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# ANIMAL MODELS OF NEURAL TUBE DEFECTS

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We reviewed the genetic variants and strains of mice that are used as models for neural tube defects (NTD) in humans. Over 40 genetic defects in mice cause obvious risk of NTD, but most are syndromic and many are lethal to embryos. Only a subset is similar to the common, nonsyndromic, genetically complex spina bifida or anencephaly in humans. The *nonsyndromic* variants that are potentially good models include homozygotes for spontaneous (*Axd* or *Lp*) or targeted (*Apob*, *Macs*, *Mrp*, or *Trp53*) mutations and five strains with spontaneous NTD of genetically complex cause, i.e., curly tail, SELH/Bc, NZW-*xid*, MT/HokIdr, and TO. Curly tail (1–5% exencephaly, 15–20% spina bifida) and SELH/Bc (15–20% exencephaly) are the best-understood developmental models for human spina bifida and anencephaly, but the genes are not yet known. The curly tail and *Cart1* gene "knockout" models show that the defect leading to NTD may be in the supporting tissues, and not in the neural tube itself. The SELH/Bc model shows that there are compensatory mechanisms that can close the neural tube despite genetic deficiency of a normal closure mechanism. The Splotch mutations have been the most studied syndromic NTD in mice and are now known to be *Pax3* gene mutations that model human Waardenberg syndrome, not common NTD. Heterogeneity of effective nutritional approaches to prevention is demonstrated by five genetically distinct models, each of which responds to a different nutrient. As in human anencephalics, an excess of females among exencephalics (of between 2–20-fold) is observed in seven mouse NTD genetically distinct models. Generally, strains with spontaneous NTD have a relatively high risk of NTD after exposure to the human teratogens, valproic acid, or retinoids. *Nonsyndromic* NTD in mice are genetically heterogeneous and often genetically complex, and we predict a similar genetic heterogeneity in human NTD. The genes contributing to the genetically complex NTD in mice, when identified, will provide candidate genes to test for association with human NTD risk. 
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**Key Words:** anencephaly; spina bifida; exencephaly; model; mouse; neural tube

Anencephaly and spina bifida aperta, defects of neural tube closure, are among the most common serious human birth defects. Often the neural tube defects (NTD) are the only primary errors in embryological development [Seller, 1994]. The cause appears to be mostly genetic, but heterogeneous with complex heredity, and some genetic liabilities interact with maternal nutrition, specifically folic acid intake, to influence the risk of NTD occurring.

The formation of the neural tube, one of the first steps in the development of the nervous system, occurs early, during the third and fourth weeks of human embryological development, and in humans is inaccessible to observation. The genes causing liability to human NTD are also not accessible by the methods used for simple Mendelian disorders, and systematic methods for identifying genes involved in human complex traits are still being developed. By default, much of the understanding of the embryology and genetics of human NTD is based on the study of other vertebrates, particularly mice, where embryos can be examined directly, and where specialized strains and crosses can be used to identify genes and their effects on neural tube closure. The process of neural tube closure is inferred to be basically the same between mouse and human, based on clinical observations [Golden and Chernoff, 1995; Van Allen et al., 1993].

NTD are seen in a wide variety of vertebrates, including monkeys [ Jerome, 1987], cattle [Cho and Leipold, 1978], horses [Rivas et al., 1996], sheep [Dennis, 1975], golden hamsters [Moffa and White, 1979], and chicks [Romanoff, 1972], but no genetic models have been well-developed except in mice. For historical [Morse, 1981] and practical reasons, mice are the genetically most well-defined vertebrate, with hundreds of well-studied mutants and cloned genes, and an extensive collection of inbred strains tracing back more than 80 years.

There are different types of genetic model (Table 1). Some give insight into the essential steps in neural tube closure. Some are also possible genetic homologs of human NTD genes. For several, there is interaction with maternal nutrition (Table 2), and it is intriguing that genetically distinct models respond to different agents.

In mouse embryos, the neural tube forms during day 8–10 of gestation. The last areas to close, the neuropores, close on day 9 at the head and during early day 10 at the base of the tail [Kaufman, 1992; Theiler, 1989]. Failure of these closures leads to exencephaly and spina bifida, respectively. Exencephaly in mice is the equivalent of anencephaly in humans. In this review, "spina bifida" means that the neural folds never met in the midline and never fused—also called spina bifida aperta. Exencephaly and spina bifida may occur together in the same embryo or independently.

Many mutations and strains of mice have a high risk of NTD [reviewed in Harris and Juriloff, 1997]. In only a handful of the mutations, but in all six of the strains affected, the NTD appears to be the only primary defect ("nonsyndromic"). There are at least 32 different genes which when mutated cause NTD as part of lethal syndromes of multiple primary defects in homozygotes. Few have been studied developmentally in detail. Of the 15 spontaneous syndromic NTD mutations, half have only exencephaly, half have both exencephaly and spina bifida,

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and one has only spina bifida. In contrast, almost all the 17 targeted or ''knockout'' mutations have only exencephaly; two have exencephaly and spina bifida, and none have only spina bifida. In many of these ''syndromic'' mutants, the embryos die during gestation, whereas fetuses with nonsyndromic exencephaly or spina bifida survive to birth. Generally, the Mendelian syndromic mutants of mice do not seem to directly model human NTD. In some, the NTD may illuminate a gene whose function is necessary for neural tube closure; in others, the neural tube may be a nonspecific morphological ''casualty'' in a failing embryo.

## SELECTED GENETIC MODELS FOR SPINA BIFIDA AND/OR ANENCEPHALY

Mouse strains with multigenic risk for nonsyndromic NTD appear to be the most promising models for common human anencephaly or spina bifida. The ''nonsyndromic'' genetic variants and gene knockouts that cause NTD in mice are listed in Table 1; all of the available mutations and the two strains that have been studied developmentally are discussed below. In addition, two ''syndromic'' mutants that may be important to understanding mechanisms of NTD, Splotch and the *Cart1* gene knockout, are discussed.

