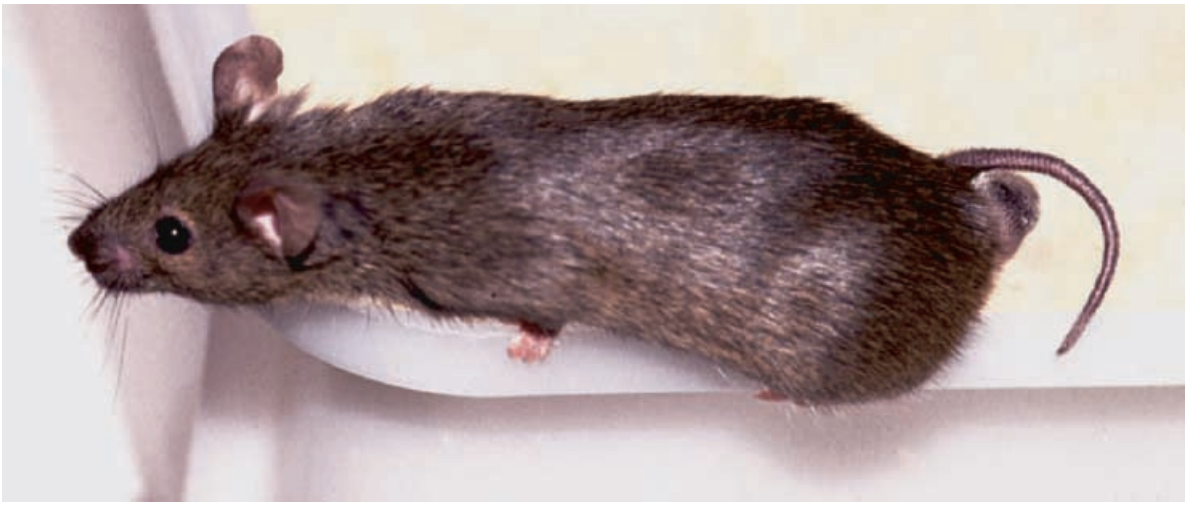
Genetics of *curly tail*

Incomplete penetrance with variable expression in *curly tail* NTD

Table 1 summarizes the variations in penetrance of the *curly tail* phenotype that have been recorded by different workers over the 50-year period of the studies. Although the overall incidence of NTD has remained around 50–60%, relatively large variations have occurred in the frequency of the most extreme NTD types: SB and EX. Interestingly, studies that report the highest EX frequency tend to observe the lowest SB frequency, and vice versa. This finding is reminiscent of the contrasting effects of excess retinoic acid administration on the frequency of EX and SB (see below) and may suggest a general 'reciprocal' relationship between the occurrence of cranial and low spinal NTD in *curly tail*.

Sex differences in the penetrance of EX and SB

Several studies (Embury et al. 1979; Seller et al. 1979, 1987a; Copp and Brook 1989) show that EX predominates in female *curly tail* fetuses, whereas SB exhibits a



