Int. J. Dev. Biol. 41: 199-212 (1997)

Mechanisms of normal and abnormal neurulation: evidence from embryo culture studies

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ABSTRACT The method of whole embryo culture has been used extensively in analyzing the mechanisms underlying formation of the mammalian neural tube. These studies have provided insight into the cell lineage of the various tissues that comprise the neurulation stage embryo, the role of microfilaments, extracellular matrix and cell proliferation in the morphogenetic events of neural tube closure and the action of specific genes and gene products in establishment of the nervous system. This information is of considerable importance not only as a means of elucidating the processes of normal embryogenesis but also to shed light on the pathogenesis of important human birth defects.

KEY WORDS: *neural tube defects, diabetes, folate, mutant genes, teratogen*

Introduction

Neurulation is a term describing the series of embryonic events by which the neural plate forms, undergoes folding of its lateral edges and subsequently fuses in the dorsal midline, to create the neural tube. Strictly, this description applies to the process of primary neurulation, in which the neural tube in the cranial and upper spinal regions is formed. Secondary neurulation is a superficially quite different process, occurring only at the lower sacral and caudal levels of the body axis, in which the neural tube forms from condensation of mesenchymal cells and cavitation of epithelia within the tail bud. In this review we concentrate on the mechanisms of primary neurulation, for which there is much more information available.

An understanding of the cellular and molecular mechanisms of neurulation is desirable for two main reasons. Firstly, neurulation is a fundamental component of embryonic development. It is important that we understand not only the mechanisms by which neurons and glia differentiate, and neuronal connections are formed, but also the morphogenetic events whereby the primordium of the central nervous system is established. Secondly, defects of neurulation result in the important category of human malformations, neural tube defects (NTD). It is essential that we gain an insight into the fundamental pathogenetic mechanisms of NTD if we are to develop more sophisticated methods for diagnosis and, ultimately, for treatment of these defects *in utero*.

In this short review, we do not attempt to be exhaustive in our coverage of the extensive field of neurulation and NTD. Instead, we consider aspects of this subject in which studies using the technique of whole embryo culture, as established by Denis New (1978), have proven particularly informative in understanding the developmental mechanisms. We concentrate on the following areas of research:

- Cell lineage analysis and the establishment of neurectodermal and non-neural tissues
- Morphogenetic principles of neurulation
  - heterogeneity of mechanism along the body axis
  - role of microfilaments in neurectodermal folding
  - role of extracellular matrix in expansion of the paraxial mesoderm
  - role of cell proliferation and curvature of the body axis
- Molecular basis of neurulation
  - gene expression and gene inactivation
  - other molecules
- Metabolic disturbances that cause NTD in humans

Cell lineage analysis: establishment of the neurectodermal and non-neural tissues

Understanding the origin of the various cell lineages that comprise the neurulation stage embryo provides a basis for investigation of the mechanisms of morphogenesis that ensue during neural tube formation. Although cell lineage studies have been performed in lower vertebrate embryos since the early decades of the 20th century, it was only with the advent of whole embryo culture that comparable studies became technically feasible in mammals. The primary requirement is an ability to mark cells within the embryo in a manner that permits the mitotic descendants of those cells to be recognized after some further period of development. This allows identification of the marked cell progeny in terms of their position in the later stage embryo and, in some cases, their state of cytodifferentiation. To date, several methods for marking cells

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0214-6282/97/$05.00

Printed in Spain

have been used in studies of cell lineage in the pre-gastrulation and gastrulation stage mouse embryo, including single cell injection with horseradish peroxidase, permitting clonal analysis (Lawson *et al.*, 1991), labeling of groups of cells using fluorescent dyes such as DiI and DiO (Smith *et al.*, 1994; Quinlan *et al.*, 1995), labeling of the entire epiblast layer with a wheat germ agglutinin-gold conjugate (WGA-gold) (Tam and Beddington, 1992) and grafting of small clumps of cells pre-labeled with ^{3}H-thymidine (Beddington, 1981, 1982; Copp *et al.*, 1986) or WGA-gold (Tam, 1989). Although no single worker has mapped cell lineage within the entire embryo, a composite of the various studies permits a good appreciation of the origin of the various parts of the neurulation stage embryo.

The basic approach is to construct a series of fate maps, ideally one for each developmental stage, in which the developmental behavior of cells and their mitotic descendants is described in terms of their future position in the embryo and their tissue derivatives. In practice, the cell lineage studies in the mouse have established fate maps for the pre-gastrulation stage embryo (6.5 days of gestation) and the gastrulation stage (7.5 days). In both cases, the position of marked cells has been followed in embryo culture until the main regions of the neural tube/plate are clearly recognizable following the start of neurulation (8.5 days).

The fate map is an indication of normal, undisturbed development and, as such, provides no information on the developmental potential of cells, or their state of commitment or determination to follow a particular lineage. These properties can be tested by challenging cells to alter their fate, for instance by transplantation to a position in the embryo which they do not normally encounter (i.e. heterotopic transplantation). Then, alteration of fate indicates lack of cell commitment, whereas maintenance of fate is consistent with, but does not prove, that the cell is already committed to a given fate, as indicated by the fate map. Although heterotopic grafting has been performed in a few studies (Beddington, 1981, 1982), there is still relatively little information on the potency of cells in the gastrulation and pre-gastrulation stage embryo.

Several important points arise from the cell lineage analysis. First, similarity between the fate maps of the mouse and those of lower vertebrates, namely the chick and frog, supports evidence from studies of gene sequence and gene expression, arguing that development in the vertebrates is homologous, with relatively minor variations on a common theme of embryogenesis. Second, all of the "germ layers" of the mouse embryo (i.e. ectoderm, mesoderm and endoderm) arise from the epiblast (also called the embryonic ectoderm) of the pre-gastrulation stage embryo. At 6.5 days, the embryo comprises two layers: the inner epiblast borders the proamniotic cavity, with the visceral endoderm (equivalent to hypoblast in the chick) on its outer surface. Cell lineage studies at the blastocyst stage have shown that the visceral endoderm has potential only to form the endodermal layer of the yolk sac, and does not contribute to the endoderm of the gut (Rossant *et al.*, 1978; Gardner and Rossant, 1979). This has been confirmed by clonal analysis of visceral endodermal cell fate in postimplantation embryos (Lawson *et al.*, 1986; Lawson and Pedersen, 1987). On the other hand, descendants of the epiblast can be found in all layers of the embryo, and in extraembryonic tissues, following labeling at pre-gastrulation and gastrulation stages (Beddington, 1981, 1982; Copp *et al.*, 1986; Tam, 1989; Lawson *et al.*, 1991; Quinlan *et al.*, 1995).

A third important point that arises from the fate map of the mouse embryo concerns the relative proportion of the epiblast that is destined to form neurectoderm, as opposed to mesoderm, at different stages of development. In the pre-gastrulation stage embryo, only the tip of the egg cylinder contains precursors of the neurectoderm (Quinlan *et al.*, 1995), whereas the majority of cells, located more proximally in the epiblast, give rise predominantly to surface ectoderm, mesoderm and extraembryonic tissues. However, in the gastrulation stage embryo where mesoderm formation is already underway via ingression of epiblast cells at the primitive streak, a much larger proportion of epiblast cells are destined to form neurectoderm and surface ectoderm. The cells fated to form mesoderm are located more posteriorly, adjacent to or within the primitive streak. It seems that, as the embryo progresses into gastrulation, the portions of the epiblast destined to form mesoderm converge towards the posterior midline of the embryo, so that cells with an ectodermal fate come to occupy an ever greater proportion of the epiblast, the future outer layer of the embryo.

