Molecular Studies of Neural Tube Defect Development in the Mouse Embryo

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Abstract

The thesis describes studies of the development of neural tube defects (NTD) in
genetically predisposed mice, specifically the *curly tail* and *loop-tail* mutants. The *curly
tail* mouse exhibits delay or failure of closure of the neural tube at the posterior
neuropore and is a model for low spinal NTD in humans. *In vivo* supplementation of
embryos with the vitamin, inositol, significantly reduces the incidence of spinal defects
raising the possibility of using inositol to prevent a proportion of NTD in humans.
Moreover, inositol treatment of embryos in culture minimises the delay in posterior
neuropore closure that is known, from previous studies, to lead directly to development
of NTD. The mechanism of action of inositol has been examined using *in vitro*
inhibitors and activators, measurements of inositol incorporation and analysis of gene
expression in cultured embryos. The findings suggest a model that involves an increased
flux through the inositol/lipid cycle which leads to activation of protein kinase C and
upregulation of expression of retinoic acid receptor-β in the hindgut, the affected tissue
in the *curly tail* mutant.

The *loop-tail* mutant mouse is a model for craniorachischisis in humans; homozygous
embryos exhibit failure of initial closure of the neural folds resulting in an open neural
tube from the midbrain/hindbrain boundary along the entire body axis. *Loop-tail*
embryos are identified by PCR analysis prior to the failure of neural tube closure.
Analysis of gene expression by whole mount *in situ* hybridisation reveals abnormal
expression of *sonic hedgehog* and *netrin-1* in the notochord and floor plate of
homozygous mutant embryos suggesting that defects in the development of these tissues
may contribute to the development of NTD.

Abbreviations

ANOVA	Analysis of Variance
AP-2	Activator protein-2
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
ConA	Concanavalin A
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinoid binding protein
DAG	sn-1,2-diacylglycerol
DEPC	Diethyl pyrocarbonate
DIG	Digoxygenin
DLHP	Dorsolateral hinge points
DNA	Deoxyribonucleic acid
DPH	Diphenylhydantoin
dpm	Disintegrations per minute
GPI	Glycosylphosphatidylinositol
EDTA	Ethylene-diamine tetra-acetic acid
HNF	Hepatocyte nuclear factor
IP$_3$	Inositol triphosphate
MARCKS	Myristoylated alanine-rich C-kinase substrate
MEM	Modified Eagles Medium
MHP	Median hinge point
MRP	MARCKS-related protein
NBT	Nitroblue tetrazolium chloride
NCAM	Neural cell adhesion molecule
NTD	Neural tube defects

OD	Optical density
PAX	Paired box
PBS	Phosphate buffered saline
PBT	PBS, 0.1% Tween-20
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PG	Prostaglandin
PI	Phosphatidylinositol
PIP$_2$	Phosphatidylinositol 4,5-biphosphate
PKC	Protein kinase C
PNP	Posterior neuropore
RA	Retinoic acid
RAR	Retinoic acid receptor
R$_f$	Relative front value
RNA	Ribonucleic acid
SDS	Sodium lauryl sulphate
SEM	Standard error of the mean
Shh	Sonic hedgehog
SSC	Sodium chloride/ sodium citrate buffer
TBE	Tris-borate EDTA electrophoresis buffer
TE	Tris-EDTA
TLC	Thin-layer chromatography
TPA	12-O-tetradecanoyl-phorbol-13-acetate
tRNA	Transfer RNA
UV	Ultra violet
ZPA	Zone of polarising activity

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CHAPTER 1

Introduction

The studies described in this thesis are concerned with the development of neural tube defects (NTD) in genetically predisposed mutant mice. In this chapter I begin by briefly reviewing the normal events of neurulation in the embryo (Sections 1.1). I then describe the occurrence of NTD which results from failure of the events of neurulation (Section 1.2). The following sections introduce the different mouse models for human NTD (Section 1.3). I first concentrate on the *curly tail* mutant mouse (Section 1.4) and then review the roles of the vitamin, inositol (Section 1.5), and retinoic acid-related molecules (Section 1.6) which interact with the *curly tail* mutation and in this study are shown to be involved in a pathway leading to prevention of NTD (Chapters 3, 4, 5 and 6). The second mutant that I have investigated is the *loop-tail* mouse which is described in Section 1.7. Section 1.8, describes the process of induction of the floor plate of the neural tube, an event which is proposed to occur abnormally in *loop-tail* embryos (Chapter 7). Finally, I introduce the strategies I have used to study the development of NTD in *curly tail* and *loop-tail* embryos (Section 1.9).

1.1 Neurulation

Neurulation is the process of embryonic development that leads to the formation of the neural tube, the primordium of the brain and spinal cord. Although the events of neurulation have been described in detail at the morphological level (Schoenwolf and Smith, 1990) the cellular and molecular mechanisms which control closure of the neural tube remain less well understood. Analysis of these events is important since formation of the neural tube, required for establishment of the entire central nervous system, is clearly a fundamental process in development. In addition, a consequence of abnormal neurulation is the development of neural tube defects, a major category of human congenital malformations (Section 1.2).

1.1.1 Events of neurulation

In mammals, neurulation can be divided, on the basis of morphology, into two phases termed primary and secondary neurulation (reviewed by Copp *et al.,* 1990; Morriss-Kay *et al.,* 1994). Primary neurulation is the process of principal interest in this study and

commences with the induction of the embryonic ectoderm to form the neural plate in the dorsal midline. The lateral edges elevate to form neural folds which continue to elevate until the apices appose. Neural tube formation is subsequently completed by fusion of the folds in the dorsal midline at which stage the neuroepithelium and surface ectoderm of the neural folds lose their continuity. The surface ectoderm from each fold fuses to form the external epithelial layer and the neuroepithelium fuses to form the roof plate of the newly formed neural tube.

In secondary neurulation, which occurs caudal to the level of the mid-sacral region, the neural tube is formed by cavitation of a condensed rod of cells in the tail bud mesenchyme (Schoenwolf, 1984).

1.1.2 Factors contributing to primary neurulation

Several different factors have been proposed to contribute to elevation and fusion of the neural folds and these may be intrinsic or extrinsic to the neuroepithelium and vary at different levels of the body axis.

Those models, which propose that the driving force is intrinsic to the neuroepithelium, commonly envisage a mechanism of microfilament contraction that causes the dorsal surface of the neural tube to shrink relative to the ventral surface, leading to bending of the neural plate (Schoenwolf and Smith, 1990). Transmission electron microscopy reveals the presence of actin-containing microfilament bundles in the apices of neuroepithelial cells during neurulation and these are associated with contractile proteins such as spectrin (Karfunkel, 1974; Nagele and Lee, 1980; Sadler *et al.*, 1986). Moreover, *in vivo* treatment of rat embryos with cytochalasin D or E, agents which inhibit microfilament polymerisation, causes failure of cranial neural tube closure (Austin *et al.*, 1982). This effect appears to be mediated through an influence on neural fold elevation since exposure of cultured rat embryos to cytochalasin D during neurulation causes the cranial neural folds to collapse, while transfer to fresh medium reverses the effect (Morriss-Kay and Tuckett, 1985). Although these observations suggest an

important role for actin-containing microfilaments in cranial neural tube closure, it is not known if this extends to spinal neurulation. Furthermore, it is not clear whether contraction of microfilaments constitutes a driving force in neurulation or is required for stabilisation of the elevated neural folds.

Morphological studies in the chick have revealed the presence of specific hinge points in the neural plate around which the neural folds elevate and converge. These are formed by localised cell wedging, which appears to involve localised alterations in the cell cycle (Schoenwolf and Smith, 1990). A single median hinge point (MHP) is located in the midline above the notochord and paired dorsolateral hinge points (DLHP) are also located in each neural fold in the future brain region. Shaping of the neuroepithelium by formation of hinge points may provide an intrinsic force for neurulation, although the MHP is not an essential factor since its extirpation does not prevent neural tube closure (Schoenwolf and Smith, 1990). Therefore, the MHP may play a greater role in the initial formation of the neural groove than in driving the elevation of the neural folds. In the mouse, hinge points are also present although their presence varies at differing axial levels (Section 1.1.4).

Axial stretch forces may play a role in neurulation since the period of closure of the neural tube correlates with rapid elongation of the midline of the neural plate in newt and chick embryos (Jacobson and Gordon, 1976; Jacobson, 1984). Moreover, UV irradiation causes a concomitant inhibition of both elongation and neural tube closure in the cranial region of chick embryos and both are normalised by photoreactivation (Jacobson, 1984).

In terms of factors extrinsic to the neural plate, the extracellular matrix provides a potential driving force. During elevation of the neural folds in the cranial region there is a large expansion of the paraxial mesoderm (Morriss and Solursh, 1978; Morris-Wiman and Brinkley, 1990) due to expansion of the mesodermal extracellular matrix. Among matrix components, increased abundance of the glycosaminoglycan, hyaluronan, appears

particularly important for expansion of the cranial mesoderm (Fleming *et al.* 1996). The expansion pressure created in the mesoderm underlying the neural plate is proposed to lead to the elevation of the neural folds on either side of the tethered midline. However, this mechanism is unlikely to play a significant role in the spinal region. At this level the mesoderm lateral to the elevating neural folds comprises epithelial somites or presomitic mesoderm (more caudally) in which expansion of the extracellular spaces does not occur in a similar manner to the cranial region.

1.1.3 Discontinuous initiation of neural tube closure

Closure of the neural folds in the mouse embryo is initiated at three sites of *de novo* contact along the body axis (MacDonald *et al.*, 1989; Copp *et al.*, 1990; Golden and Chernoff, 1993). Initial closure of the neural tube, designated closure 1, occurs at the cervical/hindbrain boundary in embryos at the 5-7 somite stage and continues in both cranial and caudal directions. At approximately the 11-12 somite stage, second and third sites of closure occur at the midbrain/forebrain boundary and at the anterior extremity of the forebrain (Figure 1.1). This discontinuous pattern of closure was originally demonstrated by isolation of embryos from the uterus at increasing gestational ages (Golden and Chernoff, 1993) and confirmed by observation of individual embryos cultured through the period of neurulation. The presence of discrete initial closure sites leads to the formation of three regions of open neural tube, the so called neuropores, which progressively shorten and close as neurulation progresses. Primary neurulation concludes with closure of the posterior neuropore (PNP) at the 28-31 somite stage.

1.1.4 Variation in neural tube closure with axial level

The mechanism of neural tube closure probably exhibits variation with axial level as indicated by differences in both the timing and morphology of neurulation events (Figure 1.1). In the spinal region of the mouse embryo, three morphological modes of primary neurulation have been identified which differ in the presence or absence of hinge points in the neural folds (Shum and Copp, 1995). In mode I, which occurs during spinal neural tube closure in embryos with fewer than 16-18 somites, there is a clearly

Figure 1.1 Stages of neural tube closure in the mouse embryo. Neural tube closure is initiated at the sites numbered 1 to 3 and continues in the direction of the arrows shown. Morphological modes of spinal neurulation as described by (Shum and Copp, 1995) are also indicated. Diagram modified from Copp *et al.,* 1994; Shum and Copp, 1995.
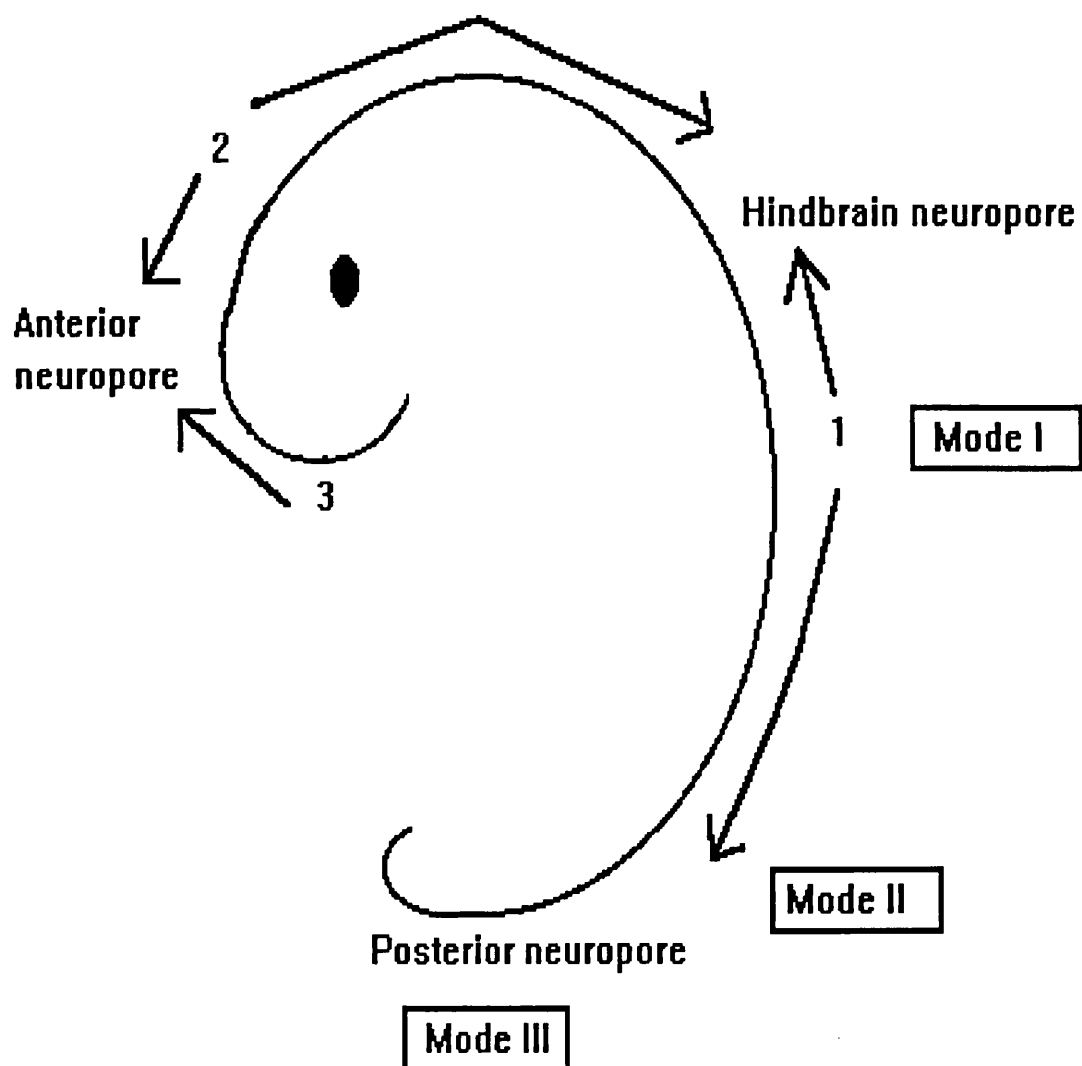
demarcated MHP but no paired DLHP. In mode II, both MHP and DLHP are present, while in mode III the neural folds exhibit uniform bending with no identifiable hinge points. Mode III occurs during spinal neurulation in embryos with more than 21-27 somites depending on the mouse strain (Shum and Copp, 1995).In addition to these differences in timing and morphology, the events at varying levels appear to require the function of specific gene products since various mouse mutants exhibit defects of neural tube closure affecting differing levels of the body axis (Section 1.3). Moreover, closure of the neural tube varies along the body axis in its sensitivity to teratogenic agents, cranial neurulation being affected much more commonly than closure of the posterior neuropore (Copp *et al.*, 1990). All of these lines of evidence suggest that different mechanisms, or different facets of a common mechanism, are critical for closure of the neural tube at different levels of the body axis.

1.2 Neural tube defects

NTD arise from failure of normal neurulation, and the subsequent exposure of the developing brain and/or spinal cord to the *in utero* environment leads to degeneration of the neuroepithelium with subsequent neural deficit. NTD are among the commonest of congenital malformations with an average frequency of approximately 1 in 1000 live births, and with marked geographical variation in prevalence. The prevalence in the British Isles is particularly high with a frequency in Wales and Ireland of approximately 4 per 1000 pregnancies for each of spina bifida and anencephaly, although this has now declined (reviewed by Lemire, 1988). The introduction of prenatal diagnosis in the 1970s has led to a decrease in the incidence of affected live births to around 1-1.5 per 1000 live births.

1.2.1 Subtypes of NTD; variation in axial level and severity

The classification of NTD is somewhat complicated by the plethora of terminology, but three general categories can be recognised depending on the level of the body axis affected (reviewed by Lemire, 1988; Copp *et al.*, 1990). The most severe type of human NTD is craniorachischisis in which the neural tube remains open throughout both cranial and spinal regions. This condition is invariably lethal and comprised 22% of all NTD in a study of affected abortuses (Seller, 1987). In the embryo, failure of closure of the cranial neural tube is referred to as exencephaly and subsequent neuroepithelial degeneration leads to anencephaly which constitutes a severe disruption of brain and skull development and is lethal at or before birth. Spina bifida is a general term applied to lesions in the spinal region (most commonly lumbar or sacral levels) in which the vertebral arches are incomplete in the dorsal midline. In spina bifida aperta, which encompasses meningomyelocele, syringomyelocele, myelocystocele and hydromyelocele, the lesion is open with neural tissue exposed, whereas spina bifida occulta generally involves the vertebral arches alone with the spinal cord covered by epithelialised skin. Spina bifida may be lethal and those children who survive beyond the neonatal period are usually severely affected by multiple handicaps of the musculoskeletal, gastrointestinal and urinary systems.

The pattern of NTD in affected human foetuses suggests that, as in mouse embryos, closure in the cranial region occurs in a discontinuous manner. Defects are clustered into discrete regions which are proposed to correlate either with sites of initiation of closure or sites of final closure of the neural tube (Van Allen *et al.*, 1993; Golden and Chernoff, 1995; Seller, 1995).

1.2.2 Aetiology of NTD

The aetiology of human NTD remains poorly understood but appears complex and multifactorial with contributions from both genetic and environmental factors (Campbell *et al.*, 1986; Lemire, 1988; Copp and Bernfield, 1994). Population studies reveal considerable variation between ethnic groups, with increased frequency for example among Celtic populations of Britain, and in Sikhs, which suggests a role for genetic factors. Moreover, family studies indicate a significantly increased recurrence risk in the siblings of affected individuals (Carter, 1974). Susceptibility is also influenced by sex of the embryo with a greater prevalence of anencephaly among females but an equal or slightly male biased sex ratio in spina bifida (Carter, 1974; Seller, 1987).

The frequency of NTD is also influenced by environmental factors which include geographical location, season of conception, maternal age and nutritional status. In addition, the susceptibility to NTD may be influenced by exposure to exogenous agents which may have a deleterious effect as in the case of teratogens such as valproic acid (Lammer *et al.*, 1987; Section 1.3.2), or an ameliorating effect as shown for folate (Section 1.2.3). Therefore, the overall incidence and severity of NTD appears to depend on genetic predisposition, involving one or more genes, with contributory environmental factors (Carter, 1974). Distinct groups of predisposing factors may be involved in the various categories of human NTD since, in view of the variation in axial level of location, they are likely to involve differing mechanisms.

1.2.3 Approaches to prevention of NTD

The frequency and severity of NTD in humans lends urgency to the requirement for clinical approaches for diagnosis and prevention of defects. Until recently, the birth of affected babies was only routinely prevented by elective termination following prenatal diagnosis of NTD. Diagnosis can be achieved by measurement of α-fetoprotein in the amniotic fluid and more recently by ultra-sonography. The frequency of affected births is reduced by this secondary prevention but a preferable approach would be primary prevention of defects.

Nutritional status is implicated in the aetiology of NTD (Laurence *et al.*, 1980) and clinical trials showed that periconceptional supplementation with a multivitamin preparation reduces the risk of recurrence of NTD following a previous affected pregnancy (Smithells *et al.*, 1981). Subsequently, specific supplementation with one of these vitamins, folic acid, was shown to significantly reduce the recurrence risk in a large scale randomised controlled trial (Wald *et al.*, 1991). Folic acid in a multivitamin supplement also reduces the first occurrence of NTD in a randomly chosen population (Czeizel and Dudás, 1992). Moreover, a number of case-control studies provide further support for the importance of folate nutritional status in determining predisposition to NTD (reviewed by Rush, 1994). Therefore, folic acid supplementation is established as a primary preventive measure for NTD and a daily dose of 0.4 mg is recommended for all women of child bearing age (Burk and Mirkes, 1994). In view of this recommendation the Food and Drug Administration in the USA announced in March 1996 that fortification of enriched foods with folic acid will be required from January 1st 1998.

The precise mechanism by which human NTD are prevented by folic acid supplementation remains unclear although evidence suggests that folate-related metabolic pathways involving methionine and homocysteine may play a role. Women with a previous affected pregnancy exhibit diminished levels of serum folate and vitamin B_{12}, the coenzyme for methionine synthase (Scott *et al.*, 1990; Kirke *et al.*, 1993).

However, these reductions do not constitute clinical deficiency and therefore appear not to cause NTD *per se* but to represent risk factors. Moreover, inhibition of methionine synthase in cultured rat embryos, using nitrous oxide, is teratogenic but does not cause NTD (Baden and Fujinaga, 1991; Fujinaga and Baden, 1994).

Conversely, elevated levels of homocysteine, which could be a consequence of decreased activity of methionine synthase, are observed in women with current or previous affected pregnancies (Steegers-Theunissen *et al.*, 1994; Mills *et al.*, 1995). However, it seems likely that elevated homocysteine levels also indicate a predisposition to NTD rather than a causative factor since homocysteine does not cause NTD in rat embryo culture (VanAerts *et al.*, 1994).

Analysis of null mutant mice for the *Cart1* homeobox gene may shed light on the possible role of folic acid in the prevention of NTD. These mice develop exencephaly, probably due to lack of forebrain mesenchymal cells (Zhao *et al.*, 1996). Interestingly, the NTD can be largely prevented by maternal administration of folic acid, although the mice still die in the early postnatal period suggesting a requirement for the function of Cart1 protein in other tissues.

Despite evidence for prevention of a significant proportion of human NTD (e.g. 70% reduction in incidence in the MRC vitamin study (Wald *et al.*, 1991)), there remains a subset which do not respond to folic acid supplementation. These NTD appear not to correspond to failure of closure at one specific axial level of the neural tube since analysis of affected foetuses shows that the common types of NTD are all represented (Seller, 1995). The occurrence of folate-resistant NTD demonstrates the need for further studies in order to develop alternative or complementary approaches to primary prevention. One of the aims of the studies described in this thesis was to examine the effect of the vitamin inositol on development of NTD in order to assess its potential efficacy as a primary preventive measure (Chapters 3 and 6).

1.3 Mouse models of neural tube defects

In order to develop and improve approaches for diagnosis, genetic counselling and primary prevention of NTD, it is important to understand the cellular and molecular mechanisms of development of NTD. The study of human embryos at the stage of neural tube formation (third and fourth weeks of gestation) is associated with major practical and ethical difficulties However, the use of model animal systems, particularly genetic mouse mutants may provide important contributions to the understanding of the pathogenesis of NTD in humans. The mouse is a useful experimental system as the embryo is accessible for study and can be cultured *in vitro* through the period of neurulation. Environmental effects on closure of the neural tube can be analysed both *in vitro* and *in vivo*. Moreover a number of genetic mutants are available which are predisposed to develop NTD and the generation of knockout mice provides the opportunity to further manipulate genetic influences.

Three distinct approaches to the study of NTD in mouse models are currently being under-taken and these can be summarised as follows:-

1. Identification of genes, whose mutation leads to neural tube defects in mice, will greatly assist in elucidating the molecular and cellular mechanisms underlying NTD and will also indicate genes which are potentially involved in genetic control of NTD in humans. For example, the *splotch* mouse displays mutations in the *Pax-3* gene (Epstein *et al.*, 1991) whose human homologue is mutated in Waardenburg's syndrome types I and III (Baldwin *et al.*, 1992; Tassabehji *et al.*, 1992). Further to analysis of genes for pre-existing mutants, gene-targeting may reveal further genes whose mutation leads development of NTD. Identification of genes in humans which predispose to NTD would facilitate genetic counselling for prevention of birth defects.

2. Comparison of cellular and molecular events between mutant and wildtype embryos may yield considerable information about the mechanisms involved in the

development of NTD. Clearly these studies are assisted by knowledge of the gene mutation which is ultimately responsible for the phenotype since potential downstream mediators will often be suggested by the nature of the genetic defect. Nevertheless, considerable progress can be made even in the absence of this information, as exemplified by the *curly tail* mutant (Section 1.4.4).

3. The study of mouse mutants enables the efficacy of potential preventive strategies to be tested, which may then be applicable to human NTD. Moreover, alteration of the frequency of defects indicates interaction with the genetic pathway leading to development of NTD and may therefore indicate a possible mechanism.

1.3.1 Genetic models for NTD

A number of mouse mutants have been identified which are genetically predisposed to develop NTD, either in isolation or in association with other abnormalities (Table 1.1). In addition, several targeted gene knockout mice also exhibit NTD (Table 1.2). Several categories of human NTD are represented since the axial level of the defect varies between mutants. Closure 1 fails in embryos homozygous for the *loop-tail* mutation (Section 1.7) leading to craniorachischisis, a severe NTD affecting the entire axis caudal to the hindbrain (Copp *et al.*, 1994). In other strains, for example SELH, closure 2 is affected leading to a 17% incidence of exencephaly (MacDonald *et al.*, 1989). Closure of the neural tube at the PNP is affected in *curly tail* (Section 1.4), *loop-tail* (heterozygotes) and *Splotch* mice (Copp *et al.*, 1990; Copp *et al.*, 1994).

Table 1.1 Mouse genetic models for NTD

Mutant (Symbol)	Gene	Chromo-some	NTD Phenoype	Other defects
Loop-tail (Lp)	-	1	Cranio-rachischisis	Heart
Cranioschisis (crn)	-	-	Exen.	-
Crooked (Cd)	-	6	Exen.	Tail
Exencephaly (xn)	-	-	Exen.	Tooth, eye, kidney, vertebrae, limb
Extra-toes (Xt, Xt^{bph})	Gli-3 [a]	13	Exen.	Limbs, craniofacial defects
Open brain (opb)	-	-	Exen.	Spinal cord, eye, skeleton, limb
Patch (Ph)	PDGF-αr [b]	5	Exen.	Neural crest, mesoderm
Rib fusions (Rf)	-	-	Exen.	Ribs
SELH	Several loci	-	Exen.	-
Trisomy 12 & 14	-	12 & 14	Exen.	-
Bent-tail (Bn)	-	X	Exen. + spina bifida	-
curly tail (ct)	-	4	Exen. + spina bifida	-
Snell's translocation (T(2;4)1Sn	-	2 & 4	Exen. + spina bifida	-
Splotch (Sp)	Pax-3 [c]	1	Exen. + spina bifida	Neural crest, limb muscles
Axial defects (Axd)	-	-	Spina bifida	-
Curtailed (T^c/t^{w5})	-	17	Spina bifida	-
Vacuolated lens (vl)	-	1	Spina bifida	Eye, neural crest

- = not reported. Exen. = Exencephaly. Table modified from Copp *et al.*, 1990; Copp and Bernfield, 1994; Günther *et al.*, 1994.

[a] Gli-3 is a zinc-finger containing transcription factor

[b] Receptor for platelet-derived growth factor α

[c] Member of *Pax* gene family of transcription factors

Table 1.2 NTD in targeted null mutant mice

Gene	Function	Chromosome	NTD Phenotype
Apolipoprotein B	Lipid transport	12	Exencephaly
Cart-1 [a]	Transcription factor	10	Exencephaly
HES-1 [b]	Transcription factor	16	Exencephaly
Hox-a1	Transcription factor	6	Exencephaly
Jumonji (jmj)	Insertion into RBP-2 homologue	13	Exencephaly
MARCKS [c]	Protein kinase C substrate	10	Exencephaly
MRP (F52, MacMARCKS) [d]	Protein kinase C substrate	4	Exen. + spina bifida
p53	Tumour suppressor gene	11	Exencephaly
RAR-α + RAR-γ [e]	Nuclear retinoid receptors	11 & 15	Exencephaly
Twist	Transcription factor	12	Exencephaly

Exen. = Exencephaly. Table modified from Copp and Bernfield, 1994; Fleming *et al.* 1996.

[a] Cartilage homeoprotein 1

[b] Homologue of *Drosophila* hairy and enhancer of split gene

[c] Myristoylated alanine-rich protein kinase C substrate

[d] MARCKS related protein

[e] Only double mutants develop NTD

1.3.2 Environmental models for NTD

In addition to genetic models a number of environmental factors, which may involve

specific deficiencies or exogenous teratogens, cause NTD in the embryo (Table 1.3,

reviewed by Copp *et al.*, 1990). Analysis of the interaction of environmental factors

with the development of NTD may indicate important factors in neural tube closure. For

example, the effect of hyperglycaemia, apparently mediated through a deficiency of

arachidonic acid, suggests a key role for arachidonic acid and downstream

prostaglandins in cranial neural tube closure (see Section 4.1).

Valproic acid, an anti-convulsant, also has teratogenic effects in the neurulation-stage

embryo of mice and rats, causing both exencephaly and spina bifida both *in vivo* and *in*

vitro (Bruckner *et al.*, 1983; Ehlers *et al.*, 1992). The action of VPA may be mediated

through a disturbance of folate metabolism which is particularly interesting in view of the

protective effect of folic acid supplementation in humans. *In vivo* co-administration of

folinic acid and VPA significantly reduces the frequency of VPA-induced exencephaly in

mice (Trotz *et al.*, 1987; Wegner and Nau, 1991) although this result is not replicated in

another study (Hansen and Grafton, 1991; Hansen *et al.*, 1995). Despite these

inconsistencies, VPA does disturb the levels of folate derivatives in the embryos whereas

a non-teratogenic analogue, 2-en VPA, has no such effect (Wegner and Nau, 1992).

Moreover, treatment of serum donors or embryo donors with a folate-related molecule,

L-methionine, has a protective effect on embryos exposed to VPA *in vitro* (Nosel and

Klein, 1992).

In this study *curly tail* and *loop-tail* mutant mice were used as genetic models for NTD

and these are considered in more detail in the following sections.

Table 1.3 Principal teratogens which cause NTD in rodents

Agent	Animal	In vitro	In vivo	NTD phenotype
β-hydroxybutyrate	Mouse	+	-	Exencephaly
Cytochalasin D	Mouse, hamster	+	+	Exencephaly
Cytochalasin E	Mouse	+	+	Exencephaly
Ethanol	Mouse	+	+	Exencephaly
Fasting	Mouse	-	+	Exencephaly
Hydroxyurea	Mouse	-	+	Exencephaly
Hyperglycaemia*	Mouse, rat	+	+	Exencephaly
Hyperthermia*	Mouse, rat, hamster	+	+	Exencephaly
Inositol deficiency	Mouse, rat	+	-	Exencephaly
Phenytoin	Mouse	+	-	Exencephaly
Retinoic acid	Mouse, rat, hamster	+	+	Exen. + spina bifida
Trypan Blue	Mouse, rat, hamster	+	+	Exen. + spina bifida
Valproic acid*	Mouse, rat	+	+	Exen. + spina bifida

Exen.= exencephaly. + = Effect of teratogen observed. - = Not tested. *In vitro* refers to whole embryo culture. *In vivo* refers to maternal treatment. Modified from Copp *et al.*, 1990.

* = Also evidence of teratogenicity in humans. **Hyperglycaemia** is a likely teratogenic cause of the elevated incidence of major birth defects, including NTD, in pregnancies complicated by poorly controlled diabetes mellitus (Kucera, 1971). **Hyperthermia** has been suggested from epidemiological evidence to cause NTD: cases of NTD have been reported following frequent use of saunas and high fever during pregnancy (Milunsky *et al.*, 1992; Lynberg *et al.*, 1994). **Valproic acid** is a known cause of spina bifida, but not cranial NTD, in humans (Lammer *et al.*, 1987).

1.4 The *curly tail* mutant

The *curly tail* mutant mouse was first described in 1954, having arisen in 1950 as a spontaneous mutation in the inbred GFF stock at the Glaxo laboratories. A founder female mutant was mated with a CBA/Gr male and the resulting offspring were maintained as a random-bred closed colony (Gruneberg, 1954).

1.4.1 Partial penetrance and variable expressivity of defect

Approximately 50% of mice homozygous for the *curly tail* gene develop NTD, indicating partial penetrance of the defect, while the remaining mice are apparently normal. The phenotypic expression is variable with NTD comprising exencephaly in approximately 1%, lumbo-sacral spina bifida in 10% and tail flexion defects in 40-50% of cases (Embury *et al.*, 1979; Copp *et al.*, 1982). The nature of spinal NTD in affected mice ranges in severity from a slight kink of the tail through increasing degrees of curly tail to open spina bifida accompanied by a tail flexion defect.

1.4.2 Genetic factors in the *curly tail* defect

Curly tail is a single gene mutation which has been mapped by linkage analysis to the distal region of mouse chromosome 4 (Neumann *et al.*, 1994). *Curly tail* was originally reported to be a recessive trait (Gruneberg, 1954), but is now classified as semi-dominant because a small percentage of heterozygous mice have been found to exhibit tail flexion defects in crosses to several other strains (Neumann *et al.*, 1994; Beier *et al.*, 1995). Predisposition to NTD is greatly influenced by unlinked modifier genes located elsewhere in the genome as indicated by the differing penetrance in various backcross populations (Embury *et al.*, 1979; Neumann *et al.*, 1994). One such locus, *mct 1* (modifier of curly-tail, 1), for which double homozygosity with *curly tail* carries greatest risk for NTD, was mapped to chromosome 17 in backcrosses to *M. spretus* and BALB/cByJ (Letts *et al.*, 1995).

Within the *curly tail* stock the genetic background appears to be relatively homogeneous since mice from the *curly tail* stock are homozygous for 22 marker loci used for genetic

quality control at the Jackson Laboratories, USA and there is no effect of parental phenotype (Neumann *et al.*, 1994). However, this has not always been the case; a study in 1979 (Embury *et al.*, 1979) indicated that there was a greater incidence of spinal NTD among the offspring of CTxCT (curly-tailed) matings compared with CTxST (straight-tailed) matings (52% compared with 42%) suggesting the existence of predisposing genetic factors segregating with the curly-tailed phenotype. In later studies this predisposition to spinal NTD was no longer observed (Copp *et al.*, 1982; Chen *et al.*, 1994; Neumann *et al.*, 1994), probably as a consequence of fixation of the genetic background which has occurred through selective inbreeding. It does appear that a slightly higher incidence of spina bifida may persist among the offspring of curly-tailed females (Chen *et al.*, 1994), which could reflect a non-genetic difference related to the uterine environment of affected versus unaffected females. However, this effect of maternal phenotype is not supported by findings described in Chapter 6 of this study.

Interpretation of experimental data can be hindered by the fact that the *curly tail* stock is not congenic with any other strain and consequently there is no control strain with an identical genetic background. This problem will be discussed more fully in the General Discussion (Chapter 8).

1.4.3 The *curly tail* mutant as a model for NTD in humans

The *curly tail* mouse is considered a good model for NTD in humans based on similarities of pathology, sex bias and axial location (Copp *et al.*, 1990). As in humans, defects in *curly tail* occur most frequently at the lumbosacral level of the body axis and are marginally more common in males than females (Copp and Brook, 1989). Hydrocephalus and increased concentration of α-fetoprotein in the amniotic fluid are also associated with open lesions in human and *curly tail* NTD (Adinolfi *et al.*, 1976; Embury *et al.*, 1979). Moreover, the female excess (4:1) in the incidence of exencephaly (which leads to anencephaly) in *curly tail* mice (Embury *et al.*, 1979) parallels the situation in human anencephaly (Carter, 1974). Finally, the aetiology of NTD in both humans and *curly tail* mice is multifactorial with the incidence and severity of defects

influenced by contributions from multiple genetic and environmental factors (Copp *et al.*, 1990; Estibeiro *et al.*, 1993; Neumann *et al.*, 1994).

In view of the multiple interacting factors it is particularly interesting that there is no ameliorating effect on the incidence of NTD in *curly tail* following treatment of pregnant females with folic acid, folinic acid or methionine (Seller, 1994; Van Straaten *et al.*, 1995). The folate-resistance of NTD in *curly tail* mice contrasts with the protective effect of folic acid supplementation on a proportion of human NTD (usually estimated at 70%) and suggests that *curly tail* may correspond to that sub-category of human NTD (up to 30%) which do not appear to respond to folic acid.

1.4.4 Development of NTD in *curly tail* embryos

Spinal NTD (spina bifida and tail flexion defects) arise in *curly tail* as a result of the delayed closure of the posterior neuropore (PNP) (Gruneberg, 1954; Copp, 1985; Van Straaten *et al.*, 1992), and not by reopening of the neural tube after initial closure (Seller and Adinolfi, 1981). The extent of PNP closure at the 27-29 somite stage gives an accurate indication of the tail phenotype following further development *in vitro*; more than 90% of embryos with a severely enlarged PNP go on to develop spinal NTD and all embryos that develop spina bifida are included in this group (Copp, 1985). PNP categories were defined on a scale of increasing size from category 1, indicating a small PNP in the distal region of the tail bud, to category 5 in which the PNP is severely enlarged, overlapping with the somites in its cranial extent.

Delayed closure of the PNP in affected *curly tail* embryos appears to be a consequence of ventral curvature of the caudal region which is suggested to oppose closure through imposition of mechanical stress on the neural folds (Brook *et al.*, 1991; Van Straaten *et al.*, 1993). Ventral curvature is significantly enhanced in embryos with an enlarged PNP. Moreover, prevention of ventral curvature by implantation of an eyelash tip into the lumen of the hindgut normalises PNP closure in embryos cultured to the 27-29 somite stage following surgery. Increased curvature is not simply a secondary consequence of

delayed closure of the PNP since non-mutant embryos in which the PNP was surgically enlarged exhibited delayed PNP closure in the absence of increased curvature (Copp, 1985; Brook *et al.*, 1991). Moreover, *Splotch* homozygous embryos in which PNP closure is also delayed exhibit no increase in curvature, emphasising that a similar phenotype (i.e. delayed PNP closure) can result from different embryonic lesions and that enhanced curvature is specific to the *curly tail* mutation (Estibeiro *et al.*, 1993). Finally, the observation that imposition of ventral curvature on the caudal region of non-mutant embryos causes a delay in closure of the PNP (Peeters *et al.*, 1996) is supportive of the proposed role of curvature in development of spinal NTD in *curly tail* embryos.

1.4.5 Cellular mechanism for development of spinal NTD in *curly tail* embryos

Affected *curly tail* embryos exhibit a cell-type specific proliferation defect in the gut endoderm and notochord, while the neuroepithelium, surface ectoderm and mesoderm exhibit no difference in cell proliferation between affected and unaffected embryos (Copp *et al.*, 1988a). The consequent imbalance in growth rates between dorsal and ventral tissues is proposed to cause enhanced ventral curvature of the caudal region as the normally proliferating neuroepithelium is tethered to the underlying defective gut endoderm. Indeed, while closure of the PNP of intact caudal regions of *curly tail* embryos is delayed, the rate of closure is similar in isolated neuroepithelia of caudal regions of *curly tail* and non-mutant embryos (Van Straaten *et al.*, 1993). The apparently normal behaviour of the *curly tail* neuroepithelium in the absence of non-neural tissues further suggests that delayed closure of the PNP is caused by an abnormality of non-neural tissues.

Evidence for a role of the cell proliferation defect in the development of spinal NTD was provided by growth retardation of *curly tail* embryos either *in vivo* by 48 hour starvation of pregnant females or *in vitro* by increasing the culture temperature from 38°C to 40.5°C for a 15-23 hour period prior to PNP closure (Copp *et al.*, 1988b). Growth retardation *in vitro* caused a reduction in proliferation rate in all tissues but the decrease

was greatest in the neuroepithelium such that the overall effect was to reduce the magnitude of the growth imbalance. Coincident with the relative correction of the growth imbalance in the caudal region, closure of the PNP was normalised by growth retardation *in vitro*. Therefore, a mechanism for development of NTD in *curly tail* embryos is proposed in which a reduced proliferation rate in the gut endoderm causes a growth imbalance. This leads to increased ventral curvature of the caudal region that opposes closure of the PNP (Figure 1.2).

1.4.6 Molecular abnormalities in the *curly tail* embryo

Several studies have revealed abnormalities at the molecular level in affected compared with unaffected *curly tail* and non-mutant embryos. There is a significant reduction in the accumulation of newly synthesised hyaluronan, an extracellular matrix molecule, in a perinotochordal distribution in the developing basement membranes of the PNP region of affected *curly tail* embryos. The effect appears specific for hyaluronan in this region of the embryo since no other differences in extracellular matrix glycoconjugates were detected in this study (Copp and Bernfield, 1988). It remains unclear whether this abnormality is a causative or secondary effect in the development of NTD.

Affected *curly tail* embryos also exhibit abnormal binding of the iron-binding growth factor, transferrin, which is taken up and localised in the hindgut of neurulation-stage mouse embryos (Copp *et al.*, 1992). Reduced binding is observed in the hindgut of affected *curly tail* embryos compared with their unaffected littermates, although the level and sites of expression of the transferrin receptor are not altered (Hoyle *et al.* 1996). Binding and uptake of transferrin may play a role in regulation of cell proliferation and reduced binding may therefore contribute to the cell proliferation abnormality in the hindgut of affected embryos. However, unaffected *curly tail* embryos also exhibit greater levels of transferrin binding in the hindgut compared with embryos of non-mutant strains (Hoyle *et al.* 1996). This observation suggests an alternative model in which uptake of transferrin is increased as a compensatory response to the genetically-determined proliferation defect in the hindgut. The affected embryos which exhibit

Figure 1.2 Summary of the mechanism of development of spinal NTD in *curly tail* **embryos.** Dotted lines indicate interactions that may be indirect or direct. Continous lines indicate demonstrated direct effects.

Curly tail

Genetic modifiers

Cell proliferation defect
in the embryonic hindgut

Growth imbalance in
in the caudal region

Increased ventral curvature
of the caudal region

Delayed closure of the
posterior neuropore
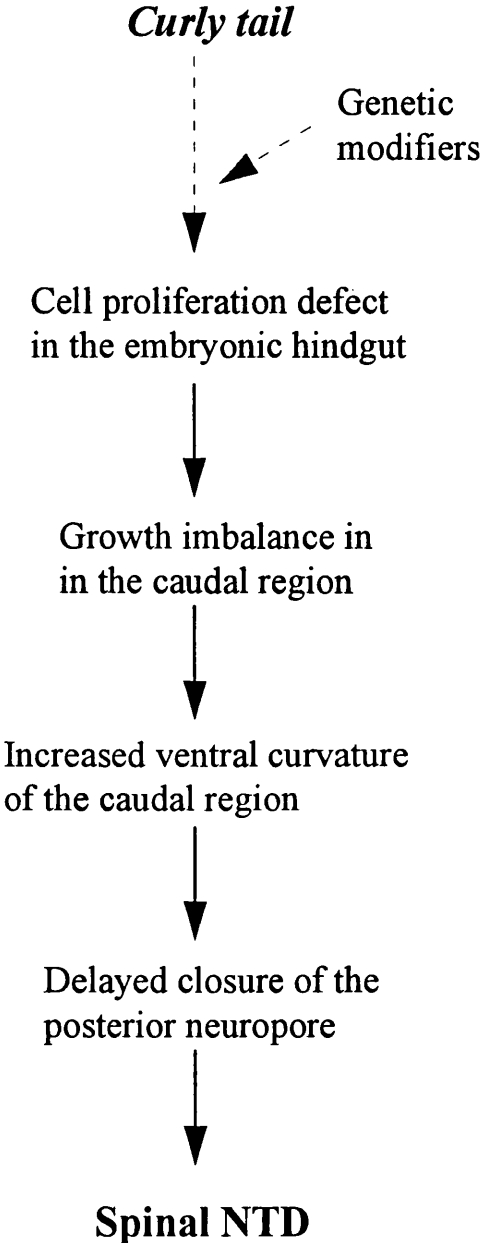
Spinal NTD

reduced binding would therefore represent those that fail to make this response and so cannot avoid developing spinal NTD.

Several studies have demonstrated an interaction between the level of retinoic acid and frequency of NTD in *curly tail* embryos (Section 1.6.1) and this has been correlated at the molecular level with altered expression of nuclear retinoic acid receptors (Section 1.6.2).

1.4.7 Interaction of environment factors with the *curly tail* mutation

In addition to the marked effect of genetic background on the penetrance of the *curly tail* defect (Section 1.4.2) there is a major influence of environmental factors. Despite the possible influence of maternal phenotype, the genetic background of the *curly tail* stock appears to be relatively homogeneous (Section 1.4.2). This implies that the incomplete penetrance observed in the *curly tail* colony (and within individual litters) is attributable to environmental (non-genetic) factors.

A wide range of teratogenic agents interfere with neural tube closure in the mouse, most frequently affecting cranial neural tube closure with induction of exencephaly (Copp *et al.*, 1990). In *curly tail* mice a differing effect of several agents is observed at cranial and caudal levels with exacerbation of exencephaly and amelioration of delayed PNP closure (summarised in Table 1.4).

Embryonic growth retardation through starvation or hyperthermia has an ameliorating effect on closure of the PNP as described in Section 1.4.5. Similar normalisation of PNP closure has been described following maternal administration of hydroxyurea, 5-fluorouracil and mitomycin C on day 9 of gestation (Seller, 1983; Seller and Perkins, 1983; Seller and Perkins, 1986). At earlier stages of pregnancy these agents have a teratogenic effect on closure of the cranial neural tube with a significant increase in the frequency of exencephaly. This phenomenon of varying effects at cranial and caudal

levels is probably related to the growth retarding properties of these cytotoxic agents which inhibits closure of the cranial neural tube while correcting PNP closure through normalisation of the growth imbalance in caudal tissues (Copp *et al.,* 1990).

Particularly relevant to the experiments described in this study are the effects of the vitamin inositol (Section 1.5.1) and retinoic acid (Section 1.6.1) on the incidence of NTD in *curly tail* mice. These topics are considered in the following sections.

Table 1.4 Interaction of environmental factors with development of NTD in *curly tail* embryos

Factor	*Frequency of Spinal NTD*	*Frequency of Cranial NTD*	*Ref.*
Hyperthermia	Decreased	Increased	a
Starvation	Decreased	-	b
Hydroxyurea	Decreased	Increased	c
Mitomycin C	Decreased	Increased	d
5-fluorouracil	Decreased	-	e
Retinoic acid	Decreased	Increased	f
Methionine	No effect	No effect	g
Folates	No effect	No effect	h

-= Not tested. a, Copp *et al.,* 1988b; b, Copp *et al.,* 1988b; c, Seller and Perkins, 1983, d, Seller and Perkins, 1986; e, Seller, 1983; f, Seller *et al.,* 1979; Seller and Perkins, 1982; Chen *et al.,* 1994; g, Van Straaten *et al.,* 1995; h, Seller, 1994.

1.5 Inositol: potential role in neural tube closure

1.5.1 Inositol deficiency and development of cranial NTD

Interest in the influence of the level of the vitamin, *myo*-inositol (referred to in this thesis as inositol), was stimulated by nutritional studies in which rat embryos were cultured in defined conditions through the period of neural tube closure (Cockroft, 1979; Cockroft, 1988). Specific omission of several individual vitamins caused growth retardation or developmental abnormalities but NTD only occurred in the absence of inositol. Deficiency of inositol in the culture medium also causes a high incidence of cranial NTD in mouse embryos (Cockroft *et al.*, 1992). In a different system, embryos exposed to hyperglycaemic conditions have been shown to be inositol deficient and are predisposed to development of NTD (Goldman *et al.*, 1985; Hashimoto *et al.*, 1990).

Curly tail embryos appear to be particularly sensitive to the level of inositol since in deficient conditions they exhibit a high incidence of cranial NTD and, compared with non-mutant strains, require a higher dose of inositol for amelioration of the effects of deficiency (Cockroft *et al.*, 1992). In addition, a small scale study demonstrated a reduction in the incidence of NTD following maternal administration of inositol at day 8 of gestation (Seller, 1994).

1.5.2 Cellular functions of inositol

One of the major aims of this study was the investigation of the role of inositol in closure of the PNP in *curly tail* embryos (Chapters 3, 4, 5 and 6). In this section I briefly review the cellular functions of inositol which are potentially involved in mediating the effect of inositol level on closure of the neural tube (Figure 1.3).

The inositol/lipid cycle

Inositol metabolism is extremely complex with multiple isomers of inositol phosphates and inositol phospholipids present in the cell. The inositol/lipid cycle plays a central role in inositol metabolism (summarised in Figure 1.4) and is involved in signal transduction leading to the regulation of many intracellular functions (Berridge, 1987; Majerus, 1992).

Figure 1.3 Summary of the cellular functions of inositol

The key functional molecules (as reviewed in Section 1.5) are indicated by double boxes. Regulatory events mediated directly by molecules derived from inositol phospholipids are indicated by double lines. PIP_2 = Phosphatidylinositol 4,5 biphosphate, PIP_3 = Phosphatidylinositol 3,4,5-triphosphate, IP_3 = inositol 1,4,5- triphosphate, DAG = 1,2-diacylglycerol, PKC = protein kinase C. Rac is a GTP binding protein.
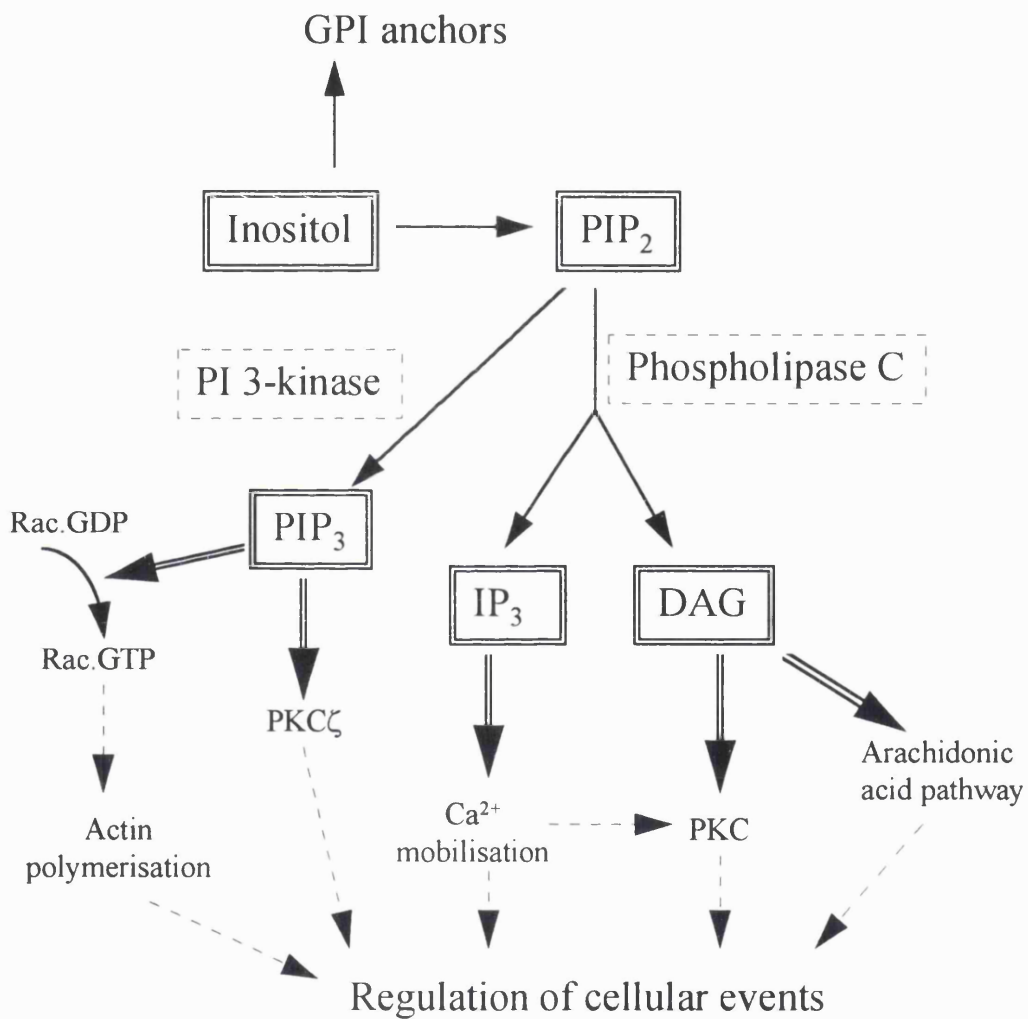
Figure 1.4 Summary of the inositol/lipid cycle

CMP-PA= Choline monophosphate-PA, Ins = inositol, Ins 4- P = Inositol 4-phosphate etc., MG = Monoacylglycerol , PA = Phosphatidic acid, PI= Phosphatidylinositol, PLC = Phospholipase C
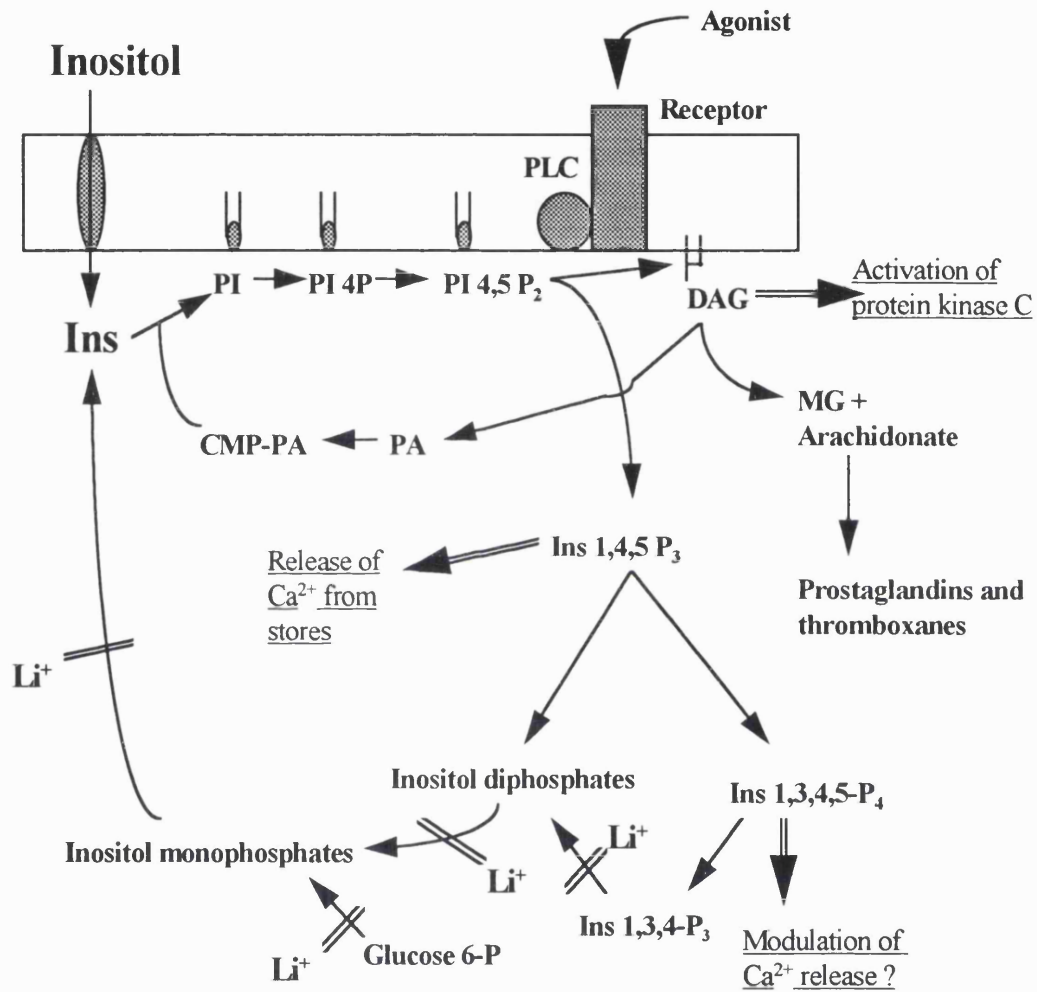
Cellular uptake of inositol occurs via a sodium-coupled transporter which is a member of the sugar transporter superfamily (Nikawa *et al.,* 1991; Batty *et al.,* 1993). Inositol is utilised in synthesis of the membrane lipid, phosphatidylinositol (PI), in which it constitutes the polar head group. Subsequent phosphorylation steps lead to generation of PIP_2 which is the major substrate for receptor activated phospholipase C. The major two pathways for activation of isoforms of PLC involve G-protein linked receptors which activate PLC-β via a GTP-binding protein and receptor tyrosine kinases which activate PLC-γ (Majerus, 1992; Berridge, 1993).