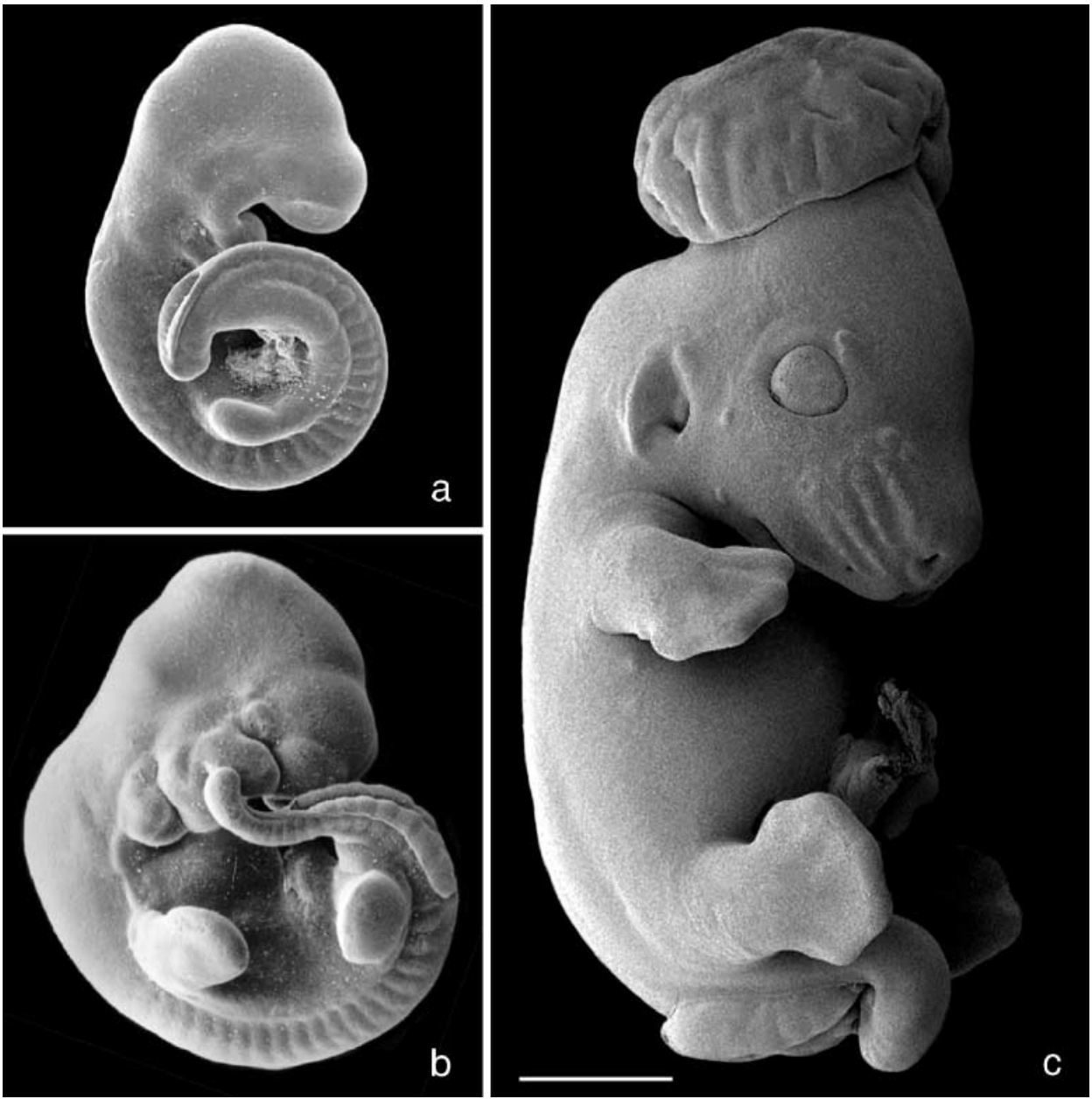


Fig. 2a–c Appearance of NTD in *curly tail* embryos. **a** *Curly tail* embryo (E10) with 30 somites. Closure of the PNP is delayed so that it remains widely open, with everted neural folds, indicating that elevation and subsequent convergence of the neural folds are failing. This neurulation delay has been demonstrated to result from enhanced curvature of the caudal body axis, which is clearly visible in this embryo. The large size of the PNP at this stage indicates that it will not close. **b** *Curly tail* embryo (E11) with 40 somites. The PNP has failed to close, with the result that the neural tube is persistently open from the level of the 28th somite onwards. A marked eversion of the neural folds, and the beginning of a curled tail are visible. This non-closed PNP will develop further into spina bifida aperta. **c** *Curly tail* embryo (E13), exhibiting all three manifestations of the *ct* phenotype: exencephaly (EX), lumbosacral spina bifida (SB) and a curled tail (CT). *Bar* 1 mm for **a–c**

slight male preponderance (Copp and Brook 1989). These sex differences have also been demonstrated for anencephaly and spina bifida in humans (Seller 1987). There does not appear to be a sex-specific prenatal loss of fetuses with EX during development, since the difference in penetrance between the sexes is already present, soon after EX can first be recognized, at E11.5. Seller and Perkings-Cole (1987b) found that the least developed embryos of a *curly tail* litter tend to be females and suggested that relative developmental retardation may increase the susceptibility of females to cranial NTD. However, although male *curly tail* embryos are, indeed, developmentally advanced relative to female embryos, the rate of progress through the stages of neurulation occurs identically in the two sexes (Brook et al. 1994). Hence, it seems unlikely that developmental retardation

Table 1 Penetrance of exencephaly (EX), spina bifida (SB) and curled tail (CT) in several colonies of *curly tail* mice over a 50-year period. All known crosses are CT×CT; in three studies (Seller and Perkins 1986; Copp et al. 1988b; Chen et al. 1994), the parent's phenotype was not reported. SB embryos were all CT, except in two studies, where SB without CT are reported to be 0.3% (Grüneberg 1954) and 0.07% in the Maastricht colony (unpublished data)

Year	Embryo age at analysis	No. of embryos	% EX	% SB+CT	% CT alone	% Total NTD	Curly tail colony
1954	E?	81	0	10	41	51	Grüneberg's[a]
1954	birth	291	0	18	18	36	Grüneberg's[a]
1979	E16	308	9	?	43	52	London, Guy's Hospital[b]
1983	E16	530	0.4	18	44	62	London, Guy's Hospital[c]
1988	E13	36	0	22	39	61	Oxford[d]
1994	E14	461	2.5	9	44	55	London, Inst. Child Health[e]
1995	E13	77	3.8	6	48	58	Maastricht[f]
1995–98	Birth	1494	0	7	48	55	Maastricht colony[g]
1998–99	E12–18	124	0	15	51	66	London, Institute Child Health[g]

[a] Grüneberg (1954) [b] Embury et al. (1979) [c] Seller and Perkins (1986) [d] Copp et al. (1988b) [e] Chen et al. (1994) [f] van Straaten et al. (1995) [g] Unpublished data

per se can cause females to be predisposed to EX, although the reason for this sex bias remains unknown.

The *curly tail* mutation is recessive and influenced by the genetic background

Grüneberg (1954) performed four types of genetic crosses with the *curly tail* mice: (1) matings between affected males and non-affected females resulted in 35% of live-born offspring exhibiting NTD; (2) outcrosses of *curly tail* mice to the inbred strain CBA/Gr yielded essentially normal F_1 offspring; (3) an intercross of the F_1 mice yielded NTD in 5% of offspring; (4) backcross of F_1 mice to *curly tail* produced 24% NTD in the offspring when the *curly tail* parent was affected (CT), but only 6% NTD when the *curly tail* parent was non-affected (ST). The results of crosses 1 and 2 suggest that the *curly tail* phenotype results from the action of a recessive gene, while crosses 2, 3 and 4 indicate that penetrance of the *ct* gene is influenced by genetic background, with the CBA/Gr strain promoting a lower penetrance than the original *curly tail* background. Grüneberg concluded that:

"*critical proof for the*..... [recessive nature of the gene].... *hypothesis would require an effort out of all proportion to the result achieved*".

Despite this warning, genetic studies of *curly tail* mice have continued in recent decades, with further progress towards a detailed understanding of *ct* genetics, as reviewed in the following paragraphs.

Is *curly tail* penetrance dependent on maternal phenotype?

Early studies (Grüneberg 1954; Embury et al. 1979) of *curly tail* mice identified a varying NTD penetrance in the offspring depending on the tail phenotype of the parents. For instance, matings in which one parent was non-affected (i.e. ST×CT) resulted in a lower NTD penetrance than matings in which both parents were affected (i.e. CT×CT). However, transfer of *curly tail* embryos at the blastocyst stage to non-mutant uterine recipients did not lead to a decrease in penetrance of NTD (Seller et al. 1981), arguing against a significant effect of maternal uterine environment. Moreover, in work performed since 1980, the dependence of penetrance on parental phenotype could not be repeated (Copp et al. 1982; Neumann et al. 1994), with the exception of studies in which *curly tail* dams were treated with retinoic acid (Seller et al. 1983; Chen et al. 1994, 1995), or with L-methionine (van Straaten et al. 1995), where a parental phenotype-specific difference in penetrance was still observed. It seems possible that polymorphic modifier gene variants were segregating in Grüneberg's original *curly tail* breeding stock, so that ST and CT individuals may have inherited different combinations of modifying genes. With successive decades of breeding, and re-derivation of the *curly tail* stock from a small number of mice on several occasions (Fig. 3), it seems likely that this genetic heterogeneity diminished or was lost. Indeed, in the early 1990s, *curly tail* mice at the Jackson Laboratories (Bar Harbor, USA) were found to be homozygous at 22 loci that are used for genetic quality control (Neumann et al. 1994). The reason why parental phenotype-specific effects continues to be observed in studies involving exogenous agents such as retinoic acid and methionine is not understood.