### Curly Tail

Curly tail is the best understood mouse model of nonsyndromic spina bifida. It arose spontaneously in the GFF stock in 1950, was crossed once to the normal CBA/Gr strain, and has been maintained as a closed colony with selection for curly tail [Embury et al., 1979]. The neural tube defects include spina bifida (15–20%), curly tail (60%; considered to be a mild form of NTD), and exencephaly (1–5%), the frequencies varying with genetic background [Copp et al., 1990].

The primary developmental defect in the curly tail model of spina bifida is not in the neural tube at all, but is in the hindgut and notochord. At the time of posterior neural tube closure, the hindgut and notochord grow too slowly, are tethered to the overlying neuroepithelium which is growing normally, and cause an abnormal ventral bend in the closing neural tube that interferes with apposition of the neural folds [Peeters et al., 1996]. Treatments that retard overall growth at this specific time, allowing the hindgut, notochord, and neuroepithelial growth to synchronize [Copp et al., 1988; Peeters et al., 1996], or that physically straighten the curvature [Brook et al., 1991], result in normal closure of the posterior neuropore (PNP) and a reduced frequency of spina bifida. In embryos with delayed PNP closure, the retinoic acid receptor RARβ is deficient in the hindgut endoderm, the tissue whose growth is deficient, and RARγ is deficient in the PNP region [Chen et al., 1995]. RARs directly regulate the activity of other genes.

Administration of the vitamin inositol both in vivo and in vitro prevents up to 70% of spina bifida through reduction of the delay in PNP closure, seen as reduced PNP length [Greene and Copp, 1997]. Curly tail mice are apparently not abnormal in inositol uptake or metabolism. The NTD-preventative effect of extra inositol appears to be through stimulating a signalling pathway that upregulates protein kinase C (PKC), which leads to upregulation of RARβ expression in the hindgut. There is no effect on RARγ. Another agent, a phorbol ester that upregulates PKC, also upregulates RARβ and causes reduced PNP length [Greene and Copp, 1997]. Retinoic acid (a form of vitamin A) given at a specific time during neural tube closure, and which can upregulate RARβ expression in the hindgut, can also prevent the tail defect in curly tail mice [Chen et al., 1994, 1995]. It is not known whether the upregulation of RARβ causes or reflects hindgut growth [Greene and Copp, 1997], and the target genes for RARβ regulation in the hindgut are still to be identified. However, the discovery of several steps in a molecular pathway underlying prevention of a neural tube closure defect offers exciting insight into a chain of events that can underlie prevention of NTD.

The less frequent exencephaly trait of the curly tail stock has not been the focus of study. We speculate that there might be an analogous relationship of

**Table 1. Genetic Variants in Mice That Result in Nonsyndromic Neural Tube Closure Defects:**

**A. Mendelian (Highly Penetrant) Mutations Resulting in Nonsyndromic Exencephaly (EX) and/or Spina Bifida (SB) or Craniorachischisis in Homozygotes**

| Gene Name | Gene Symbol | Neural Tube Defect Frequency | References[d] |
|---|---|---|---|
| Spontaneous mutations: | | | |
| Axial defects | *Axd* | 50–100% SB | 1, 2 |
| Exencephaly[a] | *xn* | 35–85% EX; (female EX:male EX = 2:1) | 3, 4 |
| Looptail | *Lp* | 100% craniorachischisis in Lp/Lp; occasional SB in Lp/+ | 5 |
| Targeted mutations: | | | |
| Apolipoprotein B[b] | *Apob* | 30% EX | 6, 7 |
| Myristoylated alanine-rich C-kinase substrate | *Macs* | 30% EX in females; 15% EX in males | 8 |
| MARCKS-related protein[c] | *Mrp* | 55% EX; 15% SB | 9 |
| | | 100% EX; no SB | 10 |
| Transformation-related protein 53[c] | *Trp53* (p53) | 30% EX in females; 0% EX in males | 11 |
| | | 20% EX in females; 1% EX in males | 12 |

**B. Strains That Have ''Multifactorial'' Nonsyndromic Exencephaly (EX) and/or Spina Bifida (SB)**

| Strain | Neural Tube Defect Frequency | References[d] |
|---|---|---|
| B10.A | 2% EX in females; 0% EX in males | 13 |
| Curly tail | 1–5% EX (80% are female); 15–20% SB | 14–16 |
| MT/HokIdr | 5–10% EX | 17 |
| NZW-*xid* | 15% EX in females; 1% EX in males | 18 |
| SELH/Bc | 20% EX in females; 10% EX in males | 19–21 |
| TO | 5% EX | 22 |

[a]Probably extinct.
[b]Truncated protein; all others in list are gene ''knockout'' or null mutations.
[c]Two knockouts, at each of *Mrp* and *Trp53*.
[d]1, Essien et al., 1990; 2, Essien, 1992; 3, Wallace et al., 1978; 4, Anderson, 1981; 5, Copp et al., 1994; 6, Homanics et al., 1993; 7, Homanics et al., 1995; 8, Stumpo et al., 1995; 9, Wu et al., 1996; 10, Chen et al., 1996; 11, Sah et al., 1995; 12, Armstrong et al., 1995; 13, Tyan, 1992; 14, Embury et al., 1979; 15, Copp et al., 1990; 16, Copp et al., 1988; 17, Matsuda, 1990; 18, Vogelweid et al., 1993; 19, Juriloff et al., 1989; 20, Harris et al., 1994; 21, Gunn et al., 1996; 22, Padmanabhan and Ahmed, 1996.