Hydrolysis of PIP_2 generates inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) which are major downstream effectors of the cycle. DAG is a physiological activator of protein kinase C (PKC), an important family of regulatory enzymes. The potential function of PKC in prevention of neural tube defects is one focus of this study and therefore reviewed in more detail in Section 1.5.3. Binding of IP_3 to its receptor leads to mobilisation of calcium from intracellular stores which may regulate cellular processes such as fertilisation, growth control, regulation of actin assembly and neuronal signalling (Berridge, 1993). A role for calcium in neurulation in the chick is suggested by the variation in intracellular calcium levels which reach a peak in the neural folds as they appose prior to fusion (Martin *et al.,* 1994).

A nuclear inositide cycle has been identified which resembles the inositol/lipid cycle at the plasma membrane (Divecha and Irvine, 1995). The role of this pathway remains unclear but may involve a nuclear function such as cell cycle control.

Phosphatidylinositol and the cytoskeleton

A property of polyphosphoinositol lipids, which is apparently distinct from that in "classical" inositide signalling, is binding to various components of the cytoskeleton such as gelsolin, profilin and cofilin (reviewed by Janmey, 1994; Divecha and Irvine, 1995). These interactions may be involved in cytoskeleton modelling. For example, it is

proposed that receptor stimulation could release the monomer-sequestering protein profilin from complexes with PIP_2 leaving profilin free to interact with actin filaments.

The possible function of polyphosphoinositol lipids in regulation of cytoskeleton assembly is intriguing in view of the potential role of actin-containing microfilaments in neurulation. This is suggested by collapse of the cranial neural folds and induction of exencephaly following treatment with cytochalasin D, an inhibitor of microfilament polymerisation (Austin *et al.*, 1982; Morriss-Kay and Tuckett, 1985).

Phosphatidylinositol 3-kinase

Aside from the inositol/lipid cycle, phosphoinositides have an additional role in signal transduction involving the enzyme, phosphatidylinositol 3-kinase (PI 3-kinase) which is recruited and concomitantly activated following stimulation of various receptor tyrosine kinases (reviewed by Kapeller and Cantley, 1994; Divecha and Irvine, 1995). Following auto-phosphorylation and dimerisation, the activated receptors for ligands such as platelet derived growth factor-α and -β, colony stimulating factor-1, c-kit and hepatocyte growth factor, are able to interact directly with PI 3-kinase . An alternative mechanism is also utilised in which the activated receptor interacts with PI 3-kinase via an adapter molecule such as insulin receptor substrate-1 and erb B3 in the case of the insulin and epidermal growth factor receptors respectively.

The active enzyme, a heterodimer of regulatory (85 kDa) and catalytic (110 kDa) subunits, phosphorylates the D-3 position of PI, PIP and PIP_2, the latter probably being the major substrate *in vivo*. Functions for the lipid products of PI 3-kinase have been proposed in activation of Ca^{2+}-independent PKC (PKC ζ), vesicular trafficking and organisation of actin (reviewed by Kapeller and Cantley, 1994). In terms of the effect on cytoskeletal organisation, the requirement of PI 3-kinase for agonist-dependent membrane ruffling has been the focus of study (Ridley, 1994; Parker, 1995). In this case the downstream function appears to be mediated through Rac, a member of the Rho class of GTP-binding proteins, which is activated by exchange of bound GDP for GTP.

46

GPI anchors

In addition to its action in multiple signal transduction pathways, inositol also has a structural role in glycosylphosphatidylinositol (GPI) anchors which tether a number of different proteins to the cell membrane (Englund, 1993). The conserved core structure of the GPI anchor (Figure 1.5), usually with additional sugar substituents on the mannose residues, is attached to the protein by an amide bond between the ethanolamine and the α-carboxyl group of the C-terminus of the protein (Ferguson and Williams, 1988).

Figure 1.5 core structure of the GPI anchor

M= mannose, Ins= Inositol, G= Glucosamine
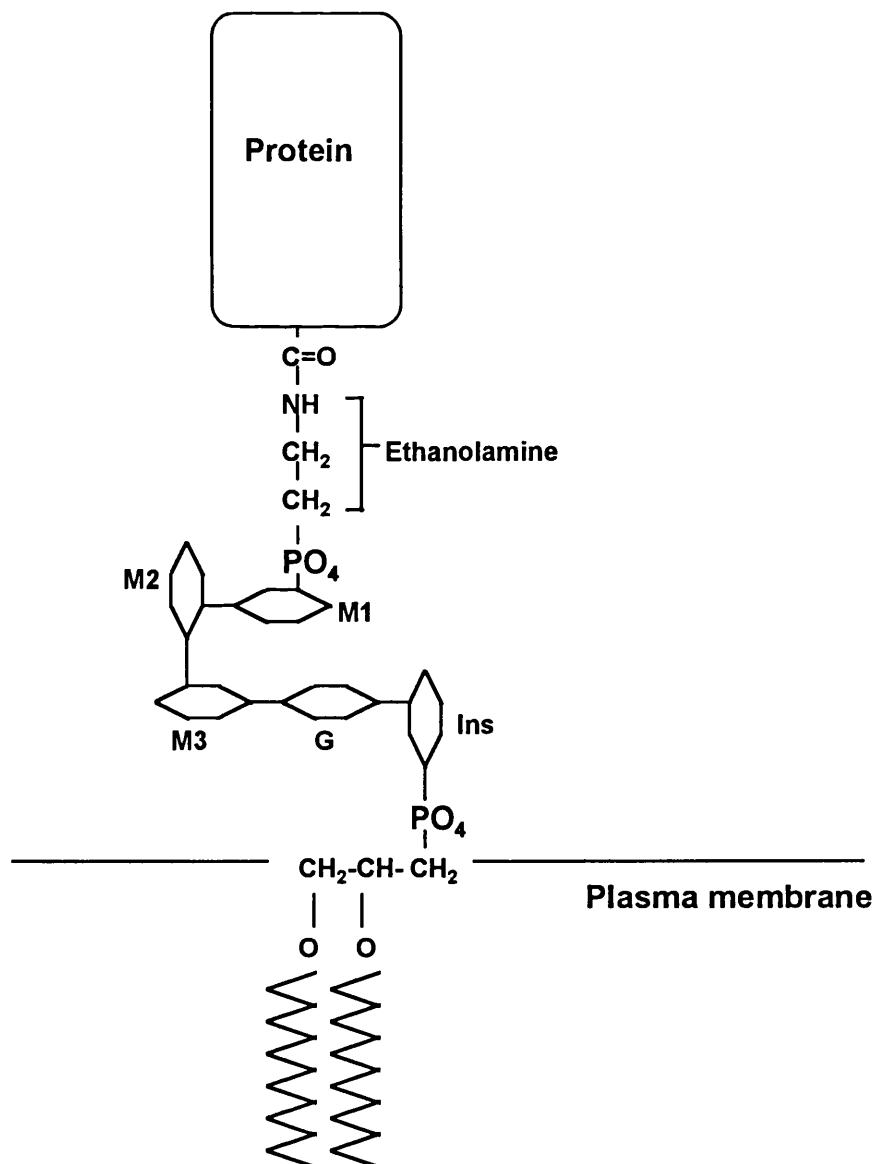
A variety of proteins utilise GPI anchors including cell surface hydrolases (e.g. acetylcholinesterase), adhesion molecules (e.g. $N\text{-CAM}_{120}$) and lymphoid antigens (e.g. Thy-1). In view of the protective effect of folic acid supplementation on NTD in humans, it is interesting to note that at least some forms of the folate receptor, including the native human placental folate receptor (Verma *et al.*, 1992), are attached to the cell membrane by GPI anchors. This observation raises the possibility that induction of NTD by inositol deficiency is associated with depletion of GPI-anchored folate receptors.

1.5.3 Protein kinase C

Diversity and structure of PKC isoforms

Protein kinase C (PKC) represents a family of serine/threonine protein kinases comprising (as presently known) twelve homologous isoforms α, βI, βII, γ, δ, ϵ, ζ, θ, ι, λ, μ and η which differ in enzymatic properties, sites of expression and cellular localisation (Nishizuka, 1988; Nishizuka, 1992; Selbie *et al.*, 1993). The general primary structure consists of four conserved domains, $C_1\text{-}C_4$, and five variable domains, $V_1\text{-}V_5$, (Figure 1.6) which are associated with different functions (Nishizuka, 1992; Hug and Sarre, 1993). The C_1 domain contains a cysteine-rich region consisting of two zinc finger motifs which are required for DAG and phorbol ester binding. C_2 is associated with Ca^{2+}-sensitivity of the enzyme, C_3 contains the ATP-binding site and C_4 the substrate recognition site.

The PKC isoforms have been divided into three groups, classical (cPKC), non-classical (nPKC) and atypical (aPKC), based on Ca^{2+}-dependence and phorbol ester binding (Nishizuka, 1992). Members of the cPKC group, α, βI, βII and γ, are activated by DAG in a Ca^{2+}-dependent manner, while the nPKC group, which comprises δ, ϵ, θ and η, are calcium independent as they lack the C_2 calcium binding domain. The aPKC (ζ, ι, λ and μ) isoforms lack both the C2 region and also one of the zinc finger domains in the C1 region and therefore do not bind or respond to DAG or phorbol esters.

Figure 1.6 Primary structure of isoforms of protein kinase C

V= variable domain, C= conserved domain (modified from Nishizuka, 1992).
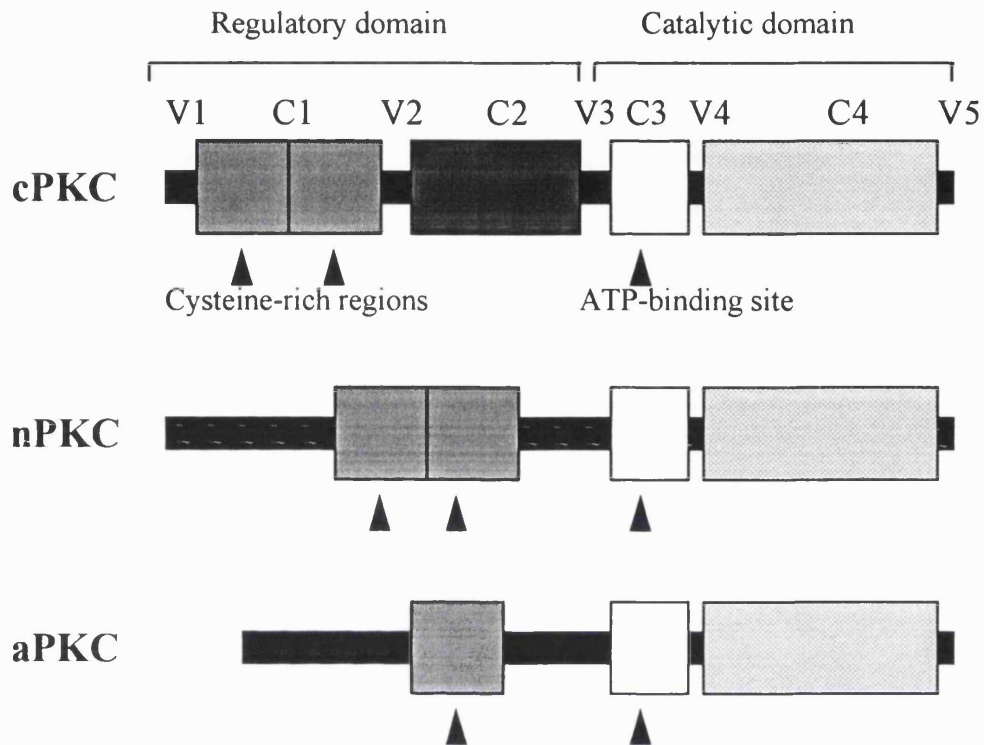
Activation of PKC

A model for activation of cPKC isoforms envisages the receptor-stimulated activity of PLC generating IP_3 and DAG by hydrolysis of PIP_2, leading to mobilisation and binding of Ca^{2+} to the C2 domain followed by translocation of the enzyme to the cell membrane. At the membrane, PKC is activated by DAG via the C1 region, with phosphatidylserine (PSer) acting as cofactor (reviewed by Nishizuka, 1988; Hug and Sarre, 1993).

Phospholipid breakdown and calcium mobilisation are related in their effects on PKC activation in that the presence of high levels of calcium increases the sensitivity to DAG while increased generation of DAG reduces the requirement for calcium (Nishizuka,

1992). Members of the nPKC group do not require calcium and are activated by DAG in the presence of PSer. Both nPKC and cPKC are constitutively activated by phorbol esters, which mimic DAG in binding and activation of PKC (Castagna *et al.*, 1982), but are stable and only slowly metabolised. In this study, use is made of this property of phorbol esters in the investigation of the role of PKC in closure of the neural tube (Chapter 4). The regulation of aPKC is less well established since there is no DAG binding site; PKC ζ appears to exhibit a low but constitutive activity (Liyanage *et al.*, 1992).

Evidence suggests that PKC may also be activated through DAG generated by phospholipase enzymes other than PLC (Nishizuka, 1992). Agonist-dependent activation of phospholipase D leads to hydrolysis of phosphatidylcholine and the phosphatidic acid produced is converted to DAG. Alternatively, phospholipase D may also catalyse the conversion of phosphatidylcholine to phosphatidylinositol which is available for the inositol/lipid cycle. The activation of this reaction by phorbol esters or membrane-permeant DAG suggests that PKC may stimulate phospholipase D which could enhance the production of DAG and contribute to prolonged activation of PKC.

At least *in vitro*, lipid molecules other than DAG may also contribute to activation of PKC. The generation of unsaturated free fatty acids through the action of phospholipase A appears to be particularly important in this respect (Nishizuka, 1992; Hug and Sarre, 1993). For example, oleic, linoleic, arachidonic and docosahexanoic acid (all generated through the action of PLA_2) reduce the Ca^{2+}-dependence and thereby enhance the DAG-dependent activation of PKC. Inositol phospholipids may also play direct roles in the regulation of PKC; PIP_2 has been reported to activate PKCα, βI and γ but not βII and in addition PI may substitute as co-factor for Pser. The *in vivo* relevance of PKC activation by arachidonic acid and other lipids remains unclear, but it seems likely that in addition to the inositol/lipid cycle several different pathways may contribute to the regulation of PKC activity, potentially enhancing and prolonging cellular responses.

Moreover, the varying sensitivity of different PKC isoforms to calcium and various activators adds another level of complexity to the system.

Therefore, the functions of PKC may be regulated at several levels due to the existence of several isoforms with differing calcium and lipid cofactor dependence, substrate specificity and sites of expression.

Expression of PKC

Several different techniques have been used to examine the expression of PKC isoforms in post-natal and adult tissues (Nishizuka, 1988; Wetsel *et al.*, 1992; Bareggi *et al.*, 1995; Hunter *et al.*, 1995). The cPKC group appears to be expressed at low levels during the late stages of gestation and early in the postnatal period and to increase during the first 2 to 3 weeks after birth. However, expression data during embryonic development are not available for mouse embryos at stages prior to day 15 of gestation.

Function of PKC

Roles for PKC have been proposed in regulation of a variety of cellular processes including function of membrane proteins, cell cycle control and transcription of various genes (reviewed by Nishizuka, 1986; Hug and Sarre, 1993). At the molecular level, the effect of PKC is mediated through phosphorylation of protein substrates on serine or threonine residues.

The functions of PKC at the cell membrane include down-regulation of target receptors such as the epidermal growth factor (EGF) receptor, modulation of ion-channel targets such as the Na^+/H^+ exchanger (Sardet *et al.*, 1990)and inhibition of potocytosis (Smart *et al.*, 1994). PKC activity also affects long-term responses such as cell proliferation and gene expression. For example, induction of expression of the transcription factor, c-fos, by insulin or insulin related growth factor-1 occurs via a PKC-dependent pathway (Heidenreich *et al.*, 1993). Phosphorylation by PKC also regulates the activity of the activator protein (AP) transcription factors, AP-1 (through phosphorylation of c-fos) and

AP-2 (Nishizuka, 1986; Imagawa *et al.*, 1987). Activation of c-Raf kinase following phosphorylation by PKC may also be involved in regulation of AP-1 (Hug and Sarre, 1993).

The major PKC substrate MARCKS (myristoylated alanine-rich C kinase substrate) may be of particular interest in relation to neural tube closure as the null mutant mouse displays exencephaly (Stumpo *et al.*, 1995). Moreover, knockout of the MARCKS family protein, MRP (also known as MARCKS-related protein, MacMARCKS or F52) also causes NTD, both spina bifida and exencephaly (Chen *et al.*, 1996; Wu *et al.*, 1996). The potential importance of these proteins will be discussed more fully in Chapter 8. Lack of PKCγ in null mutant mice has no apparent effect on embryonic development but a role in memory was suggested by mild defects in learning tasks associated with function of the hippocampus (Abeliovich *et al.*, 1993a; 1993b).

1.6 Retinoic acid and related molecules

1.6.1 Retinoic acid: prevention of spinal NTD in the *curly tail* mouse

Several studies have demonstrated an *in vivo* effect of all-*trans* retinoic acid (RA), a metabolite of vitamin A (retinol), on the incidence of NTD in the *curly tail* mouse. Treatment of both homozygous (*ct/ct*) and heterozygous (*ct/+*) embryos on day 8 of gestation causes a dose-dependent increase in the frequency of exencephaly (Seller *et al.*, 1979; Seller *et al.*, 1983). In contrast there is a reduction in the frequency of NTD, specifically spinal NTD, following treatment on day 9 of gestation (Seller *et al.*, 1979; Seller and Perkins, 1982). A separate study (Chen *et al.*, 1994) also demonstrated a stage-specific reduction in spinal NTD (spina bifida and tail flexion defects) following low dose (5 mg/kg) RA treatment although in this case the maximal effect was obtained with dosing at 10 days 8 hours. A protective effect, indicated by reduction in mean PNP length, was observed within 6 hours of RA treatment.

The dose of RA which is sufficient to reduce the frequency of spinal NTD had no apparent effect on overall embryonic growth (Chen *et al.*, 1994) which suggests a specific protective mechanism, in contrast to the growth retardation that is proposed to account for the reduction in spinal NTD caused by other agents such as hyperthermia and hydroxyurea (Copp *et al.*, 1988b). Litter size and resorption rate are also unaffected (Seller *et al.*, 1979; Chen *et al.*, 1994) showing that RA does not cause an apparent preventive effect through increased mortality of affected embryos. Therefore, it appears that RA has a specific differential effect on the phenotypic expression of the *curly tail* defect in terms of cranial and spinal NTD which probably reflects differing mechanisms of neural tube closure at these axial levels.

RA effects on the penetrance of the *curly tail* defect appear to be influenced by both maternal phenotype and genotype. At day 8 of gestation the increased incidence of cranial NTD in RA-treated heterozygous embryos is more pronounced with a *curly tail* mother (Seller *et al.*, 1983), suggesting an influence of maternal genotype. Maternal

phenotype in *curly tail* homozygous embryos also influences penetrance with a higher incidence of spina bifida but not total NTD (spina bifida and tail flexion defects) in the offspring of curly-tailed mothers (Chen *et al.*, 1994). In addition, these embryos are more resistant to RA prevention of spina bifida than offspring of straight tailed mothers (Chen *et al.*, 1994).

1.6.2 Retinoid nuclear receptors

The effects of RA are mediated through two families of retinoid binding nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each of which currently comprises three types, α, β and γ. These receptors, members of the steroid/thyroid hormone receptor superfamily, regulate the transcriptional activity of target genes in a ligand-dependent manner. A number of isoforms of each RAR, (RAR-α, -β and -γ) are generated by differential usage of promoters and alternative splicing of primary transcripts (Zelent *et al.*, 1989; Kastner *et al.*, 1990; Leroy *et al.*, 1991; Zelent *et al.*, 1991). Specificity for RA isoforms varies, RARs being activated by all-*trans* RA or 9-*cis* RA while RXRs respond only to 9-*cis* RA (Allenby *et al.*, 1993).

Function of RARs

Ligand-activated RARs function as heterodimers with RXRs; RAR/RXR complexes regulate transcription through binding to specific DNA sequences, known as retinoic acid response elements (RAREs), in the promoter regions of target genes (Leid *et al.*, 1992). On binding 9-*cis* retinoic acid, RXRs can also function as homodimers, binding to retinoid x response elements (RXREs). Activated RARs modulate expression through up- or down-regulation of transcription of a range of targets including Hox B genes (Simeone *et al.*, 1990), laminin B1 (Vasios *et al.*, 1989), transforming growth factor-β1 (Salbert *et al.*, 1993), and proteases of the extracellular matrix such as stromelysin (Nicholson *et al.*, 1990). In addition, several isoforms of RARs (α-2, β-2 and γ-2) are auto-regulated by all-*trans* RA since their promoters contain an RARE (De Thé *et al.*, 1990; Leid *et al.*, 1992). The positive feedback provided by up-regulation of RAR

expression in the presence of their ligand suggests that a small increase in the level of RA could lead to a proportionately greater response in terms of regulation of target genes.

Developmental expression of RARs

The specific spatio-temporal patterns of expression of the RARs in the mouse embryo suggests that they play important and diverse roles in development (Dollé et al., 1990; Ruberte et al., 1990; Ruberte et al., 1991; Ruberte et al., 1993). RAR-α expression is essentially ubiquitous whereas RAR-β and -γ are expressed in more restricted domains. Interestingly, with respect to neural tube development, neurulation stage embryos exhibit complementary patterns of expression of RAR-β and -γ in the trunk region. RAR-β is expressed in the neuroepithelium only in regions where the neural tube is closed whereas RAR-γ is expressed in the region of open PNP and most strongly in the tail bud (Ruberte et al., 1991; Chen et al., 1995). This boundary of expression moves caudally as neural tube formation progresses until the PNP finally closes at the 28-30 somite stage when RAR-γ expression becomes absent from neural tissue, although it continues to be expressed in the tail bud for a further period of development.

Null mutants of RARs

The important function of RARs in development is supported by the phenotypes of mice which are null mutants for the RARs (Table 1.5). The apparent normality of null mutants for the predominant type of the individual isoforms, RAR-α1, RAR-β2 and RAR-γ2 (Lohnes et al., 1993; Lufkin et al., 1993; Mendelsohn et al., 1994), suggests a high degree of functional redundancy between RAR isoforms. Disruption of the whole RAR-α or -γ gene does cause a phenotype and also early post-natal lethality in null homozygotes. There appears to be some redundancy even between RAR family members since even in the total gene knockout for RAR-γ the range of abnormalities is rather limited considering the specific expression pattern (Lohnes et al., 1993). In contrast, RAR double mutants exhibit numerous developmental abnormalities and, with

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Table 1.5 Phenotypes of targeted mutations in RARs and RA-binding proteins

Locus	Phenotype	Ref.
RAR-α1	No apparent defects	a
RAR-α	Neonatal lethal; Testis degeneration	a
RAR-β2	No apparent defects	b
RAR-γ2	No apparent defects	c
RAR-γ	Neonatal lethal; Growth retardation; Cartilage, axial skeletal & glandular defects; Male sterility	c
RAR-α1 + RAR-β2	Heart, eye, thymus, thyroid, parathyroid, tracheal, urogenital defects; Middle ear fusions; Postnatal lethal.	d
RAR-α + RAR-β2	Skeletal abnormalities; homeotic transformations; Heart, eye, thymus, thyroid, parathyroid, tracheal, urogenital defects; Middle ear fusions; Postnatal lethal.	d
RAR-α1 + RAR-γ	Skeletal abnormalities; homeotic transformations; gland defects. Postnatal lethal.	d
RAR-α + RAR-γ	Embryonic lethal (day 13-16); reduced size; exencephaly; Craniofacial & skeletal abnormalities; homeotic transformations; Heart, eye, thymus, thyroid, parathyroid, urogenital, gland, and umbilical defects; Middle ear fusions	d
RAR-β2 + RAR-γ	Skeletal and cartilage abnormalities; homeotic transformations; Eye, thyroid, gland & urogenital defects; Postnatal lethal.	d
RAR-β2 + RAR-γ2	Eye defects (retinal dysplasia); Viable and fertile	e
RXR-α	Embryonic lethal (day 13-16); Eye, heart, liver defects	f
RAR-α + RXR-α	Embryonic lethal (day 13-16); Eye and heart defects	g
RAR-γ + RXR-α	Embryonic lethal (day 13-16); Eye and heart defects	g
CRABP I	No apparent defects	h
CRABP II	Post-axial polydactyly in forelimbs	i
CRABP I + CRABP II	Post-axial polydactyly in forelimbs	j

References: a, Lufkin et al., 1993; b, Mendelsohn et al., 1994b; c, Lohnes et al., 1993; d, Lohnes et al., 1994a; Mendelsohn et al., 1994a; e, 13392; f, Kastner et al., 1994; Sucov et al., 1994; g, Kastner et al., 1994; h, Gorry et al., 1994; i, Fawcett et al., 1995; Lampron et al., 1995; j Lampron et al., 1995

the exception of the RARβ2/RARγ2 mutant (Grondona *et al.*, 1996), are lethal *in utero* or in the early post- natal period (Lohnes *et al.*, 1994a; Mendelsohn *et al.*, 1994a). The phenotypes of these compound null mutants suggests roles for RARs in multiple systems and at differing stages of development (Table 1.5). The compound RAR-α/γ knockout appears to be particularly severely affected since, in addition to malformations affecting other RAR double mutants, these embryos also specifically exhibit exencephaly and craniofacial skeletal defects associated with neural crest abnormalities (Lohnes *et al.*, 1994).

Abnormal RAR expression in the *curly tail* mutant

RAR-β and -γ have been implicated in the *curly tail* defect since both exhibit abnormal expression correlating with delayed closure of the PNP in affected embryos (Chen *et al.*, 1995). Neither RAR-α, -β or -γ are candidates for the *curly tail* gene (localised to chromosome 4) since they are present on different chromosomes; 11, 14 and 15 respectively (Mattei *et al.*, 1991). The level of expression of RAR-β is deficient in the hindgut endoderm, a tissue which is known to exhibit reduced proliferation in affected *curly tail* embryos. RAR-γ expression is also reported to be down-regulated in the tail bud and open neuropore region (Chen *et al.*, 1995). In addition, the protective effect of RA treatment on spinal NTD is proposed to be mediated through up-regulation of transcription of these RARs. Following low-dose RA treatment, embryos exhibit specific up-regulation of RAR-β expression in the hindgut endoderm both in the region of closed neural tube and also beneath the open PNP. Transcription of RAR-γ is also partially corrected in the tail bud and PNP region (Chen *et al.*, 1995). These observations suggest a potential mechanism for development of spinal NTD in *curly tail* embryos in which deficiency of RAR transcripts in the caudal region causes aberrant transcriptional regulation of target genes leading to the specific proliferation defect which is responsible for increased ventral curvature and thus delayed closure of the PNP.

1.6.3 Cellular retinoid binding proteins

In addition to the nuclear retinoid receptors, two groups of cytosolic low molecular weight retinoid binding proteins are also involved in the molecular mechanism of action of RA in the embryo (Blomhoff *et al.*, 1990; Giguere *et al.*, 1990). The cellular retinoic acid binding proteins, CRABP I and CRABP II, and cellular retinol binding protein I, CRBP I (CRBP II is not expressed in the embryo), exhibit specific spatio-temporal patterns of expression in the embryo (Perez-Castro *et al.*, 1989; Dollé *et al.*, 1990; Maden *et al.*, 1992; Ruberte *et al.*, 1992). The RA-binding activity of CRABP I and II probably contributes to regulation of the function of RA in development. For example, the temporal alteration in expression of both genes in the hindbrain appears to be related to segmentation (Ruberte *et al.*, 1991; Ruberte *et al.*, 1992).

CRABP I and II, are proposed to modulate the cellular level of free RA. The binding of RA by CRABP I appears to limit the availability of RA to nuclear RARs by binding and sequestering RA in the cytoplasm and by facilitating its degradation (reviewed by Morriss-Kay, 1993). For example, overexpression of CRABP I in F9 cells significantly decreases the RAR mediated transcriptional activation induced by RA (Boylan and Gudas, 1991). In addition, CRABP I transcripts are distributed in tissues such as neural crest, limbs and hindbrain which are susceptible to teratogenic effects of exogenous RA (Maden *et al.*, 1992; Ruberte *et al.*, 1992; Lyn and Giguère, 1994). It is proposed that the function of CRABP I in these sensitive tissues is to limit the level of free RA but that in conditions of excess RA the binding capacity is saturated and the protective effect is diminished, since the level of CRABP I is not elevated in response to elevated RA levels (Lyn and Giguère, 1994). The function of CRABP II is unclear since expression is observed both in tissues which are sensitive to excess RA and in other tissues which are apparently unaffected (Ruberte *et al.*, 1992).

This interpretation of a protective role of CRABPs in sensitive tissues is probably an over-simplification. Detailed comparison of the sites of teratogenesis and expression patterns of CRABPs suggests that there is not really a clear correlation. There is also no

relationship between CRABP expression and the quantitative level of RA in tissues following administration of exogenous RA (Horton and Maden, 1995). Moreover, the phenotype of CRABP knockout mice does not suggest any functional role for CRABPs in normal development except in the limb (Table 1.5), and there is also no alteration in the response to exogenous RA. CRABP I null mice are apparently normal (Gorry *et al.*, 1994) while the only abnormality in CRABP II homozygous null mice is an isolated forelimb outgrowth (Fawcett *et al.*, 1995; Lampron *et al.*, 1995). Absence of mutant phenotypes is apparently not due to functional redundancy in CRABP I and II since double mutant mice exhibit no detectable abnormalities additional to the limb defect observed in CRABP II null mutants (Lampron *et al.*, 1995). The presence of an additional unidentified CRABP, which compensates for the loss of CRABP I and II in double mutant embryos is also unlikely since no cytoplasmic RA-binding activity is detected in the null embryos. Therefore, the function of the CRABPs remains unclear since they are apparently not required for either regulation of RA levels during normal development or in conditions of RA excess.

CRBP I is involved in the synthesis of RA; the protein binds to maternally derived retinol and mediates its conversion to retinaldehyde and then to RA (Blomhoff *et al.*, 1990; Morriss-Kay, 1993). Therefore, the expression domains of CRBP I may indicate tissues which require and therefore synthesise RA (Dollé *et al.*, 1990; Ruberte *et al.*, 1991). In the trunk of late day 8 and day 9 mouse embryos, CRBP I expression sites include the most recently formed somites, gut endoderm and tail bud mesoderm (Ruberte *et al.*, 1991). Expression in the PNP region suggests that this is a site of RA synthesis. This raises the possibility that the mechanism leading to alteration in level and distribution of RA-inducible RAR transcripts in the caudal region of affected *curly tail* embryos may involve reduced synthesis which could be related to abnormal expression or function of CRBP I. The phenotype of null mutants for CRBP I, which have not so far been reported, will be of interest, particularly in the case of compound mutants with other retinoid binding proteins and RARs.

1.7 The *loop-tail* mutant mouse

Mouse embryos homozygous for the *loop-tail* (*Lp*) mutation exhibit NTD in which the neural tube fails to close for the entire length of the body axis caudal to the midbrain and thus is a model for the severe human malformation, craniorachischisis. *Lp/Lp* embryos die around the time of birth probably due to degeneration of the exposed nervous tissue (Strong and Hollander, 1949; Stein and Rudin, 1953). In addition to NTD, a circulatory defect involving coarctation of the aorta has also been observed in approximately 50% of homozygotes (Conway, Greene and Copp, Unpublished). Heterozygous mice also develop NTD comprising tail flexion defects with occasional spina bifida, head wobbling behaviour, enlarged brain ventricles and imperforate vagina in approximately 30% of females.

1.7.1 The *loop-tail* gene

The semi-dominant *loop-tail* mutation has not been identified thus far but has been mapped by linkage analysis to the distal region of mouse chromosome 1 which is syntenic with human 1q21-23 (Stanier *et al.*, 1995). The close linkage of the *Lp* mutation and the *Crp* microsatellite enables genotyping of embryos by PCR analysis of their DNA (Section 2.5), since Crp has polymorphic variants of 95, 140 and 150 base pairs in different mouse strains (Copp *et al.*, 1994). This technique allows determination of the embryonic genotype prior to development of identifiable defects.

1.7.2 Embryonic development of NTD in *loop-tail*

The basic embryonic defect leading to NTD in homozygous embryos is the failure of closure 1 (Section 1.1.3), the initial closure at the hindbrain/cervical boundary (Smith and Stein, 1962; Copp *et al.*, 1994). Closure 1 is also affected in heterozygous embryos with delay of closure by 2-3 somite stages compared to wildtype embryos. Heterozygotes also exhibit delayed closure of the PNP which is presumably responsible for the tail flexion defect. The mechanism underlying failure of closure 1 has not been determined and neither has the tissue or tissues which are responsible for development

of NTD. One of the aims of this study was to address the tissue localisation of the defect by analysis of gene expression (Chapter 7).

Closure of the neural tube at the midbrain/forebrain boundary (closure 2) is unaffected in both *Lp/Lp* and *Lp/*+ embryos which supports the idea that neural tube closure at the various "closure" sites involves different mechanisms. Further evidence was provided by *in vivo* treatment of *loop-tail* embryos with retinoic acid on day 8 of gestation (Wilson *et al.*, 1990). Forebrain exencephaly was induced in both heterozygous and wildtype embryos but there was no incidence of closure 1 failure. Therefore, RA does not summate with the *loop-tail* mutation, suggesting that the aetiology of RA-induced NTD is distinct from that of *loop-tail*. In fact *loop-tail* is the only mutant known to affect closure 1 while several mutations, e.g. *curly tail* and *Splotch*, and many teratogens, affect closure 2.

1.7.3 Additional abnormalities in *Lp/Lp* embryos

An early study suggested that the body length of *Lp/Lp* embryos is reduced compared with litter mates, suggesting that abnormal elongation contributes to failure of elevation of the neural folds (Smith and Stein, 1962). This is supported by the observation that the proportion of longitudinally oriented mitoses, which may contribute to axial elongation, is significantly reduced in the neuroepithelium of *Lp/Lp* embryos at 8.5 to 9 days of gestation (Wilson and Wyatt, 1994). A reduction in elongation could potentially affect neurulation through a mechanical effect on the neuroepithelium such as the imposition of abnormal curvature which inhibits closure of the PNP in *curly tail* embryos and influences neural tube closure in the chick (Van Straaten *et al.*, 1993). However, in both of the previous studies of *loop-tail* mice, the embryos were not genotyped and therefore *Lp/Lp* homozygotes could only be identified following the failure of neural tube closure. In our laboratory, recent analysis of genotyped embryos before initiation of closure 1 demonstrates that there is no significant difference in either axial length or cellular proliferation rates in *Lp/Lp* compared with *Lp/*+ or +/+ embryos (Gerrelli & Copp,

Unpublished). The reduction in axial elongation thus appears not to be causative but to be a result of failure of closure 1.

A number of morphological abnormalities have also been observed in *Lp/Lp* embryos which may be secondary to the presence of the persistently open neural tube. The spatial relationship of the neuroepithelium to the surrounding tissues is distorted and the thoracic skeleton is abnormal (Stein and Rudin, 1953; Stein and Mackensen, 1957). At the ultrastructural level, the neuroepithelium of abnormal embryos exhibits structural disorganisation with increased intercellular space and excessive number of gap junctions (Wilson, 1978; Wilson and Finta, 1980). The ventral and ventrolateral neural basal lamina is also discontinuous with disorganisation of the fibrils which span it (Wilson, 1985b). Comparison of extracellular matrix molecules revealed that the basal lamina sulphated proteoglycans appear prematurely in abnormal embryos (Copp and Wilson, 1981; Wilson, 1985). In contrast, the distribution of laminin and fibronectin in the neural basement membrane and midaxial structures ventral to the midline appears unaffected (Wilson and Wyatt, 1989a; Wilson and Wyatt, 1989b). Again, these studies were hampered by the absence of genotype information, mutant embryos being identified only after NTD became apparent. Therefore, it is likely that some of the celluar and molecular defects are secondary to the failure of neural tube closure.

Immunocytochemical analysis, using neural cell adhesion molecule (NCAM) and the lectin, concanavalin A (Con A), as histological markers, suggests that the floor plate at 10-12 days of gestation is similar in affected and normal embryos (Wilson and Wyatt, 1995). This observation was proposed to indicate that, despite the abnormal formation of the neural tube, the dorsoventral pattern polarity of the neuroepithelium is essentially normal in *loop-tail* homozygotes. The use of genetic markers in the present study suggests an alternative theory in which the specification of the floor plate occurs abnormally in *loop-tail* embryos (Chapter 7).

1.8 Induction of neural tube pattern by the notochord

One of the initial steps in closure of the neural tube is the formation of the MHP which forms the fulcrum about which the neural folds elevate at the site of closure 1. Formation of the MHP, the precursor of the floor plate of the neural tube, is dependent on signals from the notochord which underlies the neural tube in the midline and is responsible for induction of different cell types within the ventral half of the neural tube. The floor plate differentiates in the ventral neuroepithelium adjacent to the notochord and motor neurons differentiate more laterally. In this section I review the events of floor plate induction, particularly focusing on the role of the *Sonic hedgehog* gene product, since I have found that this gene is abnormally expressed in the notochord and floor plate of *loop-tail* embryos (Chapter 7).

Studies in which the notochord of chick embryos was experimentally manipulated have implicated this structure in the patterning of the neural tube. Extirpation of the notochord results in the absence of the characteristic MHP and failure of expression of floor plate markers such as *netrin-1* (Placzek *et al.*, 1990; Van Straaten and Hekking, 1991; Yamada *et al.*, 1991). Conversely, notochord grafts induce cell wedging (as at the MHP) and differentiation of floor plate cells and motor neurons at ectopic locations in the neural tube (Van Straaten *et al.*, 1988; Placzek *et al.*, 1990; Yamada *et al.*, 1993). Once it has been induced by notochordal signals, the floor plate exhibits similar inducing properties to the notochord. Implantation of a floor plate graft adjacent to the neural tube induces ectopic differentiation of floor plate cells in the neuroepithelium adjacent to the graft and motor neurons at locations further from the graft (Yamada *et al.*, 1991; Placzek *et al.*, 1993; Yamada *et al.*, 1993).

In addition to induction of ventral (floor plate) and ventrolateral (motor neurons) cell types, the notochord appears to regulate dorsoventral patterning throughout the neural tube through suppression of expression of dorsally expressed molecules such as the transcription factor, *Pax-3*. Implantation of a notochord graft at an ectopic site lateral to

the neural tube of chick embryos causes a striking dorsal restriction of the *Pax-3* expression domain (Goulding *et al.*, 1993).

Subsequent studies have addressed the nature of the signals that mediate the inductive properties of the notochord. Differentiation of neuroepithelial cells to form the floor plate in the neural tube immediately adjacent to the notochord suggests that the induction of the floor plate by notochord or floor plate-derived signals may be contact dependent. This was confirmed by studies in which neural tube explants were cultured either in proximity or in contact with notochord or floor plate. Induction of floor plate markers in neural explants was dependent on contact with the inducing tissue (Placzek *et al.*, 1993). Moreover, separation of notochord and neural plate explants by a filter inhibits the differentiation of floor plate in response to notochord (Tanabe *et al.*, 1995).

In contrast to the floor plate, the induction of motor neurons (as indicated by expression of Islet-1) is contact-independent. Motor neurons can differentiate in neural explants at some distance from the boundary with notochord or floor plate grafts and, unlike floor plate, induction can also occur when the tissues are separated by a filter (Yamada *et al.*, 1993; Tanabe *et al.*, 1995). In addition, motor neurons differentiate in neural explants exposed to medium conditioned by notochord and floor plate, further suggesting that a diffusible factor is responsible for motor neuron induction (Yamada *et al.*, 1993).

At a molecular level the inductive properties of the notochord and floor plate have been shown to involve the product of the *Sonic hedgehog* gene (Placzek, 1995).

1.7.1 Sonic hedgehog

Sonic hedeghog (*Shh*, also known as *Vhh-1* or *Hhg-1*) is one member of a family of genes which are homologous to the *Drosophila* segment polarity gene *hedgehog* (*hh*). In the mouse, three family members, *Sonic hedgehog, Desert hedgehog* and *Indian hedgehog* have been identified (Echelard *et al.*, 1993; Chang *et al.*, 1994) and *hh* homologues have also been identified in chick, human, rat and zebrafish (Krauss *et al.*,

1993; Riddle *et al.*, 1993; Chang *et al.*, 1994; Roelink *et al.*, 1994). The phenotype of *Shh* functional mutant embryos suggests roles in patterning of several tisuues. Embryos exhibit defects of axial structures such the notochord and floor plate, absence of ventral cell types in the neural tube, cyclopodia and abnormalities affecting development of the limbs, ribs and spinal column (Chiang *et al.*, 1996).

Induction of floor plate and motor neurons

Shh has been implicated in mediating the ventralising effect of the notochord and floor plate on the neural tube. This is based on its expression in the notochord and floor plate and the observation that misexpression of *Shh* leads to differentiation of floor plate cells at ectopic sites in the neural tube (Echelard *et al.*, 1993; Roelink *et al.*, 1994). Moreover, *Shh*-expressing COS cells induce differentiation of floor plate cells and motor neurons in adjacent neural tube explants (Roelink *et al.*, 1994; Tanabe *et al.*, 1995). Conversely, disruption of *Shh* function leads to the failure of development of both the floor plate and motor neurons (Chiang *et al.*, 1996).

Shh protein processing

The *Shh* primary transcript is cleaved through an autoproteolytic activity in the carboxy-terminal domain into amino-terminal and carboxy-terminal proteins, Shh-N and Shh-C (Chang *et al.*, 1994; Yang and Niswander, 1995) of which Shh-N mediates both the floor plate and motor neuron inducing functions. Both these cell types are induced in neural tube explants cultured in contact with COS cells expressing Shh or Shh-N (but not Shh-C) or in medium conditioned by COS cells expressing Shh-N (Roelink *et al.*, 1995; Tanabe *et al.*, 1995).

Independent induction of floor plate and motor neurons

As described previously for the notochord, floor plate induction by *Shh* is contact-dependent since separation of neural tube explants from *Shh*-expressing COS cells, by a filter, results only in differentiation of motor neurons (Tanabe *et al.*, 1995). Moreover, *in vitro* induction of the distinct cell types by conditioned medium from Shh-N

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expressing cells requires a five-fold higher concentration for induction of the floor plate compared with motor neurons (Roelink *et al.*, 1995). Thus, motor neuron induction is independent and does not depend on prior differentiation of floor plate with generation of a second floor plate-derived signal. These observations suggest a model in which expression of Shh-N at the surface of the notochord results in a high local concentration which induces floor plate differentiation. Additional neural cell types would then be induced either by diffusion of secreted Shh-N from notochord and induced floor plate or by an as yet unidentified intermediate signalling molecule (reviewed by Fietz *et al.*, 1994; Placzek, 1995).

Additional functions of *Shh*

Shh is also implicated in inductive interactions during patterning of the somites (Fan and Tessier-Lavigne, 1994), the anterior-posterior axis of the limb bud (Riddle *et al.*, 1993) and induction and regionalisation of the hindgut (Roberts *et al.*, 1995).

In addition to the patterning of the neural tube, the notochord also influences dorsal-ventral patterning of the somites in chicks and mammals, inducing sclerotome, the ventral mesenchymal component as opposed to dermomyotome, the dorsal epithelial component which is induced by dorsalising signals, possibly *Bmp-4* (Dietrich *et al.*, 1993; Fan and Tessier-Lavigne, 1994). Similar to the effect on the neural tube, the ventralising effect of the notochord and floor plate on somite development is mediated by a diffusible factor, proposed to be *Shh*. *Shh* mimics the effect of the notochord in that sclerotomal markers are induced in mouse presomitic mesoderm explants exposed to Shh or Shh-N expressing cells (or their conditioned medium) (Fan and Tessier-Lavigne, 1994; Fan *et al.*, 1995). Furthermore, mis-expression of *Shh* in chick embryos by an injected retrovirus induces expression of sclerotomal markers such as *Pax-1* while dermatome (the dorsal derivative of the dermomyotome) markers such as *Pax-3* are diminished (Johnson *et al.*, 1994). These observations are supported by the complementary experiment in which *Shh* signals are absent following targeted disruption. In these embryos the expression of *Pax-1* in the sclerotome is

progressively reduced and then lost while *Pax-3* expression in the dermomyotome expands ventrally (Chiang *et al.*, 1996).

In the limb bud *Shh* has been implicated in the function of the zone of polarising activity (ZPA), a region at the posterior margin of the limb bud which appears to be responsible for normal anteroposterior patterning. *Shh* expressing cells implanted into the anterior margin will cause digit duplications in a similar manner to ZPA transplants (Riddle *et al.*, 1993).

During formation of the chick hindgut *Shh* is expressed in the definitive endoderm adjacent to the mesoderm which will differentiate into the visceral mesoderm. The *Abd-B* related *Hox* genes are proposed to play a role in regionalisation of the hindgut due to their expression in the mesoderm in a nested pattern whose boundaries correspond to morphologic boundaries. *Shh* is proposed to constitute an endodermal signal involved in hindgut patterning, since mis-expression causes ectopic expression of the *Abd-B*-like *Hox* genes and *Bmp-4*, another mesodermally expressed molecule which may be involved in mesoderm induction (Roberts *et al.*, 1995).

1.9 Experimental approaches

The experiments described in the following chapters focus on development of NTD in two mouse genetic mutants, *loop-tail* and *curly tail*, which exhibit mutations in different genes, present on chromosomes 1 and 4 respectively. These mutations affect specific and distinct sites of closure of the neural tube leading to development of spinal NTD in the *curly tail* mouse and craniorachischisis in the *loop-tail* mouse.

In *curly tail* embryos, the basic embryonic (increased ventral curvature) and cellular (reduced hindgut proliferation) mechanisms of the defect have been established (Section 1.4). The initial aim in this study was to perturb the typical development of spinal NTD using an environmental factor, inositol (Chapter 3). This approach was based on the premise that an alteration in incidence of NTD may give further insight into the mechanism of the defect and/or into possible protective measures. Following the observation of a protective influence of inositol, the biochemical and molecular basis of this effect was investigated (Chapters 4 and 5). The potential protective effect of inositol was also assessed following *in vivo* treatment of *curly tail* embryos (Chapter 6).

In contrast to *curly tail*, cell proliferation abnormalities are not apparent in *loop-tail* embryos and very little is known of the mechanism underlying development of NTD (Section 1.7). In this case, a gene expression study was carried out with the aim of identifying affected tissues in *Lp/Lp* embryos. The notochord and floor plate of the neural tube are identified as likely sites of the *loop-tail* developmental defect on the basis of their abnormal patterns of gene expression (Chapter 7).

CHAPTER 2

Materials and Methods

All the reagents used in this chapter were obtained from Sigma unless specified. The components of individual solutions are described in the text except for certain general solutions which are listed in Section 2.8.

2.1 Maintenance of mouse stocks and generation of experimental litters

2.1.1 *Curly tail* mice

The *curly tail* mice are maintained as a closed, random-bred stock with breeding pairs of male and female mice, all with tail flexion defects. The light/dark cycle is modified such that the dark period occurs from 8 p.m. to 8 a.m.. Experimental litters were produced by matings between males with tail flexion defects and females with either straight tails or tail flexion defects. Females of both tail phenotypes were used since the incidence of NTD is the same in both types of experimental litter (Copp *et al.*, 1982). In order to allow accurate estimation of gestational age, timed matings were performed by pairing two females with one male, at approximately 8.30 a.m. and checking for copulation plugs 3-4 hours later. Mating was presumed to occur at the mid-point of this period (10 a.m.) and this was designated 0 hours of gestational age.

In vivo **inositol supplementation**

Inositol supplementation of embryos *in vivo* was achieved by intra-peritoneal injection of pregnant females at the appropriate stage of gestation (Section 6.2). Mice were weighed immediately prior to injection and a 30 or 150 mg/ml stock solution of *myo*-inositol in PBS was used in order to give a dose of 400 or 2000 mg/kg in a volume of approximately 0.4 ml. Control mice were injected with the appropriate volume of PBS.

2.1.2 *Loop-tail* mice

The *loop-tail* muatation is carried by mice of the LPT/Le inbred strain obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice of the inbred LPT/Le strain breed very poorly so inbred males are crossed with CBA/Ca females to produce F1 *Lp*/CBA mice.

The mice are maintained under the same conditions as the *curly tail* mice. Embryos for expression studies were produced from inter-crosses of *Lp*/CBA. Male and female mice (two per male) were paired overnight and females checked for copulation plugs the following morning, mating was assumed to occur at the mid-point of the dark period, 2 a.m.

2.1.3 CBA/Ca mice

The inbred CBA/Ca strain is used as a non-mutant control for *curly tail* mice because it is closely related to the CBA/Gr strain which contributed to the original *curly tail* stock (Gruneberg, 1954). Mice are maintained under the same conditions as the *curly tail* mice. Experimental litters were generated by overnight matings, one male paired with two females and mating was assumed to have occurred at the mid-point of the dark period, 2 a.m. Overnight matings were used in order to obtain earlier mating and therefore compensate for the fact that CBA/Ca embryos are developmentally delayed in comparison to *curly tail* embryos.

2.2 Dissection and *in vitro* culture of mouse embryos

2.2.1 Culture medium

Materials

Reagents to be used in embryo culture were all of cell culture grade.

Table 2.1 Constituents of dialysing saline (after Cockroft, 1990)

Reagent	g/l
Sodium chloride (NaCl)	13.8
Potassium chloride (KCl)	0.2
Magnesium sulphate, heptahydrate ($MgSO_4.7\ H_2O$)	0.2
Magenesium chloride, hexahydrate ($MgCl_2.6\ H_2O$)	0.1
Sodium phosphate monobasic ($NaH_2PO_4.2\ H_2O$)	0.2
Calcium chloride ($CaCl_2$)	0.4
Sodium bicarbonate ($NaHCO_3$)	4.0

Two litres were prepared, filtered through a γ-sterilised 0.22 μm filter (Falcon) and stored in 800 ml tissue culture flasks (Nunc) at 4°C.

Modified Eagles Medium (MEM) Vitamins (1000x concentrate)

1.0 mg/ml pantothenic acid, choline chloride, folic acid, niacinamide, pyridoxal hydrochloride, thiamine hydrochloride (vitamin B), 0.1 mg/ml riboflavin (vitamin B_2)

Preparation of rat serum

Blood was collected from the abdominal aorta of male Wistar rats anaesthetised with diethyl ether and was immediately centrifuged at 3500 rpm for 5 minutes to pellet the red cells. The fibrin clot generally formed in the plasma following centrifugation was compressed to expel the serum and recentrifuged at 3500 rpm for 5 minutes. Supernatant serum was pipetted into fresh 15 ml tubes (Falcon) at which point serum

from several rats was pooled. A final centrifugation at 3500 rpm for 5 minutes pelleted any remaining red cells. The serum was again decanted, heat inactivated at 56°C, allowing much of the dissolved ether to be vaporised, cooled and stored at -20°C. Before use serum was thawed and filtered through a 45µm filter (Millipore), gassed with the appropriate gas mixture (Section 2.2.2) and warmed to 38°C.

Serum dialysis (based on Cockroft, 1990)

In certain experiments, serum was dialysed to remove small molecules and then supplemented with nutrients to allow control of the concentration of vitamins, specifically *myo*-inositol. Approximately 40-50 ml of serum was filtered through a 45µm filter, pipetted into a length of approximately 20 cm of dialysis tubing (12-14 kDa exclusion limit, Gibco BRL) which had been washed in running tap water for 24 hours and rinsed three times in Milli-Ro water. The ends of the tubing were tied to avoid the use of potentially toxic plastic clips. The tubing containing serum was suspended in a glass beaker, used solely for this purpose to avoid contamination, containing 500 ml of dialysis saline. Serum was dialysed against daily changes of 500 ml dialysis saline with stirring at 4°C over a period of four days, decanted, stored at 4°C and used within 7 days. Using this protocol a 10:1 ratio of volumes of saline and serum gives a theoretical dilution of dialysable serum molecules of 10000:1. In the case of *myo*-inositol this would reduce the concentration in the medium from approximately 5 µg/ml (Hashimoto *et al.*, 1990) to 0.5 ng/ml.

Supplementation of dialysed serum

Immediately before use, serum was supplemented with 1.75 mg/ml D-glucose, 1x MEM essential amino acids (50x stock), 1x MEM vitamin mixture and 2 mM glutamine (200 mM stock). In different studies additional reagents were added to the dialysed serum as described in Section 2.2.3.

2.2.2 Dissection and *in vitro* culture of embryos

Pregnant females were killed by cervical dislocation at the appropriate stage of gestation, 9 days 4 hours for *curly tail* mice and 9 days 12 hours for CBA/Ca mice. The uterus was explanted into Dulbecco's Modified Eagles Medium (Gibco) containing 10% foetal calf serum (Advanced Protein Products). Embryos were dissected from the uterus using fine watchmakers forceps (Weiss). The decidua, trophoblast and Reichert's membrane were removed leaving the yolk sac and ectoplacental cone intact. In experiments where embryos were not to be cultured the dissection was continued with removal of the yolk sac and ectoplacental cone followed by the amnion. Embryos were then treated according to the analytical technique to be used.

Embryos to be cultured were briefly rinsed in PBS and placed in 30 ml plastic universal tubes (Nunc) with 3 embryos and 3ml of medium (whole or dialysed rat serum) per tube. In general there was a delay of 2 hours between killing the female mouse and putting the embryos into culture. Cultures started at 9 days 6 hours (*curly tail*) or 9 days 14 hours (CBA/Ca) of gestation were gassed for 1 minute with 20% O_2, 5% CO_2, 75% N_2 (all gases from Cryoservice Ltd.) and incubated at 38°C in a roller bottle oven (Cockroft, 1990). After approximately 17 hours, the cultures were again gassed for 1 minute with 40% O_2, 5% CO_2, 55% N_2 and incubated for a further 7 hours.

In some cases the head-length of the embryo was measured before culture since this gives an accurate approximation of the number of somites in the range 17 to 23 somites (Peeters *et al.*, 1996). The head-length can be measured since the head is visible through the yolk sac using under-stage illumination on the microscope, whereas it is impossible to accurately assess the number of somites when the yolk sac is still intact. At 9 days 6 hours of gestation, explanted embryos were found to be in the range of 15-21 somites based on measurements of head-lengths.

Measurements after culture

After a total of 24 hours in culture, embryos were assessed for yolk sac circulation while still in the culture serum and scored on a scale of 1 to 3. A score of 3 refers to a vigorous circulation across the entire yolk sac, 2 indicates vigorous circulation with small static areas and a score of 1 indicates a strong heart beat and some yolk sac circulation. In the absence of a heart beat embryos were discarded from further analysis. Embryos were transferred to cold PBS, the extraembryonic membranes removed and the following observations made. Measurements were made using an eyepiece graticule on a Zeiss SV6 stereomicroscope.

1) Crown-rump length was measured as an indication of growth of the embryo during culture.

2) Number of somites was counted as a measure of the developmental progression of the embryo. For those embryos whose somite number was recorded before culture the number and rate of somite addition could be calculated.