Penetrance of *curly tail* is influenced by crosses to other strains

The influence of genetic background on the penetrance of the *ct* gene (Grüneberg 1954) was confirmed by Embury et al. (1979) using outcross and backcross experiments. Neumann et al. (1994) also performed various backcrosses, producing a NTD frequency that was highest with C57BL/6 (18.5%) and lowest with DBA/2 (2.2%). In another type of cross, Estibeiro et al. (1993) found a summation effect between *curly tail* and another NTD mutant, *splotch* (*Pax3* mutant). Mice heterozygous

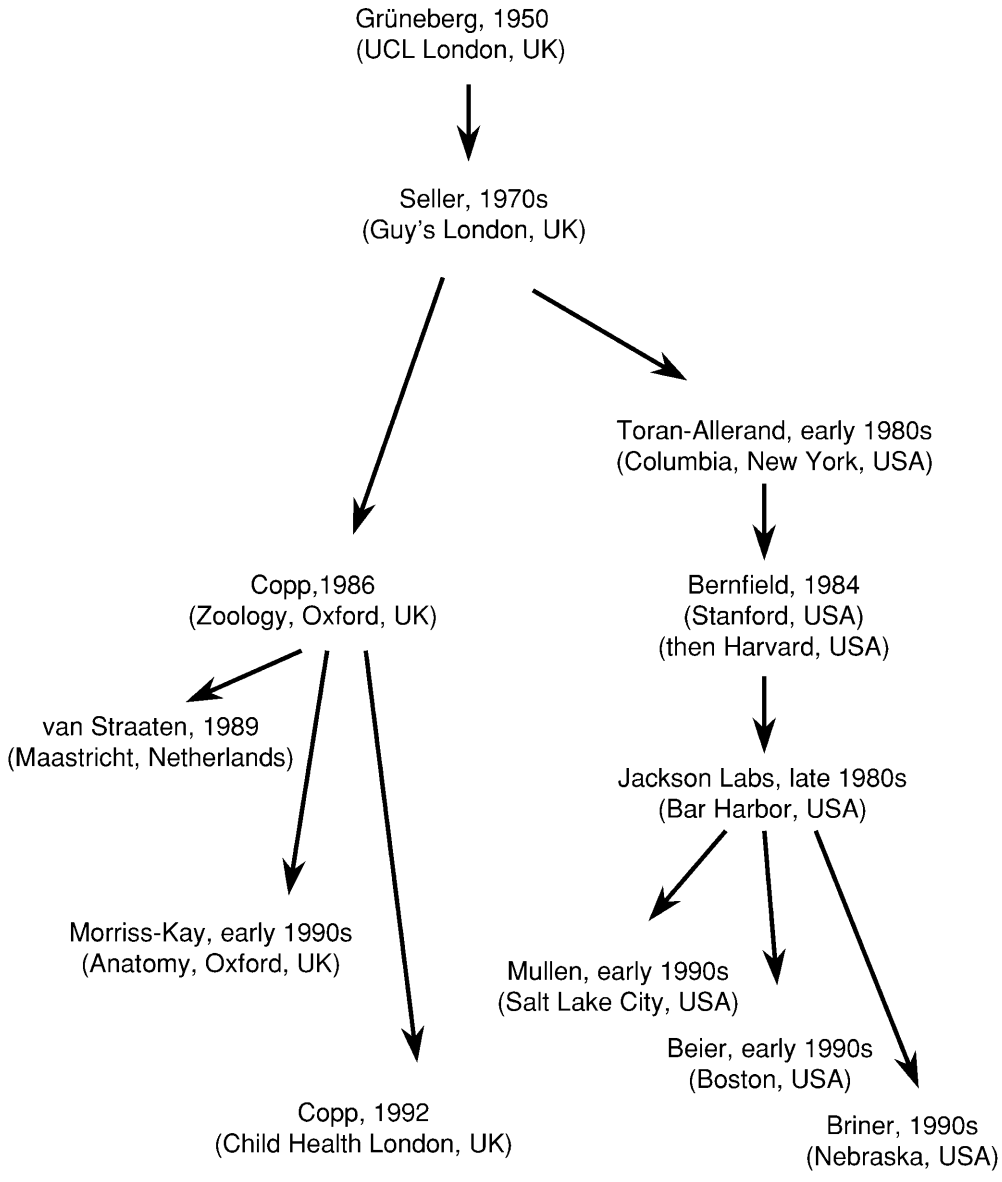


Fig. 3 Movement of *curly tail* mice 1950–2000

for either *curly tail* or *splotch* very rarely exhibited NTD, whereas double heterozygotes (*ct*/+, *Sp*/+) exhibited the CT phenotype in 10% cases. Moreover, backcross of double heterozygotes to *curly tail* mice generates the genotype *ct/ct*, *Sp*/+, among which a spinal NTD phenotype (SB or CT) had an almost 100% penetrance. Thus, the interaction between *ct* and *Sp* represents a summation of the effects of the mutants to produce a disturbance of spinal neurulation that is greater than with either mutation alone.

Is the *ct* gene recessive or semidominant?

Grüneberg (1954) suggested, and Embury et al. (1979) confirmed, that the *curly tail* phenotype is observed only in *ct* homozygotes. Moreover, 6 generations of selecting for the ST phenotype was not able to reduce the incidence of CT offspring, indicating homozygozity for the *ct* gene in all mice within the *curly tail* colony (Embury et al. 1979). On the other hand, Neumann et al. (1994) and Beier et al. (1995), both using backcrosses of (*curly tail* x inbred) F1 mice to the *curly tail* parent strain, produced evidence of phenotypic expression in a small proportion of *ct*/+ heterozygotes. In the congenic strain in Maastricht we also observed an average penetrance of 0.46% (10/2157) in the successive crosses between *curly tail* and BALB/c, although the phenotypic expression (small tail kinks) appeared minimal. Moreover, 11.9% (14/117) of heterozygotes in the London congenic strain had the CT phenotype. Hence, it is clear that the *ct* mutation can exhibit dominance in the presence of particular combinations of modifier alleles, despite the fact that penetrance among homozygotes is incomplete.

Mapping and evaluating candidates for the *ct* gene and its modifiers

Neumann et al. (1994) were the first to describe a linkage analysis that localized the *ct* gene to distal Chromosome 4, approximately 65 cM from the centromere, in the vicinity of the D4Mit13 marker. Beier et al. (1995) independently mapped the *ct* gene to 63.4 cM, close to D4Mit69. Among the several candidate genes considered in these two studies (e.g. *Hspg2*, *Synd3*, *Fgr*, *Cdc2l1*), *Pax7* was selected for detailed analysis, in view of its expression in the neural tube. Although multiple sequence differences were found between *curly tail* and C57BL/6 wild type mice, none of these resulted in amino acid substitutions, suggesting that *Pax7* is an unlikely candidate for the *ct* gene (Beier et al. 1995). In a further study, Peeters et al. (1998a) evaluated *Glut-1* as a candidate for *ct*, but no sequence differences were demonstrated in a comparison with two non-mutant strains. The *ct* gene remains uncharacterized.