**Table 2. Preventative Effect of Nutritional Supplementation on Risk of Exencephaly (EX), Spina Bifida (SB) or Curly-Tail Defect (CT) in Mouse Genetic NTD Models***

| Genetic Model | NTD | Folic Acid | Thymidine | Retinoic Acid | Methionine | Inositol | Purina #5001 vs. Purina #5015 |
|---|---|---|---|---|---|---|---|
| Cartilage homeoprotein 1 mutant (*Cart1*−/−) | EX | Yes[1] (85%) | — | — | — | — | — |
| SELH/Bc strain | EX | No[2] | — | No[3] | No[3] | — | Yes[2] (70%) |
| Axial defects mutant (*Axd/Axd*) | SB | No[4] | — | No[5] | Yes[4,6] (50%) | — | — |
| Curly tail stock | SB | — | — | ns[7] | — | Yes[8] (70%) | — |
| | CT | — | — | Yes[7] (50%) | — | — | — |
| | CT and/or SB | No[9] | No[9] | Yes[10] (50%) | No[9] | ns[9] | — |
| Splotch mutants ($Sp^d/Sp^d$) | SB | — | — | Yes[11] (50%)[a] | — | — | — |
| ($Sp^{2H}/Sp^{2H}$) | SB | Yes[12] (40%) | Yes[12] (40%) | — | — | — | — |

*Includes substances that have been shown to have a preventative effect on NTD in at least one mouse genetic model in vivo. —, means not known to have been done. Yes, agent causes a decrease in NTD frequency (approximate percent prevented); ns, effect not significant. References shown as numeric superscripts: [1]Zhao et al., 1996; [2]Juriloff and Harris, unpublished data; [3]Tom et al., 1991; [4]Essien and Wannberg, 1993; [5]Haviland and Essien, 1990; [6]Essien, 1992; [7]Chen et al., 1994; [8]Greene and Copp, 1997; [9]Seller, 1994; [10]Seller et al., 1979; [11]Moase and Trasler, 1987; [12]Fleming and Copp, 1998.
[a]For $Sp^d/Sp^d$, the decrease in spina bifida frequency after retinoic acid treatment appears to be due to differential mortality of spina bifida embryos. Results for the *Sp* allele are inconsistent [Kapron-Bras and Trasler, 1985; Moase and Trasler, 1987].

cranial closure with foregut growth or with PKC activity.

Genetically, NTD susceptibility appears to be multifactorial and complex. Two genes have been mapped, one called ''curly tail'' (*ct*) to a broad 20-cM region of distal Chr 4 [Neumann et al., 1994], and a second, called ''modifier of curly tail 1'' (*mct1*), to Chr 17 [Letts et al., 1995]. Potential human homologs would likely be on human chromosomes 1p and 6p, respectively. No genes have yet been identified and the RARs map elsewhere. There is evidence that there are more ''modifier'' loci to be mapped [Neumann et al., 1994]. The mapping studies used segregants after a cross, scored 1–3 days after birth, and the NTD scored was the tail flexion defect, not spina bifida. More precise mapping, towards cloning of the genes, is complicated by the lack of categorical effect of the *ct* gene. For example, in one backcross set, although only 20% of the *ct/ct* segregants had curly tails, a surprising 5% of *ct/+* heterozygotes were also affected [Beier et al., 1995]. Heterozygote expression interferes with recognition of recombination between the trait and marker genes, necessary for mapping. It is important to identify the nature of the NTD genes in this model, and it would be useful to know if heterozygote expression would be removed if spina bifida, rather than the tail defect, were used as the indicator trait.

### *Macs* and *Mrp*

There may be a molecular link, through PKC, between the mechanism leading to NTD in curly tail and in homozygotes for *Macs* or *Mrp* gene knockouts. Knockout of either *Macs* or *Mrp* causes a high frequency of exencephaly in homozygotes [Stumpo et al., 1995; Wu et al., 1996; Chen et al., 1996]. *Macs* (myristoylated alanine-rich protein kinase C substrate or MARCKS) and *Mrp* (MARCKS-related protein) code for similar cell-membrane proteins that respond to protein kinase C-induced signals and that also bind and cross-link actin molecules, thus regulating the arrangement of actin molecules at the cell membrane. *Macs* is known to be expressed in cells of the cranial neuroepithelium and supporting tissues at the time of cranial neural tube closure [Blackshear et al., 1996], and *Mrp* is expressed throughout the developing neural tube, most abundantly in the cranial neural folds [Wu et al., 1996]. The link of *Macs* and *Mrp* with both PKC signaling and actin distribution, combined with their expression patterns, makes them good candidates for active roles in cranial neural fold elevation. An actin-based change in neuroepithelial cell shape is thought to provide the major force for elevation of the neural folds [e.g., Sadler et al., 1982], and the signal to redistribute the actin to produce the wedge shape likely involves PKC [Chen et al., 1996].

The *Macs* and *Mrp* gene knockouts are among the very few that appear to cause mostly ''nonsyndromic'' NTD. Nonexencephalic homozygotes for either the *Macs* or *Mrp* knockouts have a midline brain defect (agenesis of the corpus callosum, the tract between the left and right halves of the forebrain) that may be a subtle form of the same defect in the neural folds that also leads to exencephaly [Wu et al., 1996]. There are non-neural tube defects in some $Macs^{-/-}$ homozygotes (failure of closure of abdominal body wall and runting) [Stumpo et al., 1995], but there are no non-neural tube defects in $Mrp^{-/-}$ homozygotes [Wu et al., 1996; Chen et al., 1996]. In one *Mrp* knockout, some homozygotes have survived to adulthood and bred [Wu et al., 1996]. In the other *Mrp* knockout, all homozygotes have exencephaly [Chen et al., 1996]. Among the three *Macs/Mrp* knockouts reported, $Macs^{-/-}$ and one $Mrp^{-/-}$ have only exencephaly, whereas the other $Mrp^{-/-}$ has both exencephaly and spina bifida. This variation in expression may be entirely due to differences in modifier genes.

The *Mrp* gene is mapped to mouse Chr 4 (homologous to human 1p) in the same region as is *ct* (curly tail) [Wu et al., 1996]. Given that the preponderance of cranial vs. caudal NTD could be controlled by modifier genes, *ct* could be a mutation of the *Mrp* gene; however, no defects were found in the *Mrp* protein encoding sequence of *ct/ct* mice [Wu et al., 1996]. The *Macs* gene is on mouse Chr 10 (homologous to human 6q), not near any other known NTD mutation [Stumpo et al., 1995].