There is also evidence of regionalization among cells that are fated to form a particular germ layer. In the pre-gastrulation embryo, marked cells in the anterior region of the distal cap provide descendants most often in forebrain and midbrain whereas cells in the posterior part of the distal cap colonize the hindbrain and spinal cord (Quinlan *et al.*, 1995). At the gastrulation stage, descendants of cells in the midline anterior epiblast are found primarily in the floor plate of the cranial neural tube, whereas those more laterally colonize the walls of the neural tube, or form surface ectoderm and/or neural crest (Tam, 1989). Similar regionalization occurs within the mesoderm-forming cells of the primitive streak: cells at the tip of the primitive streak (the node) form mainly notochord and endoderm, those behind the tip form paraxial mesoderm whereas those at caudal levels within the streak form lateral plate and extraembryonic mesoderm (Smith *et al.*, 1994).

In conclusion, the availability of embryo culture has permitted a number of cell labeling studies that provide a fairly detailed fate map of the pre-neurulation stage mouse embryo. A challenge for the future will be to determine at what stage in the divergence of the lineages cells become irreversibly committed to a differentiated fate, and how these developmental decisions are achieved in terms of differential gene expression.

Morphogenetic principles of neurulation

Over the decades, there have been numerous speculations about the morphogenetic basis of neural tube closure and the underlying mechanisms of NTD (see reviews: Copp, 1983; Gordon, 1985; Schoenwolf and Smith, 1990). Arguments have centred upon whether NTD arise primarily from failure of closure of the neural tube or secondary re-opening, whether the ability to form a neural tube is intrinsic to the neurectoderm or requires the participation of adjacent, non-neural tissues, and whether differential rates of cell proliferation play an important role in neural tube closure. In this section, we review evidence that sheds light on these arguments, which has arisen largely from the ability to observe and manipulate mouse and rat embryos undergoing neurulation *in vitro*.

Heterogeneity of neurulation mechanism along the body axis

Although neurulation can be described as a craniocaudal sequence of morphogenetic movements resulting in the formation of the neural tube, when analyzed more closely, the process is seen

to be discontinuous. In the mouse, neural tube closure begins at discrete sites of *de novo* contact (Fig. 1) and fold fusion proceeds from these initiation sites in both cranial and caudal directions leaving neuropores – regions of open neural tube between closure points – that ultimately close, completing primary neurulation. Although direct evidence from human embryos is lacking, the pattern and variety of human NTD suggest the discontinuous mode of neural tube closure is conserved among mammals (Van Allen *et al.*, 1993; Golden and Chernoff, 1995; Seller, 1995).

The discontinuous pattern of mouse neural tube closure was described originally by observations on embryos removed from the uterus at varying times between 8.5 and 10.5 days of gestation (Golden and Chernoff, 1993). More recently, using the whole embryo culture system, it has been possible to confirm that the events shown in Figure 1 do indeed occur sequentially in individual embryos in culture. Thus, closure 1, in which the neural folds contact each other *de novo* at the cervical/hindbrain boundary in embryos with 5-7 somites, has been observed in cultured embryos, and this event is known to fail in embryos homozygous for the *loop-tail (Lp)* mutation leading to the severe NTD, craniorachischisis (Copp *et al.*, 1994). Closure 2 has been observed in normal cultured embryos at the 11-12 somite stage, with failure of this closure event in all embryos of the SELH strain, resulting in a 17% incidence of exencephaly (MacDonald *et al.*, 1989). Closure of the posterior neuropore can also be observed *in vitro* at the 28-30 somite stage in normal embryos and this event is disturbed in embryos homozygous for the *curly tail (ct)* mutation, leading to development of spina bifida and tail defects (Copp *et al.*, 1993).

These observations lead to two important conclusions. Firstly, there are dramatic variations in the timing and mode of neural tube closure at different levels of the body axis. The evidence from the mutant studies shows that the different neurulation events require different gene products for successful completion, pointing to fundamental variations in the mechanism of neurulation along the body axis. Isolation of the mouse genes that control the various events of neurulation will provide a starting point for understanding the genetic causes of human NTD. Secondly, most of the major categories of human NTD, which affect both the cranial and spinal regions, occur also in mouse mutant strains, arising by failure of primary neural tube closure at different levels of the body axis.

Role of actin-containing microfilaments

Proponents of the view that the ability to form a neural tube is an *intrinsic* property of the neurectoderm most frequently cite contraction of apically arranged microfilaments as the mechanism of neural folding (Karfunkel, 1974). Thick subapical microfilament bands have been detected by transmission electron microscopy at the time and in the position at which the cranial neural plate undergoes folding (Baker and Schroeder, 1967; Nagele and Lee, 1980; Wiley, 1980). The distribution of spectrin (fodrin), an actin-binding protein, also correlates with appearance of microfilaments. It is deposited as the cranial neural folds converge and is most prominent in regions of greatest bending (Sadler *et al.*, 1986).

These findings are consistent with a role for actin-containing microfilaments in neural fold elevation. To test this idea experimentally, several workers have used cytochalasins or calcium chelating agents to inhibit microfilament polymerization in cultured rat embryos during neurulation *in vitro*. Short term exposure to cytochalasin D causes the cranial neural folds to collapse, followed by recovery if the embryos are transferred to drug-free medium (Morriss-Kay and Tuckett, 1985). Exencephaly is also observed when embryos are treated with cytochalasins D and E on days 7 and 9 of gestation *in vivo* (Austin *et al.*, 1982). Moreover, EDTA, calcium antagonists or calmodulin antagonists all produce exencephaly in a reversible manner in rat embryo cultures (Smedley and Stanisstreet, 1985). Although these findings provide evidence for an important role of actin-containing microfilaments in mammalian cranial neural tube closure, it is important to note the following. Firstly, microfilaments may not be required for neural fold elevation *per se*, but rather to stabilise the neural folds once they have reached an elevated configuration. Second, it is not known whether spinal neurulation is dependent on actin-containing microfilaments. Third, a careful dose-response study of cytochalasin D treatment in the hamster has shown that exencephaly can occur at doses that are too low to cause microfilament disassembly, suggesting that cytochalasins may exert their effects via cellular targets other than microfilaments (Wiley, 1980). Therefore, the precise role of actin-containing microfilaments in neurulation remains to be determined.