Progress has also been made in mapping modifier genes for *ct*. Using recombinant inbred strains, at least three modifier loci were identified, two of which mapped to Chromosomes 3 and 5 (Neumann et al. 1994). Subsequently, a third *ct* modifier locus was identified on Chromosome 17 (Letts et al. 1995). The existence of genetically-defined modifier genes provides definitive evidence of multifactorial inheritance in *ct*, an important parallel between NTD in *curly tail* and in humans.

Development of *curly tail* congenic strains: a tool for pathogenesis studies

Since all mice in the *curly tail* colony are homozygous *ct/ct*, a difficulty arises in interpreting the results of *curly tail* research, owing to the lack of a co-isogenic wild type control strain. Differences between *curly tail* and a given control strain might point to strain differences rather than to a specific *curly tail* effect, and minor DNA sequence differences might represent polymorphisms, rather than causative mutations. In some studies, unaffected *curly tail* embryos have served as controls for affected littermates, but these studies may have indicated secondary differences not related to the *ct* gene effect, or resulting from the expression of modifier genes, rather than differences that are direct effects of the *ct* major gene.

The solution to this problem was to produce a congenic strain in which both wild type and mutant alleles of *ct* are present. In Maastricht such a strain was constructed by backcrossing the *ct* mutant gene onto a BALB/c inbred background for 5 generations. These mice, with BALB/c inbred mice as a control, were successfully used in several recent studies (Peeters et al. 1998a, b). In London, a complementary congenic strain was produced by backcrossing the wild type *ct* allele, derived from the SWR inbred strain, onto the *curly tail* background for four generations. This strategy has succeeded in producing a partially congenic strain ($+^{ct}$) which shares more than 90% of its genetic background with the *curly tail* stock, but which is wild type throughout the *ct* genetic region on Chromosome 4 (G. Pavlovska and A.J. Copp, unpublished). Both of these congenic strains are available for distribution to interested workers.

Interaction of *curly tail* with environmental factors

Effect of teratogens, nutrients, anti-mitogens and hyperthermia

In the 1980s, Seller and colleagues evaluated the effect of a variety of exogenous agents on *curly tail* penetrance. Both teratogenic agents and nutrients, including trypan blue, methotrexate, cortisone, progesterone and vitamin E, were tested but none had any substantial effect on the incidence of NTD (Seller and Adinolfi 1981).

In contrast to these negative findings, the anti-mitogens hydroxyurea, 5-fluorouracil, mitomycin C and cytosine arabinoside all exhibited striking effects on the penetrance of NTD in *curly tail* mice (Seller 1983; Seller and Perkins 1983, 1986). Treatment of pregnant females at E8.5, the critical time for cranial neurulation, led to an increase in the frequency of EX, whereas treatment at E9.5, when the low spinal neural folds are closing, led to a reduction in the frequency of SB and CT. The frequency of EX was also increased, to a total incidence of 20%, by subjecting pregnant *curly tail* mice to hyperthermia (Seller and Perkins-Cole 1987a). Although no effect of hyperthermia on the frequency of caudal NTD was seen in this study, subsequent work showed an ameliorating effect of hyperthermia on delayed posterior neuropore closure, the developmental precursor of CT and SB (Copp et al. 1988b). Hence, environmental factors that produce embryonic growth retardation have contrasting effects on NTD in *curly tail* mice, exacerbating the cranial phenotype, but lessening the frequency and severity of the spinal phenotype. This subject is discussed further below.

Cell proliferation-related factors: glucose and transferrin

At embryonic stages of neural tube closure, glucose acts as the main energy source, and is important for cell proliferation. Determination of glucose handling by cultured *curly tail* embryos indicated a normal glucose uptake per cell (*curly tail* congenic vs control; Peeters et al. 1998a). Iron is another factor that is essential for cell proliferation. In its transferrin-bound form, iron is taken up and localized in the hindgut of midgestation mouse embryos (Copp et al. 1992). Immunostaining revealed a reduced binding and/or uptake of transferrin by hindgut epithelial cells in affected *curly tail* embryos compared with their unaffected littermates, but no difference in distribution or level of expression of the transferrin receptor was seen (Hoyle et al. 1996). Although transferrin receptor is expressed specifically in the hindgut, mRNA is present in all posterior tissues, indicating post-transcriptional

control. Diminished transferrin uptake may be implicated in the decreased rate of cell proliferation observed in *curly tail* hindgut endoderm (see below), although this possibility has not been examined in detail.

Folate metabolism is not disturbed in *curly tail* embryos

When, in the early 1980s, a possible preventive role of folic acid emerged, in relation to the occurrence and recurrence of human NTD, Seller (1994) tested folic acid and other folate derivatives in *curly tail*, but found no remedial effects. Methionine is an amino acid intricately related to folate metabolism. It is important for normal neurulation in rat embryos (Coelho et al. 1989), and reduces the penetrance of NTD in the *Axd* mouse mutant (Essien and Wannberg 1993). However, methionine supplementation did not cause any alteration in penetrance in the *curly tail* embryo, while a methionine-loading test did not result in an aberrant rise of the serum homocysteine levels, when compared with three non-mutant strains (van Straaten et al. 1995). Recently, use of the deoxyuridine suppression test, a biochemical screening test for defects of folate metabolism, found normal folate cycling in *curly tail* embryos, in contrast to *splotch* mice in which there is a defect of folate metabolism (Fleming and Copp 1998). These findings indicate that folic acid is not preventive in the *curly tail* mouse, and that folate metabolism is undisturbed. Hence, *curly tail* has become known as a genetic model of folate-resistant NTD.

Effects of retinoic acid on the *curly tail* phenotype

Vitamin A, and its most bioactive metabolite retinoic acid (RA), are potent teratogens producing NTD as well as other birth defects. When RA was administered to pregnant *curly tail* mice at E8.5, an increase in NTD was observed, comprising a specific increase in the frequency of EX, but no alteration in SB incidence. When administered at E9.5, in contrast, the overall incidence of NTD was reduced, as a result of a specific preventive effect on CT and SB, whereas the frequency of EX was not altered (Seller et al. 1979, 1983; Seller and Perkins 1982). Prevention of low spinal NTD by RA was confirmed and extended in a further study (Chen et al. 1994) in which RA was administered in utero, at the stage of posterior neuropore closure. A preventive effect was observed on both tail defects and SB (50% and 36% reduction in frequency, respectively). These results point to a narrow teratogenic time window in which RA appears to act, and to radically different mechanisms of interaction of RA with cranial and low spinal neurulation.

