See the corresponding editorial in this issue, pp 211–212.

J Neurosurg (3 Suppl Pediatrics) 106:213–221, 2007

Fetal spina bifida in a mouse model: loss of neural function in utero

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Object. The devastating neurological deficit associated with myelomeningocele has previously been assumed to be a direct and inevitable consequence of the primary malformation—failure of neural tube closure. An alternative view is that secondary damage to the pathologically exposed spinal cord tissue in utero is responsible for the neurological deficiency. If the latter mechanism were shown to be correct, it would provide an objective rationale for the performance of in utero surgery for myelomeningocele, because coverage of the exposed spinal cord could be expected to alleviate or perhaps prevent neurodegeneration. To examine this question, the authors studied the development of neuronal connections and neurological function of mice during fetal and neonatal stages in a genetic model of exposed lumbosacral spina bifida.

Methods. The persistently exposed spinal cord of mouse fetuses carrying both curly tail and loop-tail mutations exhibited essentially normal anatomical and functional hallmarks of development during early gestation (embryonic Days 13.5–16.5), including sensory and motor projections to and from the cord. A significant proportion of fetuses with spina bifida at early gestation exhibited sensorimotor function identical to that seen in age-matched healthy controls. However, at later gestational stages, increasing neurodegeneration within the spina bifida lesion was detected, which was paralleled by a progressive loss of neurological function.

Conclusions. These findings provide support for the hypothesis that neurological deficit in human myelomeningocele arises following secondary neural tissue destruction and loss of function during pregnancy.

Key Words • myelomeningocele • spina bifida • neurological deficit • neural tube • fetal surgery • mouse

Myelomeningocele is the most severe type of spina bifida, and in most cases leads to lifelong and devastating physical and developmental disability. These disabilities include, but are not limited to, paraplegia, hydrocephalus, incontinence, sexual dysfunction, various skeletal deformations, endocrine disorders, and impaired mental development.[6,7,15] Both genetic and environmental factors play a role in the etiology of myelomeningocele; in the majority of cases, a disorder of primary neurulation leads to failure of neural tube closure.[4,7]

A widely supported hypothesis is that neurological deficiency in survivors with myelomeningocele is related to neurodegeneration in utero of the exposed spinal cord. Results from studies of therapeutically aborted fetuses with myelomeningocele have demonstrated damage to the open spinal cord that appeared to be acquired in utero.[16,21,23] These findings have been supplemented by surgical creation of myelomeningocele-like lesions in utero in the monkey,[26] sheep,[24,25] rabbit,[14,30,31] and rat,[5,11] all of which demonstrated progressive loss of spinal cord tissue with increasing gestation. Furthermore, there is direct evidence that in vitro survival of rat spinal cord cells is diminished by exposure to human amniotic fluid.[8] These findings led Heffez and colleagues[11] to propose a "two-hit" hypothesis in which failure of neural tube closure (the first hit) is followed by in utero spinal cord degeneration (the second hit), resulting in the typical neurological deficit of myelomeningocele.

One condition of the two-hit hypothesis is that the early dysraphic spinal cord initially develops with normal function. The rationale for human prenatal surgical repair in early myelomeningocele is to prevent neurodegeneration, thereby preserving the functional competence present in the early exposed spinal cord. In seeking evidence for functional development of the dysraphic spinal cord, one cannot rely on data from animal models in which a myelomeningocele is created artificially, but must instead look to naturally occurring myelomeningocele lesions. Early gestation human embryos and fetuses have been found to exhibit normally differentiated spinal cord tissue at the site of the myelomeningocele lesion.[23] Moreover, ultrasound examination has detected leg movements in 16- to 17-week-old fetuses with myelomeningocele.[20] Mouse genetic models provide the

Abbreviations used in this paper: DiI = 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FCS = fetal calf serum; PBS = phosphate-buffered saline; TBS = Tris-buffered saline.

primary source of data for naturally occurring spina bifida among experimental animals.[4] Several studies have provided evidence showing that the dysraphic mouse neural plate is initially composed of healthy neuroepithelial cells that appear to undergo neuronal differentiation according to normal developmental progression.[33]

To date, what is lacking is any evidence on whether mouse fetuses with spina bifida develop neurological function at the site of the lesion, and then lose this function as neurodegeneration occurs in utero. Alternatively, it is possible that neurological function is absent throughout gestation in mice with spina bifida. In this study, we used a genetic model of mice with naturally occurring spina bifida[32,34] to distinguish between these two possibilities.