*Macs* and *Mrp* knockouts are very promising models for study of the molecular events necessary for neural-fold elevation, and as candidate genes contributing to human NTD. It would be interesting to know if they respond to vitamin supplementation.

### SELH

The SELH/Bc inbred strain is the best-understood mouse model of development of nonsyndromic anencephaly (exencephaly). Exencephaly occurs in 10–20% of SELH embryos. There are no other known unrelated defects, and the exencephalics usually survive until just after birth. All SELH embryos have an abnormal mechanism of cranial neural tube closure (Fig. 1). The primary abnormality is failure of elevation of midbrain neural folds at the normal time to initiate closure at the forebrain/midbrain boundary (Closure 2) [Macdonald et al., 1989; Gunn et al., 1995]. The

folds are in an abnormally flat shape from very early in their development, although the distribution of actin in the neuroepithelial cells, thought to have a role in neural-fold shape, appears to be normal [Gunn et al., 1993]. Another normal site of initiation of a zipper-like closing of the neural folds, Closure 3, starts at the extreme end of the neural tube, in the region that will later underlie the face. Normally the advancing ''zipper'' from Closure 3, moving caudally, meets the advancing front of Closure 2, moving towards Closure 3. In SELH, where there is no Closure 2, the advancing ''zip'' from Closure 3 continues through the midbrain, enabled by a late elevation of the midbrain neural folds, and closes the entire region that should have been closed earlier from the Closure 2 initiation site, passing last through the ''rhombic lips'' that will later form the cerebellum [Harris et al., 1994]. Most SELH embryos (75–80%) successfully close their neural tube by this abnormal mechanism and grow up to be normal healthy mice. In 10–20%, the midbrain folds never elevate, and the closure mechanism fails, leading to exencephaly. Presumably there is a ''threshold'' of elevating force which is barely adequate in SELH embryos, and that some embryos fail to attain. The insights gained from SELH mice are that a late extension of closure from the most rostral initiation site can compensate for lack of initiation of closure at the forebrain/midbrain boundary, that the closure initiation sites have some redundancy, and that normal adults can often emerge from abnormal embryology, having run perilously close to NTD in their development.

Spina bifida has not been seen in SELH embryos, nor in the segregants after crosses to three normal strains [ Juriloff et al., 1989; Gunn et al., 1992, 1995]. This indicates that there are genetic causes of NTD that affect only the head and supports the view that neural tube closure mechanisms differ along the length of the embryo.

The genetic cause of exencephaly in SELH mice is complex and involves 2 or 3 gene loci acting codominantly and additively [ Juriloff et al., 1989; Gunn et al., 1992; Gunn, 1996]. The consequence of this type of heredity is that embryos have some risk of exencephaly if they have any of these genes, the risk increasing with each additional liability gene. Some of the gene loci are being mapped; none is identified, but it is likely that one is on Chr 13 [Gunn, 1996], and by linkage homology the human equivalent would likely be on chromosome 9q or 5q [ Justice and Stephenson, 1997].

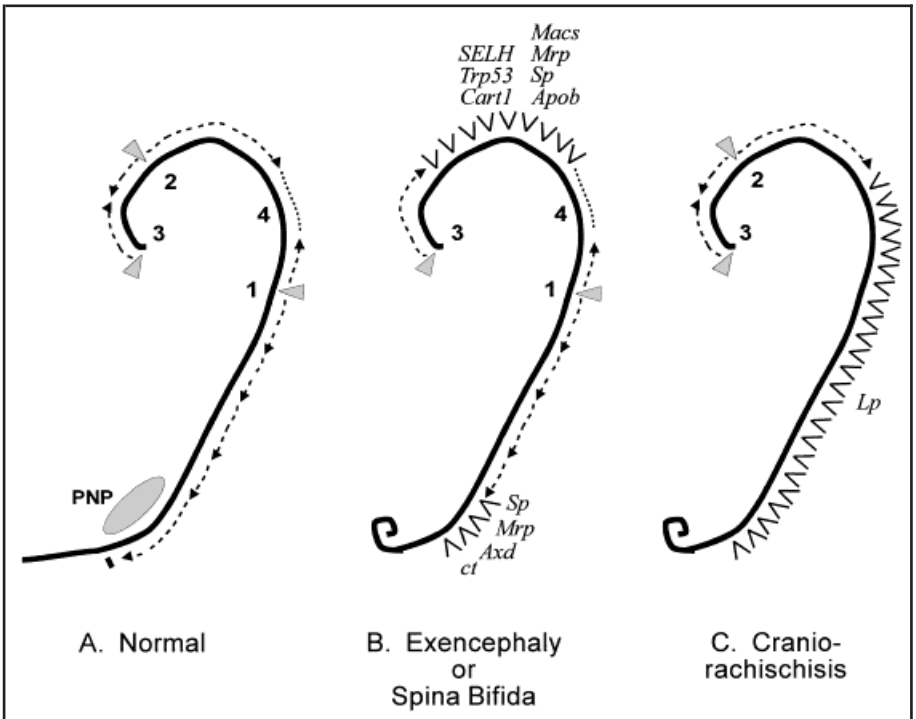


Fig. 1. Location of open neural tube defects (marked by rows of Vs) in mouse NTD models, in relation to initiation sites of normal neural tube closure. Lateral diagrammatic views show patterns of closure from sequential points of initial contact and fusion that are present in normal (**A**) and absent in abnormal (**B** and **C**) genotypes. In B and C, the genes or strains discussed in this review are listed at the location of their characteristic NTD. 1, first point of contact between neural folds, in future cervical region; fusion proceeds towards head and tail; 2, initiation site at boundary between future forebrain and midbrain; fusion proceeds both ways; 3, most rostral initiation site; 4, closure over hindbrain; PNP, posterior neuropore, the last caudal region to close.