Inositol can prevent spinal NTD in *curly tail*

Myo-inositol plays a vital role in the inositol/lipid cycle, as a source of metabolic intermediates for processes including signal transduction, steroid synthesis and intracellular calcium regulation. The role of inositol in the *curly tail* has been tested in three studies. Cockroft et al. (1992) found that inositol deficiency increases the incidence of EX to 71% in *curly tail* embryos, whereas non-mutant embryos exhibit EX in 61% (inbred CBA/Ca strain) or 26% (outbred PO stock) of embryos cultured under identical inositol-deficient conditions. Addition of exogenous *myo*-insitol was able to greatly ameliorate this effect, but *curly tail* embryos required a higher concentration of inositol than non-mutant embryos to achieve the same rescuing effect. Inositol deficiency did not lead to spinal defects in this study, although embryos were not cultured to the stage of low spinal closure. Subsequently, Seller (1994) reported preliminary data suggesting that inositol supplementation reduces the frequency of NTD in *curly tail*. This work was extended and confirmed in a third study by Greene and Copp (1997) who found a 70% reduction in the frequency of SB after a single injection of *myo*-inositol at E9.5. Inositol was also able to normalize posterior neuropore closure after addition to embryo cultures, an effect that could be mimicked by the protein kinase C (PKC) agonist, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and blocked by the protein kinase C inhibitor GF109203X. Hence, the protective effect of inositol appears specific and involves stimulation of protein kinase C, but does not appear to be mediated by growth retardation, unlike the protective effect of cytotoxic factors.

Pathogenesis of NTD in *curly tail*

Exencephaly

Relatively few studies have addressed the pathogenesis of EX in *curly tail* mice, although its penetrance can be increased by exposure of embryos to retinoic acid, antimitogens, hyperthermia and inositol-deficient culture conditions. This increase in the frequency of EX has been explained on the basis of a temporary reduction in cell proliferation rate (Seller and Perkins 1986), although the pathogenetic mechanism underlying EX in *curly tail* has not been studied further. The EX phenotype appears independent of the caudal phenotypes.

Spina bifida in *curly tail* is due to primary failure of closure, not reopening

Grüneberg (1954) described the morphological defect of SB as comprising a region of open neural tube extending from the low thoracic level to the base of the curled tail. Was SB the result of reopening of an already closed neural tube, or did closure fail to occur? Copp et al. (1982) studied this question by means of dye injection into the closed part of the neural tube, whereby leakage at its caudal end would indicate an open posterior neuropore (PNP), even when very small. In non-mutant embryos,

the PNP appeared closed at the 30-somite stage, whereas 50% of *curly tail* embryos with 30–34 somites still showed an open PNP (Fig. 2a). Even at more advanced stages, 20% of *curly tail* embryos had an open PNP, apparently corresponding to the 15–20% of fetuses that exhibit SB later in development. These data were confirmed in another study (van Straaten et al. 1995). To examine the relationship between delay of PNP closure and development of SB in more detail, the technique of in vitro embryo culture technique was used (Copp et al. 1982; Copp 1985). This enabled individual embryos to be followed during PNP closure. SB proved to be always preceded by non-closure of the PNP, whereas CT was preceded by PNP closure that was delayed, but ultimately completed. In a morphometrical study (van Straaten et al. 1992), the length of the PNP in *curly* tail embryos during development was measured in detail. Some embryos exhibited an increase of up to five times the original PNP size, but the rostral border of the PNP still proceeded in caudal direction, although much more slowly than the caudal border. This phenomenon indicated that reopening did not occur, and explained the increase in PNP length.

It was concluded, therefore, that SB in the *curly tail* mouse does not arise from reopening of a closed neural tube, but from a primary failure of neural tube closure. This was one of the first direct demonstrations of non-closure in spina bifida studies.

Spina bifida is a defect of primary neurulation, not canalization

A related question concerned the site of failure of closure: was this in the region of primary neurulation, or in the region where secondary neurulation occurs? This question was prompted by the suggestion that lumbosacral spina bifida in humans is mostly a defect of secondary neurulation (Toriello and Higgins 1985; Seller 1987). During primary neurulation, neural tube formation is achieved by elevation, convergence and fusion of the neural folds, whereas in secondary neurulation the neural tube forms by cavitation of a solid cord of progenitor cells (so-called ‘canalization’). To resolve this question, Copp and Brook (1989) mapped the level of final closure of the posterior neuropore in mice and found the transition point between primary and secondary neurulation to be at the level of somites 32–34, corresponding to the future second sacral segment. Grüneberg (1954) had already localized the rostral margin of SB defects in *curly tail* embryos to low thoracic levels, indicating a failure of primary neurulation. In two subsequent, more detailed studies (Copp and Brook 1989; van Straaten et al. 1992), the rostral margin of the spina bifida was located at the level of somites 27–32 in a series of *curly tail* embryos (Fig. 2b), demonstrating unequivocally that SB originates rostral to the level of final PNP closure (i.e. in the region of primary neurulation). Careful analysis of PNP closure in human embryos revealed a close similarity in level of closure to the mouse, suggesting strongly that in humans, as well, SB usually represents a closure failure during primary neurulation (Copp and Brook 1989).

Spina bifida and tail defects are alternative outcomes of delayed PNP closure

The question arose: since almost all *curly tail* embryos exhibit delayed PNP closure, why do some progress to develop SB+CT, whereas others develop only CT? The answer to this question came from a study of individual *curly tail* embryos developing in whole embryo culture (Copp 1985). While a moderate delay in PNP closure was compatible with eventual completion of closure, giving rise only to the CT phenotype, extreme delay was incompatible with eventual closure, and the neural tube remained open, resulting in the development of SB. Furthermore, experimental reopening of the PNP was used to impose a delay of PNP closure on non-mutant embryos, with the result that a proportion developed the CT phenotype, although SB was not seen in this study.

Hence, SB is the result of a permanent closure delay, whereas CT is the result of a temporary delay. These studies supported Grüneberg’s suggestion that delayed closure is the main developmental caudal lesion leading to SB and CT in *curly tail* mice.

How do tail defects develop and are they always associated with spina bifida?

The mechanism by which the tail curls dorsally following transient delay of PNP closure is poorly understood, although it has been demonstrated that the neural tube is abnormally positioned in curled tails, lying on top of the somites rather than between them (Copp et al. 1982). The study by Grüneberg (1954) revealed a strong association between SB and CT: of 417 affected mice, 262 had SB with CT and 151 had CT alone. Only 4 mice (0.3%) had SB without CT, indicating that the association of SB with CT is strong but not absolute. In the Maastricht Colony, over a 4 year period, only 1 out of 1494 neonates was found to exhibit SB without CT. Chen et al. found, however, that 13% of RA-treated embryos with SB show a straight tail (ST), indicating that the developmental association between SB and CT can be uncoupled (Chen et al. 1994). Although SB is clearly a defect of primary neurulation, and a curled tail follows delayed PNP closure, the region of tail that curls is formed during secondary neurulation. Hence, RA appears to have different effects on primary and secondary neurulation in *curly tail* embryos, with a greater ameliorating effect on the tail defect than on SB.