3) Length of the PNP was measured from the tail tip to the rostral end of the PNP.

4) PNP category was assigned on a scale of 0 to 5 for embryos with 27-31 somites (Copp, 1985).

> 0 = Closed posterior neuropore
>
> 1 = Small neuropore restricted to the expanded distal region of the tail bud.
>
> 2 = Larger neuropore but still restricted to the expanded distal region of the tail bud and extending cranially less than half the distance to the somites.
>
> 3 = Intermediate size PNP extends beyond the distal expansion but does not reach the level of the hindlimb bud.
>
> 4 = The cranial extent of the enlarged PNP extends to the level of the hindlimb bud and approaches the level of the somites.
>
> 5 = The most severely enlarged PNP category in which the open PNP overlaps with the somite rows.

5) Embryos were checked for teratogenic effects following culture as judged by external morphology. The assessed features (Table 2.2) were yolk sac circulation, neural tube

closure, axial rotation, regularity of the somites and development of the branchial arches and dorsal recess of the otocyst.

Table 2.2 Features of external morphology assessed for teratogenic effects following embryo culture (based on Brown, 1990)

Morphological feature	Expected development following culture	Potential abnormality
Yolk sac circulation	Most embryos with category 2 or 3 (see above)	No yolk sac circulation No heart beat
Axial rotation	Embryo fully turned, caudal region on left side of head	1. Turning incomplete 2. Caudal region on right side of head (situs inversus)
Somites	Regular, clearly defined pairs of somites	1. Irregular shape 2. Fused or undefined somites
Neural tube	Closed neural tube apart from PNP	Regions of open neural tube Wavy or distorted neural tube
Otocyst	Dorsal recess developed	Dorsal recess absent
Branchial arches	Arches 1, 2 and 3 distinct. Arch 2 overgrowing 3	Development retarded

2.2.3 Treatment of embryos in whole embryo culture

A range of treatments was applied to embryos *in vitro* (whole embryo culture).

Inositol - *Myo*-inositol was added immediately before use as an aqueous 1% addition to either whole or dialysed serum to give final concentrations ranging between 0 and 1000 µg/ml.

Lithium chloride - added to dialysed serum as an aqueous 1% addition to a concentration of 200 µg/ml.

Arachidonic acid - added (dissolved in ethanol) to dialysed serum as a 0.1-0.5% addition to give final concentrations of 20 or 50 µg/ml. Embryos in both the arachidonic acid treatment group and the control group, were cultured in the dark as arachidonic acid is light sensitive.

TPA - added to whole serum from stock solutions (dissolved in ethanol) of 0.1 and 0.5 mM as a 0.1% addition (1 µl/ml) in order to give final concentrations in the medium of 100 and 500 nM. Embryos in the control group were cultured in rat serum with the appropriate concentration of ethanol added. In studies utilising TPA treatment, embryos of all experimental groups (treatment and control) were kept in the dark during the period of TPA exposure since TPA is light sensitive.

Bisindolylmaleimide I, Hydrochloride (GF109203X, Calbiochem) - A water-soluble form of the protein kinase C inhibitor GF109203X was used as a 0.5 % addition to the culture medium. When applied in conjunction with another treatment the GF109203X was added to the culture medium 15 minutes prior to the other reagent.

2.3 [³H] inositol labelling of embryos *in vitro*: extraction of water- and lipid-soluble fractions

2.3.1 Embryo culture

[³H] inositol was added to the culture medium (Section 3.2.4) in order to measure uptake and incorporation of inositol into the embryo. [³H] inositol was used from a 117 Ci/mmol stock aqueous solution of *myo*-[³H] inositol with PT6-271 stabiliser (Amersham).

2.3.2 Tissue preparation and extraction of water- and lipid-soluble fractions

The method is modified from that of Estibeiro et al. (1990). Following 24 hours culture each embryo was dissected free of its yolk sac and both embryo and yolk sac were rinsed twice in ice-cold PBS and stored in microfuge tubes at -70 °C until required for further processing.

Protein content and total [³H] inositol uptake

Excess PBS was removed with a pulled glass pipette and the sample resuspended in 200 μl of methanol. Tissue was disrupted by sonication (Soniprep) for 20 seconds at approximately 15 μm, vortexed and kept on ice. Two 5 μl samples were removed and assayed for protein content (Section 2.4) and two 5 μl samples added to 2 ml scintillant (UniverSol, ICN) and the [³H] counts measured to give total counts incorporated (Total counts (dpm) = mean counts in 5 μl sample × 40).

Extraction of water- and lipid-soluble phases

Chloroform (670 μl) and methanol (150 μl) were added to give a final volume of 1 ml (chloroform: methanol, 2:1) and the mixture was agitated gently overnight at 4 °C. The mixture was centrifuged at 13000 rpm for ten minutes at 4°C and the supernatant transferred to a fresh tube with a pulled glass pipette. The crude mixture was extracted by addition of 200 μl of 0.73% sodium chloride and separated into two phases by

standing for two minutes followed by centrifugation at 13000 rpm for two minutes. The upper, water-soluble phase was transferred to a fresh tube. The lipid-soluble lower phase was then re-extracted twice by adding of 200 µl 'pure solvents upper phase' (the upper phase from an 8:4:3 mixture of chloroform/ methanol/ 0.58% aqueous sodium chloride), standing to allow separation and centrifugation at 13000 rpm for two minutes. The lower phase was retained, washed again with 'pure solvents upper phase' and the supernatants were added to the water-soluble fraction.

[^{3}H] inositol counts in the water- and lipid-soluble phases

The pooled water-soluble and lipid-soluble phases were made up to 1 ml volumes with water and methanol respectively and duplicate 50 µl samples were added 2 ml scintillant and [^{3}H] counts measured . Remaining water-soluble phase was stored at -20 °C. The lipid-soluble phase was dried under nitrogen gas and stored at -70 °C. Water-soluble and lipid-soluble counts were calculated as:-

Counts in fraction (dpm) = mean counts in 50 µl (minus background) × 22.22 (accounts for dilution and removal of samples)

The recovery of [^{3}H] from total tissue homogenate, using this extraction method, was approximately 96% for embryos and approximately 83% for yolk sac. The water-soluble phase contains free inositol and inositol phosphates, the lipid-soluble phase contains inositol phospholipids and the pellet contains inositol associated with insoluble tissue residue (cytoskeleton, DNA and protein material; Strieleman *et al.*, 1992).

2.3.3 Thin-layer chromatography (TLC)

Approximately 150 ml of solvent mixture, chloroform/ methanol/ H_2O/ acetic acid (50: 43: 3: 1), was poured into a TLC tank (Sigma) and left with the lid on for 5 hours to equilibrate. Lipid samples were resuspended in 110 µl chloroform/ methanol/ H_2O (75: 25: 2). Duplicate 50 µl samples were taken and spotted, using glass capillary tubes, at intervals along a line 2 cm from the base of a 25 cm square TLC plate (Silica Gel 60

on aluminium, Aldrich Chemical Co.). In order to facilitate detection of phosphatidylinositol (PI) in the lipid sample 50 µg of standard PI, from a 2 µg/µl stock solution (dissolved in the same solvent as the lipid samples), was spotted on top of each lipid sample.

Samples were allowed to air dry. The TLC plate was placed into the TLC tank and allowed to run for approximately 4 hours until the solvent front was approximately 2 cm from the top of the plate. The plate was removed, the position of the solvent front marked and the plate air-dried. A molybdenum blue spray reagent was used to develop the plates and the position of the PI spots was marked. In this solvent system, the PI standard runs close to the solvent front with a relative front value (R_f) of approximately 0.95 (R_f = distance run by sample ÷ distance run by solvent front).

An approximately 2 cm square of TLC plate encompassing each PI spot was cut out, the silica scraped into a scintillation tube, 2 ml of scintillant added and the [³H] counts measured. A similar size region of TLC plate which was not in a lipid lane was cut out and counted to give a control value for background counts on the TLC plate. Total counts in PI for each embryo sample were calculated as:-

Total counts (dpm) = mean counts in 50 µl sample run on TLC (minus background) × 2.716 (this accounts for dilution and removal of samples for protein and counts).

2.4 Protein Assay

Assay for protein content of samples was carried out using a Bicinchoninic acid (BCA) protein assay reagent (Pierce). Samples were diluted in order to give approximately 3-10 μg of protein in the 5 μl assay volume. For example, following [^{3}H] labelling of embryos in culture (Section 2.3.2), single embryos at approximately 10.5 days of gestation (expected protein content= approximately 200 μg) were sonicated in 200 μl volumes and duplicate 5 μl samples (expected protein content= approximately 5μg) were taken for protein assay. Samples were diluted to 50 μl with Milli-Ro H_2O.

A 200 μg/ml standard protein solution (bovine serum albumin, Pierce) was used to make a series of standards containing 0, 2, 4, 5, 7.5 and 10 μg protein in a 50 μl final volume. A fresh set of standards was made up and assayed with each set of samples in order to control for variability between experiments.

BCA working reagent (Pierce), a mixture of solutions A and B (50:1) was made up fresh for each experiment and 1 ml was added to each sample and standard. Following a thirty minute incubation at 60°C the OD_{562} of sample was read against an H_2O blank using an Ultrospec III spectrophotometer (Pharmacia). A standard graph of OD against protein concentration was plotted using the values from the standard protein solutions and the amount of protein in the assay volume was calculated.

2.5 Genotyping of *loop-tail* embryos by the polymerase chain reaction (PCR)

2.5.1 Approach

This technique exploits the fact that the *Crp* microsatellite sequence is closely linked to the *Lp* locus and displays polymorphic variation between different mouse strains (Copp *et al.*, 1994). Therefore, the genotype of embryos generated from *Lp*/CBA crosses can be identified by PCR analysis of their DNA.

2.5.2 Extraction of DNA for PCR

The tissue, typically yolk sac, from which DNA was to be extracted was rinsed twice in PBS and stored at -20°C until required.

Any PBS was removed from the tissue and 0.25 ml of 300 mM glucose in PBS (filtered through a 0.22 μm filter) was added and incubated at room temperature for approximately one hour to allow cells to lyse. After centrifugation at 13000 rpm for ten minutes the supernatant was discarded and the pellet resuspended in 20-40 μl of Milli-Ro H_2O depending on the quantity of pellet: e.g. 20 μl for yolk sac from embryos at 8 days of gestation and 40 μl for yolk sac from embryos at 10 days of gestation.

A 10% volume of 20 mg/ml proteinase K solution was added, the mixture vortexed and incubated at 37°C for fifteen minutes followed by vigorous vortexing. The reaction was stopped by incubation at 100°C for five minutes. After cooling on ice for five minutes, the mixture was centrifuged at 2000 rpm for five minutes at 4°C and stored at 4°C. The supernatant was used as the source of DNA for PCR.

2.5.3 PCR reactions

Primers specific for *Crp* microsatellite (Hearne *et al.*, 1991)

Crp-1 = AGAATCTGACTTACCCATGGT

Crp-2 = GAGGGAGAAGAATTATGTCTG

Reaction mix and PCR conditions

The following mixture was prepared.

Reagent	Volume per sample (µl)
10x NH_4 reaction buffer (Bioline)	2.5
dNTP mix (2.5mM each dC, dA, dG, dT in H_2O)(Promega)	2.5
50mM $MgCl_2$ (Bioline)	1.5
Crp primers (*Crp*-1 and *Crp*-2)	0.325 each
Taq DNA polymerase (Bioline)	0.1
H_2O (0.22 µm filtered Milli-Q)	16.75

24 µl of reaction mix was added to 1 µl of DNA sample, pulse centrifuged to mix and the mixture overlaid with two drops of mineral oil. Samples were placed in the PCR machine (Hybaid Omnigene) and incubated as follows:-

    94°C for 1 minute

    94°C for 1 minute (denaturing)⌉

    57°C for 1 minute (annealing) ⎬ 30 cycles

    72°C for 1 minute (extending) ⌋

    72°C for 8 minutes

Separation of PCR products

PCR products (9 µl sample + 1 µl bromophenol blue loading buffer) were run out on 4% agarose gels (LMP agarose: normal agarose, 3:1) with 1x TBE buffer containing approximately 0.5 µg/ml ethidium bromide solution with an extra lane containing molecular weight pGEM markers (Promega). The gel was run at 80-100V for 2-3 hours, visualised by ultra-violet transillumination and photographed on a Mitsubishi Video Copy system.

The *loop-tail* allele at the Crp locus gives a band of 140 bp and the CBA allele a band of 150 bp enabling differentiation between tissue genotypes of homozygous mutant (one band of 140 base pairs), heterozygous (140 base pair band and 150 base pair band) and homozygous CBA, i.e. wildtype at *Lp* locus (one band of 150 base pairs).

2.6 Preparation of RNA probes for whole mount *in situ* hybridisation

Digoxygenin-labelled RNA probes were prepared by transcription from plasmids containing a cDNA insert of the gene of interest. All of the plasmids were obtained from suppliers as solutions, with the exception of *Sonic hedgehog* for which 500 ng DNA was obtained blotted on a filter paper. In this case the DNA was resuspended by addition of 100 μl 10 mM Tris (pH 7.6), gentle mixing and incubation for 5 minutes at room temperature in order to allow rehydration. All the solutions used in Section 2.6 and 2.7 were prepared using diethyl pyrocarbonate (DEPC)-treated H_2O.

2.6.1 Preparation of plasmid DNA

Transformation

Plasmid DNA was used to transform DH5α competent cells (Gibco BRL). Cells were thawed on ice and 100 μl pipetted into a 2 ml round-bottom tube (Sarstedt) for the transformation procedure. 20 ng of plasmid DNA was added and the cells were incubated on ice for 30 minutes followed by heat-shock at 37 °C for 45 seconds. The transformed cells were cooled on ice, 0.9 ml LB-Broth (20 g/l, Gibco) added and incubated for 1 hour at 37°C followed by 30 minutes at 37°C with shaking. Cells were pelleted by centrifugation at 6000 g for 10 minutes at 4°C, the supernatant discarded and cells were resuspended in 200 μl of fresh LB-Broth. 50-70 μl portions were streaked onto agar plates (32 g/l LB-Agar, Gibco) containing 50 μg/ml ampicillin (the cells are ampicillin sensitive, but the plasmid contains an ampicillin-resistance gene, allowing selection of transformed cells).

After incubation for 5 minutes at room temperature, plates were inverted and incubated at 37°C overnight. Colonies were picked (four from each plate) into 10 ml LB-Broth containing 50 μg/ml ampicillin in a 50 ml Falcon tube and grown up by incubation with shaking at 37°C overnight.

Glycerol stocks were prepared by mixing 0.5 ml glycerol with 0.5 ml of cell suspension and storage at -70°C. When required, a 0.1 ml volume of glycerol stock was added to 10 ml LB-Broth and grown up as described above.

Isolation of plasmid DNA

A 10 ml volume of transformed cell suspension (grown up as above in 50 ml Falcon tubes) was centrifuged at 6000g for 10 minutes at 4°C to pellet the cells. The supernatant was discarded and the pellet resuspended in 2 ml of ice-cold solution 1 (50 mM glucose, 25 mM Tris.HCl (pH 8.0), 10 mM EDTA (pH 8.0)), incubated on ice for 30 minutes followed by addition of 4 ml of solution 2 (0.2 M sodium hydroxide, 1% SDS). After 10 minutes incubation on ice, 3 ml of ice-cold solution 3 (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O) was added. The tube was inverted to mix and stored at -20°C for at least two hours.

The sample was centrifuged at 6000g for 10 minutes at 4°C, the supernatant transferred to a fresh tube and the same volume of phenol/ chloroform (1:1 mixture) was added and thoroughly mixed. The mixture was centrifuged at 6000g, for two minutes at room temperature, mixed again and re-centrifuged as before. The upper aqueous phase was transferred to a fresh tube and plasmid DNA precipitated by addition of a one-tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of 100% ethanol followed by incubation at -20°C for at least 30 minutes.

Plasmid DNA was pelleted by centrifugation at 6000g for 10 minutes at 4°C and the supernatant discarded. The pellet was washed with 15 ml of 70% ethanol, re-centrifuged, washed with 10 ml of 100% ethanol and, following a final centrifugation, the supernatant was discarded. The pelleted DNA was allowed to air dry for five minutes at room temperature followed by five minutes at 65°C and resuspended in 200 μl TE. The concentration of DNA was calculated by dilution of a 4 μl sample to 1 ml with water and determination of optical density (OD) against a water blank.

Concentration of plasmid DNA (μg/ml) = OD_{260} × 250 (dilution) × 50 (constant)

Determination of insert size

In order to check that the plasmid contained the cDNA insert of interest, digestion of plasmid DNA was carried out using an appropriate restriction enzyme. A reaction mixture was prepared containing 1 μg plasmid DNA, 4 μl restriction enzyme buffer, 1μl restriction enzyme and made up to 40 μl total volume with Milli Ro-H$_2$O. The mixture was incubated at 37°C for two hours. A 20 μl portion was taken, added to 2 μl bromophenol blue loading buffer and run out for 2-3 hours at 80V on a 1% agarose gel (made up with TBE) using TBE buffer containing ethidium bromide (approximately 0.01 μg/ml). The size of the insert was determined following visualisation under UV light by comparison with a control lane containing approximately 1 μg of a 1 Kb ladder (Gibco).

2.6.2 Synthesis of RNA probes

Linearisation of plasmid DNA

A reaction mixture was prepared containing 10 μl of plasmid DNA (1 μg/μl), 10 μl restriction enzyme buffer, 5 μl restriction enzyme (Table 2.3) and 75 μl DEPC-H$_2$O. The mixture was vortexed and incubated at 37°C for 2-4 hours. A sample was prepared for electrophoresis containing 5 μl linearised DNA, 1 μl bromophenol blue loading buffer and 4 μl Milli-Ro H$_2$O. A sample was included on the gel containing 1μl uncut plasmid DNA, 1 μl bromophenol blue loading buffer and 8 μl Milli-Ro H$_2$O. Samples were run out on a 1% agarose gel (as above) and visualised under UV light. Linear DNA runs at an apparently different molecular weight to uncut DNA and only one band is present (compared with three bands for uncut plasmid which correspond to supercoiled, coiled and open circular DNA).

Isolation of linear plasmid DNA

Provided the linearisation reaction was successful, approximately the same volume of phenol/chloroform (1:1 mixture) was mixed with the remaining 95 μl of linearisation reaction mixture and the layers separated by centrifugation at 13000 rpm for two minutes. The upper layer was transferred to a fresh microfuge tube and the lower phenol

Table 2.3 Anti-sense RNA probes used for whole mount *in situ* hybridisation

Plasmid	Size of insert (bp)	Insert site	Linearised with:	Transcribed with:	Source
CRABP I	800	EcoRI	BamHI	T7	P. Chambon
CRABP II	830	EcoRI/ HindIII	EcoRI	T7	P. Chambon
CRBP I	640	EcoRI	Bam HI	T7	P. Chambon
Netrin-1	1900	EcoRI/ XhoI	PstI	T7	S. Conway
Pax-3	519	PstI/ HindIII	HindIII	T7	P. Gruss
Pax-3*	519	Pst1/ HindIII	PstI	T3	P. Gruss
RAR-β	1800	BamHI	HindIII	T7	P. Chambon
RAR-β*	1800	BamHI	XbaI	T3	P. Chambon
RAR-γ	1800	EcoRI	BamHI	T7	P. Chambon
Shh	642	EcoRI	HindIII	T3	A. McMahon
Shh*	642	EcoRI	PstI	T7	A. McMahon

All details correspond to antisense probes unless specified.

* = Sense orientation (used as controls).

layer was re-extracted by mixing with 50 μl TE followed by centrifugation for two minutes at 13000 rpm. The supernatants were pooled and linear DNA precipitated by addition of one tenth volume (approximately 15 μl) 3 M sodium acetate and three volumes (approximately 600 μl) 100% ethanol. The solution was mixed and stored at -70°C for one hour. Precipitated linear DNA was pelleted by centrifugation at 13000 rpm for ten minutes at 4°C, the supernatant discarded and the pellet washed successively with 500 μl volumes of 70% ethanol and 100% ethanol. The supernatant was discarded and the DNA pelleted by centrifugation at each step. The pellet was allowed to drain with the tube inverted, dried at 65°C for five minutes, resuspended in 10 μl TE at 65°C for 15 minutes and stored at-20°C.

Synthesis of digoxygenin-labelled probes

The following transcription reaction mixture (probe labelling mix) was prepared. The appropriate polymerase was included depending on the particular probe (Table 2.3).

Probe labelling mix

1.0 μl linear DNA (1μg/μl)

2.0 μl DIG RNA Labelling Mix (10x, Boehringer Mannheim)

2.0 μl Transcription Buffer (10x, Boehringer Mannheim)

1.0 μl RNAsin (Boehringer Mannheim)

2.0 μl RNA Polymerase (T7, T3 from Boehringer Mannheim)

12.0 μl DEPC-H_2O

The reagents were mixed and incubated at 37°C for 2-3 hours. In order to assess the efficiency of the transcription reaction, a 1 μl sample was taken from the reaction mixture, added to 5 μl DEPC-H_2O and 1 μl bromophenol blue loading buffer, and run out on a 1% agarose gel. The size of the transcript was assessed by comparison to 1 Kb ladder (Gibco) run in a control lane. The relative intensity of bands for linear DNA and RNA transcripts gave an indication of the degree of amplification in the transcription

(generally ten times) enabling an approximation to be made of the amount of transcript produced.

Isolation of labelled probe

The transcription reaction was stopped by mixing with 4 µl 0.2 M EDTA. The labelled probe was precipitated by addition of 4 µl 4M lithium chloride, 1 µl tRNA and 100 µl 100% ethanol and storage at -20°C overnight. The precipitate was pelleted by centrifugation at 13000 rpm for ten minutes at 4°C, the supernatant discarded and the pellet washed by successive addition of 0.5 ml volumes of 70% ethanol and 100% ethanol with centrifugation (as above) and discarding of supernatant at each step. Finally the labelled probe was dissolved at 65°C for 15 minutes in an appropriate volume (based on the approximate efficiency of transcription) of TE to give a final concentration of 0.1 µg/µl. 1 µl of RNAsin (Boehringer Mannheim) was added and the probe was stored at -20°C.

2.7 Whole mount *in situ* hybridisation

The protocol is based on the protocols (provided as Personal Communications) of R. Conlon (Mt. Sinai, Toronto), P. Ingham (ICRF, London) and D. Wilkinson (NIMR, Mill Hill) with some modifications. Unless specified all washes were carried out in 5-10 ml volumes in 15ml Falcon tubes for ten minutes at room temperature with rocking.

2.7.1 Embryo preparation and pre-treatment

Embryos were dissected out of the yolk sac in ice-cold DEPC-PBS and rinsed in two further changes of DEPC-PBS. Embryos were fixed in 5 ml of 4% paraformaldehyde (PFA) in DEPC-PBS at 4°C with rocking overnight.

Embryos were washed twice in PBT (PBS, 0.1% Tween-20) at 4°C and dehydrated by successive washes at 4°C in 25%, 50% and 75% methanol in PBT followed by two washes in 100% methanol. Dehydrated embryos were stored in 100 % methanol at -20°C for up to several weeks.

Embryo pre-treatment

Rehydration was carried out by successive washes in 75%, 50% and 25% methanol in PBT followed by two washes in PBT. The embryos were then bleached in 6% hydrogen peroxide in PBT for one hour. Following three washes in PBT, embryos were treated with 10μg/ml proteinase K (from a 20 mg/ml stock solution) in PBT. 10.5 day embryos were treated for 8-10 minutes, 9.5 day embryos for 4-5 minutes and 8.5 day embryos for 1 minute. The reaction was stopped by washing in freshly prepared 2mg/ml glycine in PBT followed by two washes in PBT.

Embryos were refixed in freshly prepared 0.2% glutaraldehyde (from 25% stock) in 4% PFA for 20 minutes and then washed twice in PBT. The PBT was removed, replaced by 2ml of prehybridisation mix (50% formamide, 5x SSC (pH 4.5), 50 μg/ml yeast RNA, 1% SDS, 50 μg/ml heparin). Once the embryos had sunk to the bottom of the tube, the

prehybridisation mix was replaced with 5 ml of fresh mix and incubated at 70°C for 3-5 hours.

2.7.2 Hybridisation

The embryos were transferred to 2 ml round-bottom microfuge tubes (Sarstedt) containing 1ml prehybridisation mix. 10µl of digoxygenin-labelled probe (0.1 µg/ml) was added, gently mixed and incubated at 70°C overnight.

2.7.3 Post-hybridisation

Hybridisation mix was removed and stored at -20°C until required in subsequent experiments. In general the probe was reused once; in the hybridisation step (Section 2.7.2); instead of adding probe to the prehybridisation mix, the prehybridisation mix was simply replaced with 1 ml of stored hybridisation mix.

Embryos were washed in solution 1 (50% formamide, 5x SSC (pH 4.5), 1% SDS) at 70 °C for 15 minutes, transferred to 15ml Falcon tubes and washed twice more for 30 minutes with solution 1 at 70°C. Following two washes for 30 minutes at 65°C in solution 2 (50% formamide, 2x SSC (pH 4.5) 1% SDS), embryos were washed three times at room temperature in TBST (8 mg/ml sodium chloride, 0.2 mg/ml potassium chloride, 25 mM Tris hydrochloride (pH 7.5), 1% Tween-20, 0.48 mg/ ml levamisole). Embryos were then pre-blocked for 90 minutes in TBST containing 10% sheep serum and meanwhile the antibody solution was prepared as described below. The 10% serum was then replaced with pre-absorbed antibody and rocked overnight at 4°C

Antibody solution preparation

Depending on the number of tubes of embryos, a suitable volume of antibody solution was prepared to give approximately 3 ml per tube. The following volumes were therefore scaled up accordingly. 0.5ml TBST was heated at 70°C for 30 minutes with 3 mg embryo powder. Embryo powder was prepared by homogenisation of 14.5 day mouse embryos, the homogenate was then washed for 30 minutes in ice-cold acetone,

pelleted by centrifugation at 6000 rpm for ten minutes and the wash repeated. The pellet was ground into powder, spread on filter paper to air dry and stored at -20°C.

After cooling on ice, 5 µl sheep serum and 1 µl anti-digoxygenin antibody (alkaline phosphatase-conjugated Fab fragments, Boehringer Mannheim) were added to the TBST/embryo powder and the mixture rocked gently at 4°C for one hour to pre-absorb the antibody. The mixture was centrifuged at 6000 rpm for ten minutes at 4°C, the supernatant (antibody solution) removed and diluted to 2 ml with 1% sheep serum in TBST. Pelleted embryo powder was stored at -20°C for re-use.

The 10% sheep serum was removed from the embryos, replaced with antibody solution (prepared as above), and incubated overnight at 4°C with rocking.

Post-antibody washes and development

Antibody solution was removed and embryos washed three times in TBST for five minutes. These washes in TBST were repeated hourly for six successive hours and overnight.

The following morning embryos were washed three times for ten minutes with NTMT (100 mM sodium chloride, 100 mM Tris.HCl (pH 9.5), 50 mM magnesium chloride, 0.1% tween-20). Embryos were transferred to glass dishes and incubated in the dark in 1 ml NTMT containing 4.5 µl NBT (nitroblue tetrazolium chloride, Promega) and 3.5 µl BCIP (5-bromo-4-chloro-3-indolyl-phosphate, Promega) with rocking for the first 20 minutes. When the colour had developed the reaction was stopped by washing twice in PBT and embryos were stored at 4°C in PBT with a small amount of sodium azide added to prevent fungal growth.

Some sets of embryos were re-developed after a few days in PBT; this increases the signal intensity without a significant increase in background staining. The embryos were simply transferred from PBT back to NTMT and developed as before.

Photography was carried out before and/or after clearing (see below) on a Zeiss SV11 microscope using Kodak Ektachrome 160T or Fuji Super G Plus (ISO 100) film.

2.7.4 Sense probes

Sense probes were used as controls in several experiments to confirm the specificity of the expression patterns observed following hybridisation with antisense probes.

Table 2.4 Use of control sense probes

Probe	Age of embryos (days of gestation)	No. of embryos	Strain	No. of experiments
Pax-3	10	6	CBA/Ca + ct	2
Shh	10	8	CBA/Ca + ct	2
	9	6	CBA/Ca + ct	2
	8	2	CBA/Ca	1
RAR-β	10	6	ct	2

Use of control sense probes consistently gave no staining at all in embryos at day 9 of gestation (Figure 7.3d) and at 10 days of gestation no staining apart from an occasional small amount of trapping in the brain ventricles of embryos (Figure 7.4a).

2.7.5 Processing for sectioning

Embryos were dehydrated and cleared by taking through increasing glycerol concentrations: 30 minutes in 50% and 75% glycerol in PBT followed by 100% glycerol overnight. Embryos were successively washed for 30 minutes in two changes of 100% ethanol, two changes of Histoclear (National Diagnostics),1:1 Histoclear/wax (at 65°C) and three changes of 100% wax (Raymond Lamb) at 65°C. Following embedding, 20 μm sections were cut (Raymond Lamb microtome), placed onto gelatin-coated slides (see below) and dried overnight at 37°C. After removal of wax by two ten minute washes in Histoclear, slides were cover-slipped using DPX mountant (BDH). Sections

were photographed on a Zeiss Axiophot microscope using Kodak Ektachrome 64T or Fuji Super G Plus (ISO 100).

Gelatin-coated slides

A 1% gelatin solution was prepared by dissolving 0.4 g gelatin in 400 ml dH_2O at 50-60°C followed by addition of 0.4 g chrome alum, and the solution cooled. Racks of slides were dipped for approximately one minute, dried at 37°C and stored at 4°C.

2.8 General solutions

Bromophenol Blue loading buffer

1.25 ml 50x TAE

3 ml glycerol

25 mg bromophenol blue

PBS

1tablet phosphate buffered saline (Oxoid)per 100 ml H_2O

Treated with diethyl pyrocarbonate (DEPC) by addition of 0.5ml/l and

incubation at room temperature for 4 hours followed by autoclaving.

SSC (20x)

175.3 g sodium chloride

88.2 g sodium citrate

to 1 litre with H_2O

TBE

0.045 M Tris

0.045 M Boric acid

0.0001 M EDTA (pH 8.3)

TE

10 mM Tris.Cl (pH 7.6)

1 mM EDTA (pH 8.0)

CHAPTER 3

Prevention of NTD in the *curly tail* mouse by inositol supplementation in whole embryo culture

3.1 Introduction

The *curly tail* mouse is a particularly good model for NTD in humans, sharing characteristics of axial location, sex bias and pathology (Section 1.4.3). In addition, susceptibility in both systems is influenced by multiple genetic and environmental factors. One approach to the study of the mechanism of the defect in *curly tail* embryos is the analysis of factors which alter the incidence of NTD. Perturbation of PNP closure (ameliorating or otherwise) indicates interaction of the factor with the pathway leading from the genetic lesion to spinal NTD. Analysis of this interaction may provide information on the mechanism of the *curly tail* defect. For example, the reduction in the frequency of NTD by growth retardation is supportive of other evidence suggesting an abnormality of cell proliferation in gut endoderm and notochord as a feature of the pathogenesis of defects in *curly tail* mice (Copp *et al.*, 1988a). Another potential benefit is that factors which are protective in this animal model may suggest possible therapeutic strategies in humans; this is important because of the practical and ethical difficulties associated with the direct study of NTD development in human embryos.

The whole embryo culture system facilitates analysis of potential protective factors since the embryo is accessible to exogenous agents in the absence of maternal effects. The latter, although important when considering the role of potential teratogens or beneficial agents, tends to complicate the dissection of metabolic pathways in the embryo itself. In addition, the accessibility of embryos in culture enables continuous treatment as opposed to intermittent maternal dosing which may cause large fluctuations in concentration.

The complementary experiment in which embryos are made deficient for a particular factor is also more feasible because the culture conditions can be tightly controlled. In this study, use is made of the fact that dialysis of the culture medium, rat serum, can be used to remove small molecules including glucose, amino acids and vitamins. Nutrients are added back to the depleted serum which is then able to sustain growth of the embryos in a comparable manner to undialysed serum (Cockroft, 1979). Individual

nutrients can be omitted or included at defined concentrations and the effect on development of the embryo determined.

Since growth retardation of *curly tail* embryos in culture causes a reduction in the frequency of NTD (Copp *et al.*, 1988b) any observed protective effects of specific exogenous factors must be treated with caution until it is established whether there is non-specific growth retardation. In the converse case, where there is no effect of exogenous agents, one explanation might be that the absence of an effect is due to poor culture conditions. However, in the case of *curly tail* embryos this can be ruled out since the result of growth retardation acts as an internal control; any sub-optimal culture conditions would be evident by an ameliorating effect on the delay of PNP closure.

A role for the vitamin inositol in closure of the neural tube is suggested by nutritional studies in which the omission of inositol from rat or mouse embryos in culture causes NTD (Cockroft, 1979; Cockroft *et al.*, 1992). *Curly tail* embryos appear to be particularly sensitive compared with other strains, exhibiting a high frequency of cranial NTD and requiring a higher level of inositol supplementation to reverse the effect of inositol deficiency (Cockroft *et al.*, 1992). In both of these studies the embryos were cultured through the period of cranial neural tube closure but the culture period did not extend to closure of the caudal neural tube. Thus, although the level of inositol is known to influence closure of the anterior neural tube, it remains to be determined whether there is also an effect on closure of the neural tube at the posterior neuropore (PNP), the site of the principal *curly tail* defect.

In this chapter the effect of the level of inositol on closure of the PNP in *curly tail* embryos is examined using the whole embryo culture system. A preventive action of inositol supplementation on spinal NTD is demonstrated and this is proposed to be mediated through a specific effect on the inositol/lipid cycle. Inositol uptake by embryos in culture is also measured and shown to be enhanced by supplementation but appears not to be rate limiting in affected *curly tail* embryos compared with unaffected embryos.

3.2 Results

3.2.1 The effect of inositol supplementation *in vitro* on closure of the PNP in *curly tail* embryos

In order to determine the effect of inositol on closure of the PNP, embryos were cultured in dialysed serum containing defined levels of inositol (Section 2.2). After a 24 hour culture period beginning at 9 days 6 hours of gestation (Figure 3.1), embryos had approximately 27-31 somites; at this stage, affected embryos could be recognised on the basis of their PNP phenotype (Section 2.2.2). PNP length was used as a measure of the degree of abnormality of the embryo following culture, an enlarged neuropore indicating delayed closure.

Since comparison of the treatment groups as a whole may mask stage-specific effects, the results were analysed more closely by division of the data into developmental stages of 25-27, 28-29 and 30-31 somites following culture. Data are shown in Tables 3.1 (complete data set), 3.1a, 3.1b and 3.1c.

Delayed PNP closure in *curly tail* embryos is reduced by inositol supplementation
Comparison of treatment groups for all stages shows that there is a statistically significant reduction in PNP length in the presence of 50 µg/ml inositol (Table 3.1) compared with 0 µg/ml or 20 µg/ml inositol.

A plot of PNP length against somite number (Figure 3.2) reveals a striking stage-specific difference between the groups. In whole serum, which contains approximately 5 µg/ml inositol (Hashimoto *et al.*, 1990) or dialysed serum containing 0 µg/ml or 20 µg/ml inositol there is essentially no reduction in PNP length at these stages. This reflects the overall delay in closure of the PNP in *curly tail* embryos and correlates with previous observations by Van Straaten et al. (1992).

Figure 3.1 Experimental strategy for culture of *curly tail* embryos in varying concentrations of inositol with and without lithium

a. Inositol dose-response

Mouse killed   All embryos in litter explanted and cultured   Embryos harvested

9 days
2 hours

9 days
6 hours

24 hours culture

10 days
6 hours

Whole serum, 0, 20 or 50 μg/ml inositol

b. Lithium treatment of embryos in culture

Mouse killed   Embryos with 18-21 somites cultured   Embryos harvested

9 days
2 hours

9 days
6 hours

24 hours culture

10 days
6 hours

0 μg/ml inositol plus 200 μg/ml lithium
or 50 μg/ml inositol plus 200 μg/ml lithium
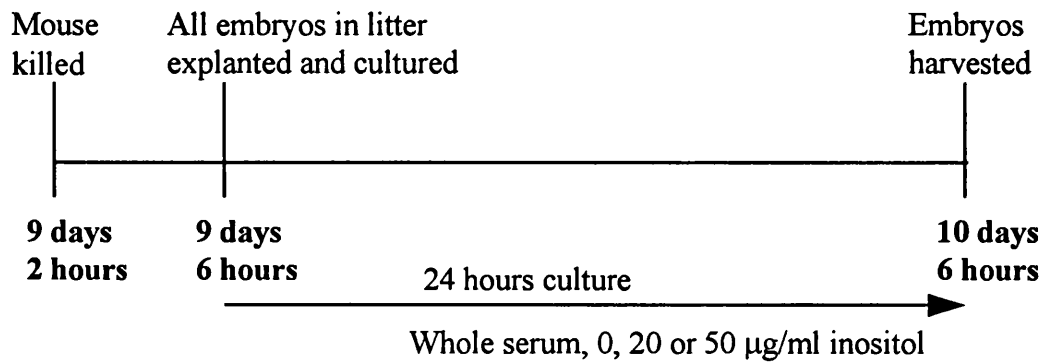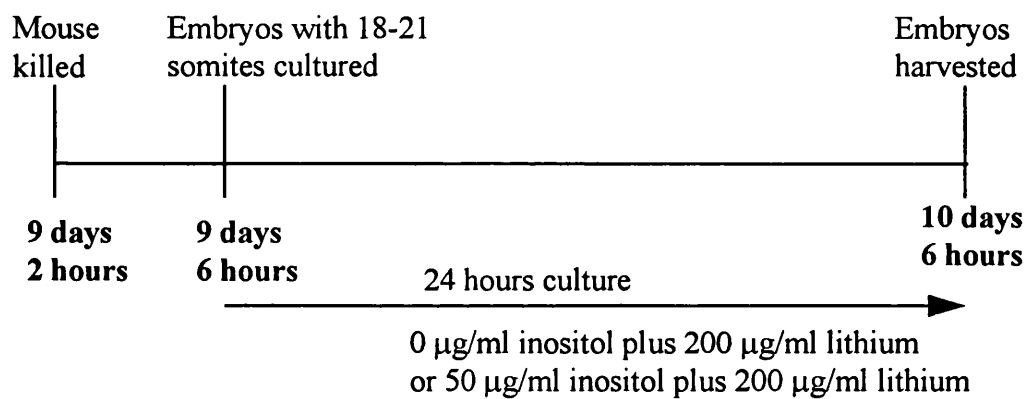
Table 3.1. Development of *curly tail* embryos (all somite numbers pooled) cultured in the presence of varying concentrations of inositol for 24 hours from 9 days 6 hours of gestation (mean values ± standard error of the mean (SEM))

Treatment	Number of embryos	Crown-rump length (mm)	Number of Somites	Mean PNP length (mm)	Rate of somite addition (no./hr)
Serum	52	3.60 ±0.07	28.73 ±0.11	0.57 ±0.04	0.46 ±0.01
0 µg/ml inos.	67	3.49 ±0.06	28.97 ±0.16	0.57 ±0.02	0.42 ±0.01
20µg/ml inos.	48	3.47 ±0.07	28.96 ±0.22	0.60 ±0.02	0.42 ±0.02
50µg/ml inos.	78	3.46 ±0.06	28.58 ±0.18	0.48 ±0.02	0.42 ±0.01
Statistically significant (p<0.05)	-	No[a] P= 0.445 F= 0.893	No[b] P= 0.422 H= 2.81	Yes[b]* P= 0.008 H= 11.8	Yes[a]** P= 0.040 F= 2.93
No culture (10.5 day embryos)	21	3.85 ±0.08	29.71 ±0.29	0.64 ±0.05	-

The embryos which had not been cultured were not included in the statistical analysis of the total number of embryos because overall they were at a later developmental stage than the cultured embryos.

[a] = One Way Analysis of Variance, [b] = Kruskal-Wallis One Way Analysis of Variance on Ranks

* = Mann-Whitney Rank Sum Test shows that there is a statistically significant difference in the median values between 50 µg/ml inositol and 0 µg/ml (P=0.0396, T= 5410.5) and between 50 µg/ml and 20 µg/ml, (P <0.0001, T= 3743.0).

** = The power of the ANOVA (Power = 0.4676) is below the desired value of 0.8 so this result should be treated with caution. Pairwise comparison by Mann-Whitney Rank Sum Test shows that the value for serum is significantly different from that for 0 µg/ml (P= 0.012, T= 464.0) and from that for 50 µg/ml (P= 0.008, T= 346.0).

Table 3.1a. Development of *curly tail* embryos to the 25-27 somite stage following culture for 24 hours in the presence of varying concentrations of inositol (mean values ± SEM)

Treatment	Number of embryos	Crown-rump length (mm)	Somites	PNP length (mm)
Serum	8	2.99 ±0.16	25.88 ±0.30	0.58 ±0.07
0 μg/ml inos.	9	2.87 ±0.11	26.33 ±0.29	0.50 ±0.03
20μg/ml inos.	7	2.83 ±0.19	26.14 ±0.34	0.60 ±0.06
50μg/ml inos.	20	3.15 ±0.07	26.40 ±0.20	0.54 ±0.02
Statistically significant (P<0.05)	-	No[a] P= 0.133 F= 1.98	No[b] P= 0.441 H= 2.70	No[b] P= 0.491 H= 2.41
No culture	2	3.17 ±0.08	27.00 ±0.00	0.60 ±0.05

[a] = One Way Analysis of Variance, [b] = Kruskal-Wallis One Way Analysis of Variance on Ranks

Table 3.1b. Development of *curly tail* embryos to the 28-29 somite stage following 24 hours culture in the presence of varying concentrations of inositol (mean values ± SEM)

Treatment	Number of embryos	Crown-rump length (mm)	Somites	PNP length (mm)
Serum	23	3.53 ±0.09	28.39 ±0.10	0.53 ±0.05
0 μg/ml inos.	34	3.35 ±0.07	28.76 ±0.07	0.57 ±0.03
20μg/ml inos.	21	3.38 ±0.09	28.62 ±0.11	0.60 ±0.02
50μg/ml inos.	30	3.40 ±0.10	28.43 ±0.09	0.57 ±0.04
Statistically significant (P<0.05)		No[a] P= 0.521 F= 0.757	Yes[b]* P= 0.021 H= 9.69	No[b] P= 0.595 H= 1.89
No culture	5	3.63 ±0.10	28.60 ±0.24	0.64 ±0.07

[a] = One Way Analysis of Variance, [b] = Kruskal-Wallis One Way Analysis of Variance on Ranks

* Using a Mann-Whitney Rank Sum Test for pairwise comparisons, the mean number of somites in the 0 μg/ml treatment group is significantly higher than in the serum (P= 0.018, T= 521.0) and 50 μg/ml (P= 0.041, T= 823.0) treatment groups.

Table 3.1c. Development of *curly tail* embryos to the 30-31 somite stage following culture for 24 hours in the presence of varying concentrations of inositol (mean values ± SEM)

Treatment	Number of embryos	Crown-rump length (mm)	Somites	PNP length (mm)
Serum	21	3.90 ±0.08	30.19 ±0.09	0.60 ±0.06
0 µg/ml inos.	24	3.92 ±0.07	30.25 ±0.09	0.60 ±0.05
20µg/ml inos.	20	3.78 ±0.07	30.30 ±0.11	0.60 ±0.05
50µg/ml inos.	28	3.74 ±0.08	30.25 ±0.08	0.35 ±0.04
Statistically significant (P<0.05)	-	No[a] P= 0.208 H= 4.54	No[a] P= 0.883 H= 0.66	Yes[b]* P= 0.0003 F= 7.02
No culture	14	4.02 ±0.07	30.50 ±0.14	0.65 ±0.06

[a] = Kruskal-Wallis One Way Analysis of Variance on Ranks, [b] = One Way Analysis of Variance

* Using t -tests for pairwise comparison, the mean PNP for the 50 µg/ml treatment group is significantly different from the values for embryos cultured in serum (P= 0.0010, t= 3.5), 0 µg/ml (P= 0.0002, t= 3.50) and 20 µg/ml (P= 0.0003, t= 3.95) inositol.

Figure 3.2 Comparison of mean PNP length of *curly tail* embryos at increasing somite stages following culture in varying concentrations of inositol
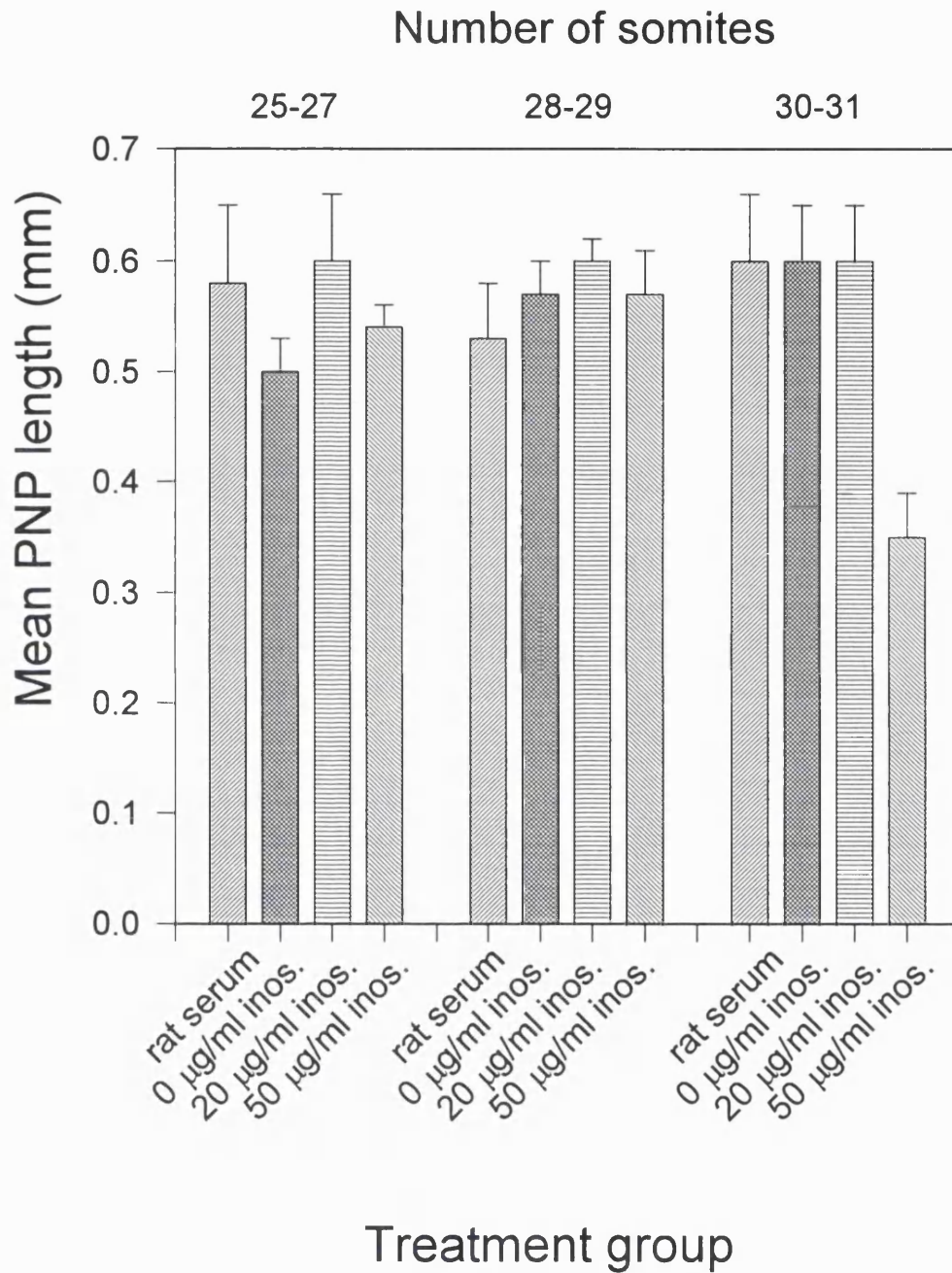
There is no apparent effect of 50 μg/ml inositol in embryos that reached the 25-27 and 28-29 somite stage following culture (Tables 3.1a and 3.1b). In contrast, embryos that reached the 30-31 somite stage, cultured in the presence of 50 μg/ml inositol, exhibit a mean PNP length which is significantly reduced compared with all the other treatment groups (Table 3.1c). This is a striking difference given the inherent variability in closure of the PNP in *curly tail* embryos, owing to partial penetrance of the defect, and suggests a strong ameliorating effect of inositol.

The proportion of 30-31 somite embryos within each PNP category was also compared for the treatment groups following culture in varying inositol concentrations (Figure 3.3). The PNP categories ranged from category 0, indicating a closed neural tube to category 5 which represents the most severely enlarged PNPs (Section 2.2.2). The frequency of PNP categories 0, 1 and 5 are very similar for embryos cultured in whole serum, 0 μg/ml or 20 μg/ml inositol but embryos exposed to 50 μg/ml inositol exhibit a strikingly different pattern. The frequency of embryos with category 5 neuropores (most enlarged) is greatly reduced in the 50 μg/ml inositol group while conversely the frequency of PNP category 0 and 1 (small PNP) is dramatically increased. Although differences in frequency are less striking for the intermediate size neuropores the data for the extremes of PNP category suggest that compared with the other treatment groups, embryos at the 30-31 somite stage following culture with 50 μg/ml inositol exhibit an overall shift in PNP size towards small or closed PNPs.

No teratogenic effects of inositol supplementation were observed in *curly tail* embryos even when the inositol dose was increased to 250, 500, or 1000 μg/ml (n =4 embryos for each). The number of embryos is too small for analysis of the effect of these high doses on closure of the PNP but there was no striking further reduction observed in PNP length at the 30-31 somite stage.

Figure 3.3 Frequency of each PNP category in *curly tail* embryos at the 30-31 somite stage following culture in varying concentrations of inositol
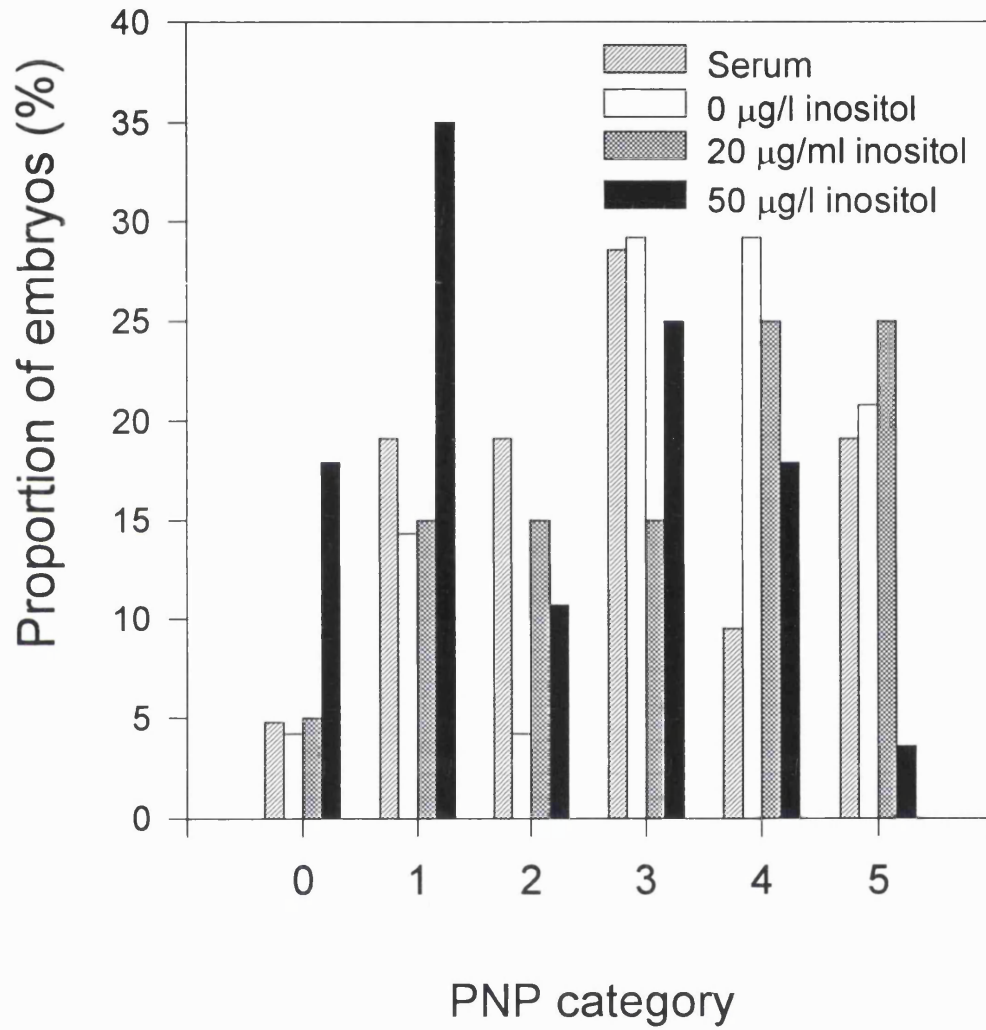
Closure of the PNP in embryos cultured in whole serum corresponds to that *in vivo*

In order to establish the effect of inositol concentration relative to the *in vivo* situation, embryos which had not been cultured were dissected and measured at 10 days of gestation. Embryos which had developed *in vivo* exhibited a mean PNP length similar to that of embryos cultured in whole serum. This was true both in comparison of the overall data (all somite stages pooled) and at the 30-31 somite stage when the effect of inositol supplementation was greatest (Tables 3.1 and 3.1c). This close correspondence between the development of embryos *in vivo* and *in vitro* supports the validity of the embryo culture system as a means of modelling the *in vivo* situation.

Closure of the PNP in a non-mutant strain

CBA/Ca embryos were also cultured in the presence of varying levels of inositol (Table 3.2). In contrast to *curly tail* embryos, all the CBA/Ca embryos with 30-31 somites had completed closure of the PNP and there was no apparent effect of varying inositol concentration. Plots of PNP length against somite number for CBA/Ca embryos and *curly tail* embryos are compared in Figure 3.4. All of the *curly tail* treatment groups show similar mean PNP lengths up to the 28-29 somite stage but, in the presence of inositol at 50 μg/ml, *curly tail* embryos show a reduction in PNP length at the 30-31 somite stage similar to that observed in CBA/Ca embryos in all treatment groups. Although the shape of the plots for CBA/Ca embryos and inositol supplemented *curly tail* embryos are similar, the mean PNP lengths of *curly tail* embryos are larger because a proportion of the embryos have enlarged PNPs at all stages examined.