Among the apparently normal SELH newborns, a further 5–10% are ataxic, with a midline cleft cerebellum, and their inability to coordinate movement becomes evident 2–3 weeks after birth [ Juriloff et al., 1993]. The cleft traces to a failure of fusion, during neural tube closure, of the neuroepithelial layer of the neural folds under the fused surface ectoderm [Harris et al., 1994], possibly due to the very delayed closure of the cerebellar region, and is caused by the same genes that cause the lack of Closure 2 and exencephaly [Gunn et al., 1995, 1996].

Dietary supplementation (Table 2) of folic acid or methionine does not reduce the risk of exencephaly in SELH mice, but substitution of one normal commercial mouse ration (Purina #5001, PMI Nutrition International, St. Louis, MO) for another (Purina #5015) can consistently reduce the risk 3-fold ( Juriloff and Harris, unpublished data), indicating that there is a dietary agent, yet to be identified, that influences neural tube closure in SELH mice.

*Axd*

Axial defects (*Axd*), a semidominant spontaneous mutation, is a potential model of nonsyndromic spina bifida [Essien et al., 1990; Essien, 1992; Essien and Wannberg, 1993]. Heterozygotes (*Axd/+*) usually have curly tails. Homozygotes (*Axd/Axd*) usually have spina bifida, but neither exencephaly nor other defects. The frequency of spina bifida is strongly affected by modifier genes [Essien, 1992; Essien and Wannberg, 1993].

The *Axd* mutant offers a unique opportunity for study of the mechanism of prevention of spina bifida by supplementation with the amino acid methionine. Either 70 mg/kg or 180 mg/kg of maternal body weight, injected on days 8 and 9 of pregnancy, appears to reduce the frequency of spina bifida in *Axd* homozygotes from about 100% to about 50% [Essien, 1992; Essien and Wannberg, 1993]. This was inferred from the frequency of spina bifida in the progeny of *Axd/+* parents, where one quarter of progeny are expected to be *Axd/Axd*. Methionine is essential for elevation and apposition of cranial neural folds in normal embryos grown in vitro [Coelho and Klein, 1990] and for the normal methylation and localization of actin and αβ-tubulin in the neuroepithelial cells. Without the added methionine the cells become round rather than columnar

[Moephuli et al., 1997]. At least one human study shows that women with a higher dietary intake of methionine are at lower risk of having a child with NTD [Shaw et al., 1997].

Although *Axd* and *ct* share similar NTD and heredity, their response to dietary agents differs. Methionine has no preventative effect on spina bifida in the curly tail mutant [van Straaten et al., 1995], whereas the reduction in frequency of spina bifida in *Axd/Axd* mice appears to be specific to methionine; neither folinic acid (the metabolically active form of folic acid) nor vitamin B12 [Essien and Wannberg, 1993] nor retinoic acid [Haviland and Essien, 1990] is effective. The *Axd* gene locus is not yet mapped. When *Axd* is mapped, linked molecular markers can be used to identify *Axd/Axd* embryos at the time of caudal neural tube closure so that the mechanisms causing failure and rescue of closure can be observed.

*Apob*

Three independent targeted mutations of the *Apob* (apolipoprotein B) gene cause exencephaly in homozygous embryos. One mutation produces a low level of truncated protein and its homozygotes are born, 30% with exencephaly but no other obvious defects. Later, an additional 30% are hydrocephalic. Excessive cell death observed in the neuroepithelium of the developing hindbrain on day 9 of gestation may underlie both the failure of neural tube closure (exencephaly) and the hindbrain defects leading to hydrocephaly. A potential protective role for dietary vitamin E remains to be resolved. Normally, apolipoprotein B contributes to the transport of cholesterol, lipids, and vitamin E in the circulation, and in these mutants as adults the plasma concentration of vitamin E is reduced [Homanics et al., 1995].

Another *Apob* mutation, a gene knockout with complete loss of protein, causes death of most homozygotes by midgestation (D10); the few that survive past day 10 of gestation have exencephaly and are small [Farese et al., 1995]. In a third *Apob* mutation, a gene knockout, all homozygotes die by day 9, before NTD can be recognized [Huang et al., 1995].

Mild loss of apolipoprotein B function in humans, due to *Apob* mutations that code for a truncated protein, is thought to protect against adult coronary vascular disease [Homanics et al., 1995]. However, from the exencephaly and hydrocephaly produced by the truncated *Apob* mutation in the mouse, it appears that what may be good for the adult is not good for the embryo.

*Trp53*

The *Trp53* gene, on mouse Chr 11 and human chromosome 17p, codes for a tumor suppressor (''p53'') involved in the cell cycle and apoptosis [Sah et al., 1995]. Two independent *Trp53* gene knockouts have each resulted in 20–30% exencephaly in homozygous females [Sah et al., 1995; Armstrong et al., 1995]. Although bias towards females is common in both human anencephaly and mouse exencephaly (see below), the bias is most extreme for *Trp53*$^{-/-}$, with 95–100% of exencephalics being female. There is no known explanation.

*The insights gained from SELH mice are that a late extension of closure from the most rostral initiation site can compensate for lack of initiation of closure at the forebrain/midbrain boundary, that the closure initiation sites have some redundancy, and that normal adults can often emerge from abnormal embryology, having run perilously close to NTD in their development.*

Null mutations at *Trp53* are among the very few gene knockouts that cause essentially ''nonsyndromic'' NTD. Exencephalic homozygotes from one knockout were reported to be otherwise histologically normal [Sah et al., 1995]. One quarter of exencephalic homozygotes from the other knockout had a single midline incisor tooth, but no defects of other major organs [Armstrong et al., 1995]. In a third *Trp53* knockout [Donehower et al., 1992], no developmental defects were observed; this knockout has subsequently been shown to have some exencephaly [Sah et al., 1995]. The developmental and molecular mechanisms causing the exencephaly have not been identified; the pattern of apoptosis in the neural folds appeared normal [Sah et al., 1995]. The variation in frequency of exencephaly produced in the three *Trp53* knockout homozygotes may be explained by genetic background effects.