A defect of cell proliferation underlies spinal NTD

Once it had been demonstrated that the primary pathogenetic defect in *curly tail* mice is faulty closure of the

PNP, the impetus was to determine the mechanism underlying this developmental abnormality. A study of cell cycle kinetics in the caudal region of *curly tail* embryos (Copp et al. 1988a) demonstrated a reduced rate of cell proliferation in the hindgut endoderm and notochord, underlying the PNP region at the 27–29 somite stage. Strikingly, however, the cell proliferation difference was not observed in the closing neural plate itself, suggesting the existence of a cell proliferation imbalance in the caudal region of *curly tail* embryos, with slower growth specifically in ventral midline tissues. This growth reduction in the caudal region was confirmed in a later study by Peeters et al. (1998a), who demonstrated a 25% reduction in the growth of the caudal region in embryos of 16–23 somites (*curly tail* congenic vs control), which is largely before the onset of PNP closure delay, and a 10% reduction in embryos of 26–33 somites, which is during PNP closure delay.

Spina bifida can be prevented by growth retardation

The studies by Seller et al. (Seller 1983; Seller and Perkins 1983, 1986) had demonstrated that penetrance of caudal NTD was reduced after application of antimitogens, and this finding was extended in a study of the effects of growth retardation on spinal NTD (Copp et al. 1988b). Growth retardation was induced either by food deprivation during pregnancy or by culture of *curly tail* embryos at 40.5°C rather than the usual 38°C. In utero growth retardation reduced the incidence of enlarged neuropores in 27–29 somite stage embryos, and the overall incidence of caudal defects, particularly SB, was diminished. Hyperthermia in vitro resulted in normalization of PNP closure. Labelling embryos in culture with [^{3}H]-thymidine showed that hyperthermia reduced proliferation to a greater extent in the neuroepithelium than in other cell types. These results led to the idea that the genetically-determined growth imbalance in *curly tail* embryos can be counteracted by growth retardation that affects the neuroepithelium to a greater extent than the hindgut endoderm and notochord. The growth balance is therefore restored in the caudal region, avoiding development of SB.

Axial curvature of the PNP region is temporarily enhanced

The next question to be asked was: what is the causal connection between the cell proliferation imbalance and the development of SB in *curly tail* embryos? Close examination of the caudal region of affected *curly tail* embryos with 27–29 somites revealed an enhanced ventral curvature (Fig. 2a) compared with unaffected embryos (Brook et al. 1991). Moreover, the angle of curvature declined, even in normal embryos as they progressed from 27 to 29 somites, immediately preceding the final phase of PNP closure. However, curvature angles continued to be greated in affected *curly tail* embryos throughout the PNP closure period. These findings suggested that decreasing curvature is associated with PNP closure and that the delay in closure in affected *curly tail* embryos may result from temporarily enhanced curvature.

In a subsequent study (Peeters et al. 1997), the development of axial curvature was studied in more detail. Affected *curly tail* embryos were found to exhibit an enhanced curvature beneath the entire neuropore region as soon as the PNP closure delay could be distinguished. Subsequently, ‘unbending’ occurred during final closure of the PNP, but at a more caudal level than in normal embryos. Thus, both the degree of curvature and the axial level at which unbending occurs appear important for correct PNP closure.

The effect of axial curvature on closure of the low spinal neural tube appears to be mediated via an effect on dorsolateral bending of the neural folds. In a study of the morphology of spinal neurulation in the mouse embryo (Shum and Copp 1996), a transition along the body axis was demonstrated from midline bending, at upper spinal levels, to dorsolateral bending at low spinal levels. In *curly tail* embryos, the transition from midline to dorsolateral bending appeared delayed, and it was proposed that this delay may be caused by stretching of the neural plate owing to enhanced curvature of the body axis.

Hypothesis: a causal relationship between growth, axial curvature and closure of the PNP

The findings on cell proliferation, axial curvature and a temporary or persistent delay of PNP closure in *curly tail* embryos provide the basis for a hypothesis, summarized in Fig. 4. This proposes that a dorso-ventral cell proliferation imbalance leads to temporarily increased ventral curvature of the PNP region of *curly tail* embryos. The curvature generates mechanical stress forces that oppose the dorsolateral bending movements of neurulation, consequently delaying closure of the PNP and leading to SB and CT. Several experiments have been performed to test the hypothesis, as described below.

In the first study (Brook et al. 1991), axial curvature was experimentally prevented by implantation of an eyelash tip into the hindgut lumen at E9.5, before the stage at which enhanced curvature is observed. This manipulation resulted in amelioration of the closure delay, consistent with a direct causal relationship between axial curvature and PNP closure delay. It was also demonstrated that experimental reopening of the PNP in non-mutant embryos does not lead to increased curvature, demonstrating that enhanced curvature is not a secondary effect of PNP closure delay.

In a second study (van Straaten et al. 1993), the caudal neural tube of the *curly tail* embryo, including the PNP region, was mechanically separated from the underlying notochord, mesoderm and hindgut. In culture, the isolated neural tube/plate fragment remained straight and exhibited PNP closure at a higher rate than was observed