3.2.2 Inositol concentration does not affect growth or developmental progression of embryos *in vitro*

Growth retardation is known to reduce the delay of closure of the PNP in *curly tail* embryos (Copp *et al.*, 1988b) and it was thus important to determine whether the effect of inositol is mediated through non-specific growth retardation. Neither growth, as

Table 3.2. Development of CBA/Ca embryos (all somite numbers pooled) following culture for 24 hours in varying concentrations of inositol and with lithium (mean values ± SEM)

Treatment	*Number of embryos*	*Crown-rump length (mm)*	*Somites*	*PNP length (mm)*
Serum	8	3.39 ±0.09	27.50 ±0.67	0.23 ±0.05
0 µg/ml inos.	14	3.45 ±0.09	27.64 ±0.48	0.30 ±0.04
50µg/ml inos.	10	3.52 ±0.11	27.50 ±0.54	0.30 ±0.04
0 µg/ml inos. + Li.	9	3.70 ±0.08	26.89 ±0.45	0.24 ±0.04
Statistically significant (P<0.05)	-	No[a] P= 0.183 F= 1.71	No[a] P= 0.751 F= 0.41	No[b] P= 0.226 H= 4.35

[a] = One Way Analysis of Variance, [b] = Kruskal-Wallis One Way Analysis of Variance on Ranks

Figure 3.4 Comparison of the change in mean PNP length of *curly tail* and CBA/Ca embryos at increasing somite stages following culture in varying concentrations of inositol

Points are offset to allow reading of error bars.
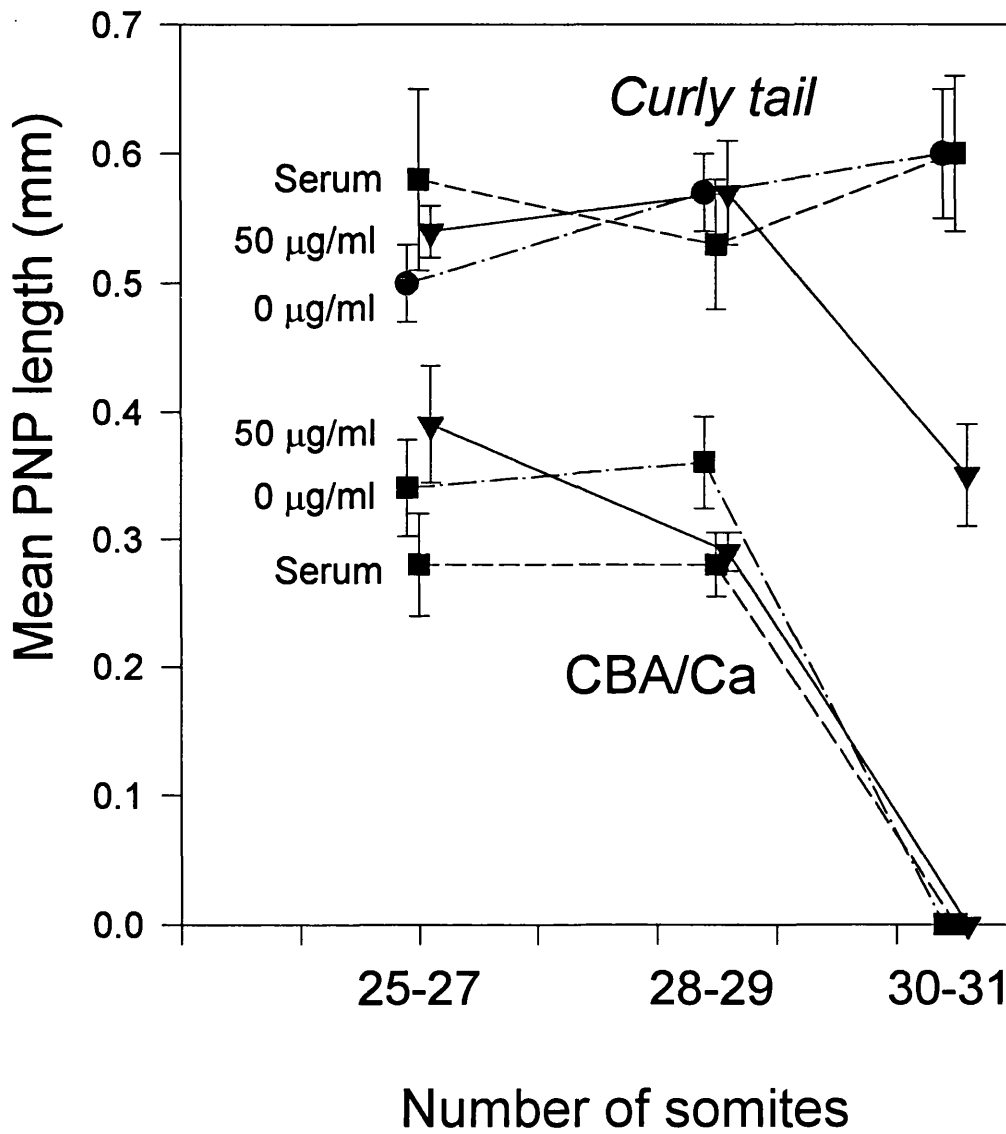
indicated by crown-rump length, nor developmental progression, as indicated by the number of somites after culture, exhibit any significant differences between treatment groups (Table 3.1). Comparison of treatment groups at each developmental stage (Tables 3.1a, 3.1b, 3.1c) also reveals no difference in mean crown-rump length showing that there is no stage-specific effect on growth which could have been masked in the analysis of the pooled embryos.

In the comparison of embryos at the 28-29 stage, the 0 μg/ml inositol group has a significantly higher mean number of somites than the serum and 50 μg/ml groups (Table 3.1.b). However, the difference amounts to less than 0.5 somites, raising a question concerning the biological significance of this finding.

The rate of development, measured in somites per hour, appears to be higher in embryos cultured in whole serum compared with those cultured in dialysed serum containing 0 μ g/ml or 50 μg/ml inositol (Table 3.1). However, the power of the statistical test is below the desired value, indicating that the result should be treated with caution.

CBA/Ca embryos are generally a little less developed at the start of culture and thus have a lower mean number of somites after culture (approximately 1 somite less). However, as with *curly tail* embryos, there was no effect of the level of inositol on growth or development during culture as determined by crown-rump length and number of somites of CBA/Ca embryos following culture (Table 3.2).

3.2.3 Lithium treatment of *curly tail* embryos *in vitro*

A specific protective effect of inositol on PNP closure is expected to be reduced by inhibition of the inositol/lipid cycle. In order to test this hypothesis, embryos were cultured in the presence of lithium (Figure 3.1) which suppresses inositol metabolism by specific inhibition of the recycling of inositol phosphates in the inositol/lipid cycle and inhibition of *de novo* synthesis of inositol 1-phosphate from glucose 6-phosphate (Berridge *et al.*, 1989).

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Embryos cultured in the presence of 200 µg/ml lithium and 50 µg/ml inositol exhibit a mean PNP length at the 30-31 somite stage which is significantly increased relative to those embryos cultured with 50 µg/ml inositol alone. The effect of inositol appears to be completely reversed by lithium such that, in terms of PNP length, these embryos resemble non-supplemented embryos (Table 3.3, Figure 3.5). In serum containing no inositol, the presence or absence of lithium does not alter mean PNP length at the 30-31 somite stage (Table 3.3, Figure 3.5). This suggests that lithium treatment reverses the protective effect of 50 µg/ml inositol rather than causing an overall increase in PNP length which counteracts the effect of inositol. In support of this conclusion, there is no effect of exposure of CBA/Ca embryos to lithium during culture in the absence of inositol (Table 3.2)

In this set of cultures, the head-length at 9 days 6 hours of gestation (prior to culture) was used to give an estimate of the number of somites (Peeters et al., 1996) allowing selection of embryos with between 18 and 21 somites which would develop to the stages of interest (30-31 somites) following the 24 hour culture period. As a consequence the mean crown-rump length and number of somites (Table 3.3) are increased relative to the previous set of cultures in which all embryos were cultured without prior selection. At the 30-31 somite stage, crown-rump length was not altered by the addition of lithium in the absence of inositol (Table 3.3). Addition of lithium plus 50 µg/ml inositol appeared to cause an increase in mean crown-rump length at the 30-31 somite stage compared with inositol alone. However, although the difference is statistically significant, the power of the test is below the desired value of 0.8 which means the result should be treated with caution.

In this set of cultures no teratogenic effects of lithium treatment were observed either in the presence or absence of inositol, as judged by external morphology (Table 2.2). The observed features included neural tube closure, yolk sac circulation and heart beat, development of the branchial arches and dorsal recess of the otocyst, regularity of the somites and axial rotation of the embryo.

Table 3.3. Development of *curly tail* embryos following culture for 24 hours in the presence or absence of lithium with varying concentrations of inositol (mean values ± SEM)

Treatment	Number of embryos	Crown-rump length (mm)	Somites	PNP length (mm)
30-31 somite stage				
0 µg/ml inos.†	24	3.92 ±0.07	30.25 ±0.09	0.60 ±0.05
0 µg/ml inos. +Li	22	3.88 ±0.08	30.09 ±0.06	0.61 ±0.05
50µg/ml inos.†	28	3.74 ±0.08	30.25 ±0.08	0.35 ±0.04
50µg/ml inos. +Li	23	4.04 ±0.07	30.09 ±0.06	0.68 ±0.05
Statistically significant (P<0.05)	-	Yes[a]* P= 0.036 F= 2.97	No[b] P= 0.232 H=4.29	Yes[a]** P< 0.0001 F= 10.8
Total embryos				
0µg/ml inos.+Li	37	3.79 ±0.07	29.49 ±0.14	0.57 ±0.03
50µg/ml inos.+Li	30	3.91 ±0.07	29.77 ±0.12	0.66 ±0.04

[a] = One Way Analysis of Variance, [b] = Kruskal-Wallis One Way Analysis of Variance on Ranks

† = The data for embryos cultured with 0 or 50 µg/ml inositol without lithium are repeated from Table 3.1a. for comparison. Lithium was included at 200 µg/ml.

* = A t-test shows a significant difference between the mean crown-rump length of the 50 µg/ml and 50 µg/ml + Li groups (P= 0.0047, t= -2.96). However, as the power of the test (0.792) is below the desired power of 0.8 the result should be treated with caution.

** = The mean PNP length in the 50 µg/ml inositol treatment group is significantly reduced compared with the 0 µg/ml (P= 0.0002, t= 3.50), 0 µg/ml + lithium (P= 0.0001, t= -4.19) and 50 µg/ml + lithium (P< 0.0001, t= -5.38) treatment groups.

Figure 3.5 Mean PNP length of *curly tail* embryos at the 30-31 somite stage following culture in 0 μg/ml and 50 μg/ml inositol both with and without concomitant lithium treatment
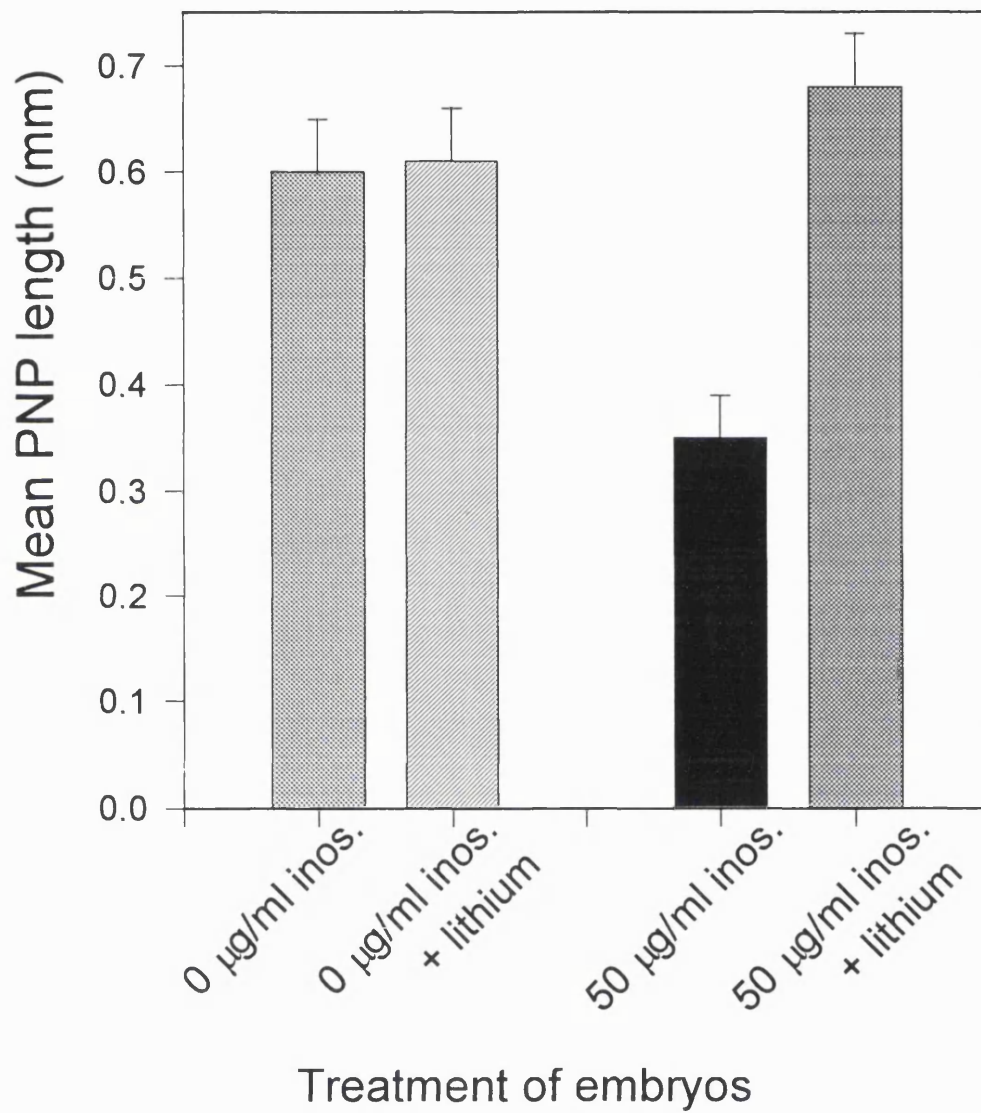
3.2.4 Uptake and incorporation of inositol by *curly tail* embryos *in vitro*

Inositol uptake and incorporation is not rate limiting in affected *curly tail* embryos

One possible explanation for the protective effect of inositol on *curly tail* embryos might be that the mechanism of the *curly tail* defect involves an abnormality of inositol uptake in affected embryos which is overcome by supplementation. In order to address this question, embryos were cultured in whole serum to reflect the *in vivo* situation and [^{3}H] inositol was included as a trace amount at 2 μCi/ml in order to allow measurement of inositol uptake by embryos during culture. [^{3}H] Inositol was used from a stock solution at 117 Ci/mmol which is equivalent to 1.54 ng/μCi. The addition of inositol to 2 μCi/ml (i.e., 3.08 ng/ml) therefore had a negligible impact on the total inositol concentration in the culture medium.

The groups of affected (PNP category 4/5) and unaffected (PNP category 1/2) embryos were comparable in terms of developmental progression and growth as shown by the similar mean number of somites and crown-rump lengths following culture (Table 3.4). As in cultures in varying concentrations of inositol (Section 3.2.2) CBA/Ca embryos were less developed after the 24 hour culture period as indicated by mean number of somites and crown-rump length values (Table 3.4). Comparison of affected and unaffected embryos reveals no differences of uptake and incorporation of [^{3}H] into either the water- or lipid-soluble fractions of embryos or yolk sacs (Table 3.5 and 3.6).

Uptake and incorporation of [^{3}H] inositol by non-mutant CBA/Ca embryos is significantly lower than for both affected and unaffected *curly tail* embryos (Table 3.5). This difference is evident in the total uptake and in each of the fractions following further separation of embryo extracts. Total uptake by the CBA/Ca yolk sac also appears to be reduced but the difference is smaller than for the embryo (Table 3.6). Moreover, in comparison of specific fractions, reduced uptake is only observed for the lipid-soluble phase of yolk sacs from affected *curly tail* compared with CBA/Ca embryos. The significance of reduced uptake by the yolk sac remains unclear since in all the statistical

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Table 3.4. Development of affected (PNP 4/5) and unaffected (PNP 1/2) *curly tail* embryos following culture for 24 hours in whole serum containing [^{3}H] inositol at 2 μCi/ml

Sample	Number of embryos	Crown-rump length (mm)	Mean no. somites	Mean PNP length (mm)	Mean protein content (μg)
PNP 1/2, embryo	15	3.77 ±0.13	29.20 ±0.37	0.32 ±0.05	180.1 ±13.1
PNP 4/5, embryo	9	4.03 ±0.11	29.78 ±0.49	0.72 ±0.14	223.3 ±16.4
CBA, embryo	5	2.78 ±0.11	26.00 ±0.55	0.20 ±0.05	102.8 ±11.8
Statistically significant (P<0.05)		Yes[a]* P<0.0001 F= 13.7	Yes[a]** P= 0.0002 F= 12.6	Yes[a]† P<0.0001 F= 25.0	Yes[a]†† P= 0.0005 F= 10.5
PNP 1/2, yolk sac	14	-	-	-	237.1 ±20.5
PNP 4/5, yolk sac	9	-	-	-	218.2 ±21.8
CBA, embryo	5	-	-	-	136.0 ±15.5
Statistically significant (P<0.05)	-	-	-	-	Yes[a]♠ P= 0.0253 F= 4.30

[a] = One Way Analysis of Variance. Pairwise comparisons are detailed below.

* = Using t-tests crown-rump length of CBA/Ca embryos is significantly reduced compared with PNP 1/2 (P= 0.0008, t= 4.03) and PNP 4/5 (P<0.0001, t= 7.17) *curly tail* embryos.

** = Number of somites of CBA/Ca embryos is significantly reduced compared with PNP 1/2 (P= 0.0003, t= 4.48) and PNP 4/5 (P= 0.0004, t= 4.83) *curly tail* embyros.

† = Mann-Whitney Rank Sum Test indicates that PNP length of CBA/Ca embryos is significantly lower than values for PNP 1/2 (P= 0.0402, T= 28.5) and PNP 4/5 (P= 0.0034, T= 15.0) *curly tail* embryos. In addition the PNP length of PNP 1/2 embryos is significantly lower than that for PNP 4/5 *curly tail* embryos (P< 0.0001, T= 179.0).

†† = t-tests indicate mean protein content of CBA/Ca embryos is significantly reduced compared with PNP 1/2 (P= 0.0046, t= 3.23) and PNP 4/5 (P= 0.0003, t= 5.03) *curly tail* embryos.

♠ = Using t-test the protein content of CBA/Ca yolk sacs is significantly lower than that for yolk sacs of PNP 1/2 (P= 0.0104, t= 2.90) and PNP 4/5 (P= 0.0239, t= 2.58) *curly tail* embryos.

Apart from mean PNP length values there were no significant differences in measured parameters in comparisons of PNP 1/2 and PNP 4/5 *curly tail* embryos.

Table 3.5. Uptake of [^{3}H] inositol by affected (PNP 4/5) and unaffected (PNP 1/2) *curly tail* embryos and CBA/Ca embryos cultured for 24 hours in whole serum containing [^{3}H] inositol at 2 μCi/ml

Sample	Number of embryos	Total counts (dpm/μg protein)	Lipid-soluble counts (dpm/μg)	Water-soluble counts (dpm/μg)	Counts in pellet (dpm/μg)
PNP 1/2	15	1029.4 ±81.1	575.1 ±42.9	157.8 ±26.9	270.3 ±28.5
PNP 4/5	9	1138.8 ±71.7	631.6 ±34.6	155.2 ±13.5	286.4 ±13.7
CBA/Ca	5	490.4 ±43.4	296.2 ±19.9	59.4 ±5.5	97.0 ±15.2
Statistically significant (P<0.05)	-	Yes[a]* P= 0.0004 F= 10.6	Yes[b]** P= 0.0019 H= 12.5	Yes[b]† P= 0.0105 H= 9.12	Yes[b]†† P= 0.0041 H= 11.0

[a] = One Way Analysis of Variance, [b] = Kruskal-Wallis One Way Analysis of Variance on Ranks. Individual embryos were analysed separately; fractionation as in Table 3.9.

* = t-test shows that total uptake of [^{3}H] inositol is reduced in CBA/Ca embryos compared with PNP 1/2 (P= 0.0016, t= 3.72) and PNP 4/5 (P<0.0001, t= 6.30) *curly tail* embryos.

** = Using pairwise t-tests uptake of [^{3}H] inositol into the lipid-soluble phase is reduced in CBA/Ca embryos compared with PNP 1/2 (P= 0.0015, t= 3.77) and PNP 4/5 (P<0.0001, t= 7.13).

† = Pairwise comparison by Mann-Whitney Rank Sum Test shows that uptake into the water-soluble fraction of CBA/Ca embryos is reduced in CBA/Ca embryos compared with PNP 1/2 (P= 0.0145, T= 24.0) and PNP 4/5 (P= 0.0034, T= 15.0) embryos.

†† = Pairwise t-tests show that uptake into the pellet of CBA/Ca embryos is reduced in CBA/Ca embryos compared with PNP 1/2 (P= 0.0032, t= 3.40) and PNP 4/5 (P<0.0001, t= 8.72) *curly tail* embryos.

There was no significant difference in any of the parameters in comparisons of affected (PNP 4/5) and unaffected (PNP 1/2) *curly tail* embryos.

Table 3.6. Uptake of [³H] inositol by the yolk sacs of affected (PNP 4/5) and unaffected (PNP 1/2) *curly tail* embryos and CBA/Ca embryos cultured for 24 hours in whole serum containing [³H] inositol at 2 μCi/ml

Sample	Number of embryos	Total counts (dpm/μg protein)	Lipid-soluble counts (dpm/μg)	Water-soluble counts (dpm/μg)	Counts in pellet (dpm/μg)
PNP 1/2, ys	14	1058.7 ±72.7	647.8 ±43.9	66.3 ±8.0	154.4 ±12.1
PNP 4/5, ys	9	1169.2 ±102.4	744.9 ±78.0	73.2 ±8.2	159.7 ±18.8
CBA yolk sac	5	762.0 ±62.6	486.4 ±49.4	47.2 ±6.9	108.8 ±9.9
Statistically significant (P<0.05)	-	Yes[a]* P= 0.0336 F= 3.92	Yes[a]** P= 0.0478 F= 3.50	No[a] P= 0.423 T= 116.0	No[a] P= 0.807 t= -0.248

[a] = One Way Analysis of Variance. ys= yolk sac. Individual yolk sacs were analysed separately and fractions separated as described in Table 3.5.

* = Using t-test the total uptake of [³H] inositol is reduced in the yolk sac of CBA/Ca embryos compared to PNP 1/2 (P= 0.030, t= 2.37) and PNP 4/5 (P= 0.0169, t= -2.77) *curly tail* embryos.

** = t-tests show that uptake into the lipid-soluble phase is reduced in the yolk sac of CBA/Ca embryos compared with PNP 4/5 *curly tail* embryos (P= 0.0349, t= 2.41) but not PNP 1/2 *curly tail* embyros (P= 0.0504, t= 2.13).

In all the statistical tests of uptake into the yolk sacs the power of the test was below the desired power (0.8) indicating that the results should be treated with caution.

comparisons the power of the test was below the desired value (0.8), indicating that results should be treated with caution.

Counts in the water-soluble phase include both free inositol and incorporated inositol phosphates while those in the lipid-soluble phase represent incorporation into the inositol phospholipids. The lipid-soluble fraction was further separated using thin layer chromatography.

Incorporation of inositol into phosphatidylinositol is unchanged in affected compared with unaffected *curly tail* embryos

In order to analyse in more detail the lipid-soluble phase of [³H] inositol labelled embryos, the material was further fractionated by thin layer chromatography. This technique allowed the separation of the specific inositol-containing lipid phosphatidylinositol due to its characteristic migration pattern. The labelled phosphatidylinositol was identified by co-migration with an unlabelled lipid standard and the counts in purified phosphatidylinositol were measured. There is no significant difference of incorporation into phosphatidylinositol of affected compared with unaffected embryos (Table 3.7).

Inositol uptake and incorporation is enhanced by *in vitro* supplementation

If the preventive effect of inositol supplementation on PNP closure in *curly tail* embryos is mediated through the inositol/lipid cycle, then supplemented embryos should be able to and the counts in purified phosphatidylinositol were measured. There is no significant difference of incorporation into phosphatidylinositol of affected compared with unaffected embryos (Table 3.7).

Inositol uptake and incorporation is enhanced by *in vitro* supplementation

If the preventive effect of inositol supplementation on PNP closure in *curly tail* embryos is mediated through the inositol/lipid cycle, then supplemented embryos should be able to take up and incorporate exogenous inositol into inositol-containing derivatives. To test

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Table 3.7. Incorporation of [³H] inositol into phosphatidylinositol by affected (PNP 4/5) and unaffected (PNP 1/2) *curly tail* embryos cultured for 24 hours in whole serum containing [³H] inositol at 2 µCi/ml

Sample	Number of embryos	Mean protein content (µg)	Mean incorporated counts (dpm/µg)
PNP 1/2	4	284.0 ±3.5	230.63 ±30.84
PNP 4/5	4	244.7 ±46.8	200.13 ±32.50
Statistically significant (P<0.05)	-	No[a] P= 0.449 t= 0.837	No[b] P= 0.400 T= 8.00

[a] = t-test, [b] = Mann Whitney Rank Sum test

this idea [^{3}H] inositol was included in the culture medium in proportion to the total

inositol concentration. The inositol treatments compared were 5 µg/ml with [^{3}H]

inositol at 0.25 µCi/ml and 50 µg/ml with [^{3}H] inositol at 2.5 µCi/ml. Thus, in both

cases [^{3}H] inositol constituted 0.0077 % of the exogenous inositol added to the cultures

(i.e, 0.05 µCi/µg inositol). Again, the inclusion of radiolabelled inositol, to 0.385 ng/ml

and 3.85 ng/ml respectively, has a negligible effect on the overall level of inositol in the

culture medium.

The mean crown-rump length and number of somites following culture were not

significantly different between treatment groups (Table 3.8). This demonstrates that the

level of inositol does not affect growth or developmental progression at this stage,

confirming the result of the previous set of cultures with varying levels of inositol

(Section 3.2.2).

In this set of cultures the mean PNP length of embryos supplemented with 50 µg/ml

inositol is lower than that for non-supplemented embryos but this is not statistically

significant. However, significance is less likely in this comparison because all the somite

stages are considered together, therefore reducing the importance of an effect at the 30-

31 somite stage. In addition, the sample number is lower than that for the analysis of the

total group of pooled embryos cultured in varying levels of inositol (Table 3.1) which

reduces the likelihood of a statistically important difference.

A significant increase in the [^{3}H] dpm taken up and incorporated into both the water-

and lipid-soluble phases (Table 3.9) was observed in embryos and their yolk sacs

supplemented with 50 µg/ml compared with 5 µg/ml. Therefore, inositol

supplementation in the culture medium is sufficient to increase the uptake of inositol by

the embryo. Inositol supplementation caused an approximately 3-fold increase in total

counts but there is variation in the degree of increase in uptake into specific fractions in

both embryo and yolk sacs. The water-soluble phase exhibited the proportionately

largest increase in uptake (approximately 8-fold), the lipid-soluble phase the lowest

Table 3.8. Development of embryos cultured for 24 hours in serum containing inositol at 5 µg/ml and 50 µg/ml and [^{3}H]inositol at 0.25 µCi/ml and 2.5 µCi/ml respectively

Sample	Number of embryos	Crown-rump length (mm)	Mean no. somites	Mean PNP length (mm)	Mean protein content (µg)
0.25 µCi/ml, embryo	21	3.88 ±0.08	28.94 ±0.37	0.67 ±0.05	189.9 ±10.2
2.5 µCi/ml, embryo	22	3.78 ±0.07	28.43 ±0.38	0.55 ±0.05	177.0 ±7.1
Statistically significant /(P<0.05)	-	No[a] P= 0.222 T= 424.0	No[b] P= 0.333 t= -0.981	No[b]* P= 0.095 t= -1.710	No[b] P= 0.298 t= -1.060
0.25 µCi/ml, ys	21	-	-	-	204.9 ±10.2
2.5 µCi/ml, ys	22	-	-	-	194.7 ±14.3
Statistically significant (P<0.05)	-	-	-	-	No[b] P= 0.558 t= -0.591

ys = yolk sac, [a] = Mann-Whitney Rank Sum test, [b] = t-test

* = The power of the test (0.2574) is below the desired power of 0.8 so this finding should be treated with a degree of caution.

Table 3.9. Uptake of [^{3}H] inositol by *curly tail* embryos cultured for 24 hours in the presence of inositol at 5 μg/ml (0.25 μCi/ml) or 50 μg/ml (2.5 μCi/ml)

Sample	Number of embryos	Total counts (dpm/μg)	Lipid-soluble counts (dpm/μg)	Water-soluble counts (dpm/μg)	Counts in pellet (dpm/μg)
5 μg/ml, embryo	21	120.3 ±3.8	71.8 ±4.6	19.1 ±1.5	27.2 ±1.7
50 μg/ml, embryo	22	425.9 ±24.4	134.9 ±8.6	166.1 ±20.6	161.83 ±11.4
Statistically significant (P<0.05)	-	Yes[a] P= 0.032 T= 271.0	Yes[a] P<0.0001 T= 96.0	Yes[a] P<0.0001 T= 153.0	Yes[a] P<0.0001 T= 136.0
5 μg/ml, ys	21	178.2 ±15.9	113.9 ±9.5	9.8 ±0.8	22.4 ±1.3
50 μg/ml, ys	22	359.6 ±15.5	170.1±11.2	80.1±8.1	85.3 ±4.9
Statistically significant (P<0.05)	-	Yes[a] P<0.0001 T= 480.0	Yes[a] P<0.0001 T= 314.0	Yes[a] P<0.0001 T= 459.0	Yes[a] P<0.0001 T= 360.0

ys = yolk sac, [a] = Mann-Whitney Rank Sum test

Fractions were separated as described in Section 2.3.2. In brief, a crude extract was prepared by sonication of samples followed by solubilisation in chloroform/methanol (1:2). After centrifugation (to remove the pellet), the extract was separated into lipid- and water-soluble phases by addition of 0.73% NaCl and the lipid phase further extracted with pure solvents upper phase. The water-soluble phase contains free inositol and inositol phosphates, the lipid-soluble phase contains inositol phospholipids and the pellet contains inositol associated with the tissue residue in insoluble cytoskeleton, DNA and protein material.

(approximately 2-fold) and the pellet an intermediate increase (approximately 5-fold).

The greater degree of uptake into the water-soluble phase probably reflects the fact that

this fraction contains free inositol in addition to inositol incorporated into inositol

phosphates in contrast to the lipid-soluble phase which contains inositol phospholipids

only.

3.3. Discussion

Inositol supplementation of *curly tail* embryos *in vitro* causes a reduction in PNP length at the 30-31 somite stage. This reflects a reduction in the genetically programmed delay of PNP closure that has been shown to lead to spinal NTD in the *curly tail* mutant mouse (Copp, 1985). Thus, inositol supplementation provides a means for prevention of spinal NTD in the *curly tail* mouse.

There are two possible explanations for the results of culture in dialysed serum; a) the reduction in PNP length caused by supplementation with 50 µg/ml inositol may represent a protective effect relative to the *in vivo* situation or b) culture in the presence of inositol concentrations of 0 or 20 µg/ml may have a worsening effect, causing a further delay in closure of the PNP in addition to that which occurs *in vivo*. These possibilities are not mutually exclusive i.e., there could be both a protective effect of inositol supplementation and a worsening effect of lower concentrations relative to the closure of the PNP *in vivo*. The data from cultures in whole serum (Tables 3.1, 3.1c) enable these possibilities to be distinguished since they reflect development *in vivo*, confirmed by measuring embryos which had not been cultured. These data support the first hypothesis since the mean PNP length of this group of embryos closely resembles that of embryos cultured in 0 µg/ml or 20 µg/ml inositol. Therefore, 50 µg/ml inositol can be seen to cause a reduction in PNP length relative to that *in vivo*. Culture of embryos of a non-mutant strain, CBA/Ca, showed no apparent effect of the level of inositol lending support to the hypothesis that the differences observed in *curly tail* embryos reflect a positive protective effect of 50 µg/ml, as opposed to a detrimental effect of lower inositol doses.

The reduction in mean PNP length at the 30-31 somite stage could be due to an effect of 50 µg/ml inositol on PNP closure in all the embryos such that there is a shift towards smaller PNPs. An alternative, although not exclusive explanation is that there is a specific effect on a subset of embryos which then exhibit a lower PNP length following culture and therefore this causes a reduction in mean PNP length. It is difficult to

differentiate between these possibilities because one cannot determine what the PNP length of an individual embryo would have been had it not been exposed to 50 µg/ml inositol. However, comparison of the frequency of embryos within each PNP category (Figure 3.3) is supportive of an overall shift in the inositol supplemented treatment group towards smaller PNPs, since there is both a reduction in the proportion of severely enlarged PNPs (category 5) and an increase in the proportion of embryos with small or closed neuropores (category 1 and 0). The altered distribution of PNP categories following culture in 50 µg/ml inositol correlates with the reduction in mean PNP length at the 30-31 somite stage and is again supportive of a protective effect of inositol on closure of the PNP.

The absence of a significant alteration in growth parameters in the presence of varying inositol concentrations shows that reduction in PNP length is not simply caused by growth retardation following inositol supplementation. A previous study reported an influence of inositol concentration on growth of mouse embryos *in vitro* (Cockroft *et al.,* 1992). In cultures beginning at day 8.5 of gestation, inositol deficiency had a retarding effect whereas there was no effect of 50 µg/ml inositol. Therefore, if inositol supplementation was to cause growth inhibition in the present study (and this retarding effect led to reduction of delay in closure of the PNP), such an effect would be predicted to occur in the absence of inositol and not in the 50 µg/ml treatment group.

The increased rate of developmental progression in whole serum compared with dialysed serum containing 0 µg/ml or 50 µg/ml inositol suggests that culture in whole serum may be superior to that obtained in dialysed serum. However, the rate is not increased compared with the 20 µg/ml group and differences in rates are not reflected in the mean crown-rump lengths in any of the treatment groups. Thus, although the increased rate is statistically significant it seems unlikely to be biologically relevant in this study particularly since the growth rates in dialysed serum are identical. Despite the difference as compared with whole serum, the rate of somite addition in dialysed serum is

unaffected by inositol concentration and all treatment groups exhibit a rate close to the expected value of approximately 0.5 somites added per hour (Brown and Fabro, 1981).

There is an increased mean number of somites in the 28-29 somite group following culture in the presence of 0 μg/ml inositol. The observed difference simply reflects an increased number of embryos with 29 compared with 28 somites at this particular stage. Therefore, the increase in mean number of somites is unlikely to be biologically relevant particularly since there is no apparent effect of inositol on closure of the PNP or on growth (as shown by crown-rump length) at this stage.

The finding that inositol deficiency did not inhibit growth in the present study can probably be attributed to the fact the cultures were initiated at 9 days 6 hours rather than at 8.5 days as in the previous study. A stage-dependent susceptibility to growth retarding influences has been shown for a number of factors, such that more advanced embryos are likely to have greater resistance. For example, hyperglycaemia (Sadler, 1980; Cockroft, 1984) and β-hydroxybutyrate (Hunter and Sadler, 1987; Moore *et al.*, 1989) cause growth retardation and malformations in rat and mouse embryos in culture and the effect is age- and dose-dependent with greater retardation of younger embryos.

In some instances, developmental changes in metabolism may play a role in altered sensitivity to growth retardation. For example, β-hydroxybutyrate has an age-dependent effect on the pentose phosphate pathway which is required for pyrimidine biosynthesis (Shum and Sadler, 1990). Inhibition of this pathway appears to mediate the teratogenic effect on embryos cultured from the 5-6 somite stage but does not occur in embryos cultured from the more sensitive 2-3 somite stage, although the pyrimidine supply in the latter group may also be reduced by inhibition of *de novo* synthesis. If the relative importance of inositol-requiring pathways differs at distinct developmental stages this could contribute to the stage-dependent effect on growth.

Several lines of evidence suggest that the effect of inositol on PNP closure is mediated through a specific effect on the inositol/lipid cycle . First, supplementation leads to raised levels in the embryo of both water- and lipid-soluble inositol-containing molecules suggesting that capacity is available for increased flux through the inositol/lipid cycle. Interestingly, a ten-fold increase in the concentration of inositol in the culture medium, 50 μg/ml compared with 5 μg/ml, is required to obtain a two-fold increase in incorporation into inositol phospholipids. This observation may explain why there is no apparent protective effect of 20 μg/ml inositol (Section 3.2.1) since this level of inositol may not cause a sufficient increase in specific incorporation of inositol to mediate a protective effect on closure of the PNP.

Secondly, lithium, an inhibitor of the inositol/lipid cycle (Berridge *et al.*, 1989), reverses the protective effect of inositol showing that the adequate function of the inositol/lipid cycle is a necessary part of the protective mechanism. The fact that lithium treatment in the absence of inositol does not worsen the delay in closure of the PNP in *curly tail* embryos or in non-mutant CBA/Ca embryos, suggests that activity of the inositol/lipid cycle is not rate-limiting for closure of the PNP in non-supplemented embryos since its inhibition has no apparent effect. This correlates with the observation that in the absence of inositol there is no worsening effect relative to closure of the PNP in whole serum or *in vivo*.

One possible explanation for the protective effect of inositol is that affected embryos have a deficiency in uptake which is overcome by supplementation. This appears unlikely for two reasons. Firstly, the absence of exogenous inositol would be expected to mimic such a deficiency of uptake and therefore worsen overall PNP closure since the embryos would not be able to take up inositol from the culture medium. However, closure of the PNP is not worsened in all embryos as shown by the absence of an overall detrimental effect relative to mean PNP lengths in whole serum or *in vivo* where exogenous inositol is available. Secondly, direct comparison of [^{3}H] inositol uptake in culture reveals no significant difference between affected and unaffected embryos. This

similarity of overall uptake also extends to specific incorporation into phosphatidylinositol the first component of the inositol/lipid cycle into which inositol is incorporated.

There is, however, a significant increase in the uptake of [³H] inositol during culture by *curly tail* embryos (of both phenotypes) compared with CBA/Ca embryos. This may be due to a strain difference in inositol uptake or could be related to the earlier stage of development of CBA/Ca embryos following culture. Alternatively, the difference in uptake potentially reflects an abnormality of inositol function in *curly tail* embryos which is compensated by increased uptake. However, the increased uptake of inositol in *curly tail* embryos does suggest that there is no deficiency in uptake. Therefore, the data do not support a mechanism of a *curly tail* mutant effect in which inositol uptake or incorporation into the inositol/lipid cycle is abnormal in affected compared with unaffected embryos. The possibility cannot be excluded that there is a deficiency of inositol function further downstream in *curly tail* (of all phenotypes) compared with other strains.

In this study, lithium at a concentration of 200 μg/ml (4.7 mM), had neither teratogenic nor growth retarding consequences whilst clearly influencing embryonic metabolism at least in inositol-supplemented conditions. This contrasts with previous studies in which rat (Hansen *et al.*, 1990; Klug *et al.*, 1992) or mouse (Hansen *et al.*, 1990) embryos were cultured in serum containing varying levels of lithium from 1.2 mM to 5.0 mM. In these cases lithium caused retardation of both growth (in terms of crown-rump length and protein content) and development (number of somites and morphological score). Rat embryos appear to be more sensitive than mice with significant retardation at all concentrations following culture from the 3-4 somite somite stage and at 2.4 mM or higher in embryos cultured from the 8-12 somite stage. Mouse embryos cultured from 8 to 10 days of gestation were also growth retarded at lithium concentrations higher than 2.4 mM. The absence of a growth retarding effect of 4.7 mM lithium in the present study is probably due to the greater resilience of mouse compared to rat embryos and the

fact that cultures are started at a later stage; more advanced embryos having been shown to be more resistant to the growth retarding effect of lithium as well as other factors (as described earlier).

In this study an increased mean crown-rump length was observed in embryos cultured in the presence of lithium plus 50 µg/ml inositol compared with 50 µg/ml inositol alone. This difference is statistically significant but the power of the test was below the desired value. It seems likely that the apparent increase in growth in this case is not biologically important since where lithium has previously been shown to affect the growth of embryos in culture, this has been a retarding effect (Hansen *et al.*, 1990; Klug *et al.*, 1992) rather than enhancement, as described above.

Teratogenic effects of lithium on cultured rat embryos have also been reported (Klug *et al.*, 1992), including blebs in the cranial region, poor yolk sac circulation and abnormal development of the otic and optic vesicles and dorsal recess of the otocyst. These effects were not reversed by addition of inositol which is perhaps not surprising because lithium is an uncompetitive inhibitor, binding only to the enzyme-substrate complex, and therefore causing a greater degree of inhibition at higher substrate concentrations. In mouse embryos, similar malformations were not reported (Hansen *et al.*, 1990) but there was an increase of cranial NTD at the highest concentration used, 5 mM. This would be predicted if lithium depletes the level of inositol by inhibition of inositol phosphate recycling, since inositol deficiency is known to cause cranial NTD (Cockroft *et al.*, 1992). Thus, the absence of teratogenic consequences of lithium treatment in this study correlates with previous observations in mouse embryos; cranial NTD are not produced because the neural tube in the cranial region has closed before the beginning of the culture period.

The observations made in this chapter show inositol supplementation of *curly tail* embryos *in vitro* has a protective effect on closure of the PNP which would lead to a reduction in spinal NTD. The effect of inositol is proposed to be mediated through

enhanced uptake from the culture medium which leads to increased flux through the

inositol/lipid cycle. The next chapter investigates the roles of potential downstream

effectors of the inositol/lipid cycle which could be responsible for the protective effect on

PNP closure.

CHAPTER 4

Preventive effect of inositol on posterior neuropore closure in the *curly tail* mouse:-

Arachidonic acid and Protein Kinase C as potential downstream mediators

4.1 Introduction

In Chapter 3, a protective effect of inositol supplementation was demonstrated and this was proposed to be mediated through the activity of the inositol/lipid cycle (Figure 1.4) which is a key component in intracellular signal transduction via phospholipase C-associated receptors in the cell membrane. The cycle also influences the availability of inositol for other cellular functions since it contains the inositol phospholipids and inositol phosphates into which exogenous inositol is initially incorporated and it also contains the enzymes for inositol phosphate recycling. Supplementation could potentially have effects on one or more of the multiple cellular functions of inositol (Section 1.5.2). These include roles as substrates in the inositol/lipid cycle, nuclear inositide cycle and phosphatidylinositol 3-kinase (PI-3 kinase) signal transduction pathways, as well as a structural role in the glycosyl-phosphatidylinositol (GPI) anchors of a number of membrane-bound proteins.

The hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) by phospholipase C generates inositol triphosphate (IP_3) and diacylglycerol (DAG) which are both responsible for downstream events. IP_3 binds to its receptor and stimulates the mobilisation of stored intracellular calcium while DAG binds and activates protein kinase C (PKC) (reviewed by Nishizuka, 1992; Berridge, 1993). PKC comprises a family of serine/threonine kinases of which 12 isoenzymes have been identified that vary in structure, tissue expression and substrate specificity (Section 1.5.3). Metabolism of DAG in the inositol/lipid cycle generates arachidonic acid, the major precursor leading to synthesis of members of the eicosanoid family which includes prostaglandins, leukotrienes, lipoxins and thromboxanes.

The arachidonic acid pathway has been implicated in the development of NTD in other systems and therefore potentially has a role in the prevention of NTD in the *curly tail* mouse. Hyperglycaemia causes a high incidence of NTD in rat and mouse embryos *in vitro* (Sadler, 1980; Cockroft, 1984) and is likely to contribute to the increased risk for congenital malformations (including NTD) associated with maternal diabetes mellitus

although the relative importance of metabolic disturbances in the diabetic state remains unresolved (Buchanan *et al.,* 1994). Several lines of evidence suggest that the abnormalities induced by raised levels of glucose are caused by depletion of inositol leading to suppression of phosphoinositide metabolism, reduced production of arachidonic acid and hence inhibition of the pathway leading to prostaglandin synthesis. This evidence can be summarised as follows.

Exposure of rat embryos to hyperglycaemia *in vitro* (Hod *et al.,* 1986; Hashimoto *et al.,* 1990) or streptozotocin-induced diabetes *in vivo* (Sussman and Matschinsky, 1988) causes depletion of inositol while levels of another hexose, sorbitol, increase probably owing to activation of the polyol pathway. Inositol appears to be the key factor since *in vitro* supplementation corrects the inositol deficiency with concomitant reduction in growth retardation and the frequency of NTD (Baker *et al.,* 1990; Hashimoto *et al.,* 1990). In contrast, aldose reductase inhibitors (e.g., Sorbinil or Statil) significantly reduce sorbitol concentrations but there is no ameliorating effect on the frequency of NTD. Inositol deficiency limits the available hydrolytic response for signal transduction via the inositol/lipid cycle (Strieleman and Metzger, 1993), because intracellular pools of both the inositol phospholipids and inositol phosphates are diminished (Strieleman *et al.,* 1992).

Reduced inositol/lipid cycle activity is proposed to lead to a functional deficiency of arachidonic acid which causes NTD. Firstly, the protective effect of inositol *in vitro* is reversed by indomethacin, an inhibitor of arachidonic acid metabolism (Baker *et al.,* 1990). In addition, arachidonic acid treatment has a protective effect against NTD in both the *in vitro* hyperglycaemia model in the mouse, and the *in vivo* streptozotocin-induced diabetic rat (Goldman *et al.,* 1985; Pinter *et al.,* 1986). The downstream prostaglandins PGE_2, $cPGI_2$ and $PGF_{2\alpha}$ which are known to be produced by cells from mouse embryos can also provide differing degrees of protection against cranial NTD caused by hyperglycaemia *in vitro* (Baker *et al.,* 1990; Goto *et al.,* 1992).

The activity of arachidonic acid in prevention of NTD is not confined to the hyperglycaemic state. The anti-convulsant diphenylhydantoin (DPH) causes dose-dependent growth retardation and teratogenic effects *in vitro* including NTD, abnormal rotation and malformations of the craniofacial region and somites (Bruckner *et al.*, 1983). Addition of arachidonic acid to the culture medium significantly reduces the frequency of abnormalities, including NTD (Kay *et al.*, 1988).

The protective role of arachidonic acid in different systems suggests that one or more prostaglandins play an important role in neural tube closure. If the arachidonic acid pathway mediates the protective effect of inositol supplementation in hyperglycaemic conditions, it must also be considered as a candidate as a downstream effector of inositol action in prevention of NTD in *curly tail* embryos. This potential role of arachidonic acid in *curly tail* embryos is examined in the present chapter.

Another possible downstream effect of inositol supplementation is the generation of DAG by hydrolysis of inositol phospholipids in the inositol/lipid cycle which, in turn, activates PKC (Nishizuka, 1986). PKC has been proposed to participate in a range of cellular functions including regulation of gene expression and the cell cycle, modulation of the activity of membrane-bound receptors and ion-channels, and activation of cells involved in the immune response (Nishizuka, 1986; Nishizuka, 1992; Hug and Sarre, 1993). Thus, PKC regulates many cellular functions, raising the possibility that the protective effect of inositol supplementation on closure of the PNP could potentially be caused by PKC activation, mediated through phosphorylation of one or more substrate molecules.

In this study use is made of the fact that phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) can substitute for DAG in activation of PKC, by binding to the same region of the C_1 structural motif (Section 1.5.3). Unlike DAG which is rapidly metabolised, TPA is metabolically stable and can thus cause sustained activation of PKC (Castagna *et al.*, 1982). PKC activation is probably responsible for the tumour

promoting activity of phorbol esters which disturb both proliferation and differentiation events. Prolonged activation of PKC by TPA causes alterations in enzyme activity, in addition to those elicited by transient generation of DAG. Extended exposure to TPA initiates degradation of activated membrane-associated PKC by proteolytic cleavage which leads to depletion of cellular PKC (Nishizuka, 1988). TPA thus has a dual action on PKC, transient positive activation followed by negative regulation owing to degradation of the enzyme. This complex regulation must be considered when interpreting the effects of TPA treatment on mouse embryos.

One approach to analysis of the contribution of PKC in cellular functions is the modulation of PKC activity using specific inhibitors. Several naturally occurring or synthetic inhibitors are known to inhibit protein kinase C, a well known example being the *Streptomyces*-derived alkaloid, staurosporine (Tamaoki *et al.*, 1986). However, although staurosporine is a potent inhibitor of PKC (K_i= 3 nM), it is not very specific, also inhibiting cAMP-dependent protein kinase and cGMP-dependent protein kinase at the same range of concentrations. Improved selectivity of PKC inhibition can be achieved using bisindolylmaleimides which are structurally similar to staurosporine but exhibit much greater specificity. In this chapter, use is made of the bisindolylmaleimide, GF109203X, which is cell-permeable and, like staurosporine, potently inhibits PKC (K_i= 10 nM) via the ATP-binding site of the enzyme (Toullec *et al.*, 1991). The specificity of this inhibitor is significantly greater than for staurosporine, with inhibition of cAMP-dependent PKA only occurring at much higher concentrations (K_i= 2 μM) and no effect on tyrosine kinases at concentrations up to 50 μM.

In this chapter, TPA treatment *in vitro* is shown to replicate the protective effect of inositol supplementation on closure of the PNP in *curly tail* embryos. Activation of PKC is therefore proposed to be involved in prevention of spinal NTD and this is supported by the reversal of the protective effect by specific inhibition of PKC.

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4.2 Results

4.2.1 Arachidonic acid treatment of *curly tail* embryos *in vitro*

In order to test the hypothesis that arachidonic acid may mimic the protective effect of inositol on PNP closure, *curly tail* embryos were cultured for 24 hours, from 9 days 6 hours, in the presence of arachidonic acid (50 μg/ml) in dialysed serum with either 0 μg/ml or 50 μg/ml inositol included (see Figure 4.1 for summary of experimental design). In all respects, other than addition of arachidonic acid, the culture conditions were identical to the previous set of cultures in dialysed serum (Section 3.2.1). This permitted comparisons to be made between the outcomes of the various treatments.

Analysis of *curly tail* embryos at the 30-31 somite stage following culture in dialysed serum containing 0 μg/ml inositol shows that the addition of arachidonic acid does not cause any reduction of PNP length (Table 4.1, Figure 4.2). Embryos treated with arachidonic acid in the presence of inositol at 50 μg/ml exhibit comparable PNP lengths to those embryos cultured in 0 μg/ml inositol (Table 4.1). This group therefore have enlarged PNPs compared with embryos treated with 50 μg/ml inositol alone suggesting that the effect of 50 μg/ml inositol is reduced by simultaneous addition of arachidonic acid (Figure 4.2).

Growth of *curly tail* embryos *in vitro* was enhanced by exposure to arachidonic acid at a concentration of 50 μg/ml. Arachidonic acid caused a significant increase in crown-rump length of embryos at the 30-31 somite stage following culture in both the presence and absence of exogenous inositol (Table 4.1).

Figure 4.1 Experimental strategy for *in vitro* treatment with arachidonic acid and TPA

a. Arachidonic acid treatment

Mouse killed | Embryos with 18-21 somites cultured | Embryos harvested

| 9 days 2 hours | 9 days 6 hours | 24 hours culture | 10 days 6 hours |

50 µg/ml Arachidonic acid plus 50 µg/ml inositol
or 50 µg/ml Arachidonic acid plus 0 µg/ml inositol

b. TPA treatment of embryos in culture

Mouse killed | All embryos explanted and cultured | Embryos harvested

| 9 days 2 hours | 9 days 6 hours | 9 days 7 hours | 9 days 23 hours | 10 days 6 hours |

1.
1 hour in serum | 1 hour, 100 nM or 500 nM TPA | 22 hours culture in whole serum

2.
17 hours culture in whole serum | 1 hour, 100 nM or 500 nM TPA | 6 hours in serum

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Figure 4.1 continued.

c. Treatment with PKC inhibitor, GF109203X

```
Mouse          Embryos with 18-21
killed         somites cultured

        |              |                                    |
        |              |                                    |
        |              |                            |       |
     9 days        9 days                        9 days    10 days
     2 hours       6 hours                       23 hours  6 hours
```

                    24 hours culture in rat serum with
        1.   and without inositol
                                          ──────────►
                                        1 hour
                                        15 minutes, GF

        2.   17 hours culture   1 hour 15   6 hours in
                    in whole serum   minutes GF,   serum
                                          plus 1 hour 500
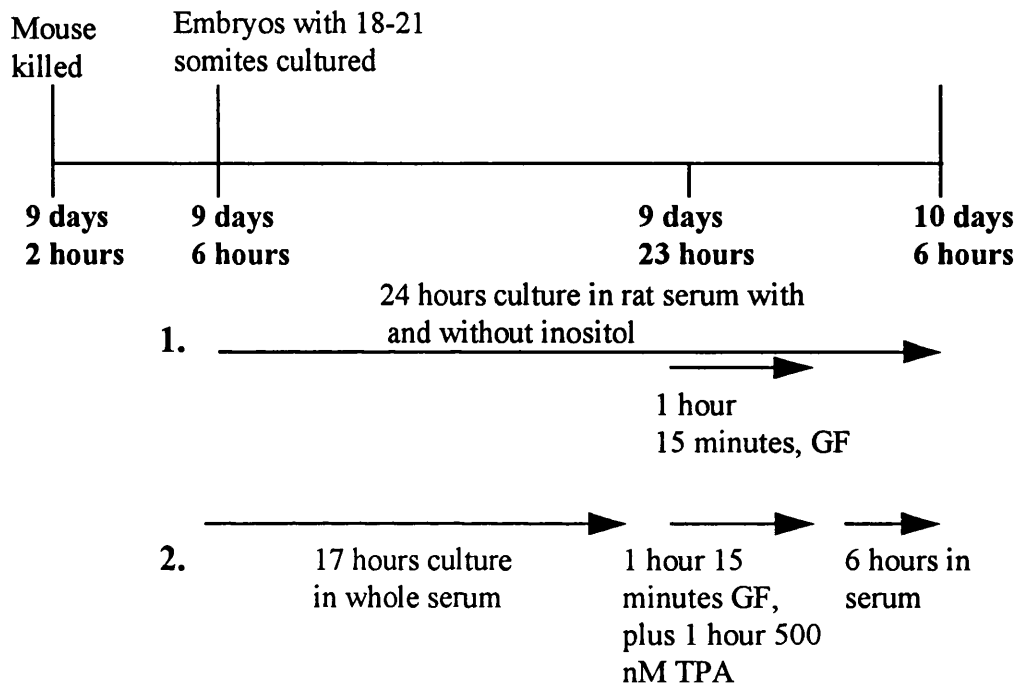   nM TPA

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Table 4.1. Development of *curly tail* embryos following culture for 24 hours in the presence of arachidonic acid (50 µg/ml) with varying concentrations of inositol (mean values ± SEM)

| Treatment	Number of embryos	Crown-rump length (mm)	Somites	PNP length (mm)
30-31 somite stage				
0 µg/ml inos. †	24	3.92 ±0.07	30.25 ±0.09	0.60 ±0.05
0 µg/ml inos. +AA	20	4.18 ±0.05	30.25 ±0.12	0.59 ±0.05
50µg/ml inos. †	28	3.74 ±0.08	30.25 ±0.08	0.35 ±0.04
50µg/ml inos. +AA	19	4.07 ±0.06	30.42 ±0.12	0.58 ±0.07
Statistically significant (P<0.05)	-	Yes[b]* P= 0.0002 H= 20.3	No[a] P= 0.480 F= 2.47	Yes[a]** P= 0.0010 F= 5.92
Total embryos				
0µg/ml inos.+AA	38	4.02 ±0.06	29.39 ±0.18	0.68 ±0.04
50µg/ml inos.+AA	44	3.89 ±0.05	29.00 ±0.23	0.70 ±0.04

[a] = One Way Analysis of Variance, [b] = Kruskal-Wallis One Way Analysis of Variance on Ranks. † = The data for embryos cultured with 0 and 50 µg/ml inositol without arachidonic acid are repeated from Table 3.1c for comparison.

* = Mann-Whitney Rank Sum Test shows a significant difference between crown rump length values in comparison of 0 µg/ml inositol ± arachidonic acid (P= 0.0052, T= 569.0) and 50 µg/ml inositol ± arachidonic acid, (P= 0.0037, T= 590.5).

** = Pairwise t-tests indicate that mean PNP length for the 50 µg/ml inositol group significantly differs from all the other treatment groups; 0 µg/ml (P= 0.0002, t= 4.01), 0 µg/ml + arachidonic acid (P= 0.0008, t= -3.59) and 50 µg/ml + arachidonic acid (P= 0.0042, t= -3.02).