*Cart1*

The *Cart1* knockout is one of two mouse NTD models that have been reported to have a preventative response to folic acid [Zhao et al., 1996]. The cartilage homeoprotein 1 (*Cart1*) gene on mouse Chr 10 [Zhao et al., 1994] codes for a transcription factor, i.e., a protein that binds to certain other genes and regulates their expression. During neural tube closure, *Cart1* is expressed in head mesenchyme but not in the neural tube. Homozygotes for the targeted gene knockout (*Cart1*$^{-/-}$) have a high frequency of exencephaly, ranging up to 100% depending on genetic background, and die at birth [Zhao et al., 1996]. Like SELH, *Cart1*$^{-/-}$ embryos lack the site of initiation of cranial neural tube closure on early day 9 at the forebrain-midbrain boundary (Closure 2), probably due to the absence of supporting mesenchyme cells in the forebrain region.

Treatment of *Cart1*$^{+/-}$ females by injection of folic acid (2.5–3.0 mg/kg) on each day from day 0–9 of pregnancy resulted in a drop in exencephaly in *Cart1*$^{-/-}$ embryos from 100% to about 15%. *Cart1*$^{-/-}$ embryos die at birth whether or not they have exencephaly. They have a syndrome of other craniofacial defects (''shortened faces'' and abnormal eyelid development) and brain defects that are thought to cause neonatal death. The folic acid treatment does not prevent these other defects [Zhao et al., 1996]. The prevention of exencephaly by folic acid in *Cart1* mutants is therefore in contrast with prevention of human NTD by folic acid treatment, where infants with closed neural tubes are normal.

**Splotch**/*Pax3*

Splotch mutations in a gene, *Pax3*, that codes for a transcription factor, are the most widely studied example of Mendelian syndromic NTD in mice and are also important basic models of genetic control of mammalian development. Splotch mutations are fully penetrant and semidominant, with NTD as part of a lethal syndrome of multiple primary defects in homozygotes. Heterozygous carriers usually have a patch of white fur on their belly, white feet, and white tail tip [Auerbach, 1954], and may also have a broadened skull between the eyes [Asher et al., 1996] and some risk (e.g., 5%) of NTD [Moase and Trasler, 1987].

Some Splotch mutations are multigene deletions that cause early embryonic death, and we shall not discuss them further. Others, where the DNA lesion is confined to within the *Pax3* gene (*Sp*, $Sp^{d}$,and $Sp^{2H}$) have not been compared in detail on the same genetic background [Vogan et al., 1993; Epstein et al., 1991, 1993], and the syndrome in homozygotes for these mutations is probably essentially the same. It includes exencephaly and/or spina bifida and defects of other systems. Homozygotes for any of these alleles may survive to birth or may die about 3 days past midgestation if a heart defect is present [Conway et al., 1997a]. The time of death and the relative frequencies of exencephaly and spina bifida are strongly influenced by ''modifier'' genes [Moase and Trasler, 1987]. In some genetic backgrounds, all *Sp/Sp* have spina bifida often combined with exencephaly [Kapron-Bras and Trasler, 1985], whereas on another background, the preponderance of NTD types is reversed and all *Sp/Sp* have exencephaly, sometimes with spina bifida [Moase and Trasler, 1987]. The exencephaly is caused by failure of elevation of the cranial neural folds [Bennett et al., 1998]; the spina bifida originates in a delay and failure of fusion of the caudal neural folds [Moase and Trasler, 1992; Auerbach, 1954]. It is thought that neural folds of Splotch mutants fail to elevate because of an unknown defect of neuroepithelium that also interferes with emigration of neural crest cells [Moase and Trasler, 1992].

Normally the neural crest cells migrate from the neuroepithelium during (cranial) or just after (trunk) neural fold elevation and closure. In Splotch homozygotes there seems to be an inhibition of migration of neural crest cells from the trunk neuroepithelium, progressively more severe and total towards the tail [Kapron-Bras and Trasler, 1988; Moase and Trasler, 1990; Conway et al., 1997b; Serbedzija and McMahon, 1997], but other neural crest cells whose emigration does not seem to be inhibited also fail to reach their destinations in the heart and face [Conway et al., 1997b; Serbedzija and McMahon, 1997; Tremblay et al., 1995]. This leads to absence of pigment cells in skin and hair (Auerbach, 1954), and of ganglia along the spine [Auerbach, 1954] and in the head [Tremblay et al., 1995], and defects of heart morphology and contraction [Conway et al., 1997a,b]. There is also a lack of migration of the myoblast cells from beside the neural tube into the limb bud, leading to a lack of limb muscles [Bober et al., 1994].

*Pax3* is expressed in the dorsal neuroepithelium of the neural folds just prior to, during, and just after neural tube closure, in various subsets of migrating neural crest cells [Goulding et al., 1991], and in dermomyotomes, the source of limb myoblasts [Dahl et al., 1997]. The abnormalities in Splotch mutants probably are due to defective expression of the genes regulated by *Pax3*, but the target genes are not known [Dahl et al., 1997]. Among 11 genes known to be expressed in the closing neural tube, two had abnormal expression, upregulated, in *Sp/Sp* [Bennett et al., 1998]: a transcription factor, *Hmx2*, and a cell adhesion molecule, *NCad*. The *Evx* genes, homologs of a target for the Pax gene in *Drosophila* [Goulding et al., 1991] whose normal expression domains do not seem related to neural tube closure [Bastian and Gruss, 1990; Dolle et al., 1994], were not included in the survey.

The histochemistry of the neural tube and surrounding tissue during neural tube closure is abnormal in Splotch homozygotes [Trasler and Morriss-Kay, 1991]. In addition, there is abnormally strong staining of the cell adhesion molecule N-CAM in lateral areas of the neuroepithelium and presence of an additional, heavier N-CAM isoform [Moase and Trasler, 1991] due to premature sialylation [Neale and Trasler, 1994].