Materials and Methods

Mouse Strains and Specimen Collection

Mutant curly tail (ct) mice were originally obtained from Dr. Mary Seller at Guy's Hospital London and have been maintained subsequently as a closed, randomly breeding colony of homozygous individual mice for more than 20 years. Loop-tail (Lp) mice were originally obtained from the Jackson Laboratory and have been maintained subsequently as an inbred strain (LPT/Le) for more than 15 years. Both strains were kept on a 12-hour light/dark cycle (lights on from 7:00 a.m. to 7:00 p.m.). Overnight matings between doubly heterozygous loop-tail/curly tail males (Lp/+; ct/+) and homozygous curly tail females (+/+; ct/ct) were determined by finding a vaginal plug the following morning, and midday was designated as embryonic Day 0.5. Full-term was typically embryonic Day 19.5, or postnatal Day 1.

Matings generated litters containing fetuses with three different phenotypes: on average, 34% of fetuses had open spina bifida of variable size, but always extending rostrally from the base of the curled tail to the different levels of the thoracic, lumbar, or sacral spine; 25.5% of fetuses had curled or looped tails in the absence of spina bifida; and 40.5% were entirely normal, with straight tails. Spina bifida lesions varied in size within the same litter; to standardize the procedure, only animals with the largest defects were selected for testing. Thus, all spina bifida fetuses tested had a lesion extending rostrally from the tail base to the upper lumbar or lower thoracic region. Animals without spina bifida were used as within-litter controls. Pregnant females were killed by cervical dislocation on embryonic Days 12.5 to 18.5 to collect experimental fetuses. Neonates were collected on the morning after nocturnal vaginal delivery, with the most severely affected living pups selected for neurological testing.

Fetuses were removed from the uterus and placed in Dulbecco modified Eagle medium (Gibco BRL) containing 10% FCS. The yolk sac and amnion were removed and the fetus was kept alive and intact while still on placental support. In each litter, one fetus with spina bifida and one control fetus was selected for neurological assessment (described later). After the neurological assessment, the umbilical cord and placenta were removed and fetuses were killed on a frozen metal plate. Neonates were killed by injection of an overdose of midazolam and fentanyl. Fetuses were rinsed in PBS, and processed for histological, immunohistochemical, or immunofluorescence studies. All animal experiments were performed in accordance with the regulations of the 1986 Animals (Scientific Procedures) Act of the United Kingdom Government.

Histological Analysis

A total of 85 fetuses or neonates were processed for histological analysis as follows: two with spina bifida, two controls (embryonic Day 13.5); four with spina bifida, three controls (embryonic Day 14.5); six with spina bifida, five controls (embryonic Day 15.5); eight with spina bifida, seven controls (embryonic Day 16.5); seven with spina bifida, eight controls (embryonic Day 17.5); six with spina bifida, seven controls (embryonic Day 18.5); and 10 with spina bifida, 10 controls (neonates). Specimens were fixed in Bouin solution (Sigma Chemical Corp.) for 2 to 7 days depending on age, serially dehydrated, and embedded in paraffin wax. Sections were cut with a rotary microtome (Microm HM330), in either a transverse or sagittal plane to the body axis, at 10- to 12-μm thickness. Slides were dewaxed with Histo-Clear (National Diagnostics), serially rehydrated to distilled water, stained with Ehrlich hematoxylin for 10 and eosin for 5 minutes, dehydrated, mounted in DPX, and coverslipped.