Figure 4.2 Mean PNP length of embryos at the 30-31 somite stage following culture in the presence of varying inositol concentrations both with and without 50 μg/ml arachidonic acid. Data for 0 μg/ml and 50 μg/ml inositol are reproduced from Figure 3.2 for purposes of comparison.
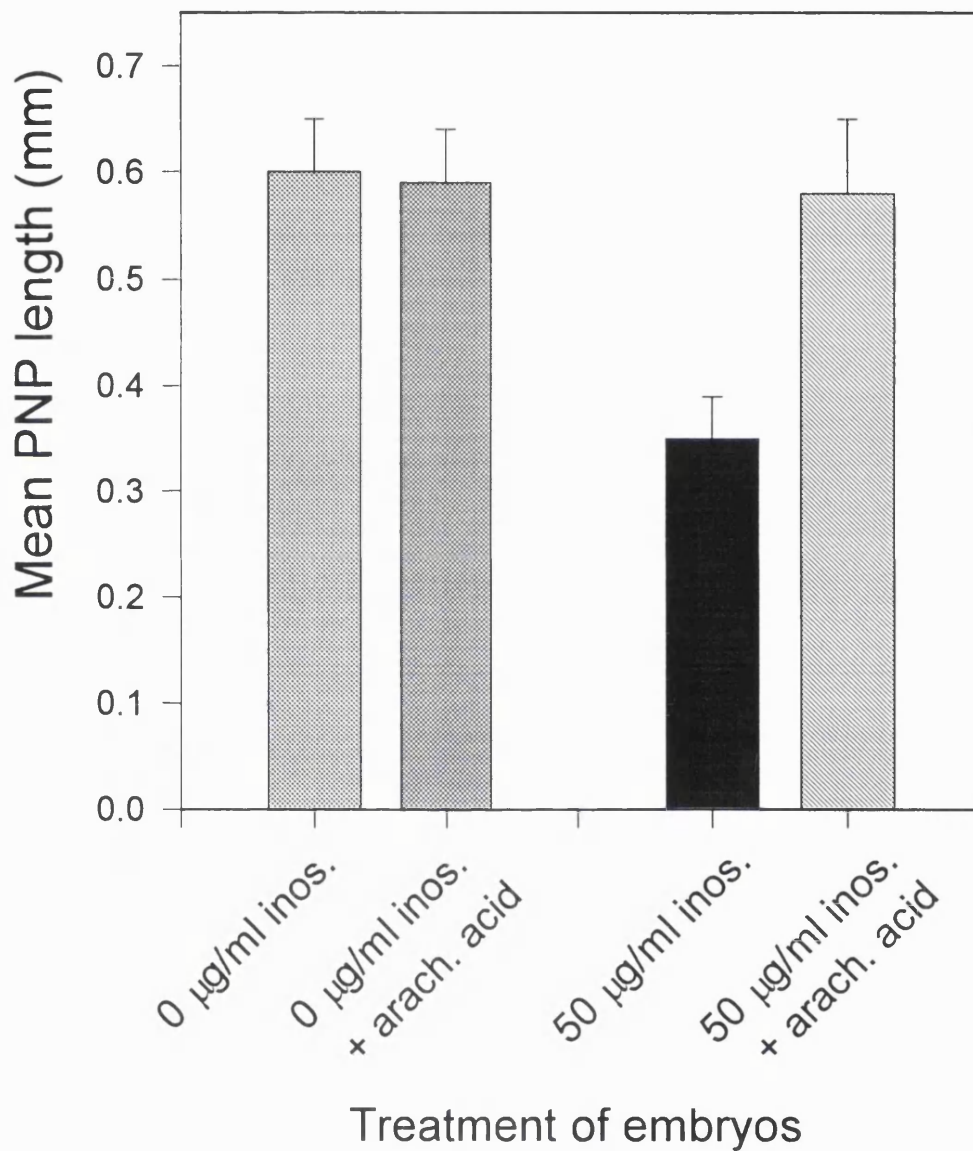
4.2.2 *In vitro* activation of protein kinase C in *curly tail* embryos

Activation of protein kinase C (PKC) is a potential consequence of increased flux

through the inositol/lipid cycle since diacylglycerol (DAG), a product of the inositol/lipid

cycle, is a physiological activator of PKC. In order to test whether activation of protein

kinase C could influence closure of the PNP, *curly tail* embryos were treated *in vitro*

with a DAG analogue, 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Unlike DAG

which is rapidly metabolised, TPA, a phorbol ester, is metabolically stable and can thus

cause sustained activation of PKC (Castagna *et al.*, 1982; Nishizuka, 1988).

TPA treatment for 24 hours is lethal to embryos *in vitro*

Embryos were cultured from 9 days 6 hours of gestation in non-dialysed rat serum with

TPA added to a concentration of 100 nM (typically used in cell culture) or 500 nM

(Figure 4.1). Exposure to TPA for 24 hours was lethal to embryos at this stage (n= 6

embryos for each concentration). This is probably due to a teratogenic effect of TPA on

the yolk sac which, following culture, was found to be smooth, lacking circulation and

showed abnormal separation of the two cell layers. This result correlates with

observations in rat embryos (Huber and Brown, 1983), in which exposure to TPA in

embryo culture causes a progressive separation of the endodermal and mesodermal cell

layers.

Short-term TPA treatment is not teratogenic to embryos *in vitro*

The lethality of 24 hours exposure of embryos to TPA can probably be attributed to the

fact that prolonged TPA treatment causes depletion of PKC due to proteolysis (Young

et al., 1987; Nishizuka, 1988). In order to overcome this problem, TPA exposure was

reduced to one hour after which the embryos were rinsed in PBS, the culture serum was

changed, and culture was continued in the absence of TPA (Figure 4.1). A one hour

TPA treatment, after either 1 or 17 hours of culture, was not teratogenic to either

embryo or yolk sac; there was a vigorous yolk sac circulation at the end of the culture

period and no abnormalities, other than an enlarged PNP, were observed.

TPA treatment mimics the effect of inositol

TPA treatment caused a significant reduction in mean PNP length at the 30-31 somite stage compared with development in whole serum (Table 4.2a, Figure 4.3). The difference in PNP length is also significant in comparison of the pooled somite stages (Table 4.2), however, although the P value is more significant for the pooled samples this is due to greater sample numbers. The percentage reduction in PNP length is greater in comparison of embryos at the 30-31 somite stage following both inositol and TPA treatment, supporting a related mechanism of prevention of NTD.

The protective effect of TPA treatment on closure of the PNP appeared dose- and possibly time-dependent. The most significant reduction in PNP length was observed following treatment with 500 nM TPA after 17 hours of culture (Table 4.2a) although the effect was not statistically different from that achieved following treatment after 1 hour. The effect of 100 nM TPA after 1 hour of culture was reduced compared with 500 nM TPA and treatment after 17 hours of culture had no protective effect on closure of the PNP. These data suggest that the more sensitive period for TPA treatment is 9 days 7 hours of gestation (after 1 hour of culture) since the lower concentration, 100 nM, has an effect on closure of the PNP at this time point but does not at 9 days 23 hours. However, this conclusion is not absolute since raising the concentration of TPA to 500 nM eliminates any time-dependence of the effect of TPA treatment.

Culture in the presence of 50 μg/ml inositol (Chapter 3) was repeated in parallel with the TPA cultures although in this case inositol (50 μg/ml) was added to whole serum rather than dialysed serum. Once again, there was a significant protective effect of 50 μg/ml inositol at the 30-31 somite stage with a similar degree of protection as was seen with TPA exposure (Table 4.2a, Figure 4.3).

Table 4.2 Development of *curly tail* embryos (all somite numbers pooled) cultured for 24 hours in the presence of 50 µg/ml inositol or with TPA treatment after 1 or 17 hours (mean values ± SEM)

Treatment	Number of embryos	Crown-rump length (mm)	Somites	PNP length (mm)
Rat serum	69	3.83 ±0.04	29.26 ±0.06	0.64 ±0.03
50 µg/ml inos.	67	3.88 ±0.04	29.15 ±0.17	0.53 ±0.03
500 nM TPA (1 hr)	28	3.98 ±0.06	29.68 ±0.18	0.55 ±0.05
100 nM TPA (1 hr)	32	3.86 ±0.05	29.38 ±0.23	0.54 ±0.03
500 nM TPA (17 hrs)	72	3.86 ±0.04	29.32 ±0.15	0.48 ±0.02
100 nM TPA (17 hrs)	32	3.89 ±0.06	29.5 ±0.26	0.56 ±0.03
Statistically significant (P<0.05)	-	No[a] P= 0.4942 F= 0.881	No[b] P= 0.6099 H= 3.59	Yes[b]* P= 0.0009 H= 20.9

[a] = One Way Analysis of Variance, [b] = Kruskal-Wallis One Way Analysis of Variance on Ranks.

* = Mann-Whitney Rank Sum Test shows a significant difference between the mean PNP length of embryos cultured in whole serum and the values for embryos cultured in serum supplemented with 50 µg/ml inositol (P<0.0001, T= 3823.5) or treated with 500 nM TPA after 17 hours (P= <0.0001, T= 5929.0) or 100 nM TPA after 1 hour (P= 0.0124, T= 1289.0) or 17 hours (P= 0.0463, T= 1358.5).

Table 4.2a Development of *curly tail* embryos to the 30-31 somite stage following culture for 24 hours in the presence of 50 µg/ml inositol or with TPA treatment after 1 or 17 hours (mean values ± SEM)

Treatment	Number of embryos	Crown-rump length (mm)	Somites	PNP length (mm)
Rat serum	32	4.00 ±0.05	30.44 ±0.09	0.61 ±0.04
50 µg/ml inos.	30	4.06 ±0.06	30.37 ±0.09	0.45 ±0.03
500 nM TPA (1 hr)	18	4.05 ±0.07	30.28 ±0.11	0.46 ±0.06
100 nM TPA (1 hr)	17	3.96 ±0.09	30.35 ±0.12	0.48 ±0.03
500 nM TPA (17 hrs)	41	4.02 ±0.04	30.29 ±0.07	0.42 ±0.03
100 nM TPA (17 hrs)	19	4.04 ±0.06	30.53 ±0.12	0.56 ±0.04
Statistically significant (P<0.05)	-	No[a] P= 0.9029 F= 0.316	No[b] P= 0.5039 H= 4.32	Yes[a]* P= 0.0031 F= 3.76

[a] = One Way Analysis of Variance, [b] = Kruskal-Wallis One Way Analysis of Variance on Ranks.

* = Using t-tests for pairwise comparison, the mean PNP length for culture in serum is significantly higher than the values for embryos cultured in serum supplemented with 50 µg/ml inositol (P= 0.0034, t= 3.05) or treated with 500 nM TPA after 1 hour (P= 0.0336, t= 2.19), 500 nM TPA after 17 hours (P= 0.0003, t= 3.76) or 100 nM TPA after 1 hour (P= 0.0343, t= 2.18).

Figure 4.3 Mean PNP length of embryos at the 30-31 somite stage following culture in serum supplemented with inositol or TPA. Embryos were cultured in serum alone, serum supplemented with 50 µg/ml inositol for the entire 24 hours or serum with TPA added for one hour after 1 or 17 hours (see Figure 4.1 for experimental strategy)
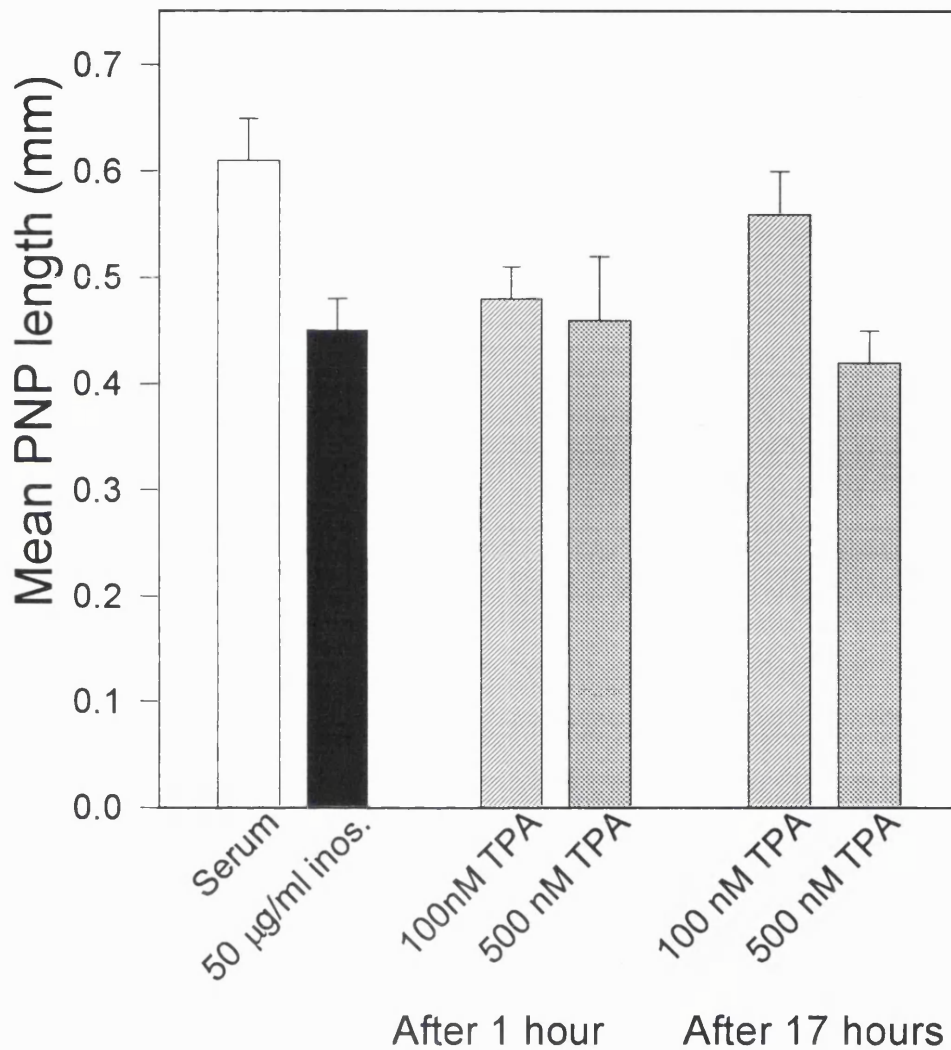
TPA treatment does not alter growth of embryos *in vitro*

It is important to determine whether TPA influences closure of the PNP through non-specific retardation of growth in culture which is known to cause a reduction in mean PNP length in *curly tail* embryos (Copp *et al.*, 1988b). This is particularly relevant in view of the observed teratogenic effect of long-term TPA treatment.

A one hour exposure to TPA had no significant effect on parameters of growth (crown-rump length) or developmental progression (number of somites) in *curly tail* embryos (all somite numbers pooled) following culture (Table 4.2). Moreover, the addition of 50 µg/ml inositol to whole serum culture did not significantly affect crown-rump length or somite number (Table 4.2) confirming the previous finding for inositol added to dialysed serum (Table 3.1). A similar finding emerged when only embryos with 30-31 somites were considered, the stage of primary interest in this study (Table 4.2a). Therefore, the action of TPA does not appear to result from non-specific growth retardation.

4.2.3 Closure of the PNP following inhibition of PKC

Experiments described in Section 4.2.2 suggest that the protective effect of inositol treatment on closure of the PNP in *curly tail* embryos is mediated via activation of PKC. In order to test this hypothesis further, embryos were treated with a water-soluble form of the specific PKC inhibitor GF109203X, which was predicted to reduce the effects of inositol and TPA if these are mediated through PKC.

In cellular models of phosphorylation, PKC activity is inhibited with IC_{50} values (half-maximal inhibitory concentration) of 0.2 to 2.5 µM . For example, in an assay of vimentin phosphorylation in Swiss 3T3 cells maximal inhibition of PKC was obtained at a concentration of 2.5-5 µM GF109203X with no inhibition of cAMP-dependent PKA even at the highest concentration (Toullec *et al.*, 1991). In another study GF109203X was added at a concentration of 10 µM to COS7 cells in order to investigate the role of PKC in RA-induced transcription (Tahayato *et al.*, 1993). Based on these data, the inhibitor was used at initial concentrations of 2 and 10 µM in cultures of *curly tail*

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embryos. The higher concentration was selected with the aim of overcoming any decreased uptake of the inhibitor by embryos with intact yolk sacs, as compared with cultured cells which are likely to be more accessible to the cell-permeable inhibitor.

The influence of PKC inhibitor on closure of the PNP in *curly tail* embryos was examined following culture in whole serum, in serum containing 50 µg/ml inositol and in serum containing 500 nM TPA for one hour after 17 hours of culture (Figure 4.1). In a preliminary set of cultures, GF109203X was added at 2 or 10 µM for the entire 24 hour culture period, but the caudal region of a number of the embryos exhibited a "crinkly" neural tube, which is indicative of a teratogenic effect (Table 2.2). Therefore, the period of treatment with inhibitor was reduced to one hour both in order to avoid the problem of growth retardation or teratogenicity and, in the case of TPA treatment, to reduce the possibility of the inhibitor being degraded before the addition of the PKC agonist.

GF109203X was added to the culture medium to 10 µM final concentration 15 minutes prior to the addition of TPA after 17 hours of culture. Following one hour exposure to TPA, embryos were transferred to fresh serum and cultured for a further 6 hours, as before (Figure 4.1). Embryos cultured in whole serum or in serum containing 50 µg/ml inositol were also treated with PKC inhibitor at the equivalent time points.

Inhibition of protein kinase C reverses the protective effect of TPA

Embryos treated simultaneously with 10 µM GF109203X and 500 nM TPA exhibited a mean PNP length at the 30-31 somite stage which was significantly increased in comparison with embryos cultured with TPA alone (Table 4.3, Figure 4.4). This increase suggests that the protective effect of TPA is reversed by inhibition of PKC.

There was no apparent effect of GF109203X on the closure of the PNP in embryos cultured in rat serum alone since mean PNP length at the 30-31 somite stage was unaffected (Table 4.3, Figure 4.4). Addition of PKC inhibitor for one hour to cultures with 50 µg/ml inositol appeared to cause an increase in the mean PNP length at the 30-31

somite stage although this difference is not statistically significant (Table 4.3, Figure 4.4). However, unlike embryos treated with 50 µg/ml inositol (Table 4.2a), the PNP length of embryos treated with both 10 µM GF109203X and 50 µg/ml inositol is no longer significantly reduced compared to embryos cultured in serum alone.

Short-term inhibition of protein kinase C does not cause growth retardation

The addition of PKC inhibitor for 1 hour 15 minutes had no apparent effect on growth at this stage of development since there was no alteration in crown-rump length of embryos following culture to the 30-31 somite stage (Table 4.3). In addition, no teratogenic effects were observed; embryos exhibited vigorous yolk sac circulation and appeared normal as judged by external morphology.

Table 4.3 Development of *curly tail* embryos cultured for 24 hours in rat serum, in the presence of 50 µg/ml inositol or with TPA treatment after 17 hours and with GF109203X (mean values ± SEM)

| Treatment	Number of embryos	Crown-rump length (mm)	Somites	PNP length (mm)
Rat serum†	32	4.00 ±0.05	30.44 ±0.09	0.61 ±0.04
Serum + 10µM GF	18	4.04 ±0.06	30.50 ±0.12	0.55 ±0.04
Statistically significant (P<0.05)	-	No[a] P= 0.6233 t= -0.494	No[b] P= 0.7231 T= 477.0	No[b] P= 0.2711 T= 404.0
50 µg/ml inos.†	30	4.06 ±0.06	30.37 ±0.09	0.45 ±0.03
50 µg/ml inos. + GF	19	3.91 ±0.05	30.26 ±0.10	0.56 ±0.05
Statistically significant (P<0.05)	-	No[a] P= 0.0685 t= 1.86	No[b] P= 0.1931 T= 705.0	No[b]* P= 0.1238 T= 550.5
500 nM TPA (17 hrs) †	41	4.02 ±0.04	30.29 ±0.07	0.42 ±0.03
500 nM TPA + GF	20	3.98 ±0.08	30.50 ±0.11	0.62 ±0.05
Statistically significant (P<0.05)	-	No[b] P= 0.7645 T= 600.0	No[b] P= 0.1931 T= 705.0	Yes[b] P= 0.0022 T= 819.5.

a= t-test, b= Mann-Whitney Rank Sum Test

GF109203X (GF) was used at 10 µM in the culture medium.

† = The data for embryos cultured with are repeated from Table 4.2a for comparison.

* = There is no significant difference between the mean PNP length for the inositol + GF treatment group compared with the inositol alone or rat serum groups.

Figure 4.4 Mean PNP length of embryos at the 30-31 somite stage following culture in serum supplemented with GF109203X and inositol or TPA. Embryos were cultured with 10 μM GF109203X in serum alone, serum supplemented with 50 μg/ml inositol for the entire 24 hours or serum with 500 nM TPA added for one hour after 17 hours (see Figure 4.1 for experimental strategy). Data for culture in the absence of GF109203X are reproduced from Figure 4.3 for comparison.
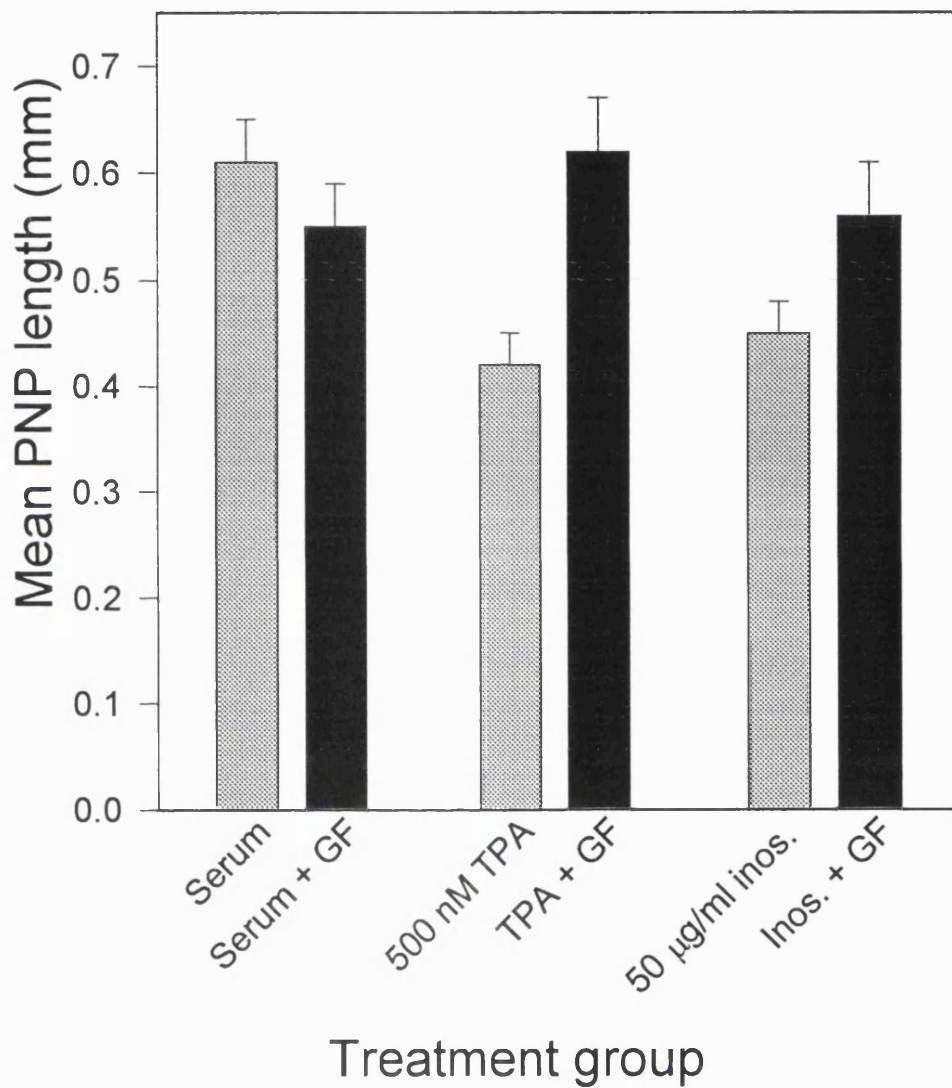
4.3 Discussion

Arachidonic acid treatment in culture does not mimic the effect of inositol in prevention of spinal NTD in *curly tail* embryos. This is in contrast to the example of hyperglycaemia-induced NTD where the protective action of inositol is apparently mediated through the arachidonic acid pathway (Goldman *et al.*, 1985; Pinter *et al.*, 1986)

However, in hyperglycaemic conditions, supplementation with inositol overcomes an induced inositol depletion, whereas in *curly tail*, a genetic lesion leads to NTD without apparent inositol deficiency as judged by the normal uptake and incorporation of [^{3}H] inositol in culture in affected compared with unaffected embryos (Section 3.2.4).

The two systems also differ in several other respects. First, the strain of mice used in the hyperglycaemia studies was an F1 hybrid between the C57BL/10 and A strains, in contrast to the study of the *curly tail* mutant which is carried on a genetic background derived principally from the CBA strain (Gruneberg, 1954). Second, the preventive effect of arachidonic acid on hyperglycaemia-induced NTD relates to closure of the cranial neural tube, not the PNP as in the present work. Third, cultures in the hyperglycaemia study were initiated at 8.3 days of gestation and continued for 48 hours (Goldman *et al.*, 1985), in contrast to the current experiments in which cultures began at 9 days 6 hours and continued for 24 hours. One or more of these differences could be responsible for the contrasting effect of arachidonic acid on the incidence of NTD in these two studies.

The strain difference is probably not significant since arachidonic acid also prevents malformations in rats both *in vivo* and *in vitro* (Goldman *et al.*, 1985; Pinter *et al.*, 1986). The later developmental stage of the *curly tail* embryos at the time of treatment may be important since embryos at later stages of gestation do appear more resistant than younger embryos to exogenous factors such as hyperglycaemia or inositol deficiency (see discussion in Section 3.3). However, the doses which failed to show a

protective effect in this study were up to fifty times higher than those which were protective in the previous study, i.e. 50 μg/ml in this study compared with 1 or 10 μg/ml at day 8.3 (Goldman et al., 1985). The axial location of the target NTD for prevention by arachidonic acid is probably an important factor since closure of the cranial and caudal neural tube involve distinct mechanisms (Copp et al., 1990). The arachidonic acid pathway seems to be particularly important in closure of the cranial neural tube affording protection against NTD caused by hyperglycaemia and DPH, whilst having no protective effect on spinal NTD in curly tail embryos. The possibility remains that arachidonic acid would reduce the incidence of cranial NTD in curly tail embryos exposed to inositol deficient conditions, although this has yet to be tested.

Although arachidonic acid is not involved in prevention of spinal NTD in curly tail embryos, the fact that inositol treatment apparently acts through this pathway in other systems (e.g., hyperglycaemia) is important in demonstrating that inositol supplementation in vitro is sufficient to generate downstream products with functional consequences.

The protective effect of inositol on spinal NTD is apparently removed by the presence of arachidonic acid in the culture medium. This may be due to stimulation of embryonic growth, as indicated by the increased crown-rump length of embryos treated with arachidonic acid, a phenomenon that also occurs in rat embryo culture (Pinter et al., 1986). Such an effect might lead to an increase in the incidence of spinal NTD, in the opposite manner to the reduction in incidence caused by growth retardation (Copp et al., 1988b). That is, while growth retardation tends to narrow the cell proliferation imbalance in curly tail embryos, by reducing growth mainly in the normal neuroepithelium, growth stimulation may specifically enhance growth in this tissue and, thereby widen the proliferation imbalance.

An alternative, although not exclusive, explanation is suggested by the observation that arachidonic acid treatment in hyperglycaemic conditions causes a reduction in total

phospholipid content compared with control embryos (Pinter *et al.*, 1988). Such a reduction in *curly tail* embryos could limit the available pool of inositol phospholipids and therefore inhibit the increased activity of the inositol/lipid cycle following inositol supplementation, which is proposed to mediate the effect on closure of the PNP. Finally, one could envisage a mechanism whereby an increased level of arachidonic acid causes feedback inhibition of phospholipase C, resulting in inhibition of DAG production and consequent down-regulation of the inositol/lipid cycle.

Downstream activation of PKC does appear to play a role in the prevention of spinal NTD in *curly tail* embryos since treatment with TPA, a PKC activator, is sufficient to cause a reduction in PNP length of embryos following culture. This effect is comparable to that observed following *in vitro* inositol supplementation (Section 3.2.1) and suggests that TPA also has a protective role since reduction in the delay in PNP closure implies a decrease in the incidence of spinal NTD (Copp, 1985).

In view of the observation that activation of PKC can cause a reduction in PNP length it is notable that there is no protective effect of arachidonic acid treatment, arachidonic acid having been shown to cause PKC activation in cell culture (reviewed by Hug and Sarre, 1993). This is probably an example of a lack of correlation between the *in vitro* (in this case referring to a cell free system) and *in vivo* (the intact embryo) properties of PKC. PKC is promiscuous in its activation *in vitro* and it is likely that not all the agents (in this case arachidonic acid) that are able to cause activation *in vitro* will do so in the whole embryo. In addition, while arachidonic acid activates the βII, γ, ϵ and α (in the presence of Ca^{2+}) PKC isoenzymes, it appears not to activate the βI or δ forms (Hug and Sarre, 1993). It may be the case that even if arachidonic acid treatment is sufficient to activate PKC in the embryo, the isoenzyme(s) species involved in the effect on closure of the PNP is/are not responsive. Similarly, comparison of the properties of PKC isoenzymes suggests that members of the aPKC group, such as PKC ζ, are not involved in the TPA stimulated pathway since they have no phorbol ester binding activity (Section

1.5.3). It remains to be determined which PKC isoenzymes are principally expressed during closure of the posterior neuropore in the mouse.

In addition to DAG, other molecules could contribute to activation of PKC following increased flux through the inositol/lipid cycle since inositol phospholipids, whose levels are raised in inositol supplemented embryos, also exhibit PKC activating properties *in vitro*. PIP_2 is able to substitute for DAG in stimulation of PKC α, βI and γ while PI can act replace phosphatidylserine as co-factor for the enzyme (Section 1.5.3).

The short-term TPA treatment that was sufficient to cause reduction in PNP length following culture had no visible adverse effect and did not alter growth, an important consideration regarding specificity of action in *curly tail* embryos, since growth retardation reduces the incidence of NTD (Copp *et al.*, 1988b). Reduction of the period of TPA treatment to one hour avoids problems of embryonic malformations and lethality associated with longer exposure (this study, Huber and Brown, 1983; Chen and Hales, 1994). The teratogenic effect on the yolk sac of *curly tail* embryos exposed to TPA for 24 hours correlates with the effect on rat embryos at approximately the same developmental stage. Huber and Brown (1983) reported that following culture there is a complete lack of yolk sac circulation and separation of the endoderm and mesoderm cell layers. In the normal yolk sac these are closely associated, allowing the blood vessels to develop in between. Abnormalities were first visible as blebs and swellings after approximately 6 hours of culture in the rat embryo.

The depletion of PKC by proteolysis that occurs with sustained TPA exposure (Young *et al.*, 1987; Nishizuka, 1988) could be responsible for the adverse consequences of such treatment. The absence of teratogenic effects on embryos treated for one hour are probably due to the avoidance of PKC depletion. It is interesting to speculate why inositol supplementation of embryos in culture does not similarly cause yolk sac abnormalities (Section 4.2.2). This may be due to differing activation of PKC since the action of TPA is non-physiological as indicated by the tumour-promoting activity of

phorbol esters. It seems likely that stimulation of PKC following inositol supplementation occurs in a more "physiological" manner, not by binding and activating PKC directly, but by acting through the inositol/lipid cycle in which DAG is the principal PKC activator.

The complex action of TPA, involving initial activation followed by depletion during prolonged stimulation, potentially complicates interpretation of the *in vitro* data. However, the beneficial effect of short-term TPA treatment strongly suggests that PKC activation is crucial in preventing spinal NTD in *curly tail* embryos. Moreover, use of the PKC inhibitor GF109203X provides support for this idea. Addition of the inhibitor just prior to, and during, the TPA treatment reverses the protective effect of TPA on closure of the PNP. The high degree of specificity of the inhibitor GF109203X for PKC shows that the effect of TPA on closure of the PNP is due to activation of PKC rather than activation of another enzyme. The inhibitor studies also suggest that the mechanism of action of TPA involves activation of PKC rather than a secondary negative feedback caused by depletion of PKC as in conditions of sustained treatment. In the latter case, the inhibitor would be expected to mimic the effect since both inhibition and depletion reduce cellular PKC activity.

The results presented in this chapter demonstrate that activation of PKC by TPA treatment of *curly tail* embryos *in vitro* has a protective effect on closure of the PNP which like inositol supplementation would lead to a reduction in spinal NTD. This suggests a possible mechanism for the prevention of spinal NTD by inositol supplementation in which there is increased flux through the inositol/lipid cycle leading to activation of PKC.

The effect of short-term PKC inhibition on embryos treated with inositol suggests a reduction of the protective effect on closure of the PNP, since there is no longer a significant reduction in PNP length at the 30-31 somite stage compared with embryos cultured in serum alone which represents the *in vivo* situation (Section 3.2.1). This

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observation supports the proposed model in which inositol supplementation leads to activation of PKC. In this case the PKC inhibitor, GF109203X, appears to have an intermediate effect, in which the protective effect of inositol is not completely reversed, since the increase in PNP length following addition of inhibitor is not statistically significant. The lesser effect is probably due to the fact that inositol is present in the culture medium, and therefore potentially causing PKC activation, for the full 24 hour culture period while PKC is only inhibited for 1 hour 15 minutes. However, the possibility cannot be ruled out that part of the protective effect of inositol is mediated through a distinct pathway from PKC, for example, stimulation of PI 3-kinase (Section 1.5.2).

The link between PKC activity and correction of PNP closure in *curly tail* embryos remains to be determined, but presumably involves phosphorylation of one or more target proteins. There are a number of potentially important PKC substrates that are proteins with direct cellular activity or transcription factors that regulate expression of further downstream proteins. For example, activation of the transcription factor AP-2 following TPA exposure of embryos may explain the up-regulation in the yolk sac of transcription of the E-cadherin gene whose upstream promoter contains two AP-2 binding sites (Chen and Hales, 1994). In the *curly tail* system, one potential target is retinoic acid receptor-beta (RAR-β). The regulation of expression of this transcription factor by inositol and TPA treatment of embryos *in vitro* is examined in the following chapter.

CHAPTER 5

The preventive effect of inositol on posterior neuropore closure in the *curly tail* mouse:-

Expression of genes encoding retinoic acid receptors, cellular retinoid binding proteins and *Sonic hedgehog*

5.1 Introduction

Abnormal expression of the genes for retinoic acid receptors (RAR)-β and -γ has previously been implicated in the pathogenesis of *curly tail* defects (Chen *et al.*, 1995). Expression of RAR-β in the hindgut endoderm extends to the level of the most recently closed neural tube in non-mutant embryos and unaffected *curly tail* embryos. In contrast, expression in affected *curly tail* embryos is absent at the same axial level where the PNP is still open suggesting a correlation between the extent of RAR-β expression and closure of the PNP. RAR-γ expression is also reported to be abnormal, being down-regulated in the tail bud and open neuropore region (Chen *et al.*, 1995).

In support of a role for RARs in the development of NTD is the finding that treatment of *curly tail* embryos *in vivo* with low doses (5 mg/kg) of all-*trans* retinoic acid (RA) causes up-regulation of RAR-β in the hindgut endoderm and RAR-γ in the PNP region with a concomitant reduction in the incidence of spinal NTD (Chen *et al.*, 1994; Chen *et al.*, 1995). Therefore, it was proposed that normal proliferation of the hindgut endoderm depends on adequate expression of RAR-β and that this may be a primary defect in the *curly tail* embryo.

The abnormal expression of the transcription factors RAR-β and -γ in affected *curly tail* embryos could be accompanied by mis-regulation of genes whose expression is dependent on RAR expression. This could include downstream genes whose expression is influenced by the transcriptional regulatory activity of RAR-β or -γ. In addition, since the *curly tail* gene product directly or indirectly causes altered expression of the RARs, there may be altered levels or patterns of expression of upstream genes in the pathway of the *curly tail* defect. Similarly, affected *curly tail* embryos may also exhibit altered expression of genes that are regulated in a related manner to RAR-β or -γ.

Evidence suggests that both the expression and transcriptional regulatory activity of RARs can be modulated by the activity of protein kinase C (PKC) which raises the possibility that the protective effect of PKC activation on closure of the PNP in *curly tail*

embryos (demonstrated in Chapter 4) involves an interaction with RAR expression or function. RA treatment is known to modulate the activity of PKC in several cell types, ranging from tumour cell lines to rat splenocytes (Snoek *et al.,* 1986; Kurie *et al.,* 1993; Zorn and Sauro, 1995). For example, activation of PKC appears to play a key functional role in the induction of RAR-β expression which follows RA treatment of the human teratocarcinoma cell line, NT2/D1 (Simeone *et al.,* 1990; Kurie *et al.,* 1993). Treatment with RA stimulates the PKC pathway prior to up-regulation of RAR-β expression, causing increased levels of diacylglycerol (DAG), a physiological activator of PKC, and localisation of PKC (γ isoenzyme) to the cell membrane (Kurie *et al.,* 1993).

In addition to a role in regulation of RAR gene expression, PKC activity can also modulate the function of RARs in transcriptional regulation. Expression of a constitutively active PKC-γ significantly enhances the response to RA induced transcription from a reporter construct containing the RA-response element (RARE) region of the RAR-β promoter (Kurie *et al.,* 1993). In a complementary study, COS cells transfected with a reporter construct containing the RARE from the RAR-β promoter were used to test the requirement of RA-inducible transcription for PKC activity. Abrogation of PKC activity by specific inhibitors or depletion by prolonged TPA treatment reduced RA-dependent transcription from this promoter and could be correlated with loss of DNA-binding activity of RAR-α containing complexes (Tahayato *et al.,* 1993). The requirement of RA-dependent transcription for PKC activity was confirmed by simultaneous overexpression of PKCα which reversed the effect of depletion of cellular PKC.

In view of the observation that PKC activation may modulate the cellular effects of RA via expression and function of RARs, it is particularly interesting that treatment with either a PKC activator (TPA) or RA has a protective effect on spinal NTD in *curly tail* embryos which exhibit abnormal expression of RARs (Chapter 4, Chen *et al.,* 1994; Chen *et al.,* 1995). This raises the possibility that the prevention of NTD by RA and TPA involves related mechanisms, and similar alterations of gene expression may be

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induced by both agents in treated embryos. Furthermore, the protective effect of inositol supplementation demonstrated in Chapter 3 is potentially mediated through activation of PKC and therefore the action of inositol could also be related to that of RA. In this Chapter, therefore, I examine the expression of RARs and certain related genes in *curly tail* embryos with and without treatment using inositol and TPA *in vitro*.

One possible explanation for the abnormal expression of RAR-β and -γ in affected *curly tail* embryos is that there is a deficiency in the level of endogenous RA in the caudal region of the embryo. The most abundant isoforms at this developmental stage are RAR-β2 and RAR-γ2 (Lohnes *et al.*, 1993; Mendelsohn *et al.*, 1994), both of which are RA-inducible, presumably via the RAREs contained in their promoter regions (De Thé *et al.*, 1990; Noji *et al.*, 1991; Leid *et al.*, 1992). A local deficiency of endogenous RA might therefore be predicted to cause down-regulation of these RARs while addition of exogenous RA would lead to up-regulation as observed following RA treatment (Chen *et al.*, 1995). Abnormal expression or function of cellular retinol binding protein I, CRBP I, which is involved in the conversion of retinol to RA (Morriss-Kay, 1993) could cause local RA deficiency. Such an effect would potentially lead to the down-regulation of RAR-β, whose expression is induced by RA (see above), in affected *curly tail* embryos. I have therefore studied expression of CRBP I in *curly tail* embryos, following development both *in vivo* and *in vitro*, to investigate this possibility.

The functions of cellular retinoic acid binding proteins, CRABP I and II, remains unclear (Section 1.6.3) but may involve modulation of the cellular level of free RA and therefore spatiotemporal regulation of the availability of RA for binding to RARs (Boylan and Gudas, 1991; Morriss-Kay, 1993). For this reason I have examined the expression of CRABP I and II in *curly tail* embryos.

CRBP I and CRABP II, like RAR-β, are inducible by RA presumably via the RAREs present in their promoter regions (Smith *et al.*, 1991; Durand *et al.*, 1992; Leid *et al.*, 1992) suggesting that these genes may, like RAR-β, be down-regulated in affected *curly*

tail embryos and up-regulated following RA treatment. Moreover, coexpression of these three genes in several embryonic tissues suggests a link in the mechanism of transcriptional regulation (Ruberte *et al.*, 1991; Ruberte *et al.*, 1992).
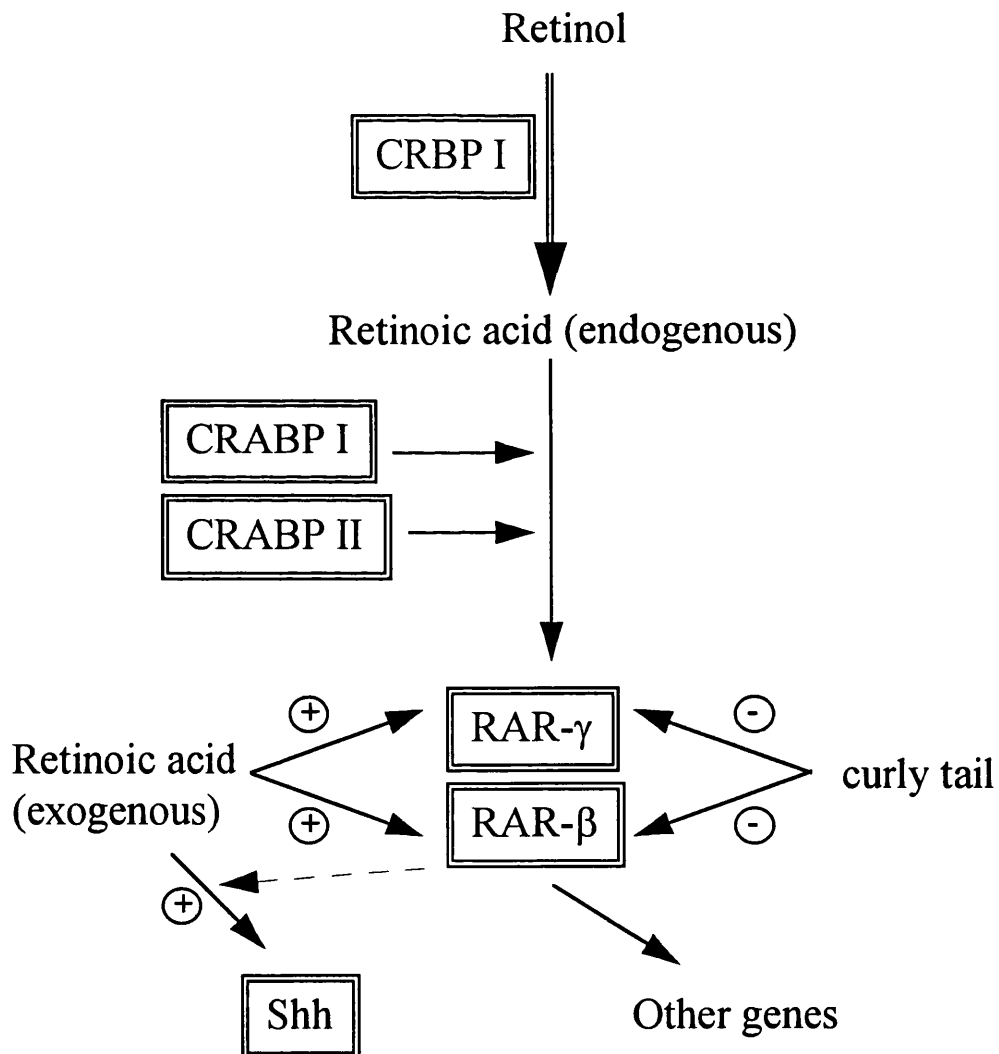
A model for development of spinal NTD in *curly tail* mice has been proposed in which down-regulation of expression of RAR-β and -γ causes altered transcription of target genes (Chen *et al.*, 1995). In order to elucidate the mechanism of development of NTD and their prevention by RA, it is necessary to identify downstream targets which mediate the effects of the RARs on closure of the PNP. One candidate for downstream regulation in this system is *Sonic hedgehog (Shh)*. *Shh* encodes a secreted protein that has been implicated in the induction of floor plate and motor neurons in the neural tube, induction of hindgut endoderm and patterning of the somites and limbs (Section 1.8). The domains and response of *Shh* expression to RA are suggestive of a potential role in the *curly tail* pathway. The sites of expression of *Shh* (Echelard *et al.*, 1993) include those tissues, the hindgut and notochord, whose proliferation deficiency is thought to cause the delay in PNP closure in affected *curly tail* embryos (Copp *et al.*, 1988a). *Shh* appears to play a role in the induction and patterning of the hindgut through induction of target genes including *Bmp-4* and *Hox* genes (Roberts *et al.*, 1995).

Interestingly, expression of *Shh*, like RAR-β, can be induced by retinoic acid. Implantation of a bead soaked in RA into the anterior margin of the limb bud induces digit duplications and ectopic expression of both RAR-β (Noji *et al.*, 1991) and *Shh* (Riddle *et al.*, 1993; Helms *et al.*, 1994). This observation raises the possibility that *Shh* expression may be up-regulated in *curly tail* embryos following treatment with RA, inositol or TPA, all of which cause up-regulation of RAR-β.

In this chapter expression of RA-related molecules (Figure 5.1) is compared between affected and unaffected *curly tail* embryos, and between embryos cultured in rat serum alone or with the addition of TPA or inositol. I demonstrate that *in vitro* treatment of *curly tail* embryos with either TPA or inositol causes the specific up-regulation of RAR-

β expression in the hindgut endoderm, although the other genes analysed are apparently unaffected.

Figure 5.1 Proposed relationships between RARs and related molecules to be examined in Chapter 5. Boxes indicate molecules whose expression is assessed by whole mount *in situ* hybridisation. Single lines indicate direct or indirect interactions, the effect of which is indicated where known. The dotted line indicates a potential interaction.

Retinol

CRBP I

Retinoic acid (endogenous)

CRABP I

CRABP II

Retinoic acid (exogenous)

RAR-γ

RAR-β

curly tail

Shh

Other genes

5.2 Results

Whole mount *in situ* hybridisation (Section 2.7) was used to analyse the expression of each gene of interest in embryos at approximately 10 days 6 hours of gestation (28-31 somites). In each section, expression was compared in affected and unaffected *curly tail* embryos and a non-mutant control strain, CBA/Ca. These embryos were obtained directly from litters *in vivo* and fixed immediately after harvesting. In addition, the expression was also compared in embryos following culture in whole serum, in serum containing 50 μg/ml inositol, or in serum with the addition of TPA (500 nM) for one hour following 17 hours of culture. These embryos were cultured from 9 days 6 hours of gestation in order to have reached the 28-31 somite stage at the end of the culture period (see Section 2.2). The number of embryos assessed for expression of each gene is indicated in Table 5.1.

5.2.1 RAR-β expression in *curly tail* embryos following development *in vivo* or *in vitro*

Expression of RAR-β is detected in the neural tube caudal to the otic vesicle and extends the length of the closed neural tube, although it is absent from the neural folds of the open PNP (Figure 5.2). In the head, expression is detected in the frontonasal mesenchyme and in a restricted domain in the mandibular part of the first branchial arch (indicated by arrow in Figure 5.2a). Staining is also detected in the splanchnopleuric mesoderm of the trunk (Figure 5.2a) and in proximal mesenchyme of the limb buds. Of particular interest, RAR-β expression is also present in the hindgut endoderm (arrows in 5.2b, c, e, f).

The overall level and pattern of expression is comparable between non-mutant CBA/Ca embryos, affected and unaffected *curly tail* embryos (Figure 5.2a, d, g) and also between embryos cultured in serum alone or treated with inositol or TPA (Figures 5.3a-c). However, detailed comparison of the isolated caudal regions reveals variation in the extent of the expression domain in the hindgut as described previously from a study using radioactive *in situ* hybridisation on embryo sections (Chen *et al.*, 1995).

Table 5.1 Numbers and treatments of embryos analysed by whole mount *in situ* hybridisation for expression of retinoic acid receptors, retinoid binding proteins and *Shh*

Number of embryos analysed (no. of experiments)

Probe	CBA/Ca	*ct, PNP 1/2*	*ct, PNP 4/5*	*ct, RS*	*ct, Ins*	*ct, TPA*
		Non-cultured embryos		*Cultured embryos*		
RAR-β	9 (2)	18 (4)	17 (4)	18 (3)	17 (3)	18 (3)
RAR-γ	5 (1)	8 (2)	9 (2)	17 (2)	11 (2)	9 (2)
CRABP I	9 (2)	14 (3)	13 (3)	10 (2)	12 (2)	11 (2)
CRABP II	10 (2)	14 (3)	13 (3)	10 (2)	11 (2)	11 (2)
CRBP I	8 (2)	17 (3)	18 (3)	10 (1)	10 (1)	10 (1)
Shh	20 (4)	11 (3)	10 (3)	5 (1)	7 (1)	8 (2)

Columns 2-4 refer to non-cultured embryos and columns 5-7 refer to embryos which were cultured for 24 hours prior to collection for whole mount *in situ* hybridisation. The *in vitro* treatment of cultured *curly tail* (*ct*) embryos is indicated: RS= rat serum, Ins= 50 μg/ml inositol, TPA= 500 nM TPA treatment for one hour after 17 hours of culture. Each group contained approximately equal numbers of embryos with 28-29 and 30-31 somites.

The number of experiments (in brackets) refers to the number of repetitions of whole mount *in situ* hybridisation.

Ventral views (Figure 5.2c, f, i) suggest that expression in the hindgut of CBA/Ca and unaffected *curly tail* embryos extends further caudally than in the hindgut underlying the open PNP of affected *curly tail* embryos. The variation in level of staining is quite subtle but is consistently observed suggesting that it represents a real difference between affected and unaffected *curly tail* embryos. The difference is also detectable, although less obvious, in side views (Figure 5.2b, e, h).

The most striking difference in expression between the groups of cultured embryos is, however, an up-regulation of RAR-β in the hindgut endoderm following culture of embryos with inositol or TPA when compared with embryos cultured in whole serum (Figures 5.3 and 5.4). Embryos cultured in whole serum exhibit expression in the hindgut endoderm which extends to, or just beyond, the level of the caudal limit of the hind limb bud (Figure 5.3d-f). RAR-β expression varies with PNP category as described previously for non-cultured embryos.

Following inositol or TPA treatment there is a distinct caudal extension of the expression domain into the hindgut of the PNP region. This increased expression is detected in both affected and unaffected *curly tail* embryos such that staining is observed in the hindgut under either open or closed neural folds (Figure 5.4d-g). The up-regulation of expression in the hindgut is most distinct when observed from the ventral surface (Figure 5.4a-c, f, g). Moreover, the intensity of staining is increased relative to embryos which were not cultured or cultured in whole serum, even those with small PNPs. A number of embryos were sectioned, confirming that the site of up-regulation of expression does in fact correspond to the hindgut endoderm (Figure 5.4h)

Figure 5.2 Expression of RAR-β in CBA/Ca and *curly tail* embryos

Expression of RAR-β as demonstrated by whole mount *in situ* hybridisation in (a, d, g) intact embryos and (b, c, e, f, h, i) isolated caudal regions (cleared in glycerol) shown as (b, e, h) a side view or (c, f, I) a view from the ventral surface. The expression pattern is compared in (a, b, c) CBA/Ca, (d, e, f) unaffected (PNP category 2) *curly tail* and (g, h, I) affected (PNP category 5) *curly tail* embryos. In this case all the embryos shown are at the 28 somite stage. Comparison of isolated caudal regions suggests that expression in ventral tissues caudal to the hind limb bud (short arrows in b and e) is greater in (b) CBA/Ca and (e) unaffected *curly tail* embryos compared with (h) affected *curly tail* embryos. This is supported by comparison of the ventral views in which expression in the hindgut (long arrows in c and f) extends more caudally in (c) CBA/Ca embryos and (f) *curly tail* embryos with small (category 1/2) neuropores than in (i) *curly tail* embryos with enlarged neuropores.

Arrow in a = staining in mandibular component of first branchial arch, FM= Frontonasal mesenchyme, F= Forelimb bud, H= hindlimb bud, S= splanchnopleuric mesoderm. The scale bar represents 1 mm in a, d, g, 0.5 mm in b, e, h and 0.4 mm in c, f, i.
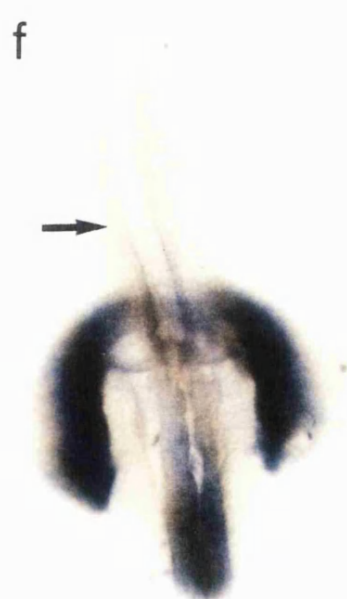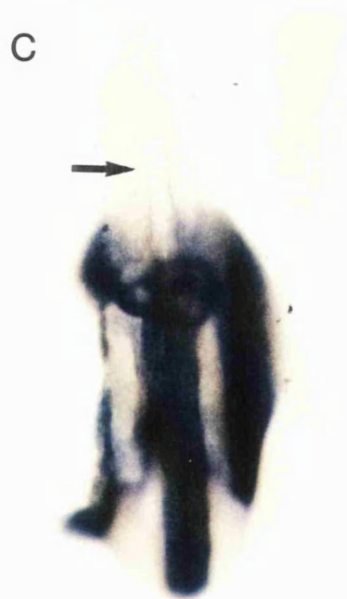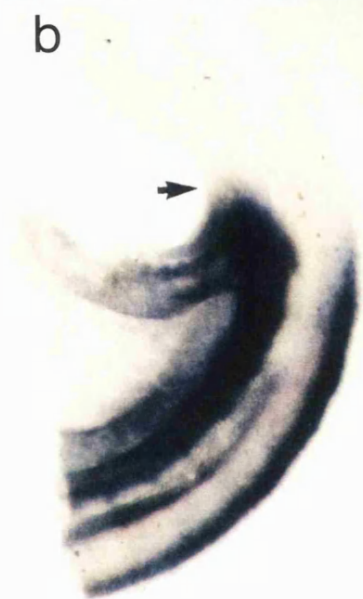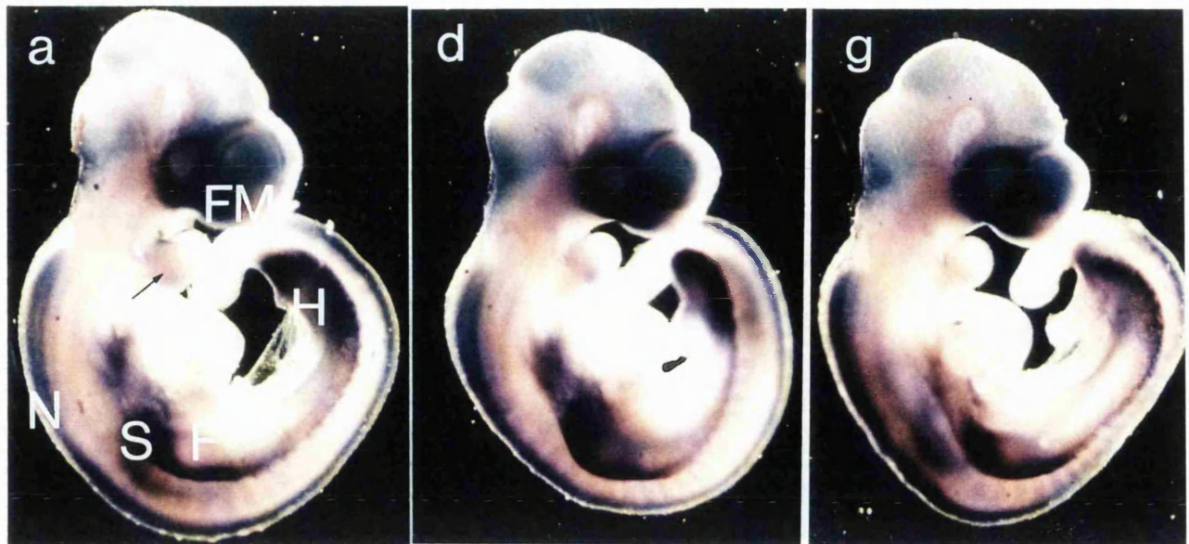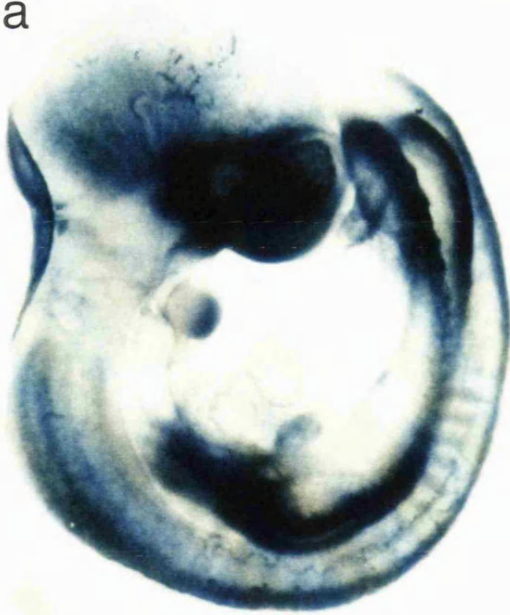
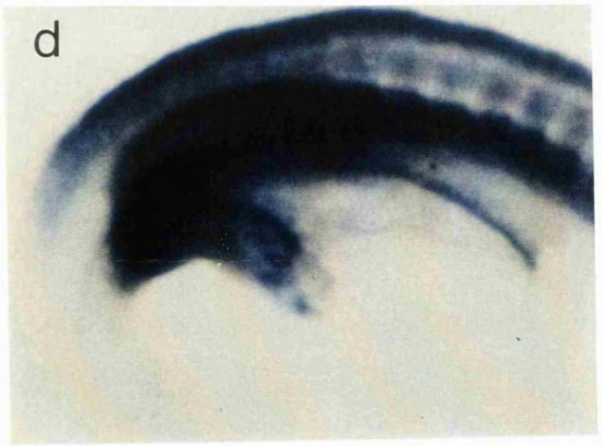**Figure 5.3 Expression of RAR-β in *curly tail* embryos following culture**

Expression of RAR-β as demonstrated by whole mount *in situ* hybridisation in (a-c) intact *curly tail* embryos and (d-f) isolated caudal regions following culture in **(a, d) rat serum**, serum supplemented with **(b, e) 50 μg/ml inositol** for the entire culture period or serum supplemented with **(c, f) 500 nM TPA** for one hour after 17 hours of culture. The expression in ventral tissues caudal to the hind limb bud extends further caudally in embryos treated with inositol or TPA (arrows in e and f).