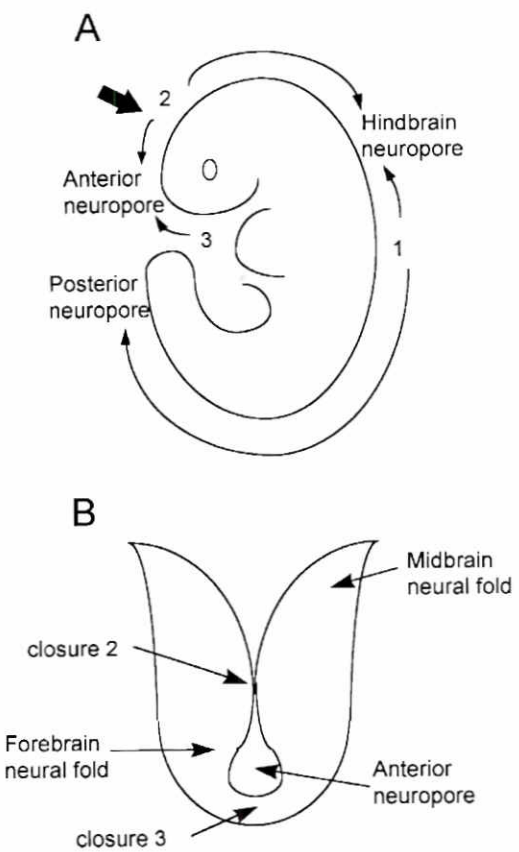

Fig. 1. *De novo* initiation sites of neurulation. (A) *Diagram of sites and directions of neural tube closure along the body axis, as projected onto an embryo following axial rotation. The hindbrain neuropore lies between closure points 1 and 2, the anterior neuropore between 2 and 3 and the posterior neuropore is the most caudal region remaining from closure 1. Modified after Copp and Bernfield (1994).* **(B)** *View from the front of the cranial region (direction of view indicated by black arrow in* **A***) of a pre-axial rotation embryo illustrating closures 2 and 3 and location of the anterior neuropore.*

Role of the extracellular matrix

The extracellular matrix (ECM) has been proposed as a potential driving force of neurulation, extrinsic to the neurectoderm. As the neural folds elevate in the cranial region, the paraxial mesoderm expands, largely owing to expansion of the mesodermal ECM which appears to create a swelling pressure within the mesenchyme (Morriss and Solursh, 1978a,b; Morris-Wiman and Brinkley, 1990b). This is not the case in the spinal region, however, where the closing neural folds are flanked either by epithelial somites (e.g. at closure site 1; Fig. 1) or by presomitic mesoderm (e.g. during posterior neuropore closure). In both cases, the mesoderm comprises relatively close packed cells, with minimal intercellular spaces.

A number of workers have described the distribution of molecular components of the ECM in relation to the neurulation process in mouse and rat embryos. Synthesis of the glycosaminoglycan (GAG) hyaluronan (HA) increases from pre-primitive streak stage to the 10 somite stage in the rat embryo, and its increasing level, and increasing state of hydration, correlate with expansion of the extracellular spaces within the cranial mesoderm during the formation of biconvex neural folds (Morriss and Solursh, 1978a; Morris-Wiman and Brinkley, 1990c). By contrast, levels of sulphated GAGs remain low during neurulation and tend to increase in the later stages of cranial neurulation, when the biconvex neural folds are converted to a V shape (Solursh and Morriss, 1977), or after neural tube closure in the caudal region (Copp and Bernfield, 1988a). Of the ECM glycoproteins, fibronectin increases in the forebrain region at the time of neural tube apposition and laminin, entactin and type IV collagen are present in the neurectodermal basement membrane, but there are no apparent alterations in the quantity of these latter glycoproteins with progression through neurulation (O'Shea, 1986; Tuckett and Morriss-Kay, 1986).

Analysis of mouse mutants with NTD provides further evidence for a role of ECM in neurulation. Both *loop-tail* and *curly tail* embryos exhibit reduced rates of HA accumulation, correlated with failure of the neural tube to close. In *loop-tail*, this abnormality was observed in the cranial region of embryos that had already failed to close their hindbrain neural tubes, raising the possibility of a secondary effect of an abnormally open neural tube (Copp and Wilson, 1981). In *curly tail*, reduced accumulation of newly synthesized HA was found in a perinotochordal distribution in the caudal region of embryos undergoing failure of posterior neuropore closure (Copp and Bernfield, 1988b). *Splotch* embryos, by contrast to the other mutants, exhibit elevated levels of sulphated GAGs, with particular increases in chondroitin sulphate in the paraxial mesoderm and heparan sulphate proteoglycan in the neuroepithelial basement membrane (Trasler and Morriss-Kay, 1991). This evidence suggests, therefore, that HA is an important component of the neurulating embryo, whereas high levels of sulphated GAGs are not conducive to neural tube closure.

Embryo culture experiments have also shed light on the role of ECM in neural tube closure, by the introduction of specific degrading enzymes and inhibitors of ECM synthesis in order to determine the effects on neurulation. Use of *Streptomyces* hyaluronidase, which specifically degrades HA, abolishes expansion of the intercellular spaces within the cranial mesenchyme and inhibits the adoption of a biconvex neural fold morphology (Morriss-Kay *et al.*, 1986). Nevertheless, neural tube closure proceeds to completion in the treated embryos, suggesting that buttressing of the neural folds by HA-rich matrix is unnecessary for neural fold closure via formation of a V-shaped neural groove. By contrast, inhibition of sulphated GAG synthesis, using ß-D xyloside does not prevent formation of biconvex neural folds, but their conversion to a V-shaped morphology is inhibited. The neural folds remain broad and closure does not occur (Morriss-Kay and Crutch, 1982). Use of heparitinase, which specifically degrades heparan sulphate proteoglycans, and chondroitinase ABC which degrades both HA and chondroitin sulphate proteoglycans, confirms the importance of sulphated GAGs in cranial neural tube closure. Heparitinase, in particular, inhibits cranial neurulation although there is no effect on trunk neural tube closure (Tuckett and Morriss-Kay, 1989). Chondroitinase ABC has only a delaying effect on cranial neurulation, but neural crest migration is more severely affected (Morriss-Kay and Tuckett, 1989).

In conclusion, the embryo culture and mutant studies suggest an important role for ECM molecules, particularly proteoglycans, in cranial neurulation. HA appears to be critical for formation of biconvex neural folds and heparan sulphate proteoglycans are required for conversion to a V-shaped neural groove and ultimate closure. The evidence pertaining to spinal neurulation is less conclusive and further studies are required in this area.

Role of cell proliferation and curvature of the body axis

The tissues of the neurulation stage embryo proliferate at a rapid rate, with cell cycle lengths of 8-10 h (Kauffman, 1968). It seems probable, therefore, that regional differences in cell proliferation will prove to be an important factor in mammalian neural tube closure. To date, evidence comes predominantly from the chick, where the formation of hinge-points within the folding neural plate (a median hinge-point overlies the notochord and paired hinge-points are located dorsolaterally) has been related to the cell cycle. Hingepoints comprise wedge-shaped cells in which the basal portion of the cell is expanded relative to the apical portion. Smith and Schoenwolf (Smith and Schoenwolf, 1987, 1988) found that this is due to the presence of cells predominantly in G_2 phase at hingepoints. Recently, we found that hinge points occur also in mouse spinal neurulation, with marked variation along the body axis (Shum and Copp, 1995). It seems certain that formation of the median hinge-point is the result of influences emanating from the notochord (Van Straaten *et al.*, 1985), which probably include the inducing molecule sonic hedgehog (Martí *et al.*, 1995; Roelink *et al.*, 1995). It will be interesting to determine whether analogous dorsoventral patterning molecules also control the position of the dorsolateral hinge-points in the closing neural tube, and whether cell cycle regulation is one of the factors controlled by these molecules.