Immunohistochemical Analysis (Neurofilament Staining)

Specimens collected from fetuses on embryonic Days 12.5 to 18.5, and from postnatal Day 1 pups (56 total; three–four fetuses of each phenotype at each stage), were fixed in 4% paraformaldehyde/PBS for 2 to 7 days, depending on age. They were dehydrated through an ethanol series and embedded in paraffin wax. Transverse sections (6 or 8 μm thick) were dewaxed, rehydrated, and unmasked with Declere (Cell Marque) in a microwave oven for 20 minutes. Endogenous peroxidase was quenched by incubation in 0.6% H_2O_2 in TBS for 7.5 minutes and nonspecific epitopes were blocked by exposure to 10% FCS/TBS. Primary monoclonal antineurofilament 68 antibody (N5139, mouse immunoglobulin G_1; Sigma) was diluted 1:400 in TBS containing 1% FCS and 0.5% Triton and applied to sections overnight at 4°C. The next day the sections were washed in 1% FCS/0.5% Triton/TBS and exposed for 1 hour to secondary biotinylated rabbit anti–mouse antibody (DAKO Corp.) diluted 1:500 in the same solution. After washing in TBS, sections were reacted with streptavidin–biotinylated horseradish peroxidase (DAKO) for 30 minutes at room temperature. Coloration of the neurofilaments was obtained by adding 3,3′-diaminobenzidine (Sigma). In negative control sections, the primary antibody was replaced by 1% FCS/0.5% Triton/TBS, with negligible background staining.

Immunofluorescent Neuronal Tracing Studies

Specimens were collected from fetuses on embryonic Days 12.5 to 18.5, and from postnatal Day 1 pups (97 total; between five and six fetuses of each phenotype at each stage), and fixed in 4% paraformaldehyde/PBS at 4°C for 2 to 5 days, depending on age. Using a light microscope, the fetal or neonatal spine was exposed and gently perforated with the tip of a forceps to create small holes at different thoracic and lumbar levels. Single crystals of DiI (Molecular Probes) were inserted into the holes. In negative control preparations, the midthigh and/or the spine rostrally and caudally to the insertion site was transected following crystal insertion, to demonstrate the prevention of dye diffusion after axonal interruption. Fetuses or neonates were then placed in 2% formalin/PBS and incubated at room temperature (for younger specimens) or 37°C (for older specimens) for 3 weeks to 4 months, depending on incubation temperature, age of specimen, and distance between insertion site and peripheral target. After incubation, 24 specimens (one–two of each phenotype at each stage) were embedded in 10% agarose and sectioned transversely at 100- to 150-μm thickness on a vibratome (Series 1000, Agar Scientific). Sections were mounted on glass slides, covered with glycerol/PBS in a 1:1 ratio, and examined under fluorescence illumination using a light microscope (Axiophot, Zeiss). Thereafter, specimens were stored at 4°C in the dark.

Neurological Assessment In Vivo

Specimens were collected from fetuses on embryonic Days 16.5 to 18.5, and from postnatal Day 1 pups (160 total; 20 animals of each phenotype at each stage). Fetuses at embryonic Day 15.5 and younger were not investigated because a pilot series revealed that no neurological reaction was elicited in unaffected controls (data not shown), probably because of physiological immaturity of the nervous system at these early stages of gestation.[19,37]

Performance of functional tests was limited to the first 4 minutes after killing the mother, while the fetus was maintained on placental support ex utero, to ensure the vital reactions of the examined fetuses. Thus, only one animal with spina bifida and one phenotypically normal fetus could be examined from each litter. To elicit a classic pain reaction, microforceps were used for a standard pinch test of both forepaws, both hindpaws, and tail in all animals. All tests were repeated three times. Results were considered conclusive, and a nor-

mal pain reaction was recorded, only if an identical response was obtained on both upper and lower limbs, and on both sides of the body, in all three consecutive test series.

Results

A combination of histological and immunohistochemical analyses and DiI tracing was used to determine the time course of spinal cord development in the region of the spina bifida lesion in affected fetuses, in comparison with the spinal cords of healthy controls.