The somite stage and PNP category of the embryos shown are as follows: (a) 30 somites, category 3, (b) 30 somites, category 2 and (c) 29 somites, category 3. The results were consistent within treatment groups for all the PNP categories and somite stages examined. For labelling of additional structures refer to Figures 5.2. The scale bar represents 0.8 mm in a-c and 0.5 mm in d-f.
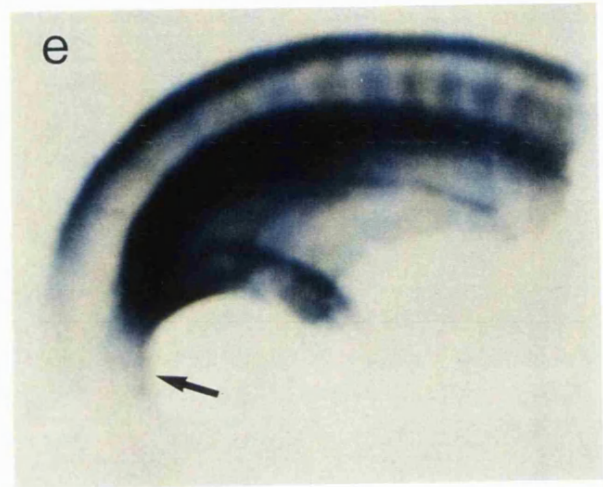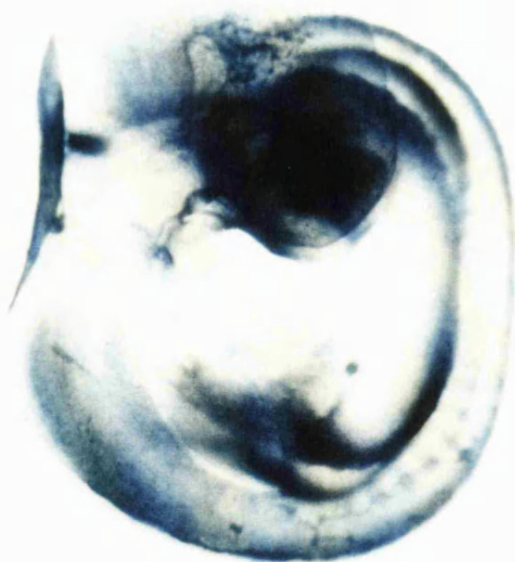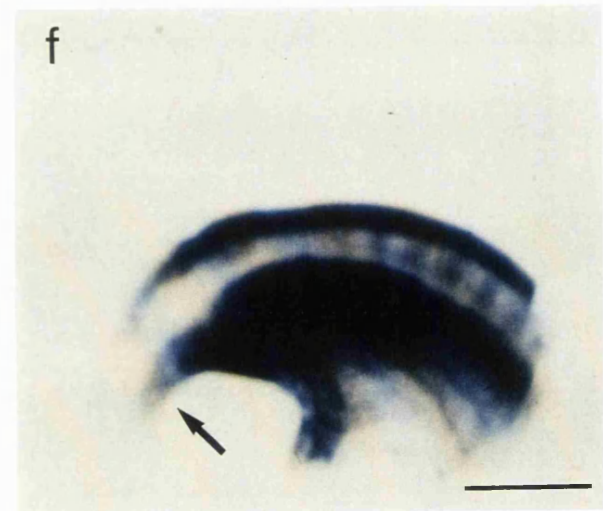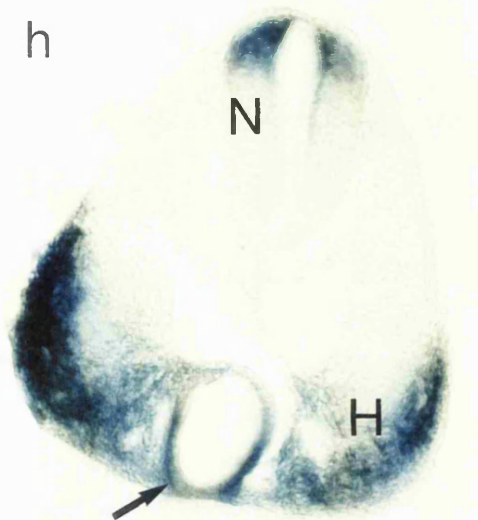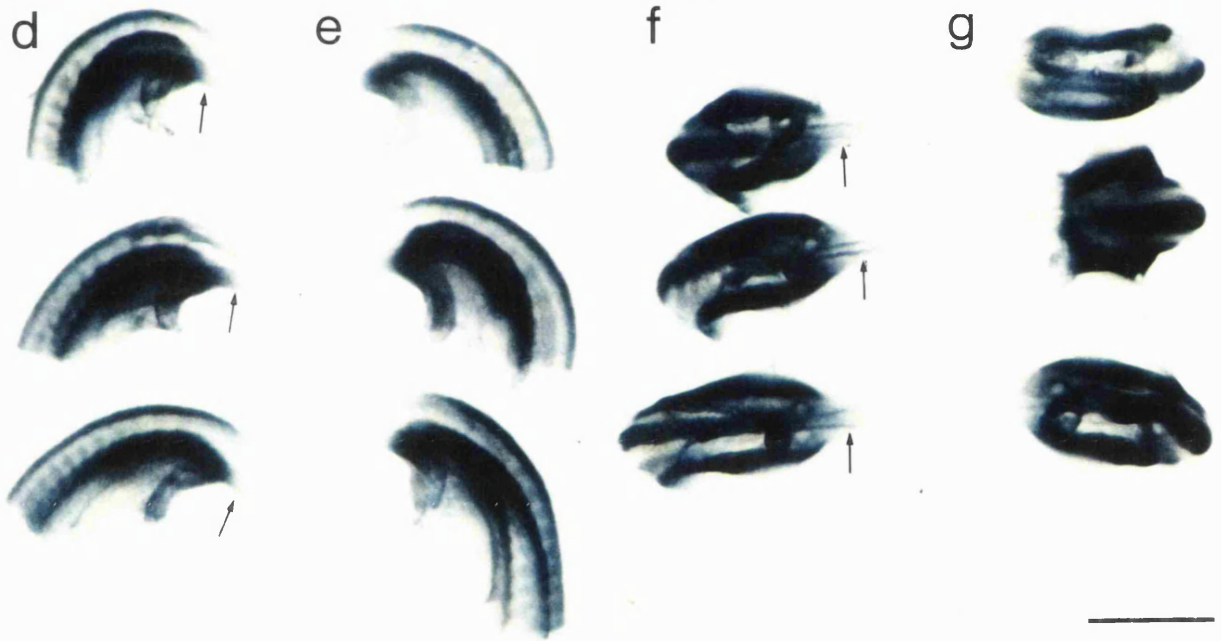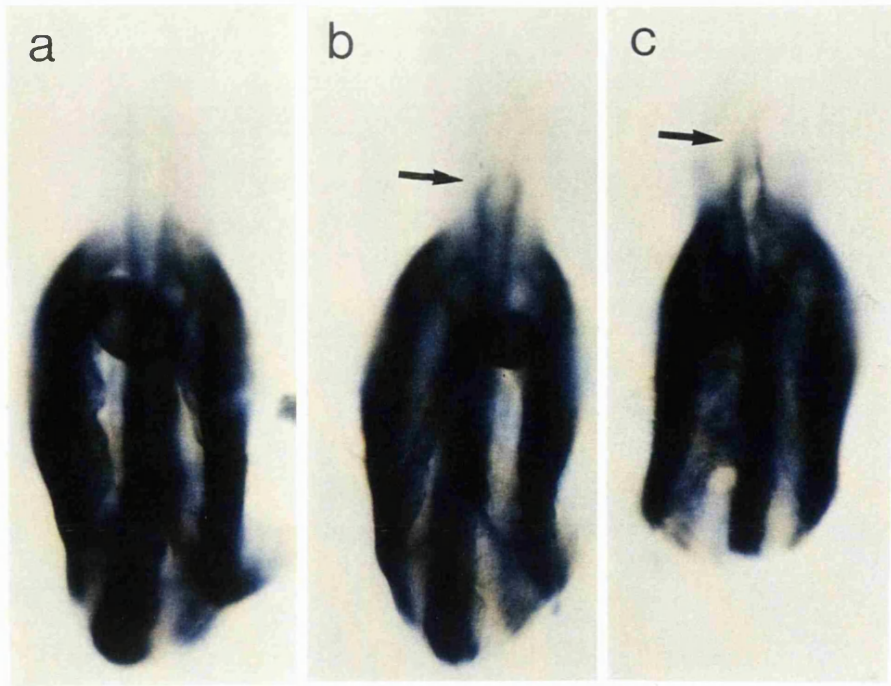
Figure 5.4 Expression of RAR-β in the caudal region of *curly tail* embryos following culture

Expression of RAR-β as demonstrated by whole mount *in situ* hybridisation in the caudal regions of embryos cultured in **(a, e, g) serum**, **(b, d, f)** serum supplemented with **50 µg/ml inositol** for the entire culture period or **(c)** serum supplemented with **500 nM TPA** for one hour after 17 hours of culture. The caudal regions of embryos in a-c correspond to those in Figure 5.9d-f.

Expression of RAR-β is up-regulated and extends further caudally in the hindgut of embryos exposed to inositol or TPA (arrows in b and c). Up-regulation of RAR-β expression following inositol treatment compared with culture in serum alone is observed in embryos of all PNP categories (arrows in d-g). The groups of caudal regions represent embryos with 30-31 somites and PNP categories 5, 3 and 2 (top to bottom in d-g) following culture in (e and g) serum or (d and f) serum supplemented with inositol. Similar results were obtained following TPA treatment (data not shown).

A 20 µm section through the caudal region (at the caudal end of the hind limb bud) of a TPA-treated embryo confirms that the site of up-regulation of expression is the hindgut endoderm (arrow in h). N= Neural tube, H= hind limb bud.

The scale bar represents 0.5 mm in a-c, 1.3 mm in d-g and 0.15 mm in h.

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5.2.2 RAR-γ expression

Strong expression of RAR-γ was detected in the frontonasal mesenchyme and first and second branchial arches. Both the fore- and hind-limb buds exhibit expression as does the lateral mesoderm of the trunk. In the caudal region RAR-γ expression is detected in the tail bud and in the ventral mesoderm caudal to the hind limb bud.

The level and sites of expression of RAR-γ are comparable in affected and unaffected *curly tail* embryos and control CBA/Ca embryos (Figure 5.5a-c). Detailed study of the caudal region revealed no apparent difference in the level of expression in the tail bud of affected and unaffected *curly tail* embryos (Figure 5.5d-f) in contrast to the previous report (Chen *et al.*, 1995). Moreover, there was no difference in expression in *curly tail* embryos following culture in serum compared with inositol or TPA treatment (Figure 5.6).

Figure 5.5 Expression of RAR-γ in CBA/Ca and *curly tail* embryos

Expression of RAR-γ as demonstrated by whole mount *in situ* hybridisation in (a-c)

intact embryos and (d-f) isolated caudal regions of **(a, d) CBA/Ca, (b,e) unaffected**

(PNP category 2) *curly tail* and **(c, f) affected (PNP category 5)** *curly tail* embryos.

Embryos are at the (a) 28 or (b, c) 29 somite stages.

TB= tail bud. For labelling of additional structures refer to Figures 5.2 and 5.7. The

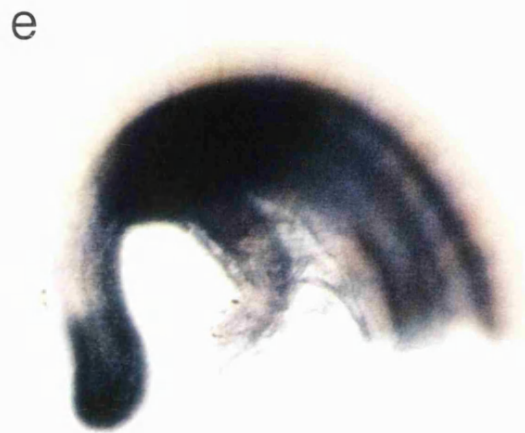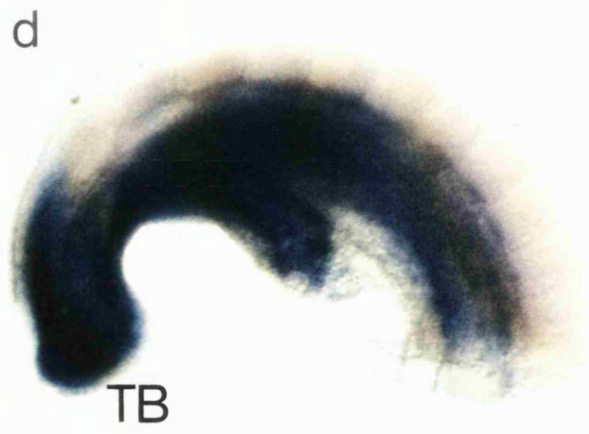scale bar represents 0.8 mm in a, 1.0 mm in b-c and 0.5 mm in d-f.

TB

Figure 5.6 Expression of RAR-γ in *curly tail* embryos following culture

Expression of RAR-γ as demonstrated by whole mount *in situ* hybridisation in (a-c) intact *curly tail* embryos and (d-f) isolated caudal regions following culture in **(a, d) rat serum, (b, e)** serum supplemented with **50 μg/ml inositol** for the entire culture period or **(c, f)** serum supplemented with **500 nM TPA** for one hour after 17 hours of culture . The somite stage and PNP category of the embryos shown are as follows: (a) 28 somites, category 3, (b) 30 somites, category 3 and (c) 29 somites, category 2. Results are consistent for all the embryos examined at the 28-31 somite stage and is unaffected by PNP category.

For labelling of additional structures refer to Figures 5.2 and 5.5. The scale bar represents 1.0 mm in a-c and 0.5 mm in d-f.
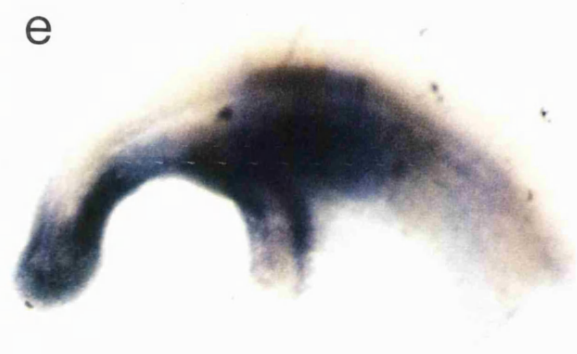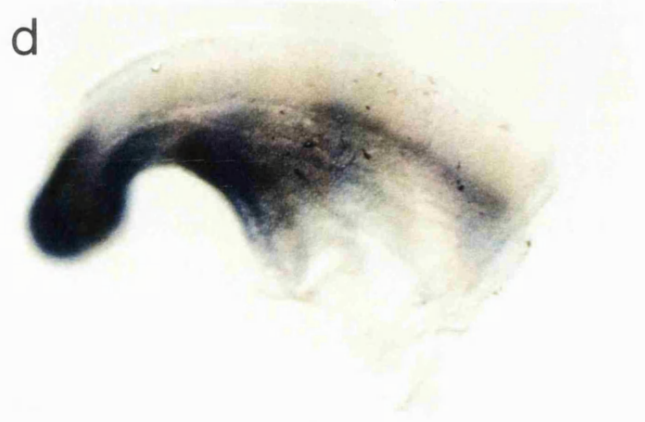
5.2.3 CRABP I expression

CRABP I expression is detected in a complex spatial pattern in embryos at the 28-31 somite stage (Figures 5.7 and 5.8). A network of CRABP I expression was observed in the brain extending from the caudal half of the diencephalon through the mesencephalon and into the rhombencephalon (Figure 5.7a). This expression correlates with that previously described in the outer layer of the mesencephalon (Ruberte *et al.*, 1992) and is proposed to correspond to developing neurons including the network formed by the axons of the mesencephalic nucleus of the trigeminal nerve (Lyn and Giguère, 1994).

As in previous studies (Lyn and Giguère, 1994; Leonard *et al.*, 1995) CRABP I expression was detected in several mesenchymal derivatives of the neural crest. Strong expression was observed in the frontonasal mesenchyme and first, second, third and fourth branchial arches. The staining in the first branchial arch was more intense in the mandibular component with a lower level of expression in the maxillary component. Streams of CRABP I expression, presumably corresponding to migrating neural crest cells, extend from the hindbrain neural tube towards the first, second and third branchial arches and towards the outflow tract of the heart (arch 4).

Immediately caudal to the hindbrain, expression is detected throughout the neural tube but in the trunk the pattern has a "speckled" appearance which extends caudally along the closed neural tube (Figure 5.7a and d). However, expression in the neural tube ceases cranial to the PNP although staining in ventral tissues extends caudal to the hind limb bud (Figure 5.7d). Expression was also detected throughout both fore- and hind-limb buds.

Comparison of the level and pattern of expression of CRABP I in whole embryos and in isolated caudal regions indicates no consistent variation between CBA/Ca embryos and affected and unaffected *curly tail* embryos (Figure 5.7). In addition, no differences could be detected between embryos following culture in whole serum or with inositol or TPA treatments (Figure 5.8).

Figure 5.7 Expression of CRABP I in CBA/Ca and *curly tail* embryos

Expression of CRABP I as demonstrated by whole mount *in situ* hybridisation in (a-c) intact embryos and in (d-f) isolated caudal regions of **(a, d) CBA/Ca , (b, e) unaffected (PNP category 1) *curly tail* and (c, f) affected (PNP category 4) *curly tail* embryos.** Embryos examined were at the 28-31 somite stage, the embryos shown having (b, c) 30 or (a) 31 somites.

D= diencephalon, M= mesencephalon, R= rhomboncephalon, FM= frontonasal mesenchyme, F= fore limb bud, H= hind limb bud, N= neural tube, V= ventral mesenchyme. Dashes in 5.7a indicate branchial arches 1-4 (arch 1 is most cranial). The scale bar represents 1.0 mm in a-c and 0.5 mm in d-f.
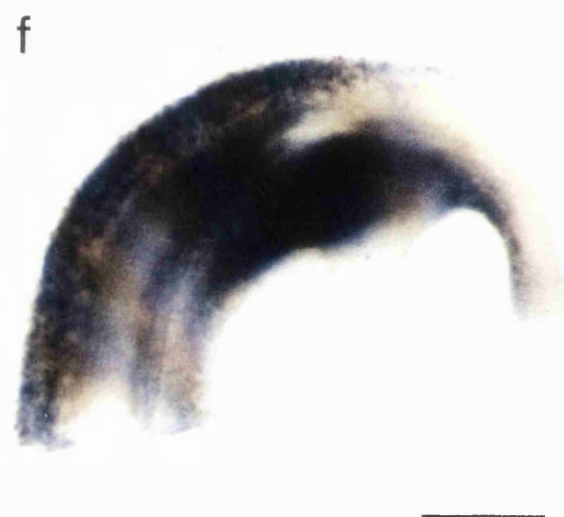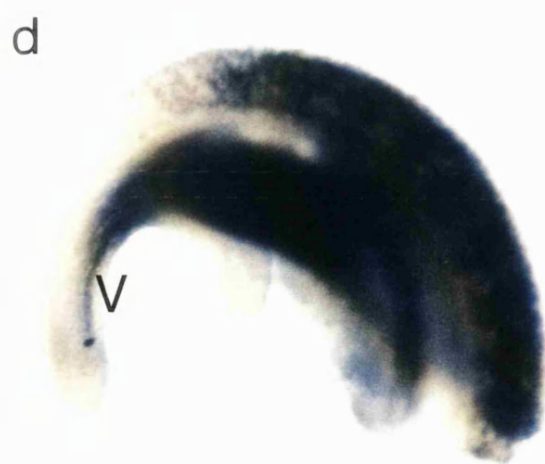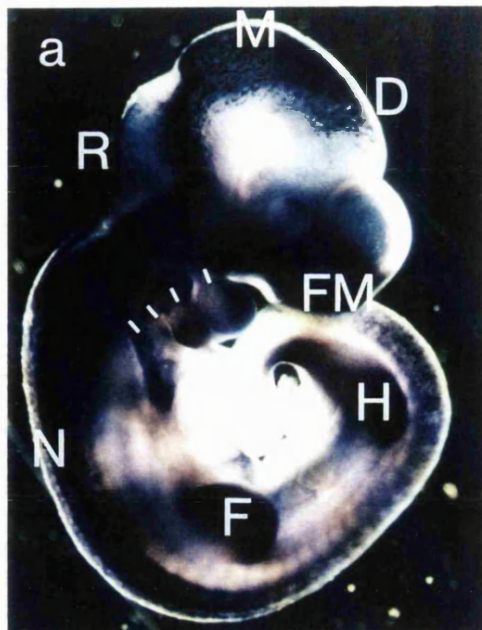
Figure 5.8 Expression of CRABP I in *curly tail* embryos following culture

Expression of CRABP I as demonstrated by whole mount *in situ* hybridisation in (a-c) intact *curly tail* embryos and in (d-f) isolated caudal regions following culture in **(a, d) rat serum, (b, e)** serum supplemented with **50 µg/ml inositol** for the entire culture period or **(c, f)** serum supplemented with **500 nM TPA** for one hour after 17 hours of culture. The embryos shown are at the (a, c) 30 and (b) 31 somite stages with PNPs designated as category (a) 4 or (b, c) 3. Results were consistent for the other PNP categories (not shown).

For labels refer to Figure 5.7. The scale bar represents 0.8 mm in a-c and 0.5 mm in d-f.
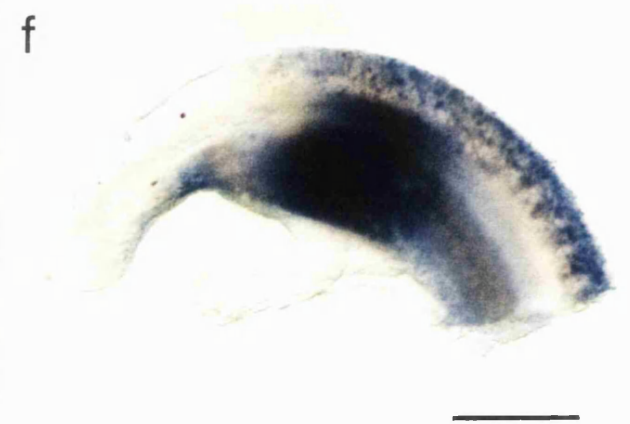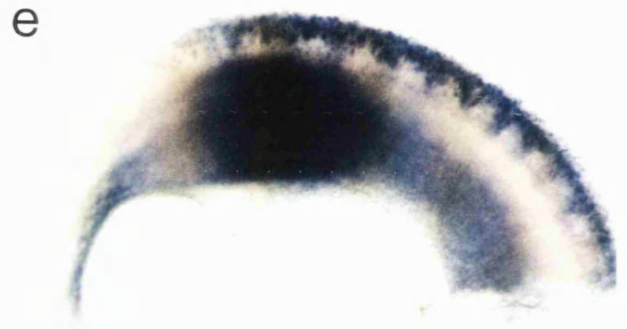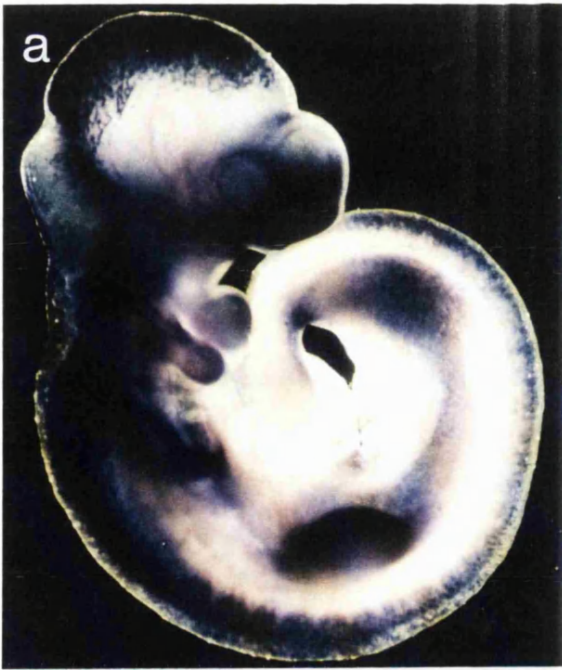
5.2.4 CRABP II expression

In the brain CRABP II expression is strong in the telencephalon and mesencephalon but weak or absent in the diencephalon (Figures 5.9 and 5.10). There is also strong staining in the fronto-nasal mesenchyme, the mandibular component of the first branchial arch and the second branchial arch. In the neural tube expression extends from the hindbrain down the length of the neural tube, and includes the open neural folds of the PNP (Figure 5.9 and 5.10).

The lateral mesenchyme of the trunk, tail bud mesenchyme and ventral mesenchyme caudal to the hind limb bud are also sites of expression. Expression of CRABP II is observed in the limb buds and appears stronger in the dorsal region than the ventral region. This pattern of expression correlates with that described in previous reports (Ruberte *et al.*, 1992; Lyn and Giguère, 1994).

Analysis of whole embryos or isolated caudal regions indicates that CRABP II expression is comparable in CBA/Ca, affected and unaffected *curly tail* embryos in terms of both the level and sites of expression (Figure 5.9). Similarly, there was no difference in expression in *curly tail* embryos following culture in serum or with inositol or TPA treatments (Figure 5.10).

Figure 5.9 Expression of CRABP II in CBA/Ca and *curly tail* embryos

Expression of CRABP II as demonstrated by whole mount *in situ* hybridisation in (a-c) intact embryos and (d-f) isolated caudal regions of **(a, d) CBA/Ca, (b, e) unaffected (PNP category 1)** *curly tail* and **(c, f) affected (PNP category 4)** *curly tail* embryos. Embryos shown have (a) 32 or (b, c) 30 somites.

T= telencephalon, TB= tail bud. For labelling of additional structures refer to Figure 5.7. The scale bar represents 1.0 mm in a-c and 0.5 mm in d-f.
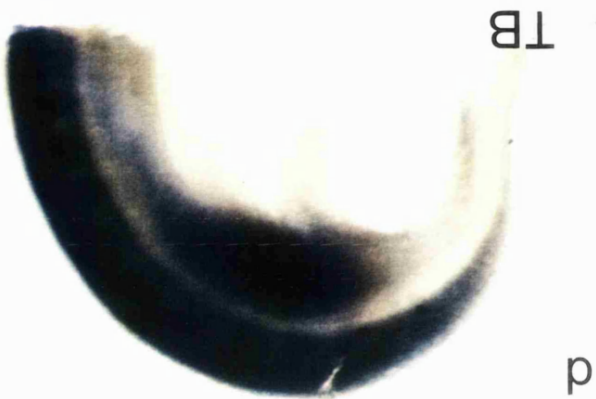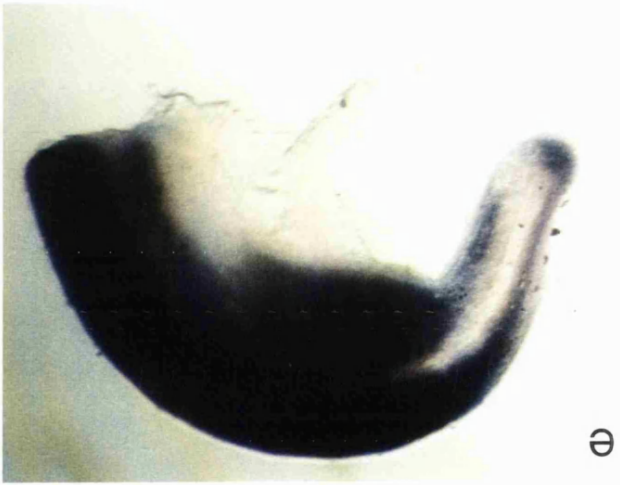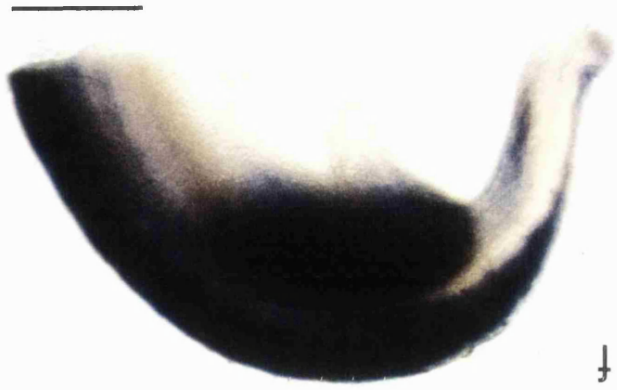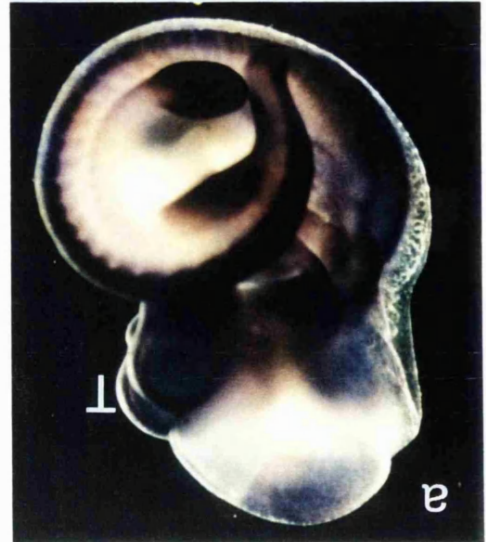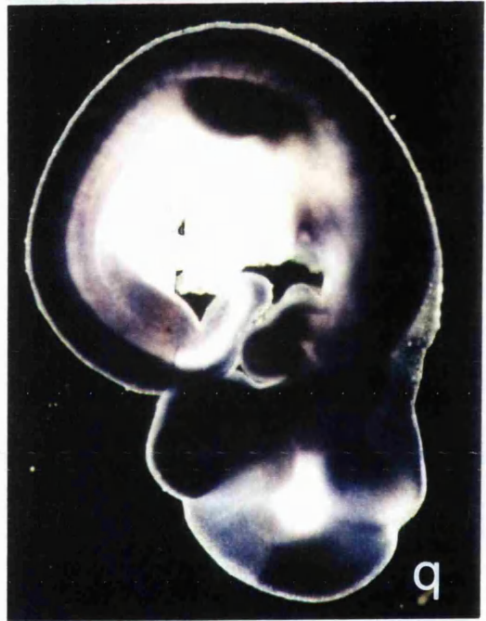
Figure 5.10 Expression of CRABP II in *curly tail* embryos following culture

Expression of CRABP II as demonstrated by whole mount *in situ* hybridisation in (a-c) intact *curly tail* embryos and (d-f) isolated caudal regions following culture in (**a, d) rat serum, (b, e)** serum supplemented with **50 μg/ml inositol** for the entire culture period or (**c, f)** serum supplemented with **500 nM TPA** for one hour after 17 hours of culture. In this case the embryos have (b) 29 or (a, c) 30 somites and PNPs of category (a, c) 1 or (b) 4. Results were consistent for embryos of all PNP categories in each treatment group.

T= telencephalon. For labelling of additional structures refer to Figures 5.7 and 5.9. The scale bar represents 1.0 mm in a-b, 0.8 mm in c and 0.5 mm in d-f.
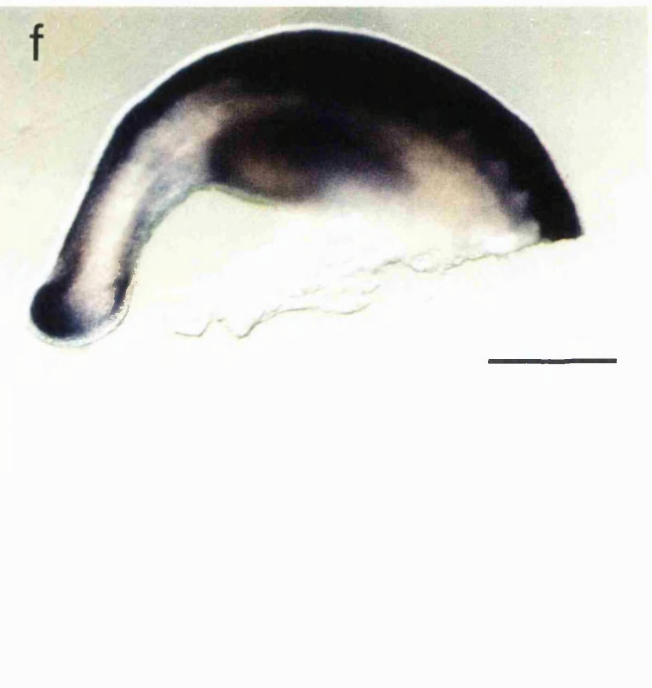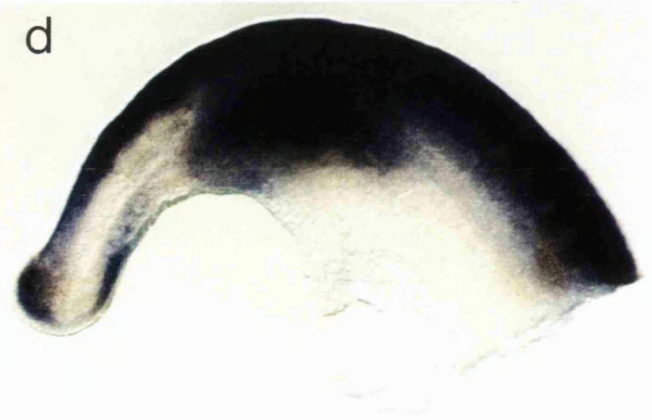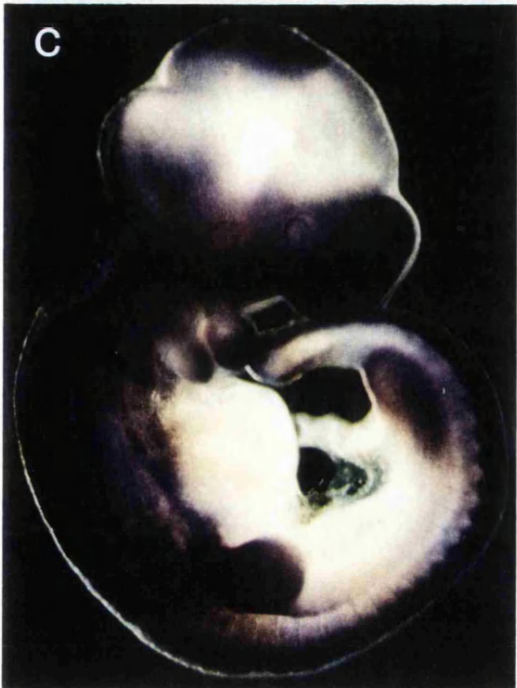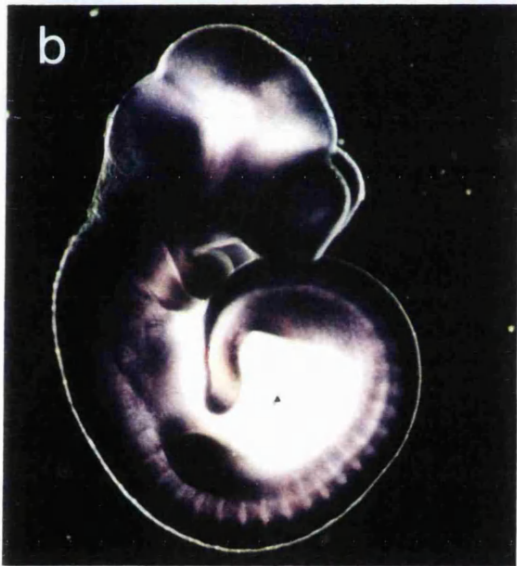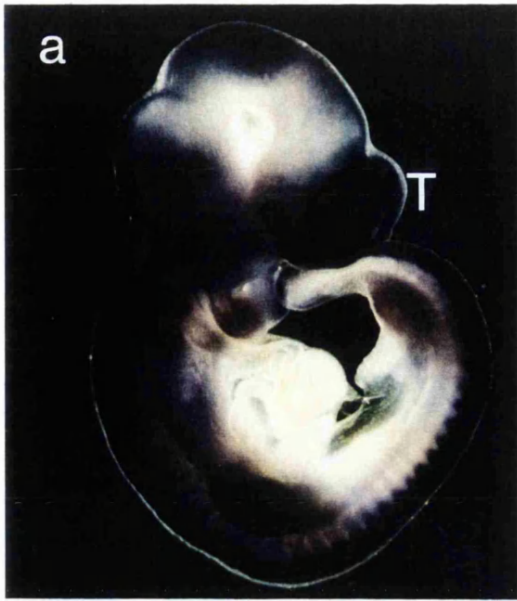
5.2.5 CRBP I expression

Expression of CRBP I can be detected in the fronto-nasal mesenchyme and the first and second branchial arches. Strong expression is present in the otic vesicle correlating with previously reported expression in the basal side of the otic pit epithelium (Ruberte *et al.*, 1991). CRBP I staining is also detected in the lateral mesenchyme in the trunk, the mesenchyme adjacent to the heart and in the tail bud (Figures 5.11 and 5.12).

No differences in CRBP I expression were apparent in comparisons of affected and unaffected *curly tail* embryos at stages from 28-31 somites. However, although the overall pattern and level of expression was similar, in a subset of non-mutant CBA/Ca embryos the staining appeared relatively weaker in the first branchial arch than in *curly tail* embryos at a comparable stage (Figure 5.11). This reduced expression was not observed in all the embryos, however, and seems likely to be an artefact of the whole mount *in situ* hybridisation technique. Inositol and TPA treatment of *curly tail* embryos in culture had no apparent effect on CRBP I expression compared to culture in whole serum (Figure 5.12). In this study particular attention was paid to the level of expression in the tail bud since this could potentially influence the availability of RA in the PNP region. However, no consistent differences were observed between any of the experimental groups (Figure 5.11 d-f, Figure 5.12 d-f).

Figure 5.11 Expression of CRBP I in CBA/Ca and *curly tail* embryos

Expression of CRBP I as demonstrated by whole mount *in situ* hybridisation in (a-c) intact embryos and (d-f) isolated caudal regions of **(a, d) CBA/Ca, (b, e) unaffected (PNP category 1)** *curly tail* and **(c, f) affected (PNP category 4)** *curly tail* embryos. The embryos shown have (b, c) 30 or (a) 31 somites and results were consistent for embryos examined between the 28 and 31 somite stages.

O= otic vesicle, L= lateral mesoderm. For labelling of additional structures refer to Figure 5.7. The scale bar represents 1.0 mm in a-c and 0.5 mm in d-f.

Figure 5.12 Expression of CRBP I in *curly tail* embryos following culture

Expression of CRBP I as demonstrated by whole mount *in situ* hybridisation in (a-c) intact *curly tail* embryos and (d-f) isolated caudal regions following culture in **(a, d) rat serum, (b, e)** serum supplemented with **50 μg/ml inositol** for the entire culture period or **(c, f)** serum supplemented with **500 nM TPA** for one hour after 17 hours of culture. The somite stage and PNP category of the embryos shown are as follows: (a) 30 somites, category 2, (b) 31 somites, category 5 and (c) 28 somites, category 3. The results were consistent between treatment groups for all the PNP categories and somite stages examined.

For labelling of additional structures refer to Figures 5.7 and 5.11. The scale bar represents 1.0 mm in a-c and 0.5 mm in d-f.
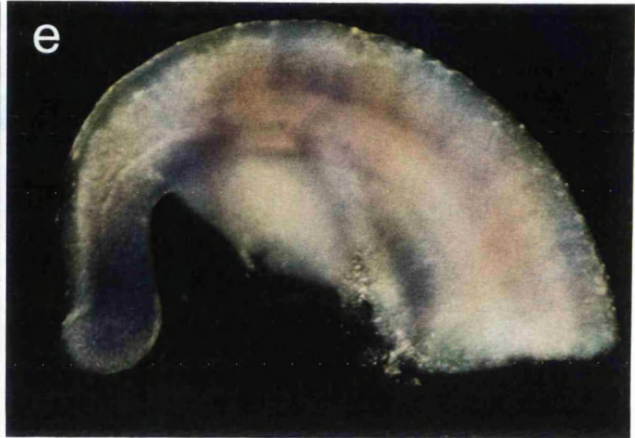
5.2.6 *Sonic hedgehog* expression

At these stages of development (27-31 somites) a distinct domain of *Shh* expression was observed from the hindbrain caudally down the length of the notochord and ventral neural tube (Figures 5.13 and 5.14). Expression also extends rostrally into the midbrain and forebrain. These patterns correlate with previous reports which describe *Shh* expression in the notochord and ventral midline of the neural tube (Echelard *et al.*, 1993). The results are consistent with previous reports that expression in the diencephalon is restricted from the ventral midline and occurs in two ventrolateral stripes (Echelard *et al.*, 1993), and this was confirmed by sectioning embryos. The fore-limb buds showed a patch of expression at the posterior margin which presumably corresponds to the zone of polarising activity (ZPA, arrow in Figure 5.13a). Strong expression was also visible in both the fore-gut and hind-gut (Figure 5.13 and 5.14).

Comparison of *Shh* expression in CBA/Ca, affected and unaffected *curly tail* embryos revealed no differences in either the level or sites of expression in either whole embryos (Figure 5.13a-c) or isolated caudal regions (Figure 5.13d-f). Similarly, there was no difference in expression in *curly tail* embryos following culture in serum or with inositol or TPA treatment (Figure 5.14a-f).

Figure 5.13 Expression of *Shh* in CBA/Ca and *curly tail* embryos

Expression of *Shh* as demonstrated by whole mount *in situ* hybridisation in (a-c)

intact embryos and (d-f) isolated caudal regions of **(a, d) CBA/Ca, (b, e) unaffected**

(PNP category 2) *curly tail* and **(c, f) affected (PNP category 4)** *curly tail* embryos.

All the examples shown are at the 30 somite stage.

N= notochord (and floor plate of the neural tube), G= hindgut. Thick arrow in (a)

indicates expression in the ZPA of the fore limb bud. For labelling of additional

structures refer to Figure 5.7. The scale bar represents 0.9 mm in a-c and 0.5 mm in
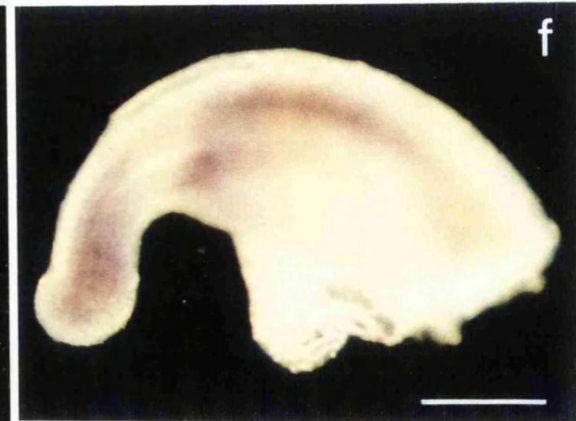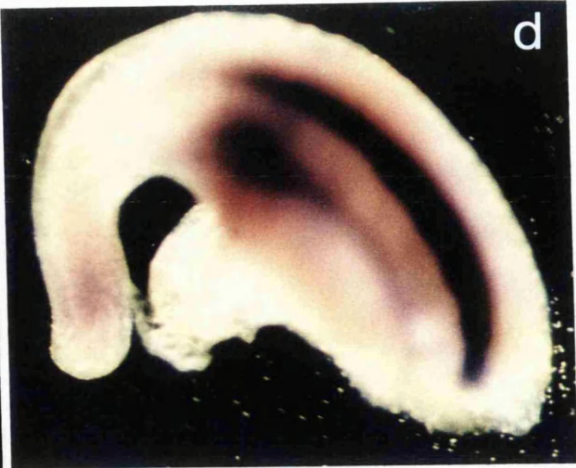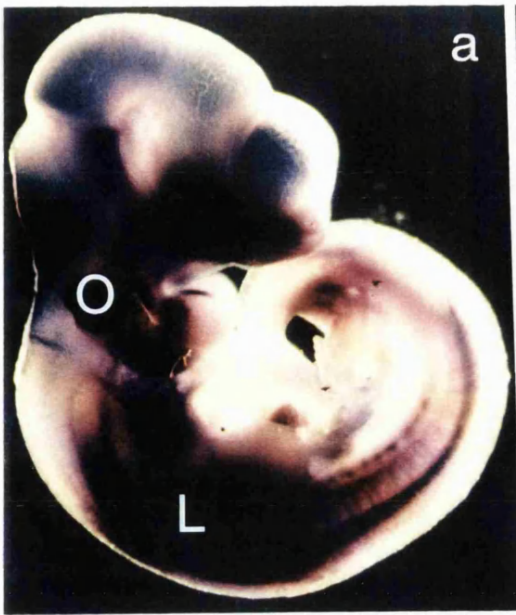
d-f.
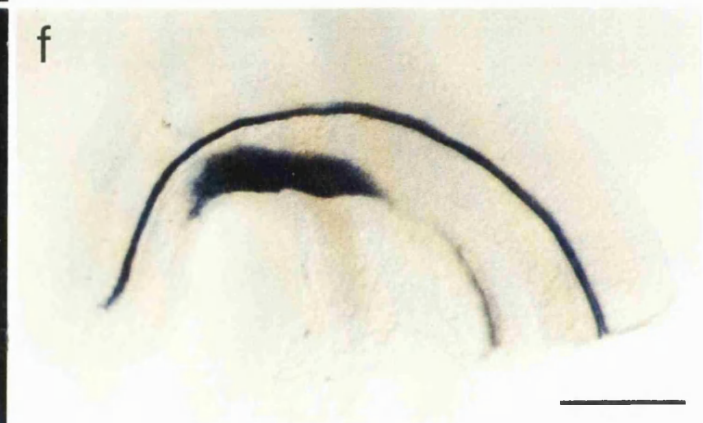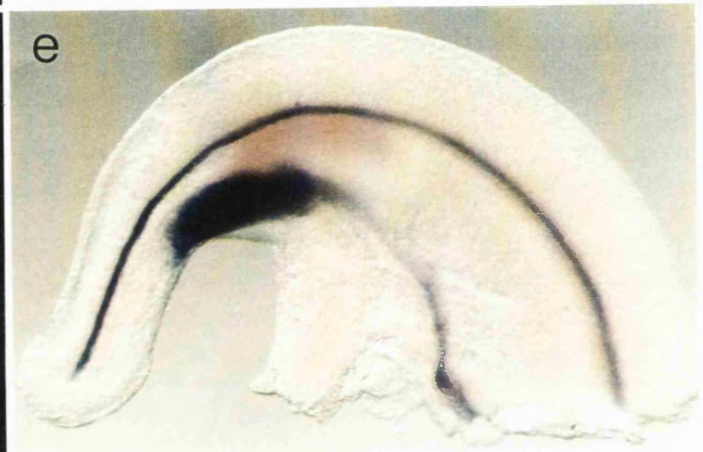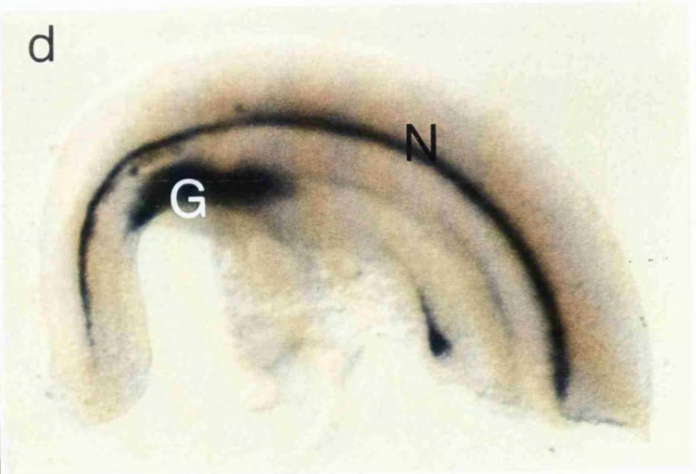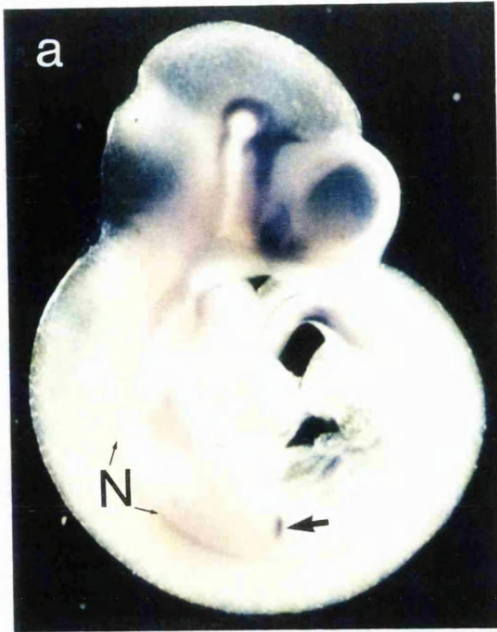
Figure 5.14 Expression of *Shh* in *curly tail* embryos following culture

Expression of *Shh* as demonstrated by whole mount *in situ* hybridisation in (a-c) intact *curly tail* embryos and (d-f) isolated caudal regions following culture in (**a, d**) **rat serum**, (**b, e**) serum supplemented with **50 μg/ml inositol** for the entire culture period or (**c, f**) serum supplemented with **500 nM TPA** for one hour after 17 hours of culture. Somite stages and PNP categories are: (a) 30 somites, category 4, (b) 30 somites, category 2 and (c) 31 somites, category 1.

For labelling of additional structures refer to Figures 5.7 and 5.13. The scale bar represents 0.9 mm in 5.13a, 1.0 mm in b-c and 0.5 mm in d-f.

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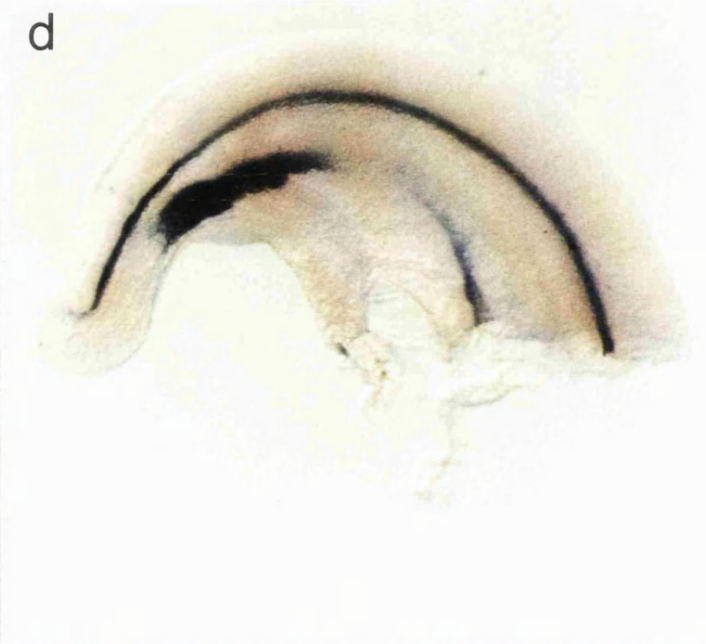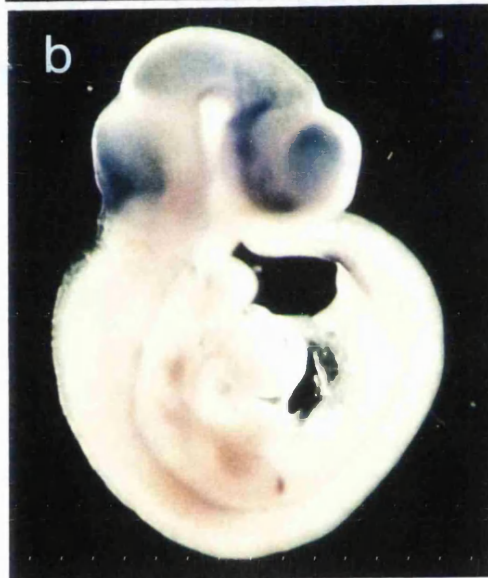
5.3 Discussion

Analysis of RAR-β expression reveals striking differences, both in quantity and spatial distribution, between affected and unaffected *curly tail* embryos and following culture with inositol or TPA compared with serum alone. In affected embryos, compared with unaffected *curly tail* or non-mutant CBA/Ca embryos, expression appears to be specifically down-regulated in the hindgut endoderm caudal to the hind limb bud. This observation corresponds with the study of Chen et al. (1995) which utilised radioactive *in situ* hybridisation. This previous report proposed that reduced expression of RAR-β may be a causal factor underlying the development of NTD (Section 1.6).