Recently, an intriguing indirect metabolic effect of the Splotch mutation was described [Fleming and Copp, 1998]. Splotch homozygotes appear to have a metabolic deficiency in the supply of folate for the biosynthesis of pyrimidine. Exogenous folic acid or thymidine appears to prevent spina bifida in about 40% of Splotch homozygotes in vivo (Table 2) and to prevent a large proportion of both spina bifida and exencephaly in vitro.

Do mutations in *PAX3* cause human NTD? Humans with one copy of *PAX3* mutated (heterozygotes) have Waardenberg syndrome type I (WSI), with symptoms similar to those of Splotch heterozygotes. They often have a white forelock, light eyes, wide bridge of the nose, and deafness [Strachan and Read, 1994]. Like *Sp/+*, WSI heterozygotes appear to have a slightly increased risk of spina bifida [Hol et al., 1995]. However, WSI is rare, and the subset with NTD would account for a tiny proportion of NTD cases. Familial spina bifida is seldom due to unrecognized WSI [Hol et al., 1995] or other *PAX3* mutations [Chatkupt et al., 1995]. Homozygotes for *PAX3* mutations are expected to be very rare. The one confirmed case had severe expression of the same anomalies as WSI, with an additional muscle ''wasting'' defect of the upper limbs, but no NTD [Zlotogora et al., 1995].

Splotch heterozygote mice are an excellent animal model for WSI, with mutations in homologous genes (*Pax3*/*PAX3*) and similar phenotypes. Splotch mice, heterozygote or homozygote, seem unlikely to be homologous to common nonsyndromic human NTD.

### Looptail

Looptail, *Lp*, a Mendelian semidominant mutation, demonstrates that the mechanisms responsible for neural tube closure differ between the head and the trunk, and points to a gene product essential for trunk neural tube closure. Heterozygotes (*Lp/+*) have a curled tail defect and occasional spina bifida, and homozygotes (*Lp/Lp*) have an open neural tube from the hindbrain to the tail (craniorachischisis) and die during late gestation or at birth [Stein and Rudin, 1953; Wilson and Finta, 1980; Copp et al., 1994]. *Lp* has been mapped on mouse Chr 1 with high resolution (homologous to human 1q) in preparation for positional cloning of the gene [Mullick et al., 1995; Stanier et al., 1995]. A closely linked molecular marker has been used to identify *Lp/Lp* and *Lp/+* embryos in segregating litters during neural tube closure, to observe the primary defect. *Lp/+* have delayed initiation of ''Closure 1'' (apposition and fusion of neural folds at the prospective cervical/hindbrain boundary) and delayed posterior neuropore closure [Copp et al., 1994]. *Lp/Lp* fail to initiate ''Closure 1,'' and the whole length of neural tube usually closed from this initiation site remains open. The cellular mechanisms causing this failure are not known. Closure cannot be induced by artificial physical apposition of the folds [Gerrelli and Copp, 1997]. Interestingly, the midbrain and forebrain regions of the neural tube do close, and initiation of closure at the forebrain/midbrain boundary (Closure 2) appears to be normal [Copp et al., 1994].

## GENDER BIAS IN EXENCEPHALY FREQUENCY

Among human anencephalics, the frequency of females is almost twice that of males. Among spina bifida cases, affecting the lower spine, the bias is opposite: the frequency of males is about twice that of females [Seller, 1995]. Among mouse genetic NTD models, gender bias is common and large for exencephaly, and rare for spina bifida (see Table 1). In all the mouse models for which gender has been reported, exen-

cephaly shows a large female excess. The ratio of females to males among exencephalics ranges from approximately 2:1 in the SELH genotype [Macdonald et al., 1989], the *Macs* gene knockout [Stumpo et al., 1995], the *xn* mutant [Wallace et al., 1978], and the *crn* mutant [Kalter, 1988], to 4:1 in the curly tail stock [Brook et al., 1994; Embury et al., 1979], to 15:1 in the NZW-*xid* strain [Vogelweid et al., 1993], and to more than 20:1 in the *Trp53* gene knockouts [Sah et al., 1995; Armstrong et al., 1995]. Gender bias, toward males, for spina bifida has been reported for only the curly tail mutant, and the bias is slight, i.e., 1:1.3 [Copp and Brook, 1989]. There is evidence for SELH, curly tail, and the *Trp53* knockout that the female excess is *not* due to prenatal death of exencephalic male embryos. Neural tube closure is completed before gonad formation. The female excess in exencephaly, particularly the extreme seen in the *Trp53* knockouts, presents an intriguing puzzle: what is the relationship between chromosomal gender, the cell cycle, and cranial neural fold elevation? Solving this puzzle by detailed study of X-inactivation, cellular behavior, and gene expression in the cranial neural folds holds the promise of profoundly deepening our understanding of the process of cranial neural fold elevation.

## MOUSE GENETIC MODELS FOR SPINA BIFIDA OCCULTA (SBO)

Confusion occurs when ''spina bifida'' is used to mean spina bifida occulta (SBO) as well as spina bifida aperta (SBA), and the question arises as to whether or not these two defects are developmentally and genetically related. In SBA, the primary defect is an early failure of the neural tube to close. In SBO, the neural tube closes (on day 9–10 of gestation in the mouse), but later the vertebrae lack bony arches that normally form from cartilaginous condensations in the dorsal midline (on day 13–15 of gestation [Kaufman, 1992]). The difference of timing and structure suggests that SBA and SBO have different developmental origins and that the relationship implied by the nomenclature is misleading.