Early Spinal Cord Development in the Spina Bifida Lesion

The normal sequence of spinal cord development between embryonic Day 13.5 and postnatal Day 1 was clearly revealed by H & E staining (Fig. 1A, C, E, and G) and immunohistochemistry analysis for neurofilaments (Fig. 2A, C, E, and G). In transverse cross-sections obtained through the lower trunk of control fetuses, the marked progression of neuronal differentiation within the spinal cord, the development of sensory and motor roots, and the establishment of dorsal root ganglia were noted.

Spina bifida fetuses at early gestation (embryonic Day 13.5) demonstrated a spinal cord lesion that was directly exposed to the amniotic cavity as a result of the neural tube failing to close. The neuroepithelium was not covered by meninges or other tissues. At this early point of gestation, the cytoarchitecture of the spinal cord appeared to have developed normally, and there were no signs of traumatic or degenerative alterations (Fig. 1B). Immunohistochemical analysis of neurofilaments showed that sensory and motor roots and dorsal root ganglia had all developed with apparently normal morphological characteristics (Fig. 2B). The only obvious deviation from normal was a marked kinking of the sensory roots, as opposed to a straighter course in controls. This deviation appeared to be the result of the extremely lateral position of the dorsal horns in the open spina bifida lesion.

Spinal Cord Characteristics During Later Spina Bifida Development

Cross-sections obtained through the spina bifida lesion stained with H & E after ongoing fetal gestation (Fig. 1D, F, and H) revealed signs of progressive neural tissue damage, ranging from superficial abrasion, erosion, and hemorrhage (embryonic Day 16.5), to tissue avulsion and degeneration (embryonic Day 18.5), and finally to complete loss of all formerly exposed spinal cord tissue at birth (postnatal Day 1). Neurofilament staining revealed good preservation of the sensory and motor roots and the dorsal root ganglia at embryonic Days 15.5 and 17.5, despite progressive alteration of the spinal cord (Fig. 2D and F). At postnatal Day 1, however, only tiny remnants of sensory and motor roots and dorsal root ganglia were identifiable in some sections (Fig. 2H), whereas in other sections no neural tissue remained at the spina bifida site.

Neuronal Connections Between the Spinal Cord and Peripheral Tissues

Tracing studies using DiI were performed (Fig. 3A and B) to demonstrate the presence or absence of functionally intact neuronal connections between the spinal cord and peripheral tissues. Nerves exiting the spinal cord and extending to the hindlimb could be identified in all control fetuses between embryonic Days 12.5 and 17.5 (Fig. 3C, E, and G). At later stages, nerves could not be identified using this technique, because of the thickness of overlying soft tissues.

In spina bifida fetuses, nerves connecting the open spinal cord to the hindlimbs were identifiable up to embryonic Day 15.5 (Fig. 3D and F), indicating the development of intact neuronal continuity between the spinal cord and peripheral tissues, despite failure of neural tube closure. In contrast, at embryonic Day 17.5, neural projections from the spina bifida lesion to the hindlimbs were barely visible, indicating a largely disrupted neuronal continuity between the spinal cord and peripheral tissues at this stage (Fig. 3H).

Sensorimotor Function in the Lower Spinal Cord

A standard set of pinching tests was performed to check for sensorimotor function in fetuses maintained on placental support ex utero (Table 1). In controls, pinching of the forepaw or hindpaw resulted in an immediate (within 2 seconds) withdrawal movement of the respective limb, squirming of the ipsilateral body side, and silent mouth opening in fetuses, or audible vocalization in neonates. Similarly, pinching of the tail resulted in an immediate motor response of all four extremities, together with trunk squirming, and silent mouth opening in fetuses or audible vocalization in neonates. All responses were immediate, simultaneous (combined withdrawal, squirming, and mouth opening), and were consistently and repetitively present in all controls at all gestational stages tested (embryonic Day 16.5–postnatal Day 1); therefore, these reaction patterns were interpreted as normal physiological fetal or neonatal pain reactions.