Misregulation of cell proliferation has been demonstrated to be a critical factor in development of NTD in the *curly tail* mutant mouse. Labeling of affected embryos both *in vitro* and *in vivo* with ^{3}H-thymidine demonstrated reduced proliferation of the hindgut endoderm and notochord compared with normal littermates (Copp *et al.*, 1988a). As neurectodermal proliferation appears normal in mutant embryos, there is a growth imbalance in the caudal region of affected embryos that causes ventral curvature in the caudal region, creating mechanical stress which delays or prevents PNP closure. Counteracting the ventral curvature, by inserting a splint (the tip of a human eyelash) into the lumen of the hindgut (Brook *et al.*, 1991), normalizes posterior neuropore closure demonstrating

that the curvature is causally related to development of spinal NTD in the *curly tail* mutant. Recently, ventral curvature of the caudal region was induced in non-mutant embryos, by reduction in volume of the amniotic cavity (Peeters *et al.*, 1996), with the result that posterior neuropore closure was delayed, further supporting the relationship between curvature of the body axis and delay of posterior neuropore closure.

When the PNP region of *curly tail* embryos is cultured in isolation, with neural tissue separated from underlying ventral structures, the PNP closes normally (Van Straaten *et al.*, 1993), demonstrating that there is no intrinsic abnormality of neurectodermal function in the *curly tail* mutant embryo. Rather, the neural folds are unable to close purely as a result of their connection with the slowly growing notochord and hindgut endoderm. Embryo culture experiments have also been used to induce re-balancing of proliferative rates within the caudal region of *curly tail* embryos. Culture under mildly hyperthermic conditions results in selective reduction of the rate of neurectodermal proliferation, minimizing the genetically-determined disparity in growth rates within the caudal region. Hence, ventral curvature is reduced and posterior neuropore closure is normalized (Copp *et al.*, 1988b; Peeters *et al.*, 1996). Similar results have been described using administration of hydroxyurea, 5-fluorouracil and mitomycin C to pregnant females on day 9 of gestation (Seller and Perkins, 1983; Seller and Perkins, 1986). Thus, coordination of cell proliferation has been shown to be a key requirement for normal closure of the posterior neuropore in the *curly tail* mouse embryo. It remains to be determined whether a similar mechanism may underlie the development of NTD in other gene- and teratogen-based NTD systems.

Molecular basis of neurulation

Gene expression and gene knockouts

Extensive studies over the past 10 years have generated a wealth of knowledge on the expression patterns of genes during mouse development. However the pattern of expression of a gene gives only an indication of its function and, since 1990, the technique of gene targeting has been increasingly used to inactivate specific genes in order to determine their precise function during development. Numerous "gene knockouts" have now been generated by homologous recombination in embryonic stem (ES) cells, followed by transmission of the new mutation through the germ line of a chimera containing cells derived from the ES cell line (Capecchi, 1989). These targeted mutations, together with naturally occurring and randomly-generated mutations (e.g. arising in mutagenesis screens) can be used to investigate the genetic control of processes such as neurulation.

Table 1 is a list of the cloned genes that cause NTD as part of their mutant phenotype. It is interesting to note that only one gene, *Pax3*, disturbs spinal neurulation whereas all of the others, including the nine gene knockouts, cause exencephaly. This suggests that neurulation, in the cranial region at least, requires close cooperation between a number of genes. One important finding to emerge from the gene targeting studies is that a number of other genes, whose patterns of expression previously suggested a role in neurulation, appear to be unnecessary for neural tube closure. For instance, *Wnt-1* is expressed at the tips of the closing neural folds throughout the hindbrain and spinal region (Wilkinson *et al.*, 1987) and yet homozygous null (i.e. loss-of-function) *Wnt-1*$^{-/-}$ embryos undergo normal spinal neural tube closure. They do, however, exhibit pattern defects in the brain, lacking the caudal midbrain and rostral hindbrain regions (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Other examples include *Notch1* which is required for coordinated formation of somites (Swiatek *et al.*, 1994; Conlon *et al.*, 1995), fibroblast growth factor receptor-1 *(fgfr-1)* which is needed for specification of paraxial mesoderm (Deng *et al.*, 1994) and *HNF-3* which is required for development of the node and notochord (Ang and Rossant, 1994; Weinstein *et al.*, 1994). In all three of these cases, although development of the early neurulation stage embryo is severely affected, neural tube closure appears to occur normally, at least for the period of development that the embryo survives. Possible explanations for these observations include: (1) functional redundancy, in which the lack of one gene is compensated for by the action of another gene with overlapping function, and (2) the non-essential nature of many genes for the neurulation process, even though they are transcribed as neurulation is progressing.

Antisense oligonucleotides to block gene expression in vitro

One problem with the gene targeting approach, as it is currently practised, is that many of the gene knockouts are early embryonic lethals. This means that the contribution of the gene to developmental events that occur *after* the stage of lethality cannot be determined by studying the knockout embryos. Methods for producing conditional gene knockouts (loss of function mutations that can be induced at varying stages of development) are now under investigation but, in the absence of such techniques, some workers have employed the embryo culture system to inactivate gene expression using antisense technology. With this approach, synthetic 15-25 base pair oligonucleotides are designed with homology to the gene sequence of interest so that, when supplied to embryonic cells *in vitro*, the antisense oligonucleotide will bind to the specific mRNA, thereby leading to its degradation by double-strand-specific RNAses. Thus, production of the protein gene product should be blocked in cells receiving the antisense oligonucleotide (Wagner, 1994). An important advantage of this approach over gene targeting technology is that the developmental stage at which the gene expression is inhibited can be varied by altering the stage at which the oligonucleotide is added to the culture. At the time of writing, however, there have been few published antisense studies in whole embryo culture, owing in part to the novelty of this technique and in part to the rather complex set of controls that have proved necessary in order to demonstrate specificity of antisense inhibition in studies on simpler systems, such as tissue culture cells (Fernandez *et al.*, 1993; Wu and Adamson, 1993).

So far, antisense technology has been applied only to genes of the *Wnt* and *En* families in mouse embryo culture. Although both sets of genes are expressed in the caudal part of the neural tube, there is a surprising lack of abnormalities in the spinal cord of homozygous null mutant embryos (Table 1), perhaps reflecting compensation between family members. That is, *Wnt-3a* may compensate for *Wnt-1* in *Wnt-1*$^{-/-}$ embryos, and *En-2* may compensate in a similar way for *En-1*. Indeed, *En-1* can be replaced by *En-2* in transgenic mice, supporting this idea (Hanks *et al.*, 1995). Antisense studies in embryo culture, adding the oligonucleotides at the start of neurulation (8.5 days), has revealed many similarities in the phenotypes of antisense inactivation of *Wnt-1* and *Wnt-3a* (Augustine *et al.*, 1993) and the null mutant mice. Moreover,