In addition to a difference between phenotypes of non-cultured embryos there is a striking up-regulation and caudal extension of RAR-β expression in the hindgut following culture in the presence of inositol (50 μg/ml) or TPA (500 nM). Embryos cultured in the presence of inositol or TPA exhibit a caudal extension of the RAR-β expression domain into the hindgut endoderm. In addition, the greater intensity of staining at this hindgut location, in embryos of all PNP categories, indicates that the level of expression is increased relative to the *in vivo* situation, even when compared with unaffected embryos. The up-regulation of RAR-β is particularly striking given that the hindgut is known to be the defective tissue in affected *curly tail* embryos and that up-regulation occurs following treatment with agents (RA, inositol and TPA) which are known to be protective against spinal NTD.

In this study, the effect of inositol or TPA treatment on RAR-β expression, concomitant with a protective effect on closure of the PNP, closely resembles the effect of low dose (5 mg/kg) RA (Chen *et al.*, 1995). Interestingly, of the genes analysed in this study, the correlation between the effects of RA and inositol/TPA appears to be restricted to regulation of RAR-β expression since no other alterations in gene expression were observed following inositol/TPA treatment (see below).

The up-regulation of RAR-β expression in the hindgut following RA or inositol/TPA treatment may be a functional step in the pathway leading to prevention of spinal NTD. An alternative explanation is that the protective effect of RA or inositol/TPA treatment is mediated through a distinct mechanism and that RAR-β is a secondary consequence of correction of the defect. At present it is not possible to distinguish between these possibilities although the likelihood that the RA effect is mediated through direct binding to RARs strongly suggests a functional role of RAR-β. One approach might be to breed the *curly tail* mutation onto an RAR-β null background; if RA or inositol treatment no longer reduced the delay in PNP closure this would suggest a role for RAR-β in the mechanism of the protective effect.

The putative protective role of RAR-β up-regulation in the hindgut endoderm would be predicted to be mediated via correction of the proliferation defect which has previously been demonstrated in this tissue in affected *curly tail* embryos (Copp *et al.*, 1988a). A mechanism involving an effect of RAR-β on cell proliferation appears plausible since RA is known to influence both growth and differentiation of a variety of cell types. For example, low concentrations of RA stimulates growth and RAR-β expression in embryonic stem cells (Pijnappel *et al.*, 1993) while high concentrations inhibit growth. Stimulation of proliferation in the hindgut of *curly tail* embryos could alleviate the growth imbalance between dorsal and ventral tissues and therefore reduce the ventral curvature of the caudal region that leads to delayed PNP closure (Figure 1.2).

The expression patterns of a number of genes, which may be functionally related to RA (as indicated in Figure 5.1), reveals comparable patterns and levels of expression in affected and unaffected *curly tail* and non-mutant CBA/Ca embryos. The absence of any detectable differences in the expression of CRABP I, CRABP II, CRBP I, RAR-γ and *Shh* suggests that abnormal expression of these genes does not play a role in the mechanism of the *curly tail* defect. Moreover, no differences in gene expression were observed between cultured and non-cultured embryos, the only exception being the up-regulation of RAR-β in the hindgut following treatment with inositol or TPA. The

consistent results obtained for embryos which have developed *in vitro* and *in vivo* is a further validation of the embryo culture technique as a means of modelling the *in vivo* situation.

The absence of any apparent difference in expression of CRBP I in affected compared with unaffected *curly tail* embryos suggests that diminished production of RA does not underlie the defects in affected *curly tail* embryos. This was previously considered as a possible cause of the reduction in RAR-β expression in the caudal region of affected embryos. However, direct assay of RA levels by chromatographic methods (Horton and Maden, 1995) or the use of RA-indicator cell lines (Colbert *et al.*, 1993) will be required to confirm this proposition.

In contrast to the previous observation of reduced RAR-γ expression in the PNP and tail bud of affected *curly tail* embryos (Chen *et al.*, 1995), no differences were observed between affected and unaffected embryos in this study. The discrepancy between these results may relate to the different techniques used: whole mount *in situ* hybridisation in this study compared with radioactive *in situ* hybridisation on sections in the former work. It is possible that radioactive *in situ* hybridisation is more sensitive in terms of quantitative detection of the level of expression. Comparison of expression levels by *in situ* hybridisation requires stringent control to ensure that conditions are uniform; certainly when using the whole-mount approach it is difficult to detect subtle changes in expression level if the spatial expression pattern is unaltered. In addition, the previously detected down-regulation of RAR-γ expression in the PNP region was not as dramatic as the difference in RAR-β expression, requiring computerised image analysis for comparison of expression levels, particularly in discrimination of non-mutant and unaffected *curly tail* embryos (Chen *et al.*, 1995). It remains to be determined, therefore, whether RAR-γ plays a significant role in the development of *curly tail* spinal NTD.

In previous studies, RA treatment of embryos *in vivo* has been shown to influence not only the expression of RAR-β, but also expression of RAR-γ, CRABP I and CRABP II (Lyn and Giguère, 1994; Chen *et al.*, 1995; Leonard *et al.*, 1995). The absence of any detectable alteration in RAR-γ expression following inositol/TPA treatment is perhaps not surprising given that no difference was observed between affected and unaffected *curly tail* embryos in this study. Moreover, 5 mg/kg RA treatment causes only partial correction of RAR-γ expression with both up- and down-regulation at varying cranio-caudal levels of the PNP region (Chen *et al.*, 1995).

In vivo exposure of non-mutant CD-1 embryos at 8.5 days or 9.5 days of gestation to a teratogenic dose of RA (50 mg/kg) causes up-regulation of CRABP II with no spatial alteration in the expression domain (Lyn and Giguère, 1994). Maternal administration of RA has also been reported to cause up-regulation and anteriorisation of expression of CRABP I (Leonard *et al.*, 1995) although no effect was reported in a separate study (Lyn and Giguère, 1994). This difference may reflect a slow response of CRABP I transcriptional activation to RA since embryos were collected six hours after RA treatment in the first study compared with 8-48 hours in the latter.

In this study, the absence of up-regulation of CRABP I and CRABP II following inositol or TPA treatment suggests that these agents do not mimic RA function *per se* but rather have a similar influence on the hindgut specifically. An alternative, although not exclusive, explanation might be that low-dose RA (5 mg/kg) is not sufficient to affect CRABP I or CRABP II expression to the extent observed following teratogenic doses of 25 or 50 mg/kg RA respectively. The expression of CRABP II is, after all, only slightly increased by a 50 mg/kg RA dose (Lyn and Giguère, 1994). Therefore, the inositol/TPA treatment which mimics the effect of low dose RA on RAR-β expression may also be insufficient to influence CRABP I or CRABP II expression. The absence of an effect of inositol or TPA treatment in culture on CRBP I expression is consistent with the observation that although RAR-β expression is increased in the hindgut there is not a

general effect on other genes, such as CRABP II, whose promoter regions contain RAREs. Moreover, these observations suggest that the mechanism of action of inositol and TPA does not involve the stimulation of synthesis of RA. Therefore, it appears most likely that the protective effects of inositol/TPA and RA on the *curly tail* defect are mediated either via up-regulation of RAR-β or through an alternative mechanism in which RAR-β is up-regulated as a downstream effect (Figure 8.1).

CHAPTER 6

Prevention of spinal neural tube defects in the *curly tail* mouse by *in vivo* inositol supplementation

6.1 Introduction

The experiments described in Chapter 3 demonstrate that inositol supplementation of *curly tail* embryos in whole embryo culture has a protective effect on closure of the PNP, as indicated by a reduction in PNP length of embryos at the 30-31 somite stage following culture. This is proposed to correspond to a reduction in the incidence of spinal NTD since spina bifida and tail flexion defects are caused by failure and delay of closure of the PNP respectively (Copp, 1985). In the previous studies, embryo culture was used in order to facilitate analysis of the mechanism of action of inositol.

In this chapter, the effect of *in vivo* inositol supplementation was tested in order to determine whether the effect of inositol on closure of the PNP *in vitro* is replicated by a corresponding alteration in the frequency of NTD. An advantage of maternal administration of exogenous agents is that embryos continue to develop *in vivo* and can thus be analysed at any later stage of development. This contrasts with *in vitro* treatment in which embryos must be analysed at the end of a defined culture period, typically 24-48 hours. Therefore, using *in vivo* studies the phenotype can be assessed at a stage when completion of neural tube closure has occurred normally in all embryos except those with spina bifida and tail flexion abnormalities. Moreover, allowing embryos to continue to develop *in vivo* should reveal any deleterious effect of inositol supplementation which may not become apparent after 24 hours in culture.

One of the requirements for a primary preventive measure for NTD is that it should be applicable to use *in vivo*. Although embryo culture is extremely useful for the analysis of the effect of exogenous agents, and the mechanism of their action, maternal effects must also be considered. In certain cases there does appear to be a discrepancy between the effect of an agent following maternal administration or after addition to embryo culture. For example, methionine supplementation of embryo donors or serum donors reduces the incidence of defects induced in embryo culture by the anti-convulsant, valproic acid (VPA), although concomitant addition of methionine with VPA *in vitro* has no such protective effect (Nosel and Klein, 1992).

A small-scale study has previously suggested a protective effect of inositol supplementation *in vivo* following maternal administration of 250 mg/kg on day 8 of gestation as indicated by a 31% incidence of NTD compared with 42% in controls (Seller, 1994). A dose of 400 mg/kg had a similar although not statistically significant effect while treatment at day 9 of gestation had no apparent effect.

In this chapter a dose of 400 mg/kg was utilised since this is the same order of magnitude as that used in the previous study and an increase in inositol dose had no apparent harmful effect in earlier embryo culture experiments (Chapter 3). The initial treatment regime consisted of a single inositol dose at 9 days 8 hours of gestation based on the fact that this corresponds to the sensitive period for treatment with TPA (Chapter 4) and falls within the 24 hour culture period in which inositol has a protective effect *in vitro* (Chapter 3). Additional treatment regimes were also used to test whether there is a specific period of sensitivity of NTD to inositol treatment and whether there is a greater effect of multiple dosage. Embryos were explanted from the uterus and assessed at 14 days of gestation by which stage the phenotype of NTD or tail flexion defects is clearly apparent.

6.2 Results

6.2.1 Maternal inositol administration has no apparent deleterious effect on viability or growth of embryos

Deleterious effects of inositol exposure *in vivo* would be expected to manifest as a reduction in litter size or increase in resorption frequency. However, litter size did not differ significantly between inositol-treated and control litters and, moreover, the frequency of resorptions was very similar between embryos exposed to inositol or PBS (Table 6.1).

The effect of inositol treatment on embryonic growth was assessed by comparison of mean crown-rump lengths at 14 days of gestation. No significant difference was observed between control and inositol treated embryos following treatment at any of the specific gestational ages. Neither was there any difference in the overall mean crown-rump length for the pooled groups of embryos (Table 6.1). Therefore, as for embryos measured following culture (Table 3.1), inositol treatment appears to have no effect on growth or survival of *curly tail* embryos *in vivo*.

6.2.2 Maternal inositol supplementation reduces the incidence of spinal NTD

Among the assessed embryos, three phenotypes were observed: straight tails, tail flexion defects and tail flexion defects accompanied by lumbo-sacral spina bifida (Figure 6.1). Embryos in the latter two groups are described as affected by spinal NTD. The frequency of spinal NTD is reduced in all three groups of embryos which were treated with inositol at 9 days 8 hours of gestation (Table 6.2, Figure 6.2). A similar reduction is observed in comparison of pooled groups of inositol- or PBS-treated embryos whose treatment regime included a dose at 9 days 8 hours of gestation (Table 6.2, Figure 6.2). Although the reduction in NTD frequency is present in all three groups it is only statistically significant for treatment at 9 days 8 hours alone and for the pooled groups of embryos.

Table 6.1 Litters of *curly tail* embryos assessed at 14 days of gestation following maternal inositol administration

Gestational age at inositol treatment	Ins/ Con	No. of litters	No. of viable embryos	Litter size*	No. Resorp- tions (%)	Crown- rump length (mm)**
9d+9d 8hrs (a)	Ins	8	63	7.88 ±0.67	1 (1.56)	10.89 ±0.07
	Con	8	66	8.25 ±0.41	4 (5.71)	11.05 ±0.07
9d 8hrs (b)	Ins	10	79	7.90 ±0.62	2 (2.47)	10.78 ±0.07
	Con	12	93	7.75 ±0.39	1 (1.06)	10.64 ±0.06
9d 8hrs+10d (c)	Ins	9	66	7.33 ±1.00	2 (2.94)	10.78 ±0.06
	Con	8	63	7.88 ±0.52	1 (1.56)	10.62 ±0.07
10d (d)	Ins	7	59	8.43 ±0.57	0 (0.00)	10.63 ±0.06
	Con	8	61	7.63 ±1.03	1 (1.61)	10.85 ±0.07
Pooled ins 9d 8hrs (a, b, c)	Ins	27	208	7.70 ±0.29	5 (2.40)	10.77 ±0.04
Pooled Con (a, b, c)	Con	28	222	7.93 ±0.25	6 (2.70)	10.85 ±0.04

Ins= 400 mg/kg inositol, Con= PBS. Timing of treatment is described in terms of the day and hour of gestation.

Pooled Ins 9d 8hrs= All embryos whose treatment included an inositol dose at 10 days 8 hours of gestation (i.e. groups a, b and c). Pooled Con = Pooled control embryos from groups a, b and c.

* Kruskal-Wallis One Way Analysis of Variance reveals no significant difference in litter size between treatment groups (P = 0.992, H= 1.95).

** Pairwise comparison by Mann-Whitney Rank Sum Test shows that there is no significant difference in crown-rump length between any of the pairs of treatment groups at specific gestational stages or between the pooled groups.

Table 6.2 Effect of maternal inositol administration (400 mg/kg) at different gestational ages on the incidence of NTD assessed at 14 days of gestation

Gestational age at inositol treatment	Ins/ Con	No. Spinal NTD (%)	No. Spina bifida (%)	No. Tail flexion defects (%)	No. Cranial NTD (%)
9d + 9d 8hrs (a)	Ins	29 (46.0)	5 (7.9)	24 (38.1)	1 (1.6)
	Con	35 (53.0)	8 (12.1)	27 (40.9)	0 (0)
9d 8hrs (b)	Ins	33 (41.8)*	3 (3.8)	30 (38.0)	0 (0)
	Con	56 (60.2)*	12 (12.9)	44 (47.3)	4 (4.3)
9d 8hrs + 10d (c)	Ins	30 (45.5)	4 (6.1)	26 (39.4)	4 (6.1)
	Con	33 (52.4)	6 (9.5)	27 (42.9)	1 (1.6)
10d (d)	Ins	33 (55.9)	4 (6.8)	29 (49.2)	1 (1.7)
	Con	31 (50.8)	6 (9.8)	25 (41.0)	1 (1.6)
Pooled ins 9d 8hrs (groups a, b and c)	Ins	92 (44.2)†	12 (5.8) ††	80 (38.5)	6 (2.1)
Pooled Con (groups a, b and c)	Con	124 (55.9)†	26 (11.7) ††	98 (44.1)	4 (1.8)

Embryos and treatments are as described in Table 6.1.

Chi-squared (χ^2) analysis with Yates correction shows that there is significant variation in the proportions of embryos with straight tails, tail flexion defects and spina bifida for treatment at 9d 8hrs (P= 0.0189) and for pooled embryos from groups a, b and c (P= 0.0179).

* = χ^2 analysis indicates significant variation in the proportions of affected (tail flexion defects with or without spina bifida) and unaffected embryos (P= 0.0239).

† = χ^2 analysis indicates significant variation in the proportions of affected and unaffected embryos (P= 0.0207).

†† = χ^2 analysis indicates significant variation between control and inositol treatment groups in the proportions of embryos with spina bifida (P= 0.0455).

Figure 6.1 Tail phenotypes of *curly tail* embryos at 14 days of gestation following *in vivo* treatment with inositol or PBS

Embryos are assessed at 14 days of gestation following inositol or PBS treatment on day 9 and/or 10 of gestation (Table 6.1). Embryos from the different treatment groups appear indistinguishable at this stage, the examples shown are PBS-treated. Three tail phenotypes are observed; **(a)** straight tail, **(b)** curly tail (tail flexion defect), **(c)** curly tail accompanied by spina bifida (arrow).

Arrow in c indicates spina bifida. The scale bar represents 2 mm.

Figure 6.2 Development of spinal NTD in *curly tail* embryos assessed at 14 days of gestation following maternal administration of inositol (400 mg/kg) at varying gestational ages

Timing of treatment
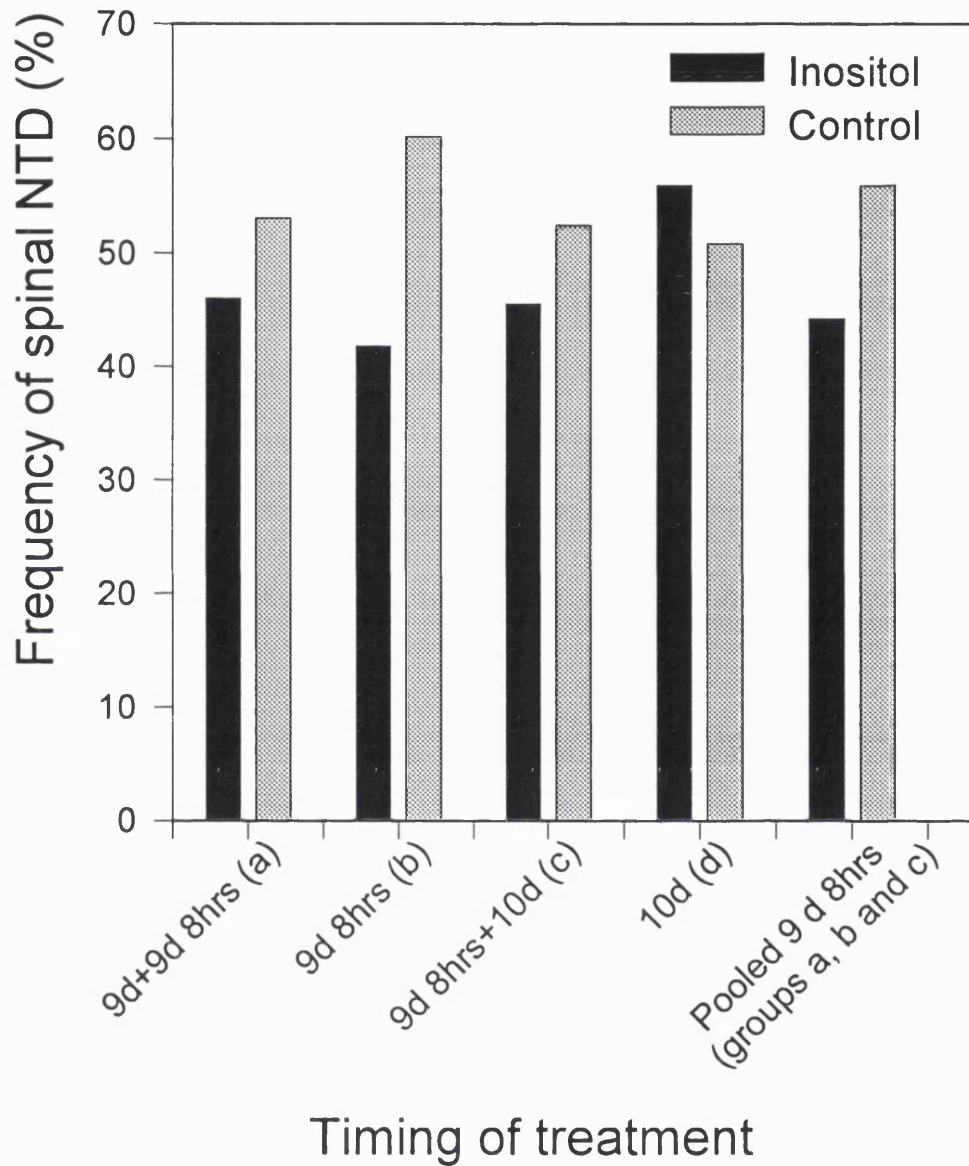
In contrast, inositol treatment at 10 days of gestation does not cause a reduction in the frequency of spinal NTD.

Inositol supplementation has a greater preventive effect on spina bifida than tail flexion defects

Separate consideration of spinal NTD as either spina bifida or tail flexion defects reveals that groups of embryos treated with inositol at 9 days 8 hours of gestation exhibit a reduction in frequency of both types of NTD (Table 6.2). However, the proportionate reduction is greater for spina bifida than for tail flexion defects with an overall reduction (pooled groups) of approximately 50% in the incidence of spina bifida (Table 6.2, Figure 6.3) compared with a 12% reduction in tail flexion defects. The reduction in spina bifida is statistically significant for the pooled embryos treated at 9 days 8 hours although not in the specific groups considered individually, probably owing to the small numbers involved.

There is an absolute decrease in the incidence of spina bifida from 0.9 per litter in controls compared with 0.45 per litter following inositol treatment (groups a, b and c pooled). This correlates with an increase in the number of straight-tailed embryos per litter following inositol treatment; 4.3 per litter following inositol treatment compared with 3.5 per litter for controls.

Inositol supplementation has no apparent effect on the frequency of cranial NTD

A low incidence of cranial NTD is seen in *curly tail* embryos but there is no apparent correlation between the frequency of exencephaly and treatment with inositol suggesting that inositol treatment has no effect on the incidence of cranial NTD. Moreover, the pooled groups of embryos exhibit a very similar frequency of cranial NTD with or without inositol treatment (Table 6.2) and this is likely to be more accurate than assessment of individual groups since the higher numbers reduce the impact of random variation.

Figure 6.3 Development of spina bifida in *curly tail* embryos assessed at 14 days of gestation following maternal administration of inositol (400 mg/kg) at varying gestational ages
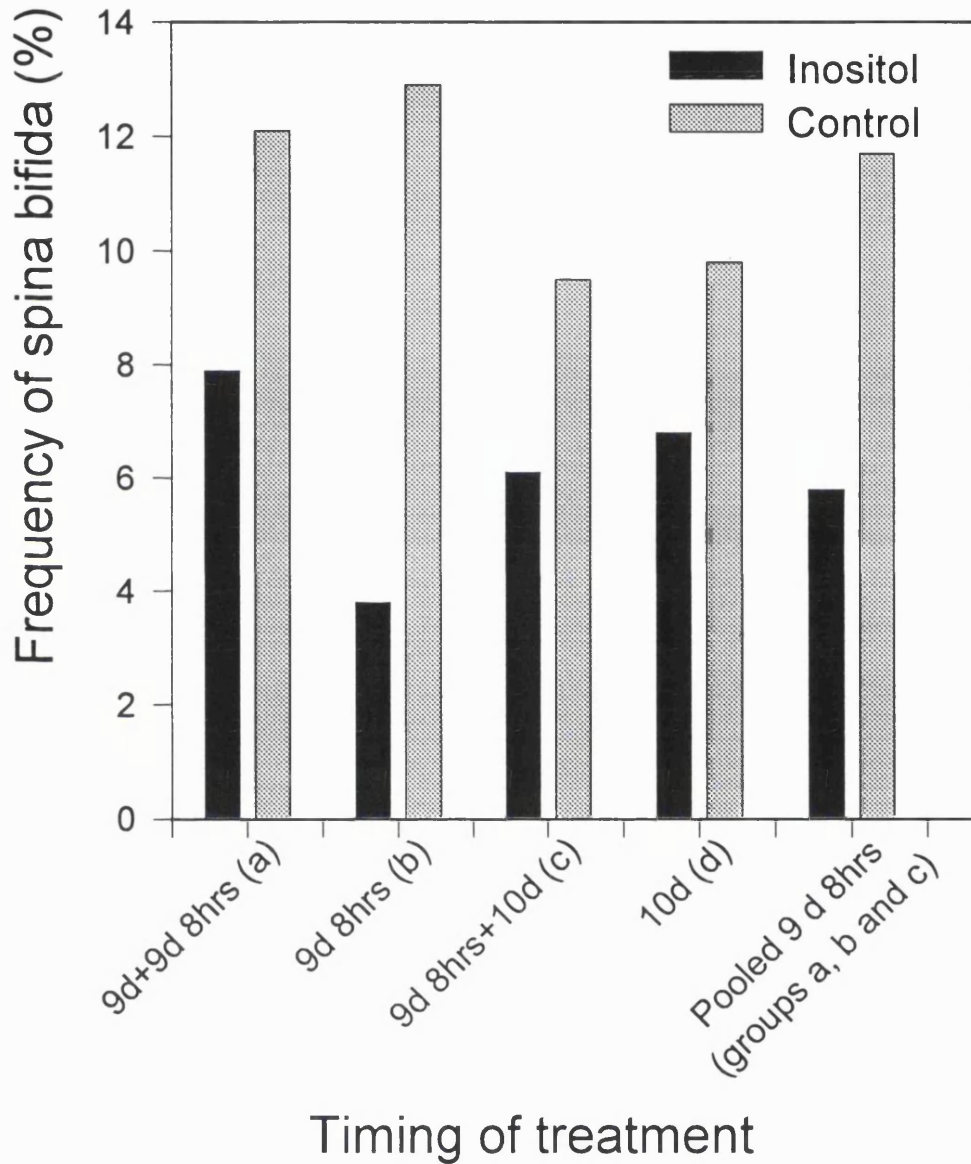
Susceptibility to spinal NTD appears not to be influenced by maternal phenotype

The overall data for the pooled treatment groups was subdivided according to the tail phenotype of the pregnant female in order to determine whether there is an influence on the incidence of NTD or the susceptibility to prevention of NTD following inositol supplementation.

Control (PBS-treated) embryos from curly-tailed females exhibit a similar incidence of spina bifida as those from straight-tailed females (Table 6.3) suggesting that, as in previous studies (Copp, 1985; Neumann *et al.*, 1994), there is no effect of maternal phenotype. Inositol treated embryos exhibit more variability in the proportions of embryos with each category of tail phenotype. There is a relatively lower incidence of tail flexion defects and a higher incidence of spina bifida in the offspring of curly-tailed females (Table 6.3) although a Chi-squared test indicates that these differences are not statistically significant. These are not definitive conclusions since in both cases the power of the statistical test was below the desired value indicating that the result should be treated with caution.

Inositol supplementation caused an apparent reduction in the frequency of spina bifida in embryos of both straight- and curly-tailed females although in neither case was this statistically significant. The relative proportions of all three phenotypes among offspring of curly-tailed females did exhibit significant variation between control and inositol treated groups, the greatest effect being on the incidence of tail flexion defects (Table 6.3).

Table 6.3 Incidence of spinal NTD in 14 day embryos of *curly tail* females with straight- and curly-tails following 400 mg/kg inositol supplementation

Treatment group	Straight tail mother (35 litters)			Curly tail mother (20 litters)*		
	ST (%)	CT (%)	SB (%)	ST (%)	CT (%)	SB (%)
Ins (Pooled 9d 8 hrs)	81 (52.9)	65 (42.5)	7 (4.6)	35 (63.6)	15 (27.3)	5 (9.1)
Con (Pooled)	62 (45.9)	58 (43.0)	15 (11.1)	36 (41.4)	40 (46.0)	11 (12.6)

Ins = 400 mg/kg inositol, Con = PBS. For both treatments embryos were pooled from groups a, b and c (treatment includes dose at 9 days 8 hours of gestation).

Phenotypes of embryos are designated as ST (straight tail), CT (tail flexion defect) and SB (tail flexion defect with spina bifida).

* = Chi square test indicates variation between treatment groups in the proportions of phenotypes displayed by offspring of curly-tailed females (P= 0.0341). For these embryos there is a significant difference between treatment groups in the proportions of affected (CT+ SB) and unaffected (ST) embryos (P= 0.0159). However, there is no apparent difference in the proportions of embryos with (SB) and without (ST + CT) spina bifida.

6.3 Discussion

Maternal administration of 400 mg/kg inositol at 9 days 8 hours of gestation causes a significant reduction in the frequency of spinal NTD in *curly tail* embryos. This protective effect correlates with the reduction in mean PNP length following *in vitro* inositol supplementation which was described in Chapter 3. This preventive effect of inositol *in vivo* was predicted, since it is known that delayed closure of the PNP leads directly to development of spinal NTD (Copp, 1985). Therefore, the protective effect of inositol in whole embryo culture is also maintained in the *in vivo* situation, an important consideration in terms of the potential application of inositol supplementation as a preventive measure for NTD, particularly folate-resistant forms.

The protective effect of inositol on spinal NTD *in vivo* is (as for the effect *in vitro*) proposed to be mediated through a specific effect on closure of the PNP. Firstly, the reduction in frequency of affected embryos is not mediated through increased lethality of this group since the mean litter size and resorption frequency are unaffected (Section 6.2.1). Moreover, there is an increase in the absolute number of straight-tailed embryos per litter (Section 6.2.2) which would not be expected if inositol action was mediated through a lethal effect on spina bifida embryos. This lends further support to the idea that inositol treatment causes a shift towards normal phenotypes rather than increased lethality of affected embryos. In addition, there is no effect on growth, indicated by mean crown-rump length, which suggests that, unlike hyperthermia or starvation (Copp *et al.,* 1988b), the reduction in frequency of spinal NTD is not mediated through growth retardation.

The reduction in frequency of NTD following *in vivo* inositol treatment is only statistically significant for embryos treated at 9 days 8 hours alone, probably due to the lower sample numbers in the other treatment groups. However, all the groups which include an inositol dose at 9 days 8 hours exhibit a reduction in NTD frequency suggesting that the effect of this treatment is not an artefact. Moreover, comparison of these pooled data with pooled data for controls indicates a statistically significant

reduction in the incidence of total spinal NTD (tail flexion defects with and without spina bifida) and also the incidence of spina bifida considered separately.

Treatment at 10 days of gestation has no apparent protective effect on spinal NTD as a whole and a much smaller effect on the incidence of spina bifida suggesting that the critical period for prevention of NTD has been missed. This contrasts with the results obtained in embryo culture in which a one hour exposure to TPA at 9 days 23 hours of gestation has a similar protective effect to inositol on closure of the PNP (Section 4.2.2). The discrepancy in timing is probably due to the fact that by day 10 of gestation, embryos which develop *in vivo* are generally approximately two hours (one somite, Table 3.1) more advanced that those which are explanted for culture. In addition, embryos treated *in vitro* are immediately exposed to inositol whereas there may be a delay following *in vivo* inositol treatment before therapeutic levels of inositol have accumulated in the embryo. Therefore, by 10 days of gestation embryos *in vivo* may have passed a critical period for intervention by inositol supplementation.

The period of sensitivity to inositol in this study differs from that described in the previous study (Seller, 1994) in which there was no effect of treatment at day 9 of gestation but a protective effect on day 8. This is likely to be due to differences in the timing and description of experimental matings. A similar discrepancy was previously observed in the timing of optimal treatment with RA between Seller's studies (Seller *et al.*, 1979; Seller and Perkins, 1982) and studies in our laboratory (Chen *et al.*, 1994). In this case maximal effects were obtained at 9 days and 10 days 8 hours of gestation respectively. Moreover, the direction of the difference compared with Seller's studies is the same for the present study and that of Chen et al. (1994).

The required stage for inositol treatment also differs from that described by Chen et al. (1994) for retinoic acid (RA) for which maximal prevention of spinal NTD was obtained following treatment between 10 days 4 hours and 10 days 8 hours. This may also be due to different timing of experimental matings, although this seems less likely since, as

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described above, this study and that of Chen et al. (1994) observed similar differences in timing of inositol- and RA-treatment compared with the Seller group. Alternatively (although not exclusively), the variation in maximal effect of inositol and RA could reflect differing mechanisms of action. For example, if the effect of both inositol and RA is mediated through upregulation of RAR-β expression in the hindgut (Section 5.3), RA may have a more immediate effect since it binds to the receptor directly rather than acting through an indirect pathway.

The mechanisms of the preventive action of inositol and RA also show further variations although ultimately both may involve up-regulation of expression of RAR-β in the hindgut. A striking variation is the differential effect on the incidence of tail flexion defects and spina bifida. In contrast to inositol, RA has a greater effect on tail flexion defects (50% reduction) than spina bifida (36% reduction). Moreover, RA treatment is able to dissociate tail flexion defects from spina bifida; 13% of treated embryos with spina bifida exhibited straight tails (Chen et al., 1994). This phenotype was not observed in any of the control or inositol treated embryos displaying spina bifida in the present study. These observations suggest that there may be a dual action of RA on the development of spinal NTD which may lead to prevention of tail flexion defects in cases where spina bifida persists (Figure 6.4). This could potentially operate through separate specific effects on closure of the PNP during primary neurulation and development of the tail bud during secondary neurulation. A normalising effect on closure of the PNP, which is indicated by the decreased mean PNP length following RA treatment (Chen et al., 1994), would be predicted to reduce both spina bifida and tail flexion defects (A in Figure 6.4). Tail flexion defects may arise as a consequence of an abnormal transition from primary to secondary neurulation following delayed closure of the PNP (Copp, 1985). A specific ameliorating effect of RA on secondary neurulation (B in Figure 6.4) would prevent tail flexion defects but not spina bifida (Chen et al., 1994). In contrast, inositol appears to have a more specific normalising effect on PNP closure such that the overall NTD phenotype is shifted towards straight tails in an analogous manner to the shift towards smaller PNP categories in embryos following culture in the presence of

Figure 6.4 Potential mechanisms for prevention of *curly tail* spinal NTD by inositol and RA

Phenotypes are shown in boxes, ST= straight tail, CT= tail flexion defect, SB= spina bifida accompanied by tail flexion defects, ST + SB= straight tail accompanied by spina bifida. In this model, the effect of inositol is mediated through enhancement of PNP closure (A) while RA affects both PNP closure (to a lesser extent than inositol) and secondary neurulation (through an effect on tail bud development, B).

Inositol

A. Enhances PNP closure (day 10)

(day 12 or later)	ST	← A —	CT	← A —	SB

ST increases   CT little changed   SB decreases

RA

(day 10)

A. Enhances PNP closure   **B. Normalises tail bud development**

(day 12 or later)	ST	← A, B —	CT	← A —	SB

ST increases   CT decreases   B

ST + SB

Small decrease in SB

ST + SB increases

220
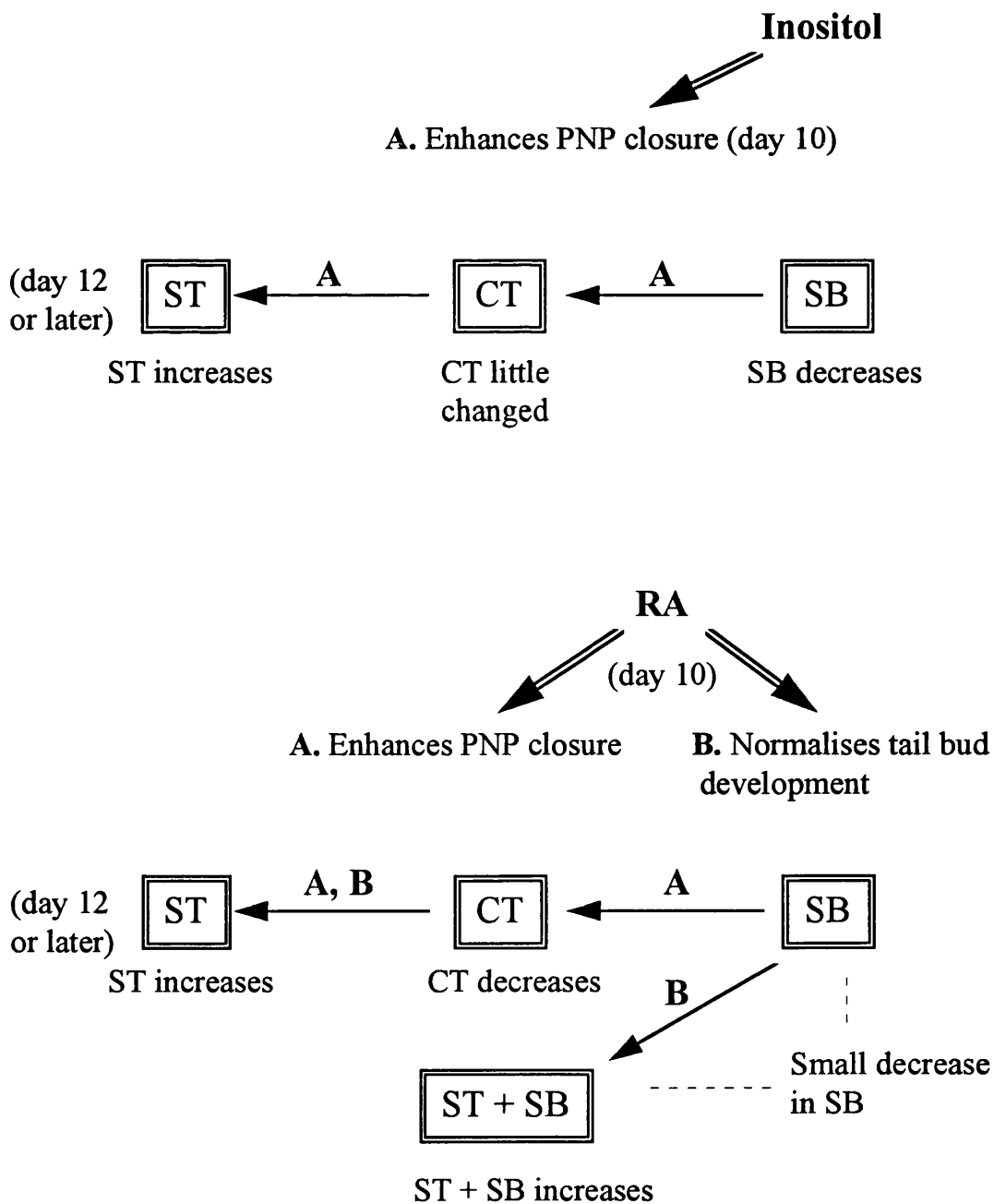
inositol (Figure 3.3). This model would explain why there is a relatively small decrease in the incidence of isolated tail flexion defects in this study since tail flexion defects can be envisaged as an intermediate state between spina bifida and normality. Thus, the effect of inositol supplementation is to reduce the number of embryos displaying tail flexion defects (they are now unaffected) which is counteracted by the additional embryos which would otherwise have developed spina bifida but, following treatment, develop only a tail flexion defect (Figure 6.4).

The protective effect of RA also appears to be influenced by the maternal phenotype. There is a statistically significant reduction in the frequency of spina bifida for offspring of straight-tailed females whereas the offspring of curly-tailed females are resistant to the prevention of spina bifida by RA (Chen *et al.,* 1994). Among inositol-treated embryos there is a greater reduction in the frequency of spina bifida in the offspring of straight-tailed compared with curly-tailed females. However, this is a small, non-significant variation compared with that observed among RA-treated embryos and suggests that inositol-treated *curly tail* embryos exhibit a reduction in the frequency of spina bifida irrespective of maternal phenotype. Moreover, there is no apparent effect of maternal phenotype on the incidence of spina bifida among control embryos, which correlates with previous studies (Copp, 1985; Neumann *et al.,* 1994) but contrasts with the findings of Chen et al. (1994).

There was no apparent effect of inositol treatment on the incidence of cranial NTD at any of the stages of inositol treatment. This observation is as expected since *curly tail* embryos should have completed closure of the cranial neural tube prior to the stages of inositol treatment.

It is possible that an increase in the dose of inositol would further reduce the incidence of NTD although, in a small-scale study, an increase of the dose to 2000 mg/kg did not eliminate spina bifida. Among embryos treated with 2000 mg/kg inositol, 2 out of 17 developed spina bifida compared with 1 out of 18 embryos treated with 400 mg/kg

inositol and 3 out of 16 embryos treated with PBS (all single doses at 9 days 8 hours of gestation). Another factor which may influence the efficacy of inositol treatment is the route of administration. In this and the previous study (Seller, 1994), mice received single or double intra-peritoneal injections. The efficiency of uptake of inositol into the maternal bloodstream has not been established. Moreover, since inositol is water-soluble, it may be rapidly cleared. Alternative approaches to inositol supplementation could include gavage or continuous dosing by the use of osmotic pumps implanted beneath the skin. Gavage treatment may be particularly applicable to a potential use in the prevention of human NTD (Section 8.3) since an inositol supplement would most likely be taken orally, as in previous clinical trials of the efficacy of inositol in treatment of panic disorder and depression (Benjamin *et al.*, 1995; Levine *et al.*, 1995).

The efficacy of inositol in prevention of spina bifida in a folate-resistant mouse strain raises the possibility of a beneficial use in humans and this will be discussed in greater detail in the General Discussion (Chapter 8).

CHAPTER 7

Development of the notochord and floor plate in the *loop-tail* mutant mouse

7.1 Introduction

The previous chapters (Chapters 3, 4, 5 and 6) describe studies of *curly tail* mouse embryos, in which spinal NTD result from the failure or delay of closure of the posterior neuropore (PNP). The embryonic defect in *curly tail* embryos has previously been localised to the hindgut endoderm which exhibits reduced proliferative ability (Section 1.4.4). The *loop-tail* mutant mouse provides a complementary model for development of NTD. Embryos that are homozygous for the *loop-tail* (*Lp*) mutation develop the severe NTD, craniorachischisis, owing to the failure of closure 1, the initial closure of the neural folds (Section 1.7.2). Therefore, elucidation of the processes underlying the *curly tail* and *loop-tail* defects may respectively provide clues to the mechanisms of development of spinal NTD and craniorachischisis. In this manner, the analysis of mutants which are defective at different closure sites should provide a more comprehensive view of the factors which are critical for closure of the neural tube in normal embryos.

In contrast to the *curly tail* mouse, although neural fold closure is affected in *Lp/Lp* embryos, the abnormal tissue or tissues which contribute to development of NTD in *Lp/Lp* embryos have not been identified. As described in Section 1.1.2, the closure (and hence failure of closure) of the neural tube in *loop-tail* could be influenced by factors that are either extrinsic or intrinsic to the neuroepithelium. For example, delayed PNP closure in *curly tail* embryos is due to mechanical forces imposed by ventral curvature arising from a defect of the hindgut, i.e. extrinsic to the neural folds. In the *Splotch* mouse, spinal NTD are not accompanied by ventral curvature of the caudal region. Instead defects are thought to be a consequence of an intrinsic abnormality of the neural folds since *Pax-3*, which is mutated in *Splotch* mice (Epstein *et al.*, 1991), is first detected in the neuroepithelium during development (Goulding *et al.*, 1991).

Several studies have addressed the question of the tissue localisation of the defects contributing to development of NTD in *Lp/Lp* embryos. However, although a number of abnormalities of the neuroepithelium have been described (Section 1.7.3), no

mechanism for the development of NTD has been suggested and no studies of gene expression have been carried out. Moreover, as described in Section 1.7.3, most of the previous investigations of abnormalities in *loop-tail* embryos relied on observation of failure of neural tube closure for identification of homozygotes. Therefore, by necessity, abnormalities which cause NTD, and must therefore be present at or preceding the time of closure 1 could not be distinguished from effects which were secondary to failure of neural tube closure. The present study takes advantage of the recent development in our laboratory of a PCR based technique that allows genotyping of the embryos prior to the appearance of NTD (Copp *et al.,* 1994).

As long ago as 1962 it was suggested that NTD in *loop-tail* homozygous embryos arise from defective growth of the notochord, neural tissue and somites which results in a reduction of axial elongation (Smith and Stein, 1962), one of the factors proposed to contribute to neural tube closure (Section 1.1.2). Analysis of genotyped embryos has since revealed that there is no apparent difference in cell proliferation or embryonic length prior to failure of closure 1, although diminished axial elongation is apparent following failure of closure 1 (Gerrelli & Copp, Unpublished). However, this study did not address the possibility that there is a specific abnormality of notochordal proliferation or gene expression in *Lp/Lp* embryos.

The notochord seems a good candidate for the location of the primary developmental defect in *loop-tail* since it extends the length of the axial midline in the trunk and therefore underlies the region affected by the *loop-tail* defect, i.e. the trunk neural tube. Previous studies of mutant embryos at day nine of gestation have indicated abnormalities of the midaxial region, albeit following (and therefore potentially secondary to) failure of neural tube closure. Light microscopy indicates that the midaxial structures are abnormally separated in affected embryos compared with embryos at the same stage in which the neural tube has closed (Wilson and Wyatt, 1989b). Ultrastructural analysis of the extracellular matrix in this region reveals the presence of basal laminar strands connecting the ventral cells of the notochord with the gut of *Lp/Lp* embryos. These

compare with short strands extending from the notochord of normal embryos and suggest that there may be a defect in the normal separation of the notochord from the gut of *Lp/Lp* embryos. Due to its multiple roles in patterning of the embryo (Section 1.8), an abnormality of the notochord could potentially influence development of several tissues, any of which could be involved in the development of NTD. Based on this premise, I carried out a study of the notochord and floor plate, in which, for the first time in *loop-tail*, these potentially abnormal tissues were assessed using molecular markers.

Sonic hedgehog (*Shh*) is expressed initially in the notochord, and subsequently in the floor plate of the neural tube (Section 1.8.1). In this chapter, *Shh* expression was examined as a marker of notochord and floor plate by whole mount *in situ* hybridisation. An abnormal pattern of expression of *Shh* is described in the notochord and at later stages, in the floor plate of genotyped *Lp/Lp* embryos.

In order to confirm the validity of *Shh* as a marker of the floor plate, the expression of another floor plate expressed molecule, *netrin-1*, was also assessed. *Netrin-1* is one of two structurally related proteins in the chick that are homologous to the UNC-6 protein of *Caenorhabditis elegans* and exhibit commissural axon outgrowth-promoting activity (Kennedy *et al.*, 1994). *Netrin-1* is expressed in the floor plate of the neural tube and is proposed to act as a diffusible chemo-attractant that is involved in the guidance of commissural axons towards the floor plate during development of the spinal cord (Kennedy *et al.*, 1994).

Shh plays a key role in the action of the notochord and floor plate in dorsoventral patterning of the somites and neural tube (Section 1.8). The somites are patterned by the induction of sclerotome and repression of development of dermomyotome (Fan and Tessier-Lavigne, 1994; Johnson *et al.*, 1994). Therefore, the observation of apparent abnormalities of *Shh* expression in the notochord and floor plate in *Lp/Lp* embryos raises the possibility that the function of these tissues may be affected. For example, a number of other mouse genetic mutants, in which the notochord develops abnormally, exhibit

disruptions in formation of the sclerotome (Dietrich *et al.*, 1993). In the present study, the dorso-ventral patterning of the somites and neural tube in *Lp/Lp* embryos was assessed on the basis of expression of *Pax-3*.

Pax-3, a member of the *Pax* gene family of transcription factors (Gruss and Walther, 1992; Noll, 1993), is expressed in the dorsal region of the neural tube and, during development of the somites, in the dermomyotome (Goulding *et al.*, 1991). Expression of *Pax-3* in the dorsal neural tube and in the adjacent mesoderm is diminished by the additional presence of a notochord explant, indicating that the expression pattern of *Pax-3* in the somites and neural tube is regulated by signals from the notochord (Goulding *et al.*, 1993; Fan and Tessier-Lavigne, 1994). As described in Section 1.8.1, *Shh* appears to play a key role in somite patterning since the suppression of *Pax-3* (a dermomyotome marker) by notochord explants is mimicked by COS cells expressing *Shh* (Fan and Tessier-Lavigne, 1994). The converse situation, in mouse mutants where the notochord is defective or missing or in a *Shh* functional mutant, leads to ventralised expression of *Pax-3* in the somites and loss of expression of *Pax-1*, a marker of the ventral somite (Dietrich *et al.*, 1993; Chiang *et al.*, 1996). An abnormality of the notochord in *Lp/Lp* embryos may therefore be predicted to alter expression of *Pax-3* in the somites and/or the neural tube.

7.2 Results

Whole mount *in situ* hybridisation (Section 2.7) was used to assess expression of *Shh*, *netrin-1* and *Pax-3* in *Lp/Lp*, *Lp/+* and *+/+* embryos, genotyped by PCR analysis of yolk sac DNA (Section 2.5). In this chapter the + allele refers to embryos which have the wild-type, CBA/Ca allele at the *Lp* locus, and with a mixture of LPT/Le and CBA/Ca background, since embryos were generated from *Lp*/CBA intercrosses (Section 2.1).

Table 7.1 Numbers and treatments of embryos analysed by whole mount *in situ* hybridisation for expression of *Shh*, *netrin-1* and *Pax-3*

		No. embryos analysed (no. of experiments)		
Probe	Stage (day of gestation)	+/+	*Lp/+*	*Lp/Lp*
Shh	8	5 (2)	11 (3)	6 (2)
Shh	9	5 (2)	8 (3)	6 (2)
Shh	10	12 (4)	10 (3)	8 (3)
Netrin-1	9	4 (2)	8 (3)	5 (2)
Pax-3	10	4 (1)	8 (2)	5 (3)

The number of experiments (in brackets) refers to the number of repetitions of whole mount *in situ* hybridisation.

7.2.1 Expression of *Shh* on day 8 of gestation

In *Lp/+* and *+/+* embryos with 4-8 somites (approximately 8 days 8 hours of gestation) *Shh* is expressed in a distinct domain in the axial midline. Staining extends from the forebrain/midbrain boundary, through the midbrain and hindbrain and along the ventral midline into the primitive streak where the expression domain broadens (Figure 7.1). In the midline caudal to the midbrain, staining is thought to correspond to notochordal expression and this is confirmed in sections (Figure 7.2). There is also some expression detectable on the ventral edge of the neuroepithelium (Figure 7.2) which suggests that

induction of the floor plate, which expresses *Shh* (Section 1.8.1), has commenced. Expression is also detected in the endoderm of both the foregut and hindgut pockets. The expression patterns closely resemble those described in previous reports (Echelard *et al.*, 1993).

Comparison of expression in *Lp/Lp*, *Lp/+* and +/+ embryos reveals no apparent difference in the level and general sites of expression. However, although side views of embryos appear very similar, comparison of ventral views (Figure 7.1b-e) indicates that there is an abnormality of the pattern of expresson in the notochord of *Lp/Lp* embryos. In a region at the level of somites 4-5, the midline expression appears broad and diffuse (Figure 7.1g) compared with the distinct continuous line of expression in *Lp/+* and +/+ embryos (Figure 7.1b, c, f). Transverse sections through this region reveals that the abnormal appearance is due to an altered expression domain in the notochord with less expression in the midline (Figure 7.2k, l) which gives the appearance of a split structure in the whole mounts. This alteration in *Shh* expression appears to correspond to an abnormal structure of the notochord, with a depletion of notochordal cells in the midline, rather than a lateral extension of expression into another tissue. At axial levels cranial to the affected region around the fourth somite, the midline expression of *Shh* in *Lp/Lp* embryos appears normal. In two-thirds (four out of six) of embryos the notochord also appears normal at levels caudal to the site of abnormal expression (Figure 7.1e and 7.2), while in the remaining embryos the diffuse expression pattern extends into the primitive streak (Figure 7.1d). No apparent differences were observed between *Lp/+* and +/+ embryos.

Figure 7.1 Expression of *Shh* in embryos on day 8 of gestation

Shh expression, as demonstrated by whole mount *in situ* hybridisation in (a, b) +/+, (c) *Lp*/+ and (d-e) *Lp/Lp* embryos with 5-7 somites. A side view, for which embryos of all genotypes appear indistinguishable, is shown in (a). Comparison of views from the ventral surface (b-e) indicates apparent abnormalities in the notochordal staining of *Lp/Lp* embryos (arrows in d, e, g). The region of potential abnormality in (b, f) +/+ and (e, g) *Lp/Lp* embryos is compared at higher magnification (photographed on a Zeiss Axiophot). The axial level of sections shown in Figure 7.2 is indicated by short arrows in 7.1e. The sections are in an anterior to posterior sequence, i.e. 7.2h to n as shown, and sections from the +/+ embryo (7.2a-g) are at equivalent levels.

N= notochord, F= foregut, H= hindgut, P= primitive streak

The scale bar represents 0.58 mm in a-e and 0.2 mm in f-g.

Figure labels visible within panels: a, b, c, d, e, f, g

Panel b: F, N, P, H

Panel e: 7·2, h, n
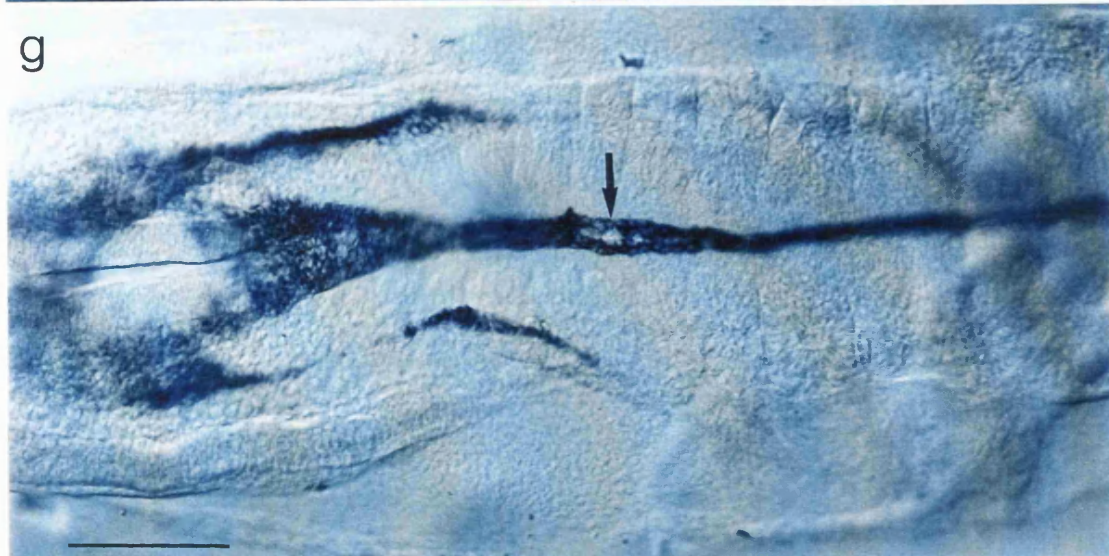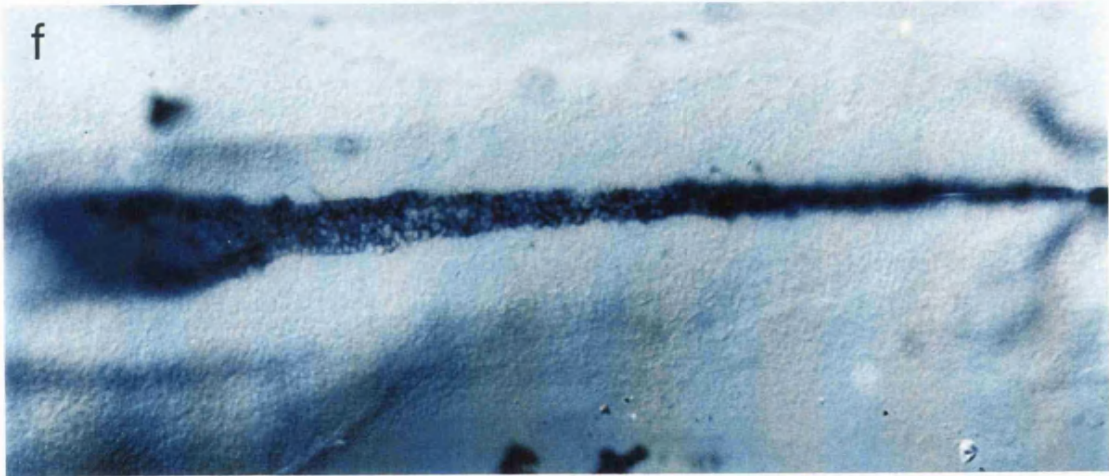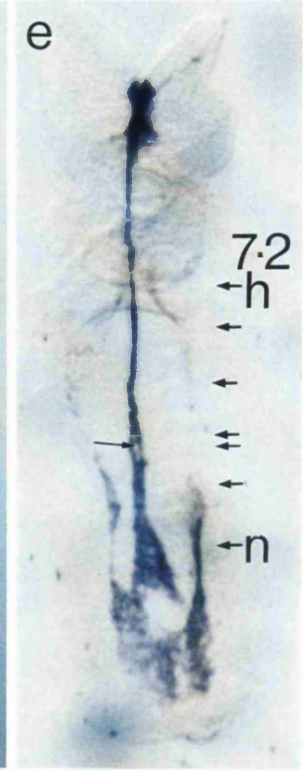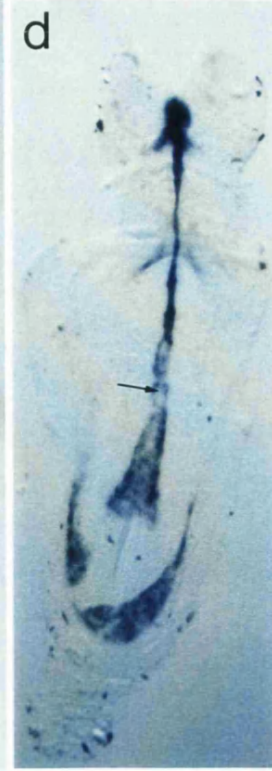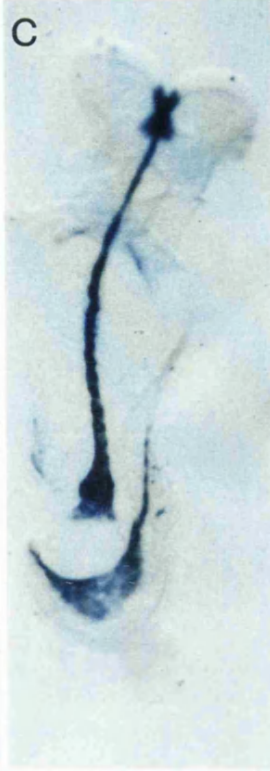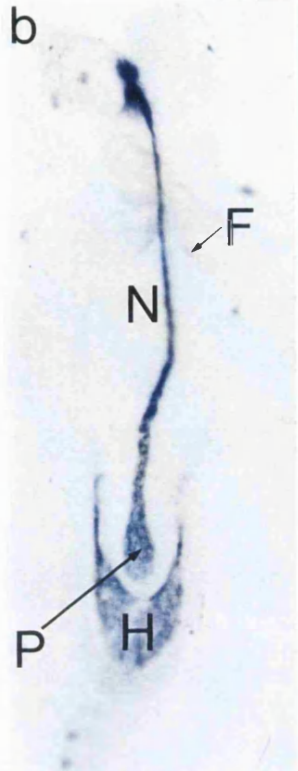
Figure 7.2 Expression of *Shh* in transverse sections of embryos on day 8 (5-7 somite stage) of gestation

Expression of *Shh*, as demonstrated in 20 μm transverse sections along the anterior-posterior axis, following whole mount *in situ* hybridisation of (a-g) +/+ and (h-n) *Lp/Lp* embryos. The axial level of sections is indicated by short arrows in 7.1e (a-g are at equivalent levels to h-n). Expression in the notochord of the *Lp/Lp* embryo appears to be depleted in the midline at the level of somite 4-5 (k, l).