Among spina bifida fetuses, forepaw pinching elicited a normal pain reaction (identical to that observed in age-matched controls) in all fetuses at all gestational stages (embryonic Day 16.5–postnatal Day 1). A normal pain reaction after hindpaw pinching was recorded in 55% of spina bifida fetuses at embryonic Day 16.5, in 50% at embryonic Day 17.5, in 45% at embryonic Day 18.5, and in 85% of neonates (Table 1). Tail pinching produced a normal pain reaction in 20% of spina bifida fetuses at embryonic Day 16.5 and in 5% at embryonic Day 17.5. All five of these spina bifida fetuses that were responsive to tail pinching also had a positive hindpaw response. In contrast, none of the spina bifida fetuses at embryonic Day 18.5 or neonates responded to tail pinching. These results of qualitative analysis of pain perception show that apparently normal neurological function is present in a considerable proportion of affected fetuses at embryonic Day 16.5 despite spina bifida, as evidenced by normal pain reactions after both hindpaw and tail pinching.

Discussion

In this study, we used a mouse genetic model in which spina bifida developed spontaneously, and with many similarities to human myelomeningocele,[32,34] to address two questions. The first question is whether spinal cord development, after the establishment of functional nerve connections, proceeds relatively normally at early gestational stages of spina bifida. The second question is whether neurodegeneration during later fetal stages ultimately leads to loss of spinal cord tissue, neuronal connections, and neuro-