TABLE 1

CLONED GENES FOR WHICH THE KNOCKOUT AND/OR MUTANT PHENOTYPE INCLUDES DEFECTIVE CLOSURE OF THE NEURAL TUBE

Gene	Function	Expression pattern in embryo	Knockout or mutant phenotype	Reference
Apolipoprotein B (apo B)	Transport and metabolism of cholesterol triglycerides and other fat soluble components in the blood	Yolk sac and fetal liver	Knockout homozygotes have exencephaly (30% of cases) with hydrocephalus in a further 30%. By day 9 there is excessive cell death in the alar plate of the hindbrain	Homanics *et al.*, 1995
csk	Widely distributed cellular substrate for protein kinase C	Strong expression at 8.5 days in cranial neuroepithelium. At 9.5 days, expressed in all tissues, particularly neuroepithelium and neural crest	Knockout homozygotes die between days 9 and 10. Cranial neural tube closure is not completed. This could be a specific defect or result from retardation of the mutant embryos	Imamoto and Soriano, 1993; Nada *et al.*, 1993
F52	Myristoylated alanine-rich substrate for protein kinase C. Also called MARCKS-related protein (Mrp) and MacMARCKS	Expressed along the entire length of the neural tube between 8.5 and 10.5 days	Knockout homozygotes have exencephaly alone (45% of cases), spina bifida alone (% of cases) or exencephaly and spina bifida (% of cases). Homozygotes also have agenesis of the corpus callosum.	Wu *et al.*, 1996
Gli-3	Zinc finger-containing transcription factors	Particularly high expression in brain, head mesenchyme and limb buds, with less intense expression elsewhere	*Extra toes* (*Xt*) mutants have *Gli-3* defects. Xt^{bph} and *Xt* / alleles have exencephaly affecting hindbrain	Hui and Joyner, 1993
HES -1	Helix-loop-helix transcription factor. Homolog of *Drosophila* hairy and enhancer of split genes	Ventricular zone of neuroepithelium and mesoderm	Knockout homozygotes have exencephaly in 70% of cases. Premature neurogenesis in brain	Ishibashi *et al.*, 1995
jumonji (jmj)	Gene trap insertion into a gene with significant homology to retino-blastoma binding protein RBP-2	Strongly expressed at mid- hindbrain boundary and in the cerebellum. Also in bulbus cordis of heart, pharynx and posterior neuropore	Exencephaly of midbrain region in 60% of homozygotes	Takeuchi *et al.*, 1995
MARCKS	Protein kinase C substrate	Strongly expressed in the nervous system particularly during development	Knockout homozygotes have exencephaly in 25% of cases	Stumpo *et al.*, 1995
p53	Tumor suppressor gene	Expressed in all cells of the neurulation stage embryo	Knockout homozygotes have exencephaly in 23% of cases	Schmid *et al.*, 1991; Sah *et al.*, 1995
Pax3	Homeobox-containing transcription factor	Expressed prior to neural tube closure along the dorsal part of the neuro-epithelium, in paraxial mesoderm and early migrating neural crest	*Splotch* (*Sp*) mutant mice have mutations in *Pax3*. Homozygotes have exencephaly and/or lumbosacral spina bifida	Auerbach, 1954
Platelet derived growth factor receptor α (PDGFα)	Cell surface growth factor receptor	Neural tube, neural crest and embryonic mesoderm	*Patch* (*Ph*) mutants are deleted for PDGFαr and have exencephaly in a proportion of cases	Smith *et al.*, 1991
RBP-jκ	Transcription factor	From 8.5 days in lateral portion of mid-brain and hindbrain, presomitic mesoderm and somites. Later becomes ubiquitous	Knockout homozygotes have open 'anterior neuropore' at 9.5 days. Could be a specific defect or result from retardation of the mutant embryos	Oka *et al.*, 1995
Retinoid acid receptors (RARs)	Nuclear retinoid receptors	Expressed during neurulation in an isoform-specific pattern	Knockout mice doubly mutant for RARα and RARγ have hindbrain exencephaly in 30% of cases	Lohnes *et al.*, 1994
Twist	Basic helix-loop-helix transcription factor	First detected at 7.5 days in the anterior lateral mesoderm underlying the head folds. By 8 days it is seen in the somites and lateral plate mesoderm. Later in sclerotome, branchial arch and limb mesenchyme	Knockout homozygotes have failure of neural tube closure in the cranial region. Posterior hindbrain and entire spinal cord close normally	Chen and Behringer, 1995

antisense inactivation of both *Wnt* genes simultaneously yields abnormalities along the length of the spinal cord that are not seen when either of the single oligonucleotides are used. Although these findings support the idea that *Wnt-1* and *Wnt-3a* can compensate for one another during development, the absence of open neural tube defects indicates that expression of these *Wnt* genes is not required for spinal neurulation.

In contrast, when antisense oligonucleotides for *En-1* are applied to cultured embryos (Sadler *et al.*, 1995), defects in the neural tube together with caudal dysgenesis have been observed, neither

of which are part of the null mutant phenotype (Wurst *et al.*, 1994). A similar finding has been reported with antisense inactivation of *En-2* (Augustine *et al.*, 1995). It is not clear how to interpret these differences in findings of the *in vitro* antisense and *in vivo* gene knockout experiments. Absence of the gene product from the earliest stages of development could permit induction of a compensatory gene expression that cannot be induced when *En* genes are inactivated later in development. Conversely, the caudal embryonic defects could represent non-specific effects of the oligonucleotides. Further studies are needed to resolve this issue.

Other molecular studies

The role of a number of other molecules in the control of neurulation has been investigated using whole embryo culture.

Cell surface glycoproteins

A number of approaches have been employed in the analysis of cell surface molecules during neurulation in cultured mammalian embryos. One of the most comprehensive studies has made use of the drug diazo-oxo-norleucine (DON), an inhibitor of GAG and glycoprotein synthesis. Culture of 8.5 day mouse embryos for 12-24 h in medium containing DON results in failure of cranial neural fold elevation, an effect that can be ameliorated by co-addition of excess glutamine (Morris-Wiman and Brinkley, 1990a). This suggests that the effect of DON is mediated through interference with normal glucosamine synthesis and, since glucosamine is incorporated into both GAGs and glycoproteins, the teratogenic action of DON is probably mediated, at least in part, through interference with HA and proteoglycan synthesis (see above). However, DON is also known to interfere with glycosylation of cell surface receptors, hence altered cell-cell interactions may also play a part in the teratogenic effect of DON. Evidence in support of this idea comes from studies with tunicamycin, a drug that inhibits protein glycosylation but not GAG addition to proteoglycans. Exposure of cultured embryos to tunicamycin prior to elevation of the cranial neural folds produces defects in neural tube closure with collapsed mesenchyme and alteration of neural tube basal lamina components, together with hypoplasia and malpositioning of the first branchial arches (O'Shea, 1982).

As well as inhibiting their synthesis, a number of studies have removed these carbohydrates from the cell surface of cultured embryos, for instance by addition of enzymes such as glucosidase, mannosidase and papain. Glucosidase and mannosidase have no effect on cultured embryos whereas brief treatment with papain appears to disrupt cell adhesion between the apposing neural folds, preventing fusion and neural tube closure (Smits van Prooije *et al.*, 1986). A similar effect has been obtained by treatment of neurulation stage embryos with phospholipase C, which removes carbohydrate-rich ECM. Exposure of cultured 8.5 day mouse embryos to phospholipase C for 12 h, during a 36 hour culture period, is compatible with normal elevation of the neural folds but fold fusion is inhibited (O'Shea and Kaufman, 1980). Glycosylated material, identified by staining with ruthenium red or lanthanum, is located on the surface of the neuroepithelium in the prospective fusion zone of normal embryos (Sadler, 1978), but this lanthanum-positive material is absent from the phospholipase C-treated embryos (O'Shea and Kaufman, 1980). The precise nature of these cell surface molecules that mediate fusion of the neural folds remains to be determined.