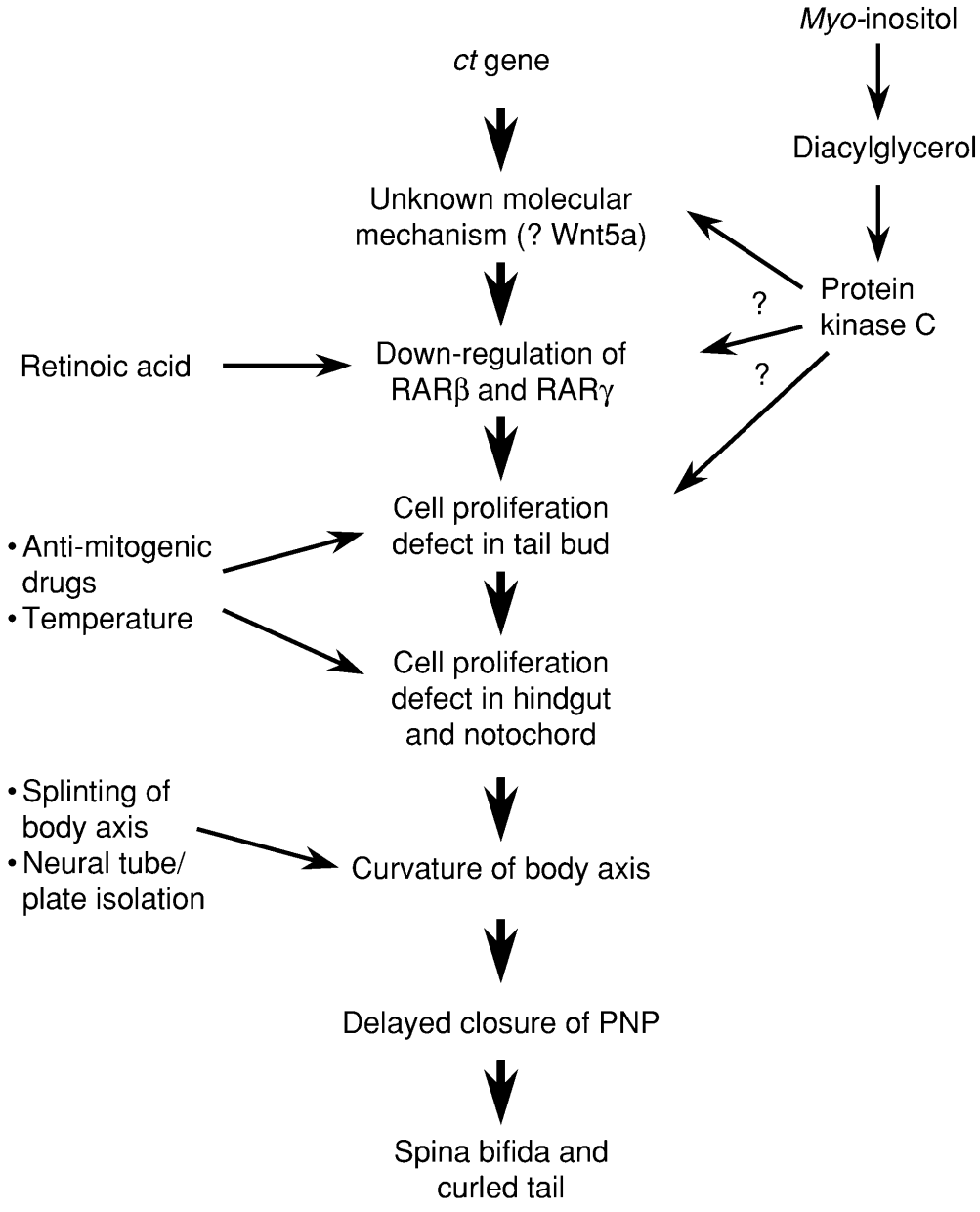


Fig. 4 A diagrammatic summary of our current understanding of the pathogenesis and prevention of low spinal NTD in the *curly tail* mouse. The central sequence depicts the molecular, cellular and tissue events that are known to occur in *curly tail* embryos. Left and right sides of the diagram show preventive agents and experimental interventions that can prevent *curly tail* NTD, with an indication of the level of the pathogenetic sequence at which these influences act

in caudal fragments of *curly tail* embryos in which the notochord, mesoderm and hindgut were left attached. This study confirmed that the primary defect in *curly tail* embryos is localized to the underlying tissues, not within the neuroepithelium. In a complementary experiment, chick embryos (which develop almost flat in ovo) were grown on a slightly curved substrate. This resulted in delay of PNP closure, demonstrating the general principle that the rate of neural tube closure can be modulated by altering axial curvature.

In a third study (Peeters et al. 1996), the amnion of non-mutant mouse embryos was punctured so that, with subsequent development in culture, curvature of the caudal region was imposed as a mechanical effect of the more or less tightly wrapped amnion. A positive correlation was found between the imposed curvature of the caudal region and the incidence of non-closed PNPs. It was also demonstrated that *curly tail* embryos, cultured at 40.5°C to induce growth retardation, exhibit a decrease in axial curvature in association with a reduced PNP length.

Hence, these experiments tested various aspects of the hypothetical developmental mechanism (Fig. 4), and provided support for its operation during the emergence of spinal NTD in *curly tail* mice.

Further evidence that the *curly tail* defect does not reside in the neuroepithelium

The above experiments indicated that the *ct* defect is present in tissues underlying the PNP, not in the neuroepithelium itself. This was confirmed in further studies, which dealt with the later developmental pathogenesis of

neural tissue in SB. Expression of several neuronal antigens, including Islet-1/2, NCAM, neurofilament and neuronal-specific nuclear protein (NeuN), was found to be normal during neuronal development and axonal outgrowth in *curly tail* mice with SB, compared with unaffected littermates (Keller-Peck and Mullen 1996). This supports the idea that the neuroepithelium is intrinsically normal in *curly tail*, and that its faulty morphogenesis results from attachment to abnormal ventral tissues. In contrast to neuronal gene expression, proliferation of the neuroepithelium within the persistently open PNP appeared abnormal (Keller-Peck and Mullen 1996). BrdU incorporation was significantly lower at E10 (when final PNP closure normally occurs), normal at E11–13 and increased at E13, compared with closed neural tube. This pattern of changing proliferation was also seen in NTD of the *splotch* mutant, suggesting that it arises as a secondary result of non-closure of the neural tube, and is not a direct effect of the *ct* mutation. A similar explanation may apply to the lack of expression of Annexin IV, a marker of dorsal midline structures, in the presumptive roof plate region of *curly tail* embryos developing SB (Hamre et al. 1996). However, another roof plate marker, *Wnt1*, is expressed normally, at the tips of the unclosed neural folds in *curly tail* embryos with SB (D.G. Wilkinson and A.J. Copp, unpublished), raising the question of whether roof plate cells, like other neural cell types, form normally in the absence of neural tube closure, in *curly tail* embryos.

Is normal cell proliferation restored by RA and inositol?

A proportion of spinal NTD in *curly tail* mice can be prevented by both RA and *myo*-inositol, raising the question of whether these agents influence cell proliferation in the tissues affected by the growth imbalance: the hindgut and notochord. Chen et al. (1995) examined the expression of nuclear RA receptors (RARs) that mediate the effect of RA intracellularly, and which are differentially expressed in the caudal region during low spinal neurulation (Ruberte et al. 1991). In affected *curly tail* embryos with 27–29 somites, RARβ showed reduced expression in the hindgut, while RARγ was down-regulated in the tail bud. Strikingly, however, both RARβ and RARγ were upregulated only 2 h after RA treatment. RARβ is known to play a role in the control of cell proliferation, suggesting that the specific upregulation of RARβ in the hindgut endoderm by exogenous RA may normalize the under-proliferation in this tissue. Support for this explanation came from the subsequent finding that *myo*-inositol also causes up-regulation of RARβ in the hindgut endoderm, prior to prevention of NTD (Chen et al. 1995).