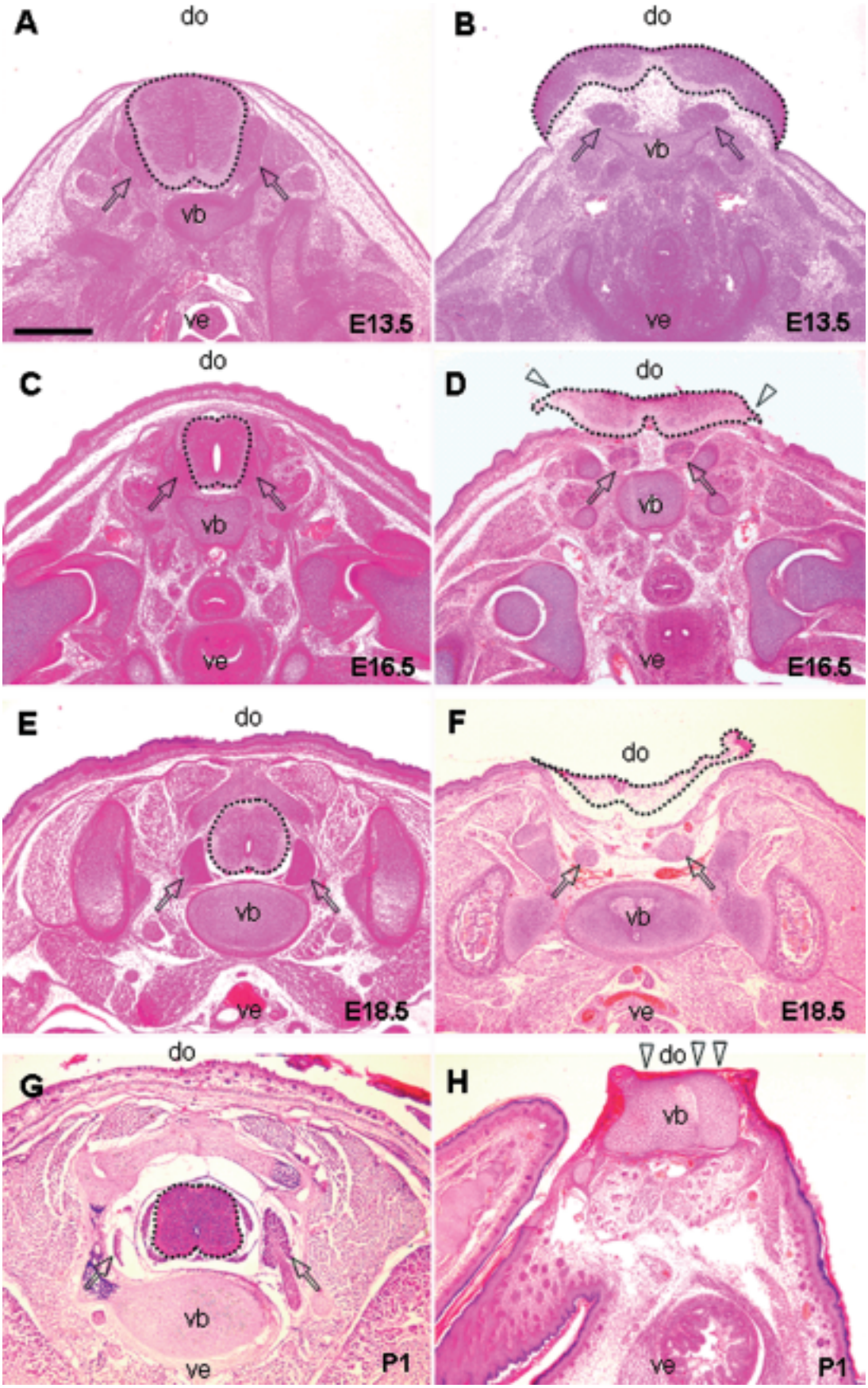

FIG. 1. Photomicrographs showing cross-sections from control (A, C, E, and G) and spina bifida (B, D, F, and H) fetuses at embryonic (E) Days 13.5, 16.5, and 18.5, and postnatal (P) Day 1. The spinal cord *(dotted line)* and dorsal (do) root ganglia *(arrows)* are indicated. Sections obtained through the lumbar spinal area in control animals show normal development of the spinal cord, with dorsal root ganglia located laterally and surrounding skeletal and soft tissues. Sections shown in panels B, D, F, and H were obtained through the central part of age-matched littermate fetuses or neonates with spina bifida. A dorsally exposed intact spinal cord faces the amniotic cavity at embryonic Day 13.5 (B). The gray matter (purple), located dorsally, can be distinguished from the ventrally (ve) located white matter (light red). Dorsal root ganglia are located ventrally as a consequence of the failure of neural tube closure. At embryonic Day 16.5, the dorsally exposed spinal cord exhibits mild to moderate superficial neural tissue abrasion and hemorrhage, mostly located laterally *(arrowheads)* and probably resulting from contact with the uterine wall (D). At embryonic Day 18.5, considerable loss of spinal cord tissue is visible, with only a few remnants of gray matter (purple) remaining. In contrast, more ventrally located white matter (light red) and dorsal root ganglia are mostly present (F). At birth, all exposed neural tissues are gone and the dorsal root ganglia are no longer discernible. The bare dorsal aspect of the vertebral body (vb) *(arrowheads)* faces the amniotic cavity (H). H & E, bar = 250 μm (A and B), 500 μm (C–H).