NCAM and N-cadherin

It is frequently claimed that these cell adhesion molecules play an important role in neural tube closure. However, gene knockouts of NCAM (Tomasiewicz *et al.*, 1993; Cremer *et al.*, 1994) and a dominant negative mutation of N-cadherin (Hermiston and Gordon, 1995) have been reported, without evidence of NTD among the mutant embryos. Use of blocking antibodies to N-CAM and N-cadherin in the chick embryo produces a grossly distorted neural tube with ectopically positioned neural crest, but the incidence of open neural tubes was elevated in embryos treated only with anti-N-cadherin, not anti-NCAM (Bronner-Fraser *et al.*, 1992). In amphibia, overexpression of N-cadherin does not prevent closure of the neural tube, although it does result in abnormalities of neural tube structure (Detrick *et al.*, 1990). Therefore, although both molecules are expressed on the cranial neural folds prior to closure of the neural tube, there is little convincing evidence that either molecule plays a critical role in neural tube closure. The significance of NCAM overexpression, as observed in mutant *splotch* embryos that develop NTD (Moase and Trasler, 1991), is unclear at present.

Growth regulating molecules

The role of growth factors and other growth-regulating molecules in neural tube closure has been the subject of relatively few studies to date. Extensive dialysis (removing molecules of less than 12-14 kDa) renders rat serum incapable of supporting development in whole embryo culture, but only glucose and certain vitamins need be added back in order to restore most of the growth promoting properties of the serum (Cockroft, 1979). This suggests that molecules smaller than 12-14 kDa are relatively unimportant for embryonic development. Nevertheless, the vitamin inositol must be added, in addition to glucose, in order to prevent cranial NTD from developing (Cockroft, 1988) (see section below on diabetes mellitus). Serum that has been exhausted by repeated culture of embryos can also be rejuvenated by addition of glucose but, in this case, a beneficial effect was also found when epidermal growth factor, insulin or transferrin was added to the cultures, indicating a potential role for these growth-promoting molecules (Pratten *et al.*, 1988).

More direct evidence for a role of transferrin in closure of the neural tube has come from studies of protein distribution in neurulation-stage mouse embryos. Closure of the neural tube in both cranial and caudal regions correlates with localization of maternally-derived transferrin in the gut at the same level of the body axis (Copp *et al.*, 1992). Uptake of ^{125}I-transferrin can be demonstrated in cultured mouse embryos, with binding and internalization by gut endodermal cells. Particularly striking has been the finding that *curly tail* embryos, developing spinal NTD, take up less transferrin at this site than unaffected *curly tail* embryos (Hoyle *et al.*, 1996). Thus, a reduction in transferrin binding and uptake could be involved in the reduced proliferation rate of the *curly tail* hindgut endoderm. However, transferrin binding and uptake is also greater in the hindgut of unaffected *curly tail* embryos than in non-mutant CBA/Ca and CD-1 embryos. This raises the alternative possibility that increased transferrin uptake by some mutant embryos is actually a compensatory response to defective cell proliferation in the hindgut, which results from an unrelated defect. If only a proportion of embryos are able to mount this compensatory response, this would lead to the observed partial penetrance of NTD in the *curly tail* mutant mouse.

NTD in metabolic disorders

The genetic basis of human NTD remains poorly understood at present. As discussed earlier, the most likely source of information in this area seems likely to come in future from the isolation of the human homologues of mouse genes that control neurulation. To date, therefore, the primary focus in human NTD research has been on the non-genetic causation of NTD, with particular emphasis on teratogenic and metabolic factors.

The occurrence of NTD in metabolic disorders (e.g. maternal diabetes mellitus), and following the use of particular teratogenic agents (e.g. valproic acid), suggests that for neural tube closure to occur there is a requirement for certain biochemical pathways to be operative. Clues to the metabolic events underlying neurulation may be gained from studying the mechanisms of action of these metabolic disorders and teratogens. Furthermore, even when the etiology of NTD is unknown, analysis of factors with an apparently preventive effect (e.g. folic acid) can also shed light on the underlying developmental mechanisms.

The whole embryo culture system facilitates analysis of biochemical events in two ways. Firstly, the embryo is accessible to exogenous agents in the absence of maternal effects which can complicate the analysis of metabolic pathways in the embryo itself. Secondly, the accessibility of embryos in culture enables continuous treatment with exogenous agents as opposed to maternal dosing which may cause large fluctuations in concentration.

Maternal diabetes mellitus

Maternal diabetes is a risk factor for congenital malformations including caudal regression syndrome, spina bifida, hydrocephalus, anencephaly, and renal and heart malformations. A complex range of metabolic disorders is associated with the diabetic state, including hyperglycemia (which is diagnostic), hypoglycemia and hyperketonemia. The relative contributions of the individual factors remains unclear but study of each in isolation using embryo culture has contributed to our overall understanding of diabetic embryopathy.

Exposure to high concentrations of glucose or 3-hydroxybutyrate *in vitro* causes growth retardation and a range of abnormalities in both rats and mice cultured through the period of cranial neural tube closure. The defects involve, most commonly, failure of closure of the cranial neural tube, but can also include open posterior neuropore (not seen in all studies), a "squirrel" like lesion involving fusion of the open anterior and posterior neural folds, failure of axial rotation and reduction in brain size. Response to these factors is dose- and stage-dependent, with embryos at earlier developmental stages being most sensitive (Cockroft and Coppola, 1977; Cockroft, 1984; Hunter and Sadler, 1987). Similarly, the culture of rat and mouse embryos in serum from diabetic women, and from streptozotocin-induced diabetic rats, also causes growth retardation and malformations similar to those described for glucose and 3-hydroxybutyrate (Moore *et al.*, 1987; Styrud and Eriksson, 1992). However, normalization of glucose and 3-hydroxybutyrate levels by insulin treatment before preparation of the diabetic rat serum does not completely prevent malformations (including NTD) in the cultured embryos suggesting that other potential teratogens are present in diabetic serum (Buchanan *et al.*, 1994). These results confirm that diabetic embryopathy is a multifactorial condition, whose effects cannot be attributed simply to the action of a single teratogenic agent.