The usual pattern in mouse models is that mutations cause either spina bifida aperta or spina bifida occulta, but not both. For example, in homozygotes for the Patch (*Ph*) mutation, the neural tube closes normally; the spinal defect is confined to the neural arches of the vertebrae and is thought to be a primary defect of somitic mesoderm rather than of neural tube [Payne et al., 1997]. Other examples are the *Tgfb2* gene knockout with 100% SBO and no SBA, snubnose (*sno*) and congenital hydrocephalus (*ch*) with ''frequent'' SBO and no SBA, and the *MHox* gene knockout with 10% SBO and no SBA [Sanford et al., 1997; Hollander, 1976; Gruneberg, 1963; Martin et al., 1995]. There are no mouse mutants reported to have a high frequency of SBO and some SBA. While some authors have hypothesized that SBO is present in mutations with SBA, the SBO has not been clearly demonstrated [Essien, 1992; Wilson and Wyatt, 1986; Embury et al., 1979]. The exception, where SBA and SBO do have common genetic ground, is a complex genetic situation based on the mutation curtailed ($T^c$), in which the $T^c/t^{w5}$ genotype gives 100% SBA and the $T^c/+$ genotype gives 100% SBO [Park et al., 1989].

*The female excess in exencephaly, particularly the extreme seen in the Trp53 knockouts, presents an intriguing puzzle: what is the relationship between chromosomal gender, the cell cycle, and cranial neural fold elevation?*

SBO is a common and often mild and undetected trait in humans. Whether or not liability to SBA and to SBO can be caused by the same genotype in humans is unclear [reviewed in Harris and Juriloff, 1997]. In mouse mutants, the liabilities to SBO and SBA are usually genetically distinct.

## SELECTED MODELS FOR NTD ASSOCIATED WITH HUMAN TERATOGENS

### Valproic Acid

Women treated with the anticonvulsant, valproic acid, have a mildly elevated risk of spina bifida in their offspring [Holmes, 1994]. Valproic acid causes exencephaly and less commonly spina bifida in genetically normal mouse embryos [Ehlers et al., 1992a; Finnell et al., 1988]. The risk differs among normal genetically different strains and the genes responsible are probably directly involved in neural tube closure, rather than in metabolism of valproic acid [Finnell et al., 1988]. Embryos with genetic risk of spontaneous NTD, in the SELH/Bc strain, have a very high frequency of exencephaly (70%) after valproic acid exposure [Hall et al., 1997]. In contrast, the genetic cause of spontaneous NTD in the TO strain does not lead to exceptionally high frequencies of NTD, the risk (30%) being similar to that of normal strains at the same dose of valproic acid [Padmanabhan and Ahmed, 1996; Padmanabhan and Hameed, 1994; Hall et al., 1997]. Valproic acid in genetically normal embryos causes disorganization of the cellular structure of the neuroepithelium [Turner et al., 1990; Ehlers et al., 1992b]. Interference with folate metabolism may be involved [Nau, 1994], and various transcription factors and cell cycle genes, including p53, may be overexpressed [Wlodarczyk et al., 1996]. It will be interesting to see whether any of the genes whose function is indirectly disrupted by valproic acid are also downstream targets of regulatory genes causing spontaneous NTD liability.

### Retinoids

Retinoids, such as retinoic acid or isotretinoin, are human teratogens. In genetically normal mouse embryos, retinoic acid causes exencephaly and/or spina bifida [Yasuda et al., 1986] through damage to neuroepithelial cell organization and cytoskeletal structure [Yasuda et al., 1987]. Genetic liability to NTD combines with retinoic acid exposure on day 8 of gestation to produce risk of NTD much higher than in normal genotypes, as demonstrated by about 50% exencephaly in the curly tail stock [Seller et al., 1979] and the SELH strain [Tom et al., 1991], compared with 0–20% in normal strains. Splotch heterozygotes also have a risk of NTD after retinoic acid exposure about twice that of genetically normal embryos [Kapron-Bras and Trasler, 1984]. Although the effect is dramatic, the mechanism may be nonspecific. These genotypes are minimally able to successfully elevate the neural folds under normal conditions and are less able to overcome any further inhibition, as demonstrated in SELH mice [Tom et al., 1991].

## CONCLUDING REMARKS

For the mammalian embryo, the complexity of the task of elevating the neural folds must rival that of the ancient Egyptians' raising immense stone obe-

lisks. The foundation, timing, and elevating forces have to be finely coordinated, and a lot can go wrong. The neural folds are built on a foundation of other tissues, and if the foundation is faulty the elevation of the folds will be jeopardized. Thus, mutations of genes that have no direct role in neural fold tissue can cause NTD. In mice there are more mutations and strains with exencephaly than for any other defect, except perhaps cleft palate, another ''elevation challenge.''

Most human NTD appear to be morphologically simple and genetically complex. Most of the mouse mutations cause morphologically complex multi-organ syndromes and are genetically simple. The NTD of the targeted and spontaneous mutants that have severe multi-organ syndromes causing death in midgestation (equivalent to the fifth week in human development) would not be detected to be included among human NTD cases, and they do not seem to be good models for common human NTD. However, setting the syndromic mutations aside, there is still considerable genetic heterogeneity of cause of NTD in mice, with various degrees of genetic complexity. The nonsyndromic NTD of mice are generally genetically complex and have multiple distinct genetic etiologies. This predicts a similar heterogeneity of complex heredity in humans. Complex heredity patterns arise from genetic mechanisms where each contributing gene adds incrementally to the risk of failure of neural fold elevation. The mouse models demonstrate that exencephaly and spina bifida can be alternative expressions of one genetic cause, with the preference controlled by modifiers, but that some genotypes can cause only one or the other. This type of heterogeneity could well be buried in the complexity of human NTD epidemiological data. In mice, various nutritional components prevent NTD caused by different genetic mechanisms, and each genotype responds to a different specific nutrient. This raises the possibility that there will be an assortment of effective preventative strategies for human NTD of different genetic origin. It should be noted that none of the mouse models is a known defect in vitamin metabolism.

We expect that in the near future the genes contributing to the more complex mouse NTD models will be mapped and identified. Because of the extensive gene and linkage homology between mouse and human, it will be possible to test for association of human NTD risk with these good candidate genes. When the gene identities are known, their function and the cellular mechanisms leading to NTD should become evident. ■

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