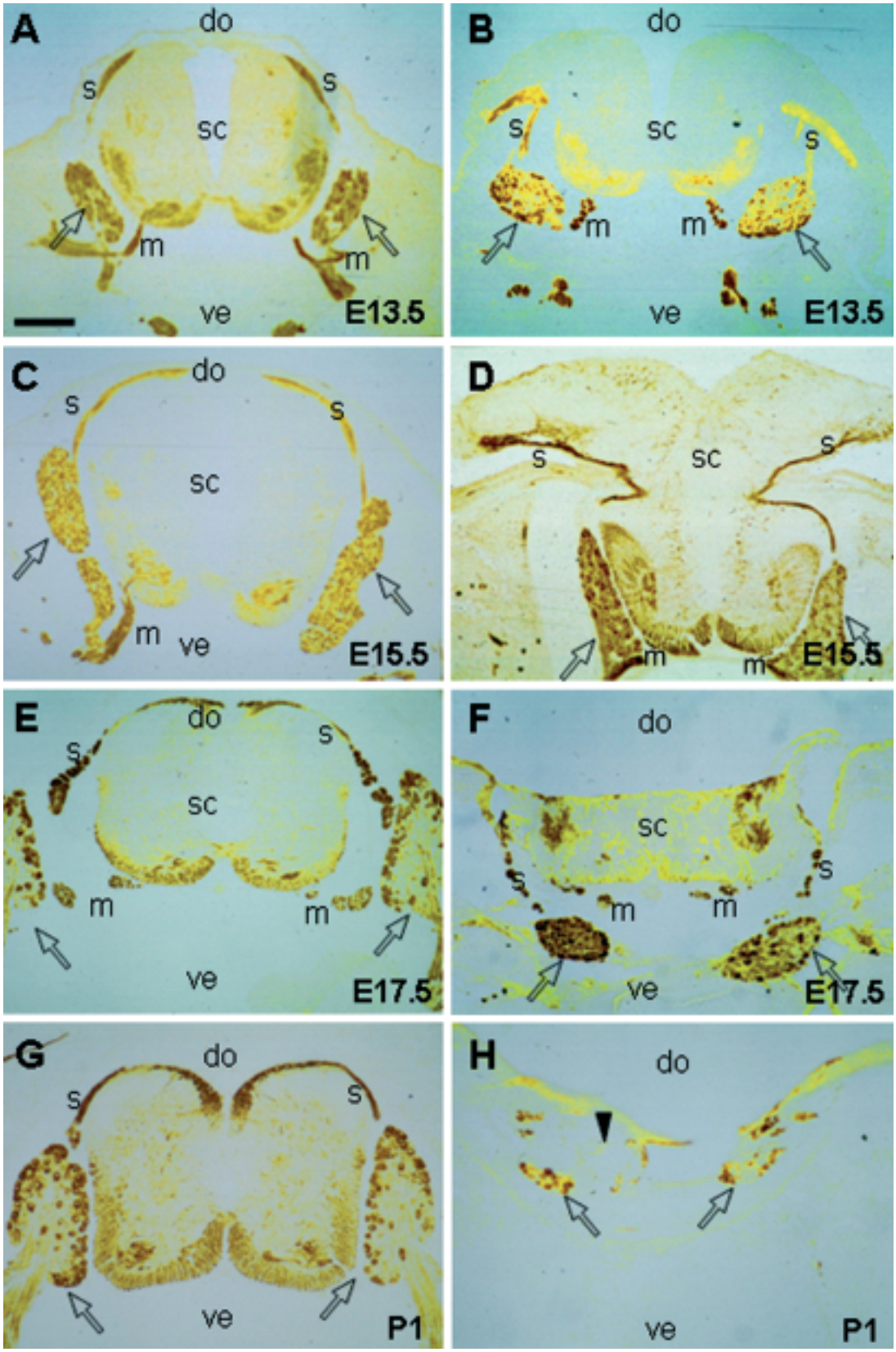

FIG. 2. Photomicrographs showing neurofilament immunostaining of cross-sections from normal (A, C, E, and G) and spina bifida (B, D, F, and H) fetuses at embryonic Days 13.5, 15.5, and 17.5, and postnatal Day 1. Cross-sections were obtained through the lumbar spinal area in control animals, and through the center in age-matched littermate fetuses or neonates with spina bifida. A, C, E, and G: The neurofilament immunostaining method provides good visualization of sensory roots (s) projecting into the dorsal part of the spinal cord (sc), motor roots (m) exiting the spinal cord ventrally, and dorsal root ganglia *(arrows)* located ventrolateral to the spinal cord. These structures characteristically exhibit a brownish coloration, whereas the spinal cord tissue stains light yellow. B, D, and F: In spina bifida fetuses at embryonic Days 13.5, 15.5, and 17.5, respectively, sensory roots, motor roots, and dorsal root ganglia are clearly visible and appear to be intact. As a consequence of failed neural tube closure, the sensory roots demonstrate an S-shaped kinking (particularly visible in panel D) and the dorsal root ganglia are located in a more ventral position than in controls. Location of motor roots is similar to that in normal mice. H: At birth, only remnants of neural tissue remain. Note the few neural bundles (probably motor roots) that persist *(arrowhead)*. Dorsal root ganglia *(arrows)* are much smaller than in controls at this stage (G). Bar = 200 μm.