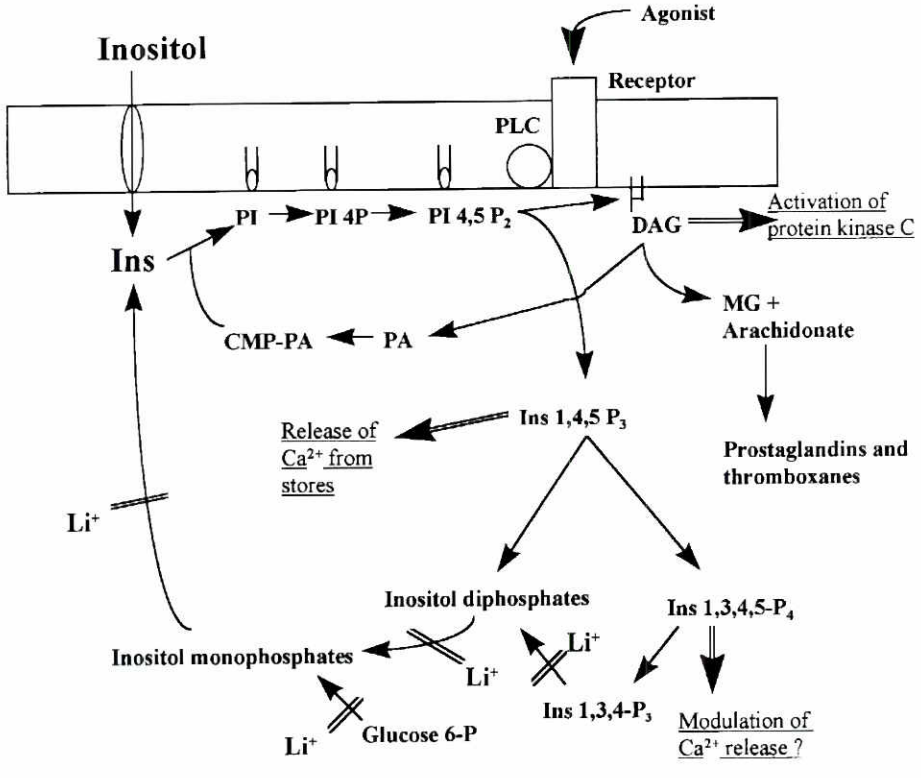

Fig. 2. The inositol/lipid cycle. *Free inositol is taken into cells via a specific receptor and converted to inositol phospholipids. Under the influence of agonist-receptor binding, the phospholipids are cleaved by phospholipase C to yield inositol phosphates and diacylglycerol, each of which has a variety of actions in the cell. Inositol phosphates are recycled back to free inositol by lithium-sensitive reactions. Abbreviations: In, inositol; PI, phosphatidylinositol; DAG, diacylglycerol; Li+, lithium ions; MG, monoacylglycerol; PLC, phospholipase C; PA, phosphatidic acid; CMP-PA, choline monophosphate-phosphatidic acid.*

Importance of yolk sac mediated teratogenesis in diabetes

During the early stages of organogenesis, rodent embryos depend on the yolk sac for nutrition. The endodermal cells of the yolk sac take up and catabolize proteins from the surrounding medium, releasing amino acids for embryonic protein synthesis (Freeman and Lloyd, 1983). This process, termed histiotrophic nutrition, is disrupted by diabetes-related metabolic disturbances including high concentrations of glucose (Pinter *et al.*, 1986b; Hunter and Sadler, 1992), 3-hydroxybutyrate (Hunter *et al.*, 1987) and somatomedin inhibitor (Hunter *et al.*, 1991), leading to protein

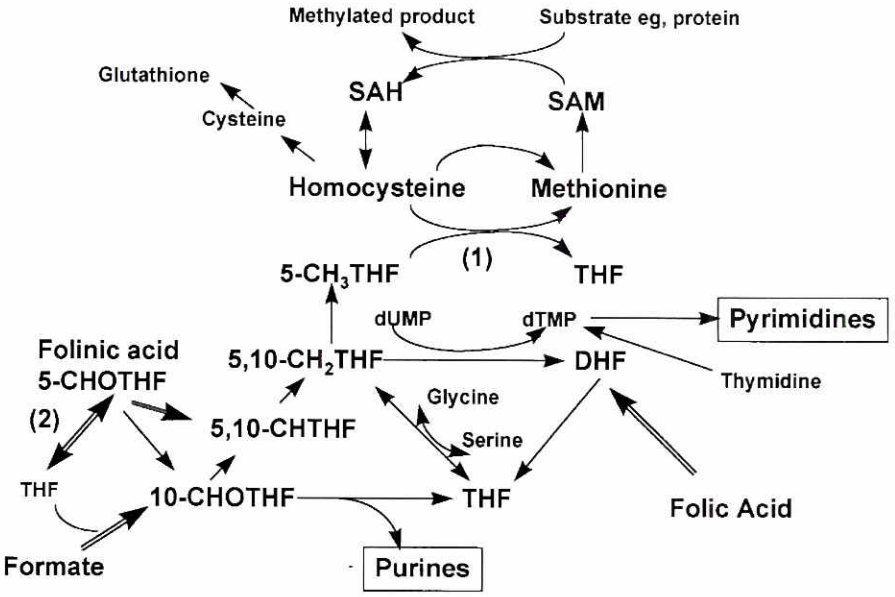

Fig. 3. The principal metabolic pathways involving folate, methionine and homocysteine. *Abbreviations: SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; DHF, dihydrofolate; 5-CH_3THF, 5-methyltetrahydrofolate; 5,10-CH_2THF, 5,10-methylenetetrahydrofolate; 5,10-CHTHF, 5,10-methenyltetrahydrofolate; 5-CHOTHF, 5-formyltetrahydrofolate; 10-CHOTHF, 10-formyltetrahydrofolate. (1), methionine synthase; (2), glutamate formyltransferase.*

starvation and growth retardation of the developing embryo (Reece *et al.*, 1993). The yolk sac exhibits disturbance of ultrastructural organization and fatty acid content (Pinter *et al.*, 1986b, 1988) and there is a rise in the generation of potentially damaging oxygen free radicals (Eriksson and Borg, 1993). It has been suggested that this yolk sac mediated effect may be an important mechanism of diabetic embryopathy, particularly in cases where growth retardation is a significant factor.

Role of inositol and arachidonic acid metabolism

A metabolic pathway that appears to be of considerable importance in diabetic embryopathy is the inositol/lipid cycle (Fig. 2), which involves downstream activation of protein kinase C, release of Ca^{2+} ions and generation of arachidonic acid, the major precursor of prostaglandins and thromboxanes. The vitamin *myo*-inositol is depleted in rat embryos exposed to high concentrations of glucose *in vitro* (Hod *et al.*, 1986; Hashimoto *et al.*, 1990), as are levels of inositol phosphates and phospholipids (Strieleman *et al.*, 1992), while another hexose, sorbitol, increases as a result of activation of the polyol pathway. Inositol appears to be the key metabolite since supplementation *in vitro* reduces the frequency of NTD and growth retardation (Baker *et al.*, 1990; Hashimoto *et al.*, 1990) whereas aldose reductase inhibitors (e.g. Sorbinil or Statil), which reduce sorbitol levels, do not have an ameliorating effect on NTD frequency (Hod *et al.*, 1986; Hashimoto *et al.*, 1990).

Several lines of evidence suggest that the effect of inositol deficiency is mediated by suppression of phosphoinositide metabolism, resulting in decreased generation of arachidonic acid (Fig. 2) and inhibition of prostaglandin synthesis. Arachidonic acid has a significant protective effect against hyperglycemia-induced cranial NTD in mouse embryos *in vitro* (Goldman *et al.*, 1985; Pinter *et al.*, 1986a). Furthermore, the protective effect of inositol *in vitro* (Baker *et al.*, 1990) is reversed by indomethacin, an inhibitor of arachidonic acid metabolism. The prostaglandins PGE_2, $cPGI_2$ and PGF_2 can provide varying degrees of protection against cranial NTD caused by hyperglycemia (Baker *et al.*, 1990), and against malformations induced by the anti-convulsant diphenylhydantoin (phenytoin) which also appears to act through inhibition of the arachidonic acid pathway (Bruckner *et al.*, 1983; Kay *et al.*, 1988).