RARγ, on the other hand, is predominantly expressed in the embryonic tail bud, where it mediates the effect of exogenous RA on tail bud development (Lohnes et al. 1993). In *curly tail* embryos, up-regulation of RARγ seems likely to underlie the putative effect of RA on secondary neurulation in *curly tail* embryos, which is able to prevent tail defects specifically, uncoupling the CT phenotype from SB (Chen et al. 1995).

Origin of the *curly tail* cell proliferation imbalance is in the embryonic tail bud

Although delayed closure of the PNP was detected in *curly tail* embryos from the 27 somite stage (Copp 1985), and later from the 24 somite stage (van Straaten et al. 1992) onwards, recent studies demonstrated that the cell proliferation imbalance, between dorsal and ventral cells, is detectable earlier, in the tail-bud region of embryos with only 21–25 somites. The tail bud represents a developmental continuation of the node, containing progenitor cells of the hindgut and other caudal structures. Peeters et al. (1998b) found a decreased rate of cell proliferation at the ventral side of the tail bud in embryos at the stage prior to the onset of enhanced curvature (21–25 somites), although this abnormality of proliferation had disappeared from the tail bud (and was now detectable in the PNP region) at the stage at enhanced curvature (26–30 somites). Hence, reduced ventral tail bud proliferation precedes the proliferation imbalance found at later stages (Copp et al. 1988a).

Molecular mechanism of the cell proliferation defect

Several molecular abnormalities have been identified in the caudal region of affected *curly tail* embryos. These correlate, in some cases, with reduced cell proliferation in the notochord and hindgut endoderm, at the 27–29 somite stage, while other molecular defects are also present in the ventral tail bud at earlier stages. The extracellular matrix proteoglycan hyaluronan exhibits reduced accumulation (Copp and Bernfield 1988), transferrin uptake by the hindgut endoderm is diminished (Hoyle et al. 1996), and RARβ and RARγ are down-regulated in the hindgut and tail bud respectively (Chen et al. 1995). A further molecular correlate of the reduced ventral tail bud proliferation was identified by Gofflot et al. (1998), who found reduced expression of *Wnt5a* in the ventral mesoderm and hindgut endoderm of the tail bud at the stage of enhanced curvature, as well as at younger stages (20–22 somites). This abnormality of gene transcription appears specific since expression of other caudal genes, *Shh*, *HNF3α*, *HNF3β*, *Brachyury*, *Hoxb1*, *Evx1*, *Fgf8* and *Wnt5b* showed no differences between affected and unaffected *curly tail* embryos. Hence, several cell proliferation-related genes (e.g.*Wnt5a*, *RARβ*) are misexpressed in the *curly tail* caudal region, although it has not yet been determined whether these genes are directly involved in the molecular pathogenesis of the cell proliferation defect.

Conclusions regarding the pathogenesis of spina bifida and tail defects in the *ct* mouse

The current view of the pathogenesis of spinal NTD in the *curly tail* mouse can be summarized as follows. The earliest sign of aberrant development is in the tail bud region at the 22 somite stage, when both cell proliferation and *Wnt5a* expression are reduced. These defects precede the onset of ventral curvature of the caudal embryonic axis, which is detectably enhanced by the 27–29 somite stage. At this later stage, both *RARβ* and *RARγ* are both down-regulated, although expression of these genes has not yet been studied in the tail bud of earlier embryos. Since both RA and *myo*-inositol can normalize PNP closure, and appear to act via up-regulation of *RARβ*, it is tempting to consider that the cell proliferation abnormality may involve the faulty regulation of RARs and/or *Wnt5a*. However, no studies have yet examined directly the effects of RA or *myo*-inositol on cell proliferation in the caudal region of *curly tail* embryos, leaving the question of the molecular regulation of the cell proliferation defect in *curly tail* unresolved.

The pathogenetic events at the 26–30 somite stage are more clearly understood. The cell proliferation imbalance in the PNP region produces enhanced ventral curvature of the caudal body axis, which, in turn, leads to delayed closure of the PNP, perhaps by counteracting the development of dorsolateral bending sites in the closing neural tube. As the angle of curvature declines with developmental progression, some embryos are able to complete PNP closure, but they develop a curled tail, perhaps as a result of deficient *RARγ* expression in the region of secondary neurulation. In other embryos, however, PNP closure delay is too severe to allow closure and, despite the 'unbending' of the caudal region, SB cannot be avoided.

General conclusions and future challenges

Over a 50-year period, a number of parallels have been drawn between NTD in the *curly tail* mutant mouse model and in the human situation. AFP level is elevated in both amniotic fluid (Adolfini et al. 1976) and maternal serum (Jensen et al. 1991), there is an association with polyhydramnios and hydrocephaly (Embury et al. 1979), females predominate amongst fetuses with EX (Seller and Perkins-Cole 1987b; Copp and Brook 1989), and the existence of modifier genes points to multifactorial inheritance (Neumann et al. 1994; Letts et al. 1995). Moreover, NTD in *curly tail* are resistant to folic acid (Seller 1994; Greene and Copp 1997), as in a proportion of human NTD, but can be prevented by *myo*-inositol (Gofflot et al. 1998), suggesting a possible alternative therapy for human folate-resistant NTD. An Arnold-Chiari malformation appeared associated with the *curly tail* mouse (Briner and Moellenberndt 1997), suggesting another similarity with human NTD. Hence, *curly tail* appears to provide a useful model for the investigation of a subtype of NTD in humans.

A new field of investigation has been initiated by Briner and Peterson (1998), who demonstrated hyperactivity, hyperreactivity, attentional disorders and learning disabilities in *curly tail* mice with spina bifida, providing an apparent parallel with behavioural studies of children with spina bifida. Further work in this area is awaited with interest.

A major goal for the immediate future is the isolation and characterization of the *ct* gene. We have learnt a great deal about the pathogenesis of NTD in *curly tail* in the absence of an understanding of the mutated gene, but further advances will undoubtedly follow the once the nature of the *ct* gene product is known. The newly developed congenic strains offer a tool to assist in precise mapping of the *ct* gene on Chromosome 4, as a preliminary to the identification of candidate genes in the genetic region. Moreover, use of congenic *ct* mice will facilitate futher studies of *curly tail* pathogenesis. Elucidation of the molecular mechanism that leads to faulty cell proliferation in the tail bud of *curly tail* embryos is a further priority, which will enable the interactions between signalling in the inositol, retinoic acid and *Wnt5a* pathways to be related to the control of cell cycle kinetics in the tail bud. We predict that the *ct* gene is responsible for stimulating or maintaining cell proliferation at a high rate in the ventral tail bud, at the 21–25 somite stage, and subsequently in the hindgut and notochord at the 26–30 somite stage. It remains to be determined exactly how the *ct* gene achieves this, and how mutation of the gene leads to under-proliferation of these tissues.

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