Thick arrow in h= floor plate, narrow arrow in h= notochord, N= neural folds, F= foregut, H= hindgut, P= primitive streak. The scale bar represents 0.1 mm.
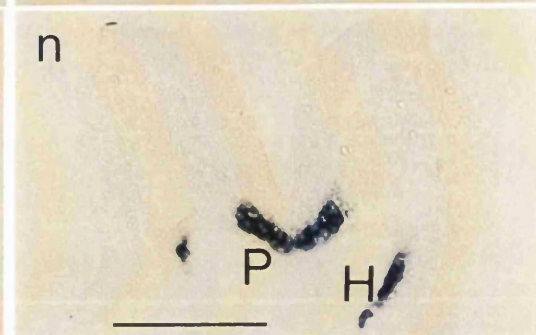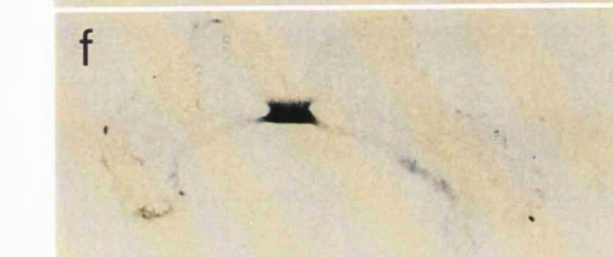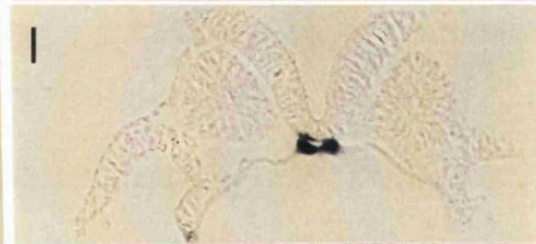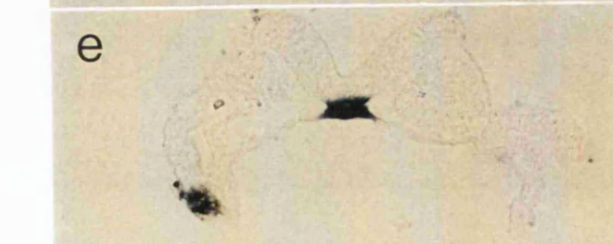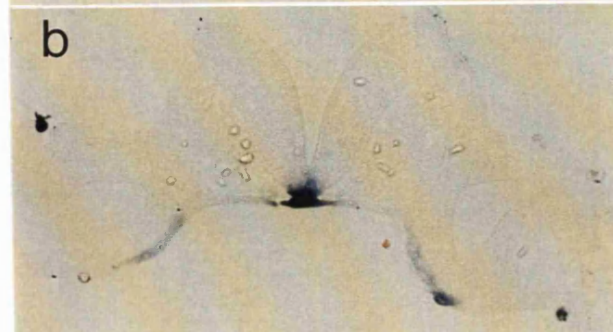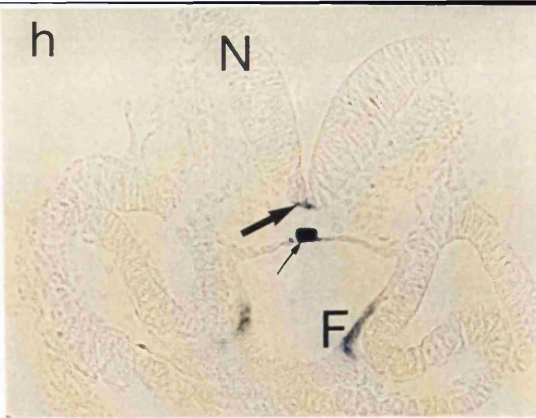
7.2.2 Expression of *Shh* on days 9 and 10 of gestation

In embryos on day 9 (15-19 somites) and day 10 (27-31 somites) of gestation *Shh* is expressed in a distinct domain in the notochord and floor plate of the neural tube extending from the hindbrain throughout the length of the neural tube. Expression also extends into the ventral neural tube of the midbrain and forebrain (Figures 7.3 and 7.4). At day 10 of gestation *Shh* expression in *Lp/+* and +/+ (Figure 7.4) closely resembles that previously observed in *curly tail* and CBA/Ca embryos (Section 5.2.6). At both these stages of gestation *Shh* is expressed in the endoderm of the foregut and the hindgut, correlating with previous reports (Echelard *et al.,* 1993). At day 10 of gestation a patch of expression can also be detected in the forelimb bud at the presumed ZPA (Figure 7.4).

Although no differences are observed between *Lp/+* and +/+ embryos at either of these stages, an altered expression pattern is detected in *Lp/Lp* embyos (Figure 7.3c and 7.4d-e). Expression is present in the same tissues as in unaffected embryos with no apparant difference of staining in the notochord, ventral neural tube in the cranial region, hindgut and foregut or ZPA of the forelimb bud (day 10 of gestation). However, at day 9 of gestation there is an apparent expansion of the expression domain in the floor plate of the neural tube in a region that is level with, and immediately caudal to, the forelimb bud (Figure 7.3c). A dorsal view, looking into the open neural folds clearly shows this broad domain of expression (Figure 7.3e). The expression in the midline of this region appears weaker so that the underlying notochord is visible, however, this may be because the walls of the neural tube appear more intensely stained as there are more positive cells between the transilluminating light source and the microscope. Transverse sections through this region of the embryo indicate that as in normal embryos, *Shh* expression extends across the floor plate of the neural tube in *Lp/Lp* embryos (Figure 7.3g). However, the expression domain in the floor plate appears to be abnormally wide in *Lp/Lp* embryos with expression extending further laterally than in wildtype embryos (Figure 7.3f-g).

By day 10 of gestation the abnormal appearance of *Shh* expression in the floor plate of the neural tube extends the entire length of the region of open neural folds (Figure 7.4d). Again, from a dorsal viewpoint the floor plate exhibits the appearance of two stripes of expression with weaker staining in the midline (Figure 7.4e). In transverse sections through the neural tube, the floor plate expression of *Shh* again appears to extend further laterally in *Lp/Lp* embryos compared with embryos in which the neural tube is closed (Figure7.3h-i). Moreover, in certain regions, which seem to vary between embryos, the expression in the midline of the floor plate appears to be weak compared with the expression in the lateral regions of the extended floor plate (Figure 7.3i). The contrast between lateral and midline expression of *Shh* in the floor plate is again exaggerated as a result of the increased number of positive cells between the light source and the microscope.

At both these stages (9-10 days of gestation) there is no apparent abnormality of the notochord. This correlates with recent observations in our laboratory that the expression of the notochord marker, *Brachyury*, appears normal on day 10 of gestation although the expression domain is abnormally broad on day 8 (and possibly also early on day 9) of gestation (Gerrelli and Copp, Unpublished).

Figure 7.3 Expression of *Shh* in whole embryos on day 9 of gestation and transverse sections of embryos on days 9 and 10 of gestation

Expression of *Shh*, demonstrated by whole mount *in situ* hybridisation, in (a) +/+, (b) *Lp*/+, and (c, e) *Lp/Lp* embryos at approximately 9 days 8 hours of gestation (approximately 19 somites). A control CBA/Ca embryo (20 somite stage) hybridised with a *Shh* sense probe is shown in d (see Table 2.x for number of embryos analysed). There is an abnormally broad domain of expression of *Shh* in *Lp/Lp* (arrow in c) compared with *Lp*/+ and +/+ (arrow in a) embryos

Transverse sections (20 μm) of embryos at approximately 9 days 8 hours (f-g) and 10 days 8 hours (h-i) of gestation are also shown. The axial level of sections in these examples are just caudal to the forelimb bud (f-g; axial level of sections indicated by arrows in a and c respectively) and at the cranial limit of the forelimb bud (h-i; axial level of sections indicated by arrows in 7.4b and d respectively). The expression domain of *Shh* appears to extend further laterally in the floor plate of (g) *Lp/Lp* compared with (f) +/+ (and *Lp*/+) embryos. At varying anterior-posterior levels (commonly just cranial to the forelimb bud) in embryos at 10 days of gestation, the expression in the midline appears weaker (arrow in i).

F= floor plate, G= hindgut, N= neural folds. The scale bar represents 1 mm in a-d, 0.5 mm in e and 0.2 mm in f-i.
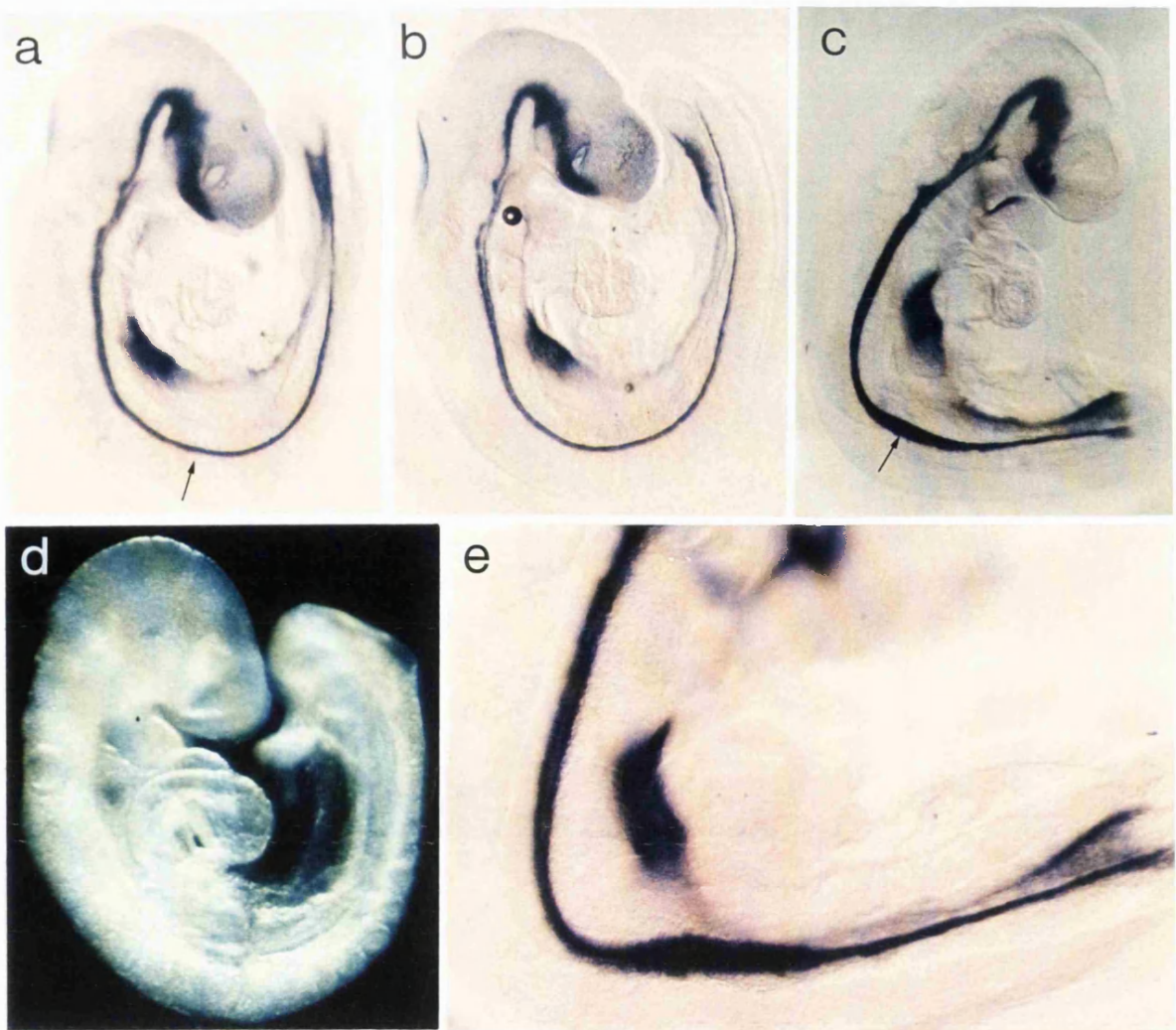
Figure 7.4 Expression of *Shh* in embryos on day 10 of gestation

Expression of *Shh* as demonstrated by whole mount *in situ* hybridisation in (b) +/+, (c) *Lp*/+ and (d) *Lp/Lp* embryos at 10 days of gestation (28-31 somites). A control CBA/Ca embryo (+/+) hybridised with the *Shh* sense probe is shown in (a). The expression in the floor plate of the *Lp/Lp* embryo appears abnormal when viewed from a lateral position (thicker region of signal) and when looking into the open neural folds (e). *Shh* is expressed in two apparent longitudinal stripes just lateral to the midline with weaker staining in the midline, so that the notochord signal is visible in between (arrow in e).

Arrows in b and d indicate the axial level of transverse sections shown in 7.3h and i. Thick arrow in b= zone of polarising activity, which does not differ between genotypes. The scale bar represents 1.1 mm in a-b and 1.0 mm in c-d and 0.5 mm in e.
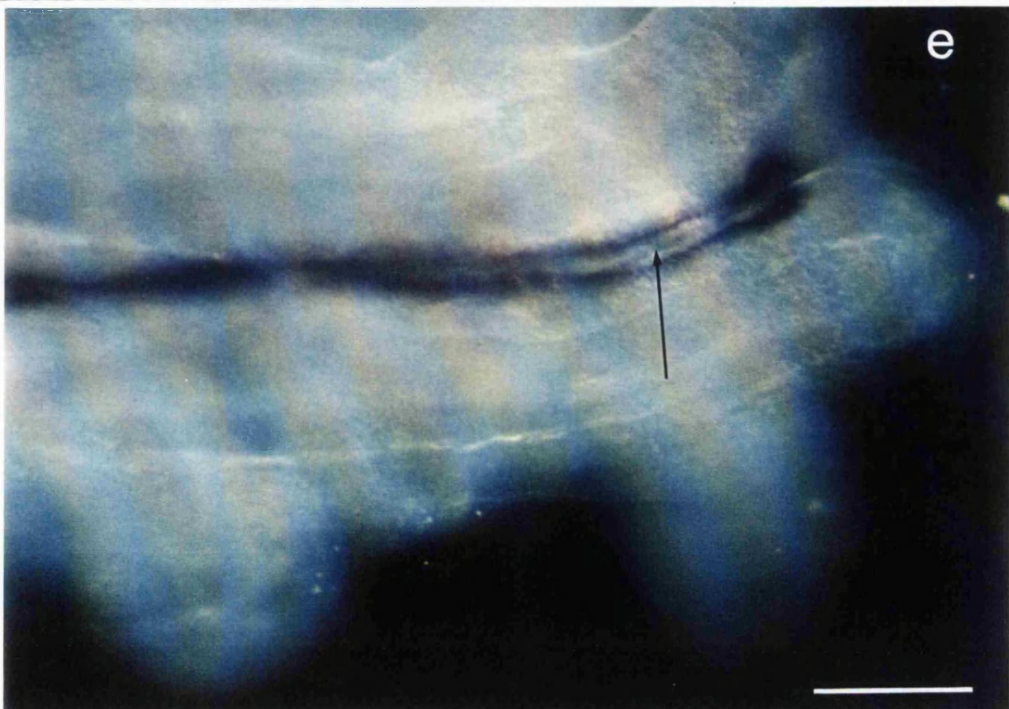
7.2.3 Expression of *netrin-1* in the floor plate of embryos on day 9 of gestation

The laterally extended domain of *Shh* expression observed in the floor plate of *Lp/Lp* embryos could represent either a real enlargement of the floor plate or a specific effect on the expression of *Shh*. In order to differentiate between these possibilities I examined the expression of *netrin-1*, another marker of the floor plate (Kennedy *et al.*, 1994).

Expression of *netrin-1* is detected in the floor plate and the somites of embryos at day 9 of gestation. No differences were detectable in comparison of *Lp/+* and *+/+* embryos but *Lp/Lp* embryos exhibit an abnormal expression pattern in the neural tube (Figure 7.5b). As observed for *Shh*, the floor plate expression in *Lp/Lp* embryos appears to be abnormally broad in a region around the forelimb bud with, again, the appearance of a laterally duplicated signal. In the case of *netrin-1*, the notochord cannot be seen between the paired floor plate signals since the notochord does not express *netrin-1*. Analysis of sections indicates that the expression in the closed neural tube is restricted to the floor plate of the neuroepithelium whereas in *Lp/Lp* embryos expression again crosses the midline but also extends laterally in the open neural folds (Figure 7.5c-d).

Figure 7.5 Expression of *netrin-1* in *Lp/+* and *Lp/Lp* embryos on day 9 of gestation

Expression of *netrin-1*, as demonstrated by whole mount *in situ* hybridisation, in **(a, c)** *Lp/+* and **(b, d)** *Lp/Lp* embryos at approximately 9 days 8 hours of gestation. The embryos shown are littermates with 23 and 18 somites respectively. The expression in the floor plate of *Lp/Lp* embryos appears broader in the region around the level of the forelimb bud **(arrow in b)**. In 20 μm transverse sections through this region **(c-d)**, the expression in the floor plate appears to extend further laterally into the neural folds of *Lp/Lp* embryos **(arrow in d)**. The apparent discontinuity in the floor plate of the *Lp/+* embryo **(c)** is a fixation artefact.

F= floor plate, S= somites. The scale bar represents 0.8 mm in a-b and 0.2 mm in c-d.

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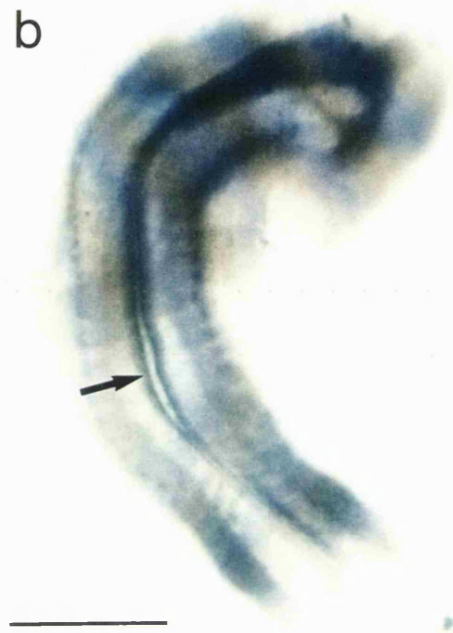
7.2.4 Expression of *Pax-3* in embryos on day 10 of gestation

Whole mount *in situ* hybridisation and sectioning of +/+ and *Lp*/+ embryos at 10 days of gestation reveals *Pax-3* expression in several sites, confirming previous reports (Goulding *et al.*, 1991). Expression extends the length of the neural tube in which it is restricted to the dorsal region (Figure 7.6). In the cranial region, expression is detected in the midbrain, hindbrain and craniofacial region. A stream of *Pax-3* positive cells is also observed in the sixth branchial arch which is thought to correspond to neural crest cells migrating into the outflow tract of the heart (Conway, Henderson and Copp, Unpublished). *Pax-3* is also expressed in the limb buds and in the somites where it is localised to the dermomyotome. Transverse sections also reveal staining in the dorsal root ganglia lateral to the neural tube (Figure 7.6c-d).

There is an altered structural relationship of the tissues in *Lp/Lp* embryos, in which the open neural folds lie flat on the dorsal surface of the embryo. Despite these differences in morphology, the overall level and sites of expression of *Pax-3* appear closely similar in *Lp/Lp* compared with unaffected embryos. However, structural abnormalities may hinder assessment of subtle expression differences. In whole mounts the staining in the somites, whose pattern is influenced by signals from the notochord, does appear to be altered in *Lp/Lp* embryos. Compared with *Lp*/+ and +/+ embryos, *Pax-3* expression is relatively more intense in the dorsal part of the somites of *Lp/Lp* embryos (Figure 7.6a-b). However, in sections the staining in the dermomyotome appears very similar between normal embryos and those with open neural tubes (Figure 7.6c-f), suggesting that the different appearance in whole mounts may be due to the altered morphology. In the neural tube there may a greater distance between the floor plate and the ventral boundary of the *Pax-3* expression domain, which suggests that the *Pax-3* expression has been dorsalised. However, the extent of *Pax-3* expression relative to the size of the neural tube may be unaltered since the neuroepithelium is flatter and extends further laterally (Figure 7.6c-f).

Whole about
PAX 3

Figure 7.6 Expression of *Pax-3* in +/+ and *Lp/Lp* embryos on day 10 of gestation

Pax-3 expression, as demonstrated by whole mount *in situ* hybridisation, in (a, c, d) +/+ and (b, e, f) *Lp/Lp* embryos at approximately 10 days 8 hours of gestation (embryos shown are littermates with 31 and 28 somites respectively). 20 µm transverse sections (c-f) indicate that expression is present in the dermomyotome of the somites, the limb buds, the dorsal root ganglia and in the dorsal region of the neural tube. The axial level of the examples shown are (c, e) at the level of the forelimb buds and (d, f) at a more caudal level between the fore and hindlimb buds. These levels of sections are indicated by white lines in a (marked c and d) and are at equivalent positions in b (e, f). +/+ and *Lp*/+ embryos were indistinguishable.

Arrow in a indicates staining of cells in the sixth branchial arch, which correspond to cardiac neural crest cells (Conway, Henderson and Copp, Unpublished). N= neural tube, L= forelimb bud, M= midbrain, D= dermomyotome, G= dorsal root ganglia. The scale bar represents 1 mm in a-b and 0.2 mm in c-f.
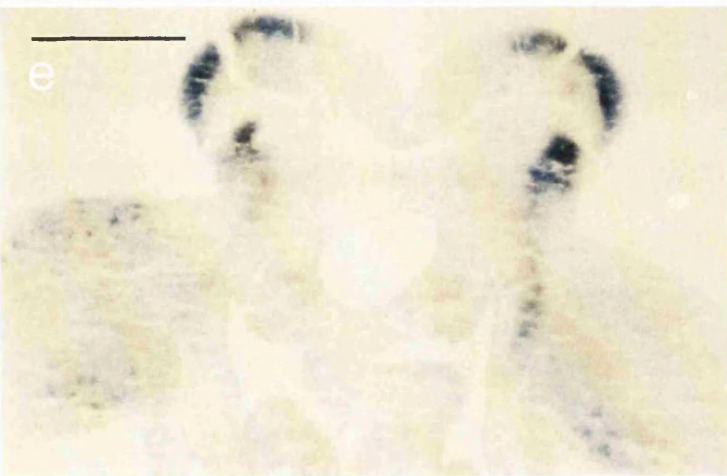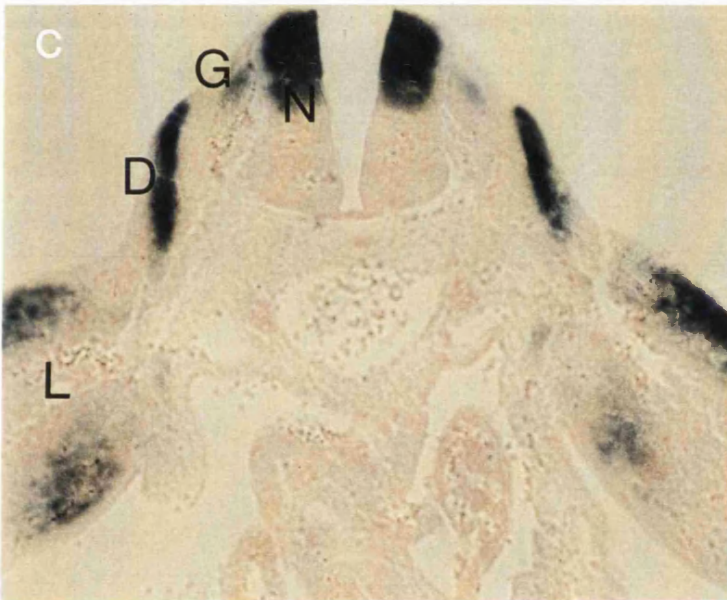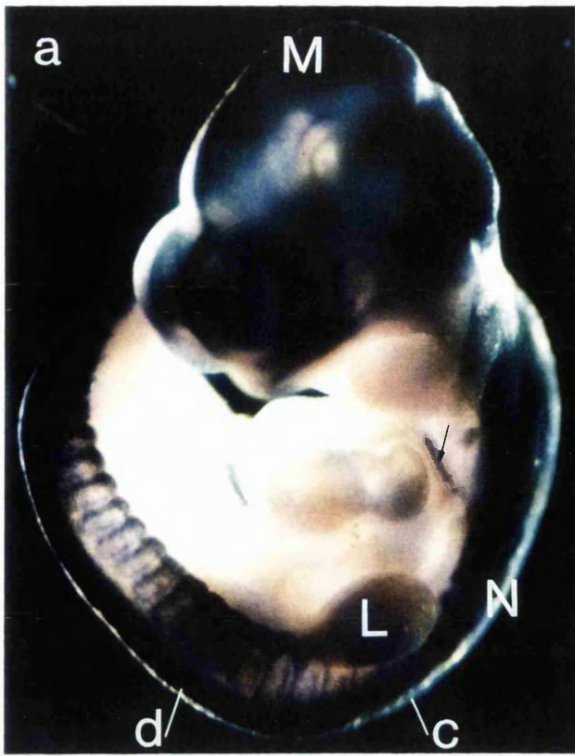
7.3 Discussion

The experiments described in this chapter demonstrate an abnormal pattern of expression of *Shh* in the notochord of *Lp/Lp* embryos on day 8 of gestation (3-8 somite stage), compared with *Lp/+* and *+/+* embryos. The domain of expression in the notochord appears broad, or even split, in a region underlying somites 3-4 (Section 7.2.1). This corresponds to the region of closure 1, the affected event in *loop-tail*, and the defect precedes the failure of neural tube closure suggesting that the abnormality of the notochord may underlie the development of NTD. One potential approach to test this hypothesis would be to ablate the notochord of *Lp/Lp* embryos. As described below the absence of a notochord does not prevent neural tube closure, and therefore notochord-ablation of *Lp/Lp* embryos may alleviate an inhibitory effect of the *loop-tail* notochord on neural tube closure and ameliorate the development of NTD.

The proposition that the abnormal expression of *Shh* in the notochord of *Lp/Lp* embryos indicates a defect in development of this tissue is supported by recent immunohistochemical studies in our laboratory using carbonic anhydrase as a marker of the notochord (Gerrelli and Copp, Unpublished). The expression of carbonic anhydrase may be down-regulated in *Lp/Lp* embryos compared with *Lp/+* or *+/+* embryos, although in transverse sections cellular material is present at the expected site of the notochord. Although, the down-regulation of carbonic anhydrase contrasts with an apparent up-regulation of *Shh* expression, these may both reflect a defect in development of the notochord. Furthermore, as described in Section 7.1, ultrastructural analysis of embryos at nine days of gestation reveals that *Lp/Lp* embryos may exhibit an alteration in the separation of the notochord from the gut (Wilson and Wyatt, 1991), perhaps indicating an alteration in surface properties of the notochord cells.

At later stages, days 9 and 10 of gestation, the expression of Shh also appears abnormal in the floor plate of the neural tube (Section 7.2.2). The expression domain appears broad, with weaker expression in the midline at certain levels of the body axis (Figures 7.3 and 7.4). By day 10, this abnormal domain of *Shh* expression extends along the

entire length of the open neural tube. The abnormal appearance of Shh expression on day 10 of gestation could potentially be a consequence of altered morphology due to the presence of a persistently open neural tube. However, sections of embryos suggest that *Shh* expression in the floor plate extends further laterally in *Lp/Lp* compared with *Lp/+* and +/+ embryos.

The expression pattern of *netrin-1* in the floor plate corresponds to that of *Shh*, with a similar broad domain of expression in the region of open neural tube, caudal to the forelimb bud on day 9 of gestation (Section 7.2.3). These observations indicate that, in *Lp/Lp* embryos, there is an abnormality of differentiation of the floor plate itself rather than an isolated defect in the expression of Shh. The expression of *Shh* at other sites, in the hindgut and limb buds, is apparently normal in *Lp/Lp* embryos, further suggesting a defect of floor plate and notochord structure rather than an anomaly of expression of *Shh per se*. Moreover, histological analysis and scanning electron microscopy (Gerrelli, Van Straaten and Copp, Unpublished) suggest that on day 8 of gestation the floor plate of *Lp/Lp* embryos is abnormally broad resulting in a U shape of the neural folds around the site of closure 1 rather than the usual V shape. These observations suggest a model in which a defect in floor plate induction arises as a consequence of the preceding abnormality of development of the notochord.

This model contrasts with the findings of Wilson and Wyatt (1995) who concluded that the floor plate and the dorsoventral polarity in the neural tube of mutant embryos is essentially normal despite its altered positional relationship to other tissues (Wilson and Wyatt, 1995). This conclusion was based on immunofluorescent analysis of the floor plate, which they recognised by its increased binding of the lectin concanavalin A (Con A), compared with the neural folds. The pattern of staining, at days 10-12 of gestation, appeared similar between normal embryos and those with an open neural tube (presumed *Lp/Lp*). In addition, use of an antibody to the neural cell adhesion molecule (NCAM) and Con A as markers of neural cell organisation indicated that the ventral motor neurons, and the commissural axons which cross the floor plate, develop normally in

Lp/Lp embryos (Wilson and Wyatt, 1995). However, this study differs from the experiments described in this thesis in several respects. The use of molecular markers in the present study indicates a subtle abnormality of the floor plate which may not be detectable by histochemical analysis. *Shh* seems likely to be a good functional marker of the floor plate since it mediates the signalling properties of the floor plate (and notochord) in dorsoventral patterning (Section 1.8). In addition, the present study addresses the early development of the floor plate (days 8-10 of gestation) whereas in the study of Wilson and Wyatt (1995) embryos were analysed only at 10-12 days of gestation. Finally, embryos in the present study were genotyped by a method independent of morphology whereas Wilson and Wyatt (1995) relied on the presence of persistently open neural folds to identify *Lp/Lp* embryos.

Previous studies involving ablation of the notochord in the chick and notochord-deficient genetic mutants in the mouse suggest that neural tube closure is not dependent on the presence of a normal notochord. *HNF3β* (*hepatocyte nuclear factor 3β*) null mouse embyros exhibit a complete lack of notochord and floor plate (Ang and Rossant, 1994). However, in these embryos the neural tube appears to close although, due to the absence of a median hinge point (MHP), it displays an uncharacteristic circular lumen reminiscent of Mode III neurulation which normally only occurs at caudal levels of the body axis (Section 1.1.4). Similarly, in embryos that are homozygous for the T^{Wis} mutation (a *Brachyury* mutation), notochord precursors form but fail to proliferate and differentiate. Although *Shh* is expressed in the region of the node and head process at 8 days of gestation, no expression can be detected in cells of the axial midline at later stages and no floor plate expression was detected at any stage. However, despite major abnormalities of somite development in these embryos, the neural tube, although severely kinked, does close (Conlon *et al.*, 1995).

The floor plate appears to be excessively broad in *Lp/Lp* homozygotes suggesting that, in contrast to the notochord mutants, in which the floor plate is absent, *loop-tail* may represent a notochord gain of function as regards floor plate induction. The notochord

abnormality is present prior to the stage of closure 1 and the induction of a broad floor plate could therefore potentially underlie the development of NTD, possibly due to a mechanical inhibition of neural fold elevation or apposition of the neural fold apices in the midline. Therefore, this model proposes that although the floor plate is not a requirement for neural tube closure, an abnormality of this structure is capable of causing NTD.

Since *Pax-3* expression in the dorsal neural tube is known to be inhibited by ectopic notochord grafts (Goulding *et al.*, 1993; Fan and Tessier-Lavigne, 1994), it might be predicted that an overabundance of notochord and floor plate material in *Lp*/Lp embryos would lead to a dorsalised expression domain of *Pax-3*. An alternative scenario would be that the apparent notochord abnormality in *Lp/Lp* embryos would lead to a reduced signalling activity in dorsoventral patterning and hence a ventralising effect on *Pax-3* expression. The loss of carbonic anhydrase reactivity in the *Lp/Lp* notochord (Gerrelli and Copp, Unpublished) indicates that the mutant notochord may display functional abnormalities, and so supports this second idea. On the other hand, the increase in the floor plate inducing activity of the notochord in *Lp/Lp* embryos argues against a general loss of notochordal function. Whole mount *in situ* hybridisation studies certainly do not demonstrate ventralisation of *Pax-3* expression and indeed may indicate a minor degree of dorsalisation (Section 7.2.4). *Pax-3* expression is first detected in a more dorsal location in the open neural folds of *Lp/Lp* compared with *Lp/+* and +/+ embryos. However, as the neural folds appear longer and flatter, the extent of the expression domain relative to the size of the neural folds may not be affected. Therefore, analysis of *Pax-3* expression in this study does not provide a definitive conclusion regarding an alteration in dorsoventral patterning in the neural tube of *Lp/Lp* embryos due to the distortion of the topological relationship of the neural folds to the other tissues. However, recent studies of *Pax-6* in genotyped *Lp/Lp* embryos (Gerrelli and Copp, Unpublished) indicates that the expression domain is shifted dorsally compared with *Lp/+* or +/+ embryos, again suggesting that *Lp/Lp* embryos may exhibit pattern dorsalisation in the neural tube.

The present study does not suggest any major effect of the *loop-tail* defect on patterning

of the dermomyotome since *Pax-3* expression in the somites appears similar between

Lp/Lp and control embryos (Figure 7.6). This suggests that, in terms of somite

patterning by the notochord and floor plate, there is neither a major loss nor a gain of

function, which would be predicted to lead to enlargement or reduction of the *Pax-3*

expression domain respectively. However, this is not a definitive conclusion as *Pax-3*

only marks a subsection of the somite. Indeed, recent sudies using *Pax-1* as a marker of

sclerotome have suggested an over production of sclerotomal cells in *Lp/Lp* embryos,

consistent with an increased inducing signal (probably *Shh*) emanating from the

notochord and floor plate (Gerrelli and Copp, Unpublished). Further analysis using

additional markers will be required to establish whether the patterning of the somites and

neural tube is altered in *Lp/Lp* embryos (see General Discussion).

CHAPTER 8

Discussion

8.1 Prevention of spinal NTD in *curly tail* mice by inositol treatment

The experiments described in this thesis demonstrate a preventive effect of inositol supplementation on spinal NTD in *curly tail* embryos treated either *in vivo* or *in vitro*. This is a particularly interesting observation since inositol is the first agent that has been demonstrated to reduce the incidence of spinal NTD in *curly tail* without any apparent deleterious effects on the embryo. Several agents have previously been shown to reduce the incidence of spinal NTD in *curly tail* mice (Table 1.4) but, with the exception of retinoic acid (RA), these are all thought to act through a non-specifc growth retarding influence which counteracts the growth imbalance in the caudal region. In contrast to the growth retardation caused by inositol deficiency (Cockroft *et al.*, 1992), there is no growth retarding effect of *in vitro* or *in vivo* inositol treatment nor of low dose RA, suggesting that both agents have a specific effect. In addition, all those agents (including RA) which reduce spinal NTD cause an increase in cranial NTD when added at an earlier stage of development, whereas inositol is required for cranial neural tube closure (Section 1.5.1). Thus, inositol appears to have a specific effect on closure of the PNP in *curly tail* embryos.

Labelling with [^{3}H] inositol indicates that *in vitro* supplementation with inositol leads to enhanced uptake and incorporation of inositol. I propose that this leads to increased flux through the inositol/lipid cycle and that this is specifically responsible for the preventive effect (Chapter 3). The downstream action of the inositol/lipid cycle appears to be mediated through the activation of protein kinase C: TPA, a PKC activator, mimics the effect of inositol while a specific PKC inhibitor abolishes the preventive effect of inositol (Chapter 4). It is possible that other inositol-dependent biochemical pathways (Section 1.5), not involving PKC, also play a role in the ameliorating effect of inositol. However, it does appear that PKC activation is sufficient to produce an equivalent reduction in PNP length as inositol in cultured embryos.

The protective actions of inositol and TPA treatment occur concomitantly with an up-regulation of RAR-β in the hindgut endoderm (Chapter 5). This up-regulation is implicated in the pathway leading to the correction of closure of the PNP, since RAR-β is known to be down-regulated in the hindgut of affected compared with unaffected *curly tail* embryos (Chapter 5, Chen *et al.,* 1995). Moreover, low-dose RA treatment which, like inositol and TPA, has a protective effect on spinal NTD also causes a similar up-regulation of RAR-β.

The mode of action of RAR-β is proposed to involve normalisation of growth rates in the caudal region, possibly mediated by stimulation of proliferation in the hindgut (Section 5.3), although this has not been tested directly. Such an effect would lead to the correction of curvature of the caudal region and hence lessen the delay in closure of the PNP. This potential model for the protective action of inositol is summarised in Figure 8.1.

8.2 The role of PKC in the protective effect of inositol

The possibility cannot be discounted that up-regulation of RAR-β in *curly tail* embryos following treatment with inositol, TPA or RA is a marker of, rather than a causal factor in, the normalisation of closure of the PNP. In addition to up-regulation of RAR-β expression in the hindgut, activation of PKC may also influence the activity of a range of other proteins involved in a variety of cellular functions. One or more of these target proteins may be involved in the protective effect on closure of the PNP and, in this respect, several proteins appear interesting, in particular MARCKS and MARCKS-related protein.

The myristoylated, alanine-rich C kinase substrate, MARCKS, is a widely expressed PKC substrate that is the major substrate in many cell types (Blackshear, 1993). The N-terminal myristoylated domain of the protein mediates binding to the plasma membrane where it can crosslink actin. PKC-dependent phosphorylation in the effector domain leads to dissociation of MARCKS from the membrane. Phosphorylated, cytosolic

Figure 8.1 Summary of a possible mechanism for the protective effect of inositol on spinal NTD

Double lines indicate potential sites of interaction of protective factors with the pathway leading to development of spinal NTD. The dotted line indicates a relationship which is predicted but has not tested experimentally thus far.

Curly tail

Inositol

Genetic modifiers

Inositol/lipid cycle

PKC activation

Either

Or

Or

RAR-β downregulation

RA

Cell proliferation defect in the embryonic hindgut

Growth imbalance in in the caudal region

Increased ventral curvature of the caudal region

Delayed closure of the posterior neuropore

Spinal NTD
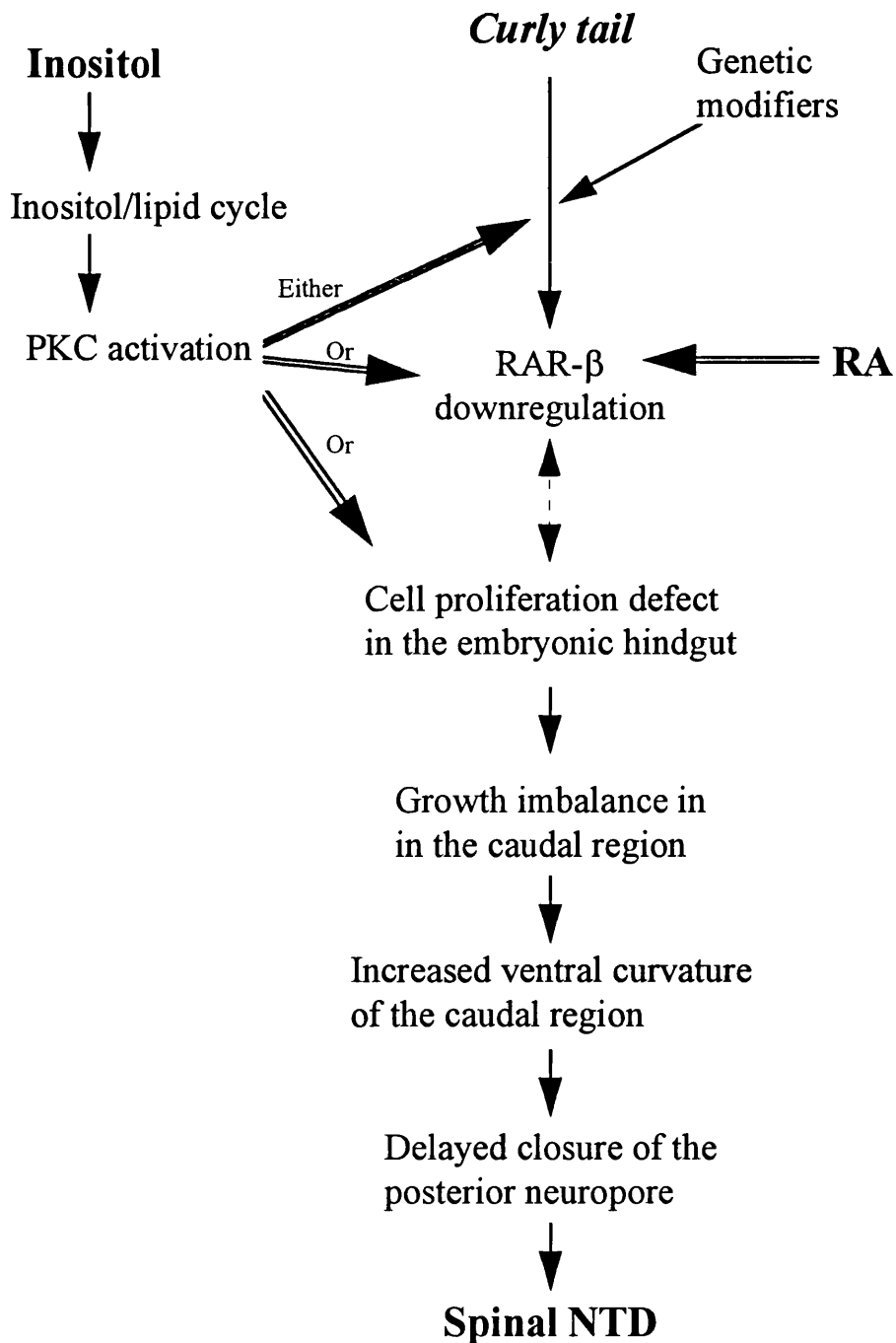
MARCKS protein still associates with, but does not crosslink actin filaments, potentially leading to local destabilisation and rearrangement of the actin cytoskeleton (Aderem, 1992; Aderem, 1995). In terms of neurulation, phosphorylation of MARCKS might be expected to lead to disassembly and rearrangement of apical microfilaments that are known to be important for neural fold elevation (Section 1.1.2).

In addition to a role in the regulation of the actin cytoskeleton, MARCKS may also be involved in the regulation of entry and exit from G_0, the quiescent state of the cell cycle. In Swiss 3T3 cells and mouse embryo fibroblasts MARCKS mRNA and protein is down-regulated following PKC activation and during rapid proliferation at low density (Brooks *et al.*, 1991; Herget *et al.*, 1993). Expression of both mRNA and protein is then markedly up-regulated as cells enter G_0 following depletion of serum-derived growth factors (Herget *et al.*, 1993). Therefore, MARCKS would be predicted to be down-regulated in the rapidly proliferating hindgut of the embryo and possibly up-regulated in the affected hindgut of *curly tail* embryos. However, the situation in fibroblasts may not be relevant in this case as evidence there is no evidence for the presence of a G_0 sub-population in the neurulation-stage embryo (Copp *et al.*, 1988a).

As a potential PKC target during neural tube closure, MARCKS appears to be particularly interesting since MARCKS null mutant mice exhibit exencephaly and die in the perinatal period (Stumpo *et al.*, 1995). In *curly tail* embryos, functions in PNP closure could be envisaged either for regulation of the actin cytoskeleton (in the neuroepithelium) or the exit of cells from G_0 (normalisation of proliferation in the hindgut). However, the absence of spina bifida in MARCKS null mutants suggests that there may be fundamental difference in the mechanism of development of NTD compared with *curly tail* embryos.

MARCKS-related protein (MRP, also called F52 or MacMARCKS) is encoded by one of a group of genes in the mouse which are related to MARCKS (Li and Aderem, 1992; Lobach *et al.*, 1993). This protein is particularly striking in terms of the *curly tail* defect,

because not only is it a cellular substrate for PKC but also the *Mrp* gene maps to mouse chromosome 4 within the *curly tail* region. Moreover, the MRP null mutant mouse exhibits NTD comprising cranial (60%) and spinal (10%) NTD (Wu *et al.*, 1996), while in another report embryos exhibit cranial NTD (100%) alone (Chen *et al.*, 1996). MRP is also regulated by retinoic acid; expression is induced in P19 embryonal carcinoma cells treated with RA (Jonk *et al.*, 1994) and this is particularly striking in view of the protective effect of RA on spinal NTD in *curly tail*.

Wu et al. (1996) suggested that *Mrp* is not the *curly tail* gene since sequencing reveals no mutation in the coding region. In addition, crosses between F52 and *curly tail* homozygotes are reported to suggest that the two mutations are non-allelic although no data have been published (Wu *et al.*, 1996). In our laboratory, *Mrp* has not so far been excluded by recombination as a candidate for *curly tail* (Pavlovska & Copp, Unpublished). Therefore, the possibility remains that *curly tail* may represent a mutation in the 5' or 3' untranslated regions of the *Mrp* gene, perhaps producing a mis-regulation of the *Mrp* gene product

8.3 Potential efficacy of inositol in prevention of human NTD

As described in Section 1.2.3, clinical trials suggest that approximately 30% of NTD in humans do not respond to folic acid supplementation, the only preventive therapy currently known. Based on the similarities to human NTD and the absence of a protective effect of folates, the *curly tail* mouse appears to provide a good model for folate-resistant spinal NTD (Section 1.4.3). In the present study the efficacy of inositol in prevention of spina bifida in a folate-resistant mouse strain raises the possibility of a beneficial use in humans.

As described above, inositol is the only agent known to have an ameliorating affect on spinal NTD in *curly tail* embryos without apparent deleterious effects in terms of either teratogenicity or growth retardation. Moreover, clinical trials in humans indicate that an inositol dose of 12g/day (compared to approximately 1g/day in the average person's diet)

provides an effective therapy for panic disorder and depression (Benjamin *et al.*, 1995; Levine *et al.*, 1995). In these studies no major clinical side effects were observed and no harmful effects of inositol treatment were indicated by assessment of kidney function, liver function and blood chemistry.

Interestingly, the greatest effect of *in vivo* inositol supplementation on spinal NTD in the mouse is the reduction in frequency of spina bifida, with only a small effect on tail flexion defects. The reduction in spina bifida appears most relevant to the human situation in which obviously there is no tail. Thus, whereas the ameliorating effect of inositol in *curly tail* may shift a putative spina bifida to a tail flexion defect the corresponding effect in humans would be proposed to produce a normal embryo.

Inositol represents a potential therapy for prevention of folate-resistant spina bifida in humans. *In vitro* and *in vivo* mouse studies and human psychiatric trials indicate that inositol is safe for human therapy although the pharmokinetics of uptake by the embryo requires further investigation. Inositol could be readily combined with folate as a periconceptional treatment and there is thus a strong case for consideration of a controlled trial in humans for supplementation with folate alone compared with combined folate and inositol.

8.4 Requirement for a congenic *curly tail* strain

As descibed in Section 1.4.2, one problem encountered in interpretation of experimental data from *curly tail* embryos is the lack of a control strain that differs only at the *curly tail* locus. There is no wildtype allele in the *curly tail* colony as the *curly tail* mutation is maintained homozygous form. Therefore, it is not clear whether "unaffected" *curly tail* mice (i.e. those that do not develop NTD) are normal relative to a wildtype strain or are in fact influenced by the *curly tail* mutation in a more subtle manner. For example, the uptake of transferrin by the hindgut of unaffected *curly tail* embryos appears enhanced compared with that seen in non-mutant embryos. This may represent a compensatory response to the *curly tail* defect (under-proliferation of hindgut cells) or alternatively

could be simply a strain difference (Hoyle *et al.* 1996, Section 1.4.6). A similar problem is associated with the interpretation of the increased uptake of [^{3}H] inositol by *curly tail* compared with CBA/Ca embryos (Chapter 3).

Differentiation between the the effects of strain differences and the *curly tail* defect could be resolved by the generation of congenic strains, pairs of strains which differ only at the *curly tail* locus as a result of successive back-crossing. In our laboratory a congenic strain is currently being generated (Pavlovska and Copp, Unpublished): *curly tail* and SWV (the strain selected to provide the wildtype, +, allele) mice were crossed to generate *ct/+* heterozygotes. These mice were back-crossed to *ct/ct* mice in order to increase penetrance of the mutant trait and *ct/+* animals generated in the cross were identified by PCR analysis on the basis of heterozygosity for a number of microsatellite markers spanning the *ct* locus. In this manner *ct/+* mice were back-crossed to *ct/ct* mice for four generations in order to fix the background as *curly tail* except at the *ct* locus where heterozygosity was maintained. A fully congenic strain will be generated by brother-sister mating of *ct/+* mice for twenty generations.

8.5 A potential role for abnormalities of notochord and floor plate in development of NTD

In the *curly tail* mouse, NTD result from delayed closure of the posterior neurpore resulting from a mechanical force imposed by increased ventral curvature of the caudal region (Section 1.4.4) and this is ameliorated following inositol supplementation (this study) . In the present study, I have also addressed the question of possible factors which could contribute to the failure of neural tube closure in a different mutant, *loop-tail*. The gene expression studies described in Chapter 7 suggest that there is a defect of *Shh* expression in the notochord of *Lp/Lp* embryos at the 4-7 somite stage. Subsequent induction of an abnormally wide floor plate is indicated by altered expression domains of *Shh* and *netrin-1*.

These observations raise questions about the origin of the notochord abnormality in *loop-tail* and its potential effect on patterning of other tissues such as the somites and neural tube. The notochord is clearly present in *Lp/Lp* embryos, on the basis of *Shh* expression, but there may be a problem in its early development which leads to altered structure and/or *Shh* expression pattern. Further investigations will be facilitated by the use of additional early markers of the notochord. The transcription factor, *HNF-3β*, is required for development of the notochord (Ang and Rossant, 1994) where it is expressed from the first stage of notochord formation (Echelard *et al.*, 1993; Ruiz i Altaba *et al.*, 1995). *HNF-3β* is also expressed in the floor plate following induction by signals from the notochord, mediated by *Shh* (Echelard *et al.*, 1993; Roelink *et al.*, 1994; Ruiz i Altaba *et al.*, 1995). Analysis of its expression will, therefore, provide not only an early marker of notochord differentiation but also another marker of the proposed floor plate defect in *Lp/Lp* embryos.

As described in Chapter 7, the observation of apparent abnormalities in the expression of *Shh* in the notochord and floor plate of *Lp/Lp* embryos raises the possibility that, as in notochord-deficient mutants (Dietrich *et al.*, 1993) and the functional mutant for *Shh* (Chiang *et al.*, 1996), there is also an influence on patterning of the somites and neural tube. In the present study, *Pax-3* has been used as a dorsal marker since it is expressed in the dermomyotome and the dorsal neural tube and is influenced by notochordal signals (Goulding *et al.*, 1991; Goulding *et al.*, 1993). However, the interpretation of results is hindered by the morphological defects of *Lp/Lp* embryos (Chapter 7).

The assessment of ventral markers will provide further information about potential alterations in dorsoventral patterning, since the expression domains of such markers may be altered in response to the abnormal notochord and floor plate in mutant embryos. Further members of the *Pax* gene family may provide useful markers (Gruss and Walther, 1992). The expression domain of *Pax-6* in the neural tube is complementary to and overlaps that of *Pax-3*, being present in a mid-lateral band in the basal plate, excluding the floor plate (Gruss and Walther, 1992). Moreover, like *Pax-3*, the

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expression of *Pax-6* becomes dorsally restricted following implantation of an ectopic notochord graft (Goulding *et al.,* 1993). As a ventral marker of the somites, *Pax-1* may be useful since it is expressed in the sclerotome (Deutsch *et al.,* 1988) in a domain which responds to notochordal signals. In mutants, such as *Danforth's short-tail*, in which the notochord is absent or degenerates *Pax-1* expression is reduced and abnormalities of sclerotome development arise (Dietrich *et al.,* 1993; Koseki *et al.,* 1993). Targeted disruption of *Shh* has a similar effect on *Pax-1* expression (Chiang *et al.,* 1996). In contrast, implanted grafts of notochord or *Shh*-expressing cells induce expression of *Pax-1* at ectopic locations (Fan and Tessier-Lavigne, 1994; Johnson *et al.,* 1994).

Does the apparent abnormality in the structure of the notochord and floor plate underlie the development of NTD? This is plausible in terms of the temporal sequence of events since alterations of gene expression (this study) and floor plate shape (Gerrelli, Van Straaten and Copp, Unpublished) precede the failure of closure 1 in *Lp/Lp* embryos. Therefore, although the floor plate is not required for neural tube closure to occur (Section 7.3), the presence of an abnormally wide floor plate may inhibit closure. In this model neural tube closure would potentially be mechanically inhibited due to the altered topology of the neuroepithelium. Neural tube closure in *curly tail* embryos is also mechanically inhibited (due to curvature of the neuroepithelium in the caudal region), raising the possibility that such a mechanism may lead to inhibition of neural tube closure in a number of NTD mutants and at different axial levels.

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