Thus, the inositol/lipid cycle may play a key role in the development of malformations in embryos of diabetic mothers. There is independent evidence from whole embryo culture studies for a role of inositol in neural tube closure. *In vitro* deficiency of specific vitamins causes growth retardation and malformation in rat and mouse embryo cultures, but only the absence of inositol causes NTD (Cockroft, 1979, 1988; Cockroft *et al.*, 1992). Moreover, the *curly tail* mouse mutant is particularly sensitive to inositol deficiency: 8.5 day embryos require a higher level of inositol supplementation to prevent cranial NTD than control strains. Recently, we found that inositol treatment also reduces the incidence of spinal NTD in the *curly tail* mouse, both *in vitro* and *in vivo* (Greene and Copp, 1997).

Since depletion of the phosphoinositide pool induces NTD in different systems, one would predict that direct suppression of inositol metabolism by lithium would elicit similar effects. In rat embryo culture, lithium treatment yields dose- and age-dependent retardation of growth and development but, unexpectedly, not open neural tubes (Hansen *et al.*, 1990; Klug *et al.*, 1992). A small number of cranial NTD, as well as growth retardation, could be induced at high doses in mouse embryos (Hansen *et al.*, 1990). The reason for this lack of effect of lithium on neurulation requires further study.

Valproic acid-induced neural tube defects

Valproic acid (VPA) treatment during pregnancy in humans has a well-established association with an increased risk of NTD, almost exclusively spina bifida (Lammer *et al.*, 1987). In mice, VPA administration on day 8 of gestation causes exencephaly while treatment on day 9 induces spina bifida (Kao *et al.*, 1981; Ehlers *et al.*, 1992a,b). Mouse (Bruckner *et al.*, 1983) and rat (Hansen and Grafton, 1991; Seegmiller *et al.*, 1991) embryos exposed to VPA in culture also exhibit NTD affecting both cranial (principally mid- and hind-brain) and caudal levels, with regions of closed but wavy neural tube also evident. The mechanism of action of VPA is not understood, although part of the effect may be mediated through the embryonic neuroepithelium which accumulates VPA after treatment *in vivo* (Dencker *et al.*, 1990) and also shows ultrastructural abnormalities including disorganization, blebbing into the lumen and increased numbers of cellular processes after treatment *in vivo* (Turner *et al.*, 1990) and *in vitro* (Bruckner *et al.*, 1983;

Seegmiller *et al.*, 1991). Although certain abnormalities such as failure of development of otic and optic vesicles seem particularly sensitive to growth retarding teratogens, the effect of VPA on the neural tube appears to be specific: for example, RA and VPA both cause growth retardation, but yield distinctive malformations of the neural tube (Seegmiller *et al.*, 1991; Ehlers *et al.*, 1992a).

There is conflicting evidence on whether VPA teratogenesis is mediated via disturbance of folate metabolism. Such an interaction would be particularly interesting considering that folic acid supplementation has been shown to prevent approximately 70% of NTD in clinical trials (Wald *et al.*, 1991; Czeizel and Dudás, 1992). Treatment of mice *in vivo* with folinic acid (Fig. 3) significantly reduces the frequency of VPA-induced exencephaly (Trotz *et al.*, 1987; Wegner and Nau, 1991). However, this protective effect has not been replicated in the mouse *in vivo*, nor in the rat embryo culture system, using supplementation with various folate derivatives (including folinic acid) or other compounds (L-methionine, L- or D-serine and sodium formate) which may interact with folate metabolism (Fig. 3). Moreover, the uptake of these compounds *in vitro* is not inhibited by VPA (Hansen and Grafton, 1991; Hansen *et al.*, 1995). It is difficult to explain the different results obtained with *in vivo* folinic acid supplementation since both studies followed the same protocol, although different mouse strains were used. Despite these inconsistencies, VPA does appear to affect levels of folate derivatives in embryos treated *in vivo* whereas the non-teratogenic analog 2-en VPA has no such effect. THF level is increased while 5-CHOTHF and 10-CHOTHF are decreased due to inhibition of the enzyme glutamate formyltransferase (Wegner and Nau, 1992).

Methionine treatment *in vitro* fails to reduce the incidence of NTD caused by VPA and there is also no protection from methionine when NTD are induced by culture in serum from VPA-treated rats. In contrast, if the animals used as either serum donors or embryo donors are previously dosed with L-methionine there is a protective effect (Nosel and Klein, 1992). The most striking reduction in the frequency of defects, from 78 to 25% is provided by supplementation of embryo donors for at least 2 weeks before mating. These studies suggest that in certain circumstances, methionine is able to reduce the frequency of VPA-induced NTD and this emphasises the importance of not considering *in vivo* and *in vitro* studies in isolation from each other.

Methionine, homocysteine and folate metabolism

Rat embryos cultured in cow serum or human serum from early head fold stages exhibit growth retardation, cranial NTD and other abnormalities, and these defects can be prevented by addition of methionine (Coelho *et al.*, 1989). Homocysteine also prevents the defects in human serum, presumably acting through the methionine pathway (VanAerts *et al.*, 1994). It is not clear whether methionine and homocysteine are overcoming a deficiency present in the serum or counteracting a different deleterious factor(s). Perhaps more significant for an understanding of human NTD is the observation that the frequency of caudal NTD caused by the mouse mutation *axial defects (Axd)* is also reduced by methionine supplementation *in vivo* at a specific developmental stage (day 8-9 of gestation) (Essien, 1992). The use of embryo culture in this system would determine whether methionine is acting directly on the embryo or via a maternal effect.

As methionine supplementation is able to prevent NTD in certain circumstances, one would predict that inhibition of methionine metabolism may cause NTD. One experimental approach is to treat embryos with nitrous oxide (N_2O) an inhibitor of the enzyme methionine synthase (Deacon *et al.*, 1978) (Fig. 3). Clinical data suggest that reduced plasma levels of vitamin B_{12}, the coenzyme for methionine synthase, is a risk factor for NTD (Kirke *et al.*, 1993). However, although N_2O is teratogenic to rat embryos in culture (from day 9 of gestation) causing growth retardation and altered laterality of the body axis, NTD were not reported (Baden and Fujinaga, 1991). This effect of N_2O appears to be mediated through methionine rather than tetrahydrofolate depletion since supplementation *in vitro* with methionine but not folinic acid protects against the abnormalities (Fujinaga and Baden, 1994).

Elevated levels of homocysteine have been observed in women with previous or current NTD-affected pregnancies (Steegers-Theunissen *et al.*, 1991; Mills *et al.*, 1995). Decreased efficiency of methionine synthase or related enzymes (Fig. 3) could lead to an accumulation of homocysteine, but the evidence from rat embryo cultures is that L-homocysteine does not cause NTD *per se*, although it is toxic at high doses (VanAerts *et al.*, 1994), possibly reducing the S-adenosylmethionine/S-adenosylhomocysteine ratio and so inhibiting methylation reactions. Therefore, homocysteinemia may be a marker of NTD risk but is unlikely to be a causal factor. At present, therefore, a variety of evidence suggests a role for folate-related metabolic pathways, particularly methionine, in control of neural tube closure, but the precise mechanism by which human NTD can be prevented by folate administration remains unknown.

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