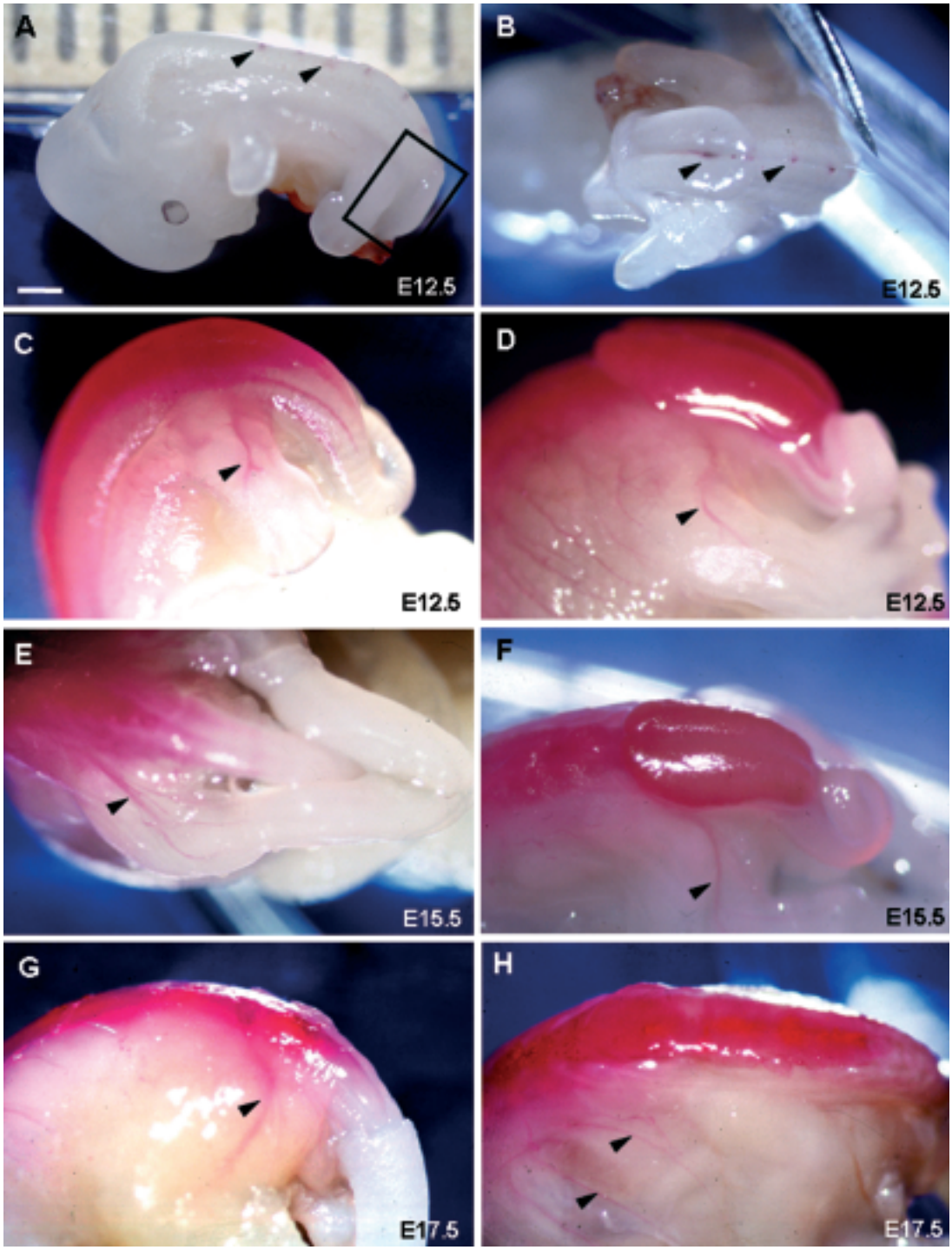

FIG. 3. Photographs from DiI tracer studies showing normal and spina bifida fetuses at embryonic Days 12.5 (A–D), 15.5 (E and F), and 17.5 (G and H). A and B: Lateral (A) and dorsal/caudal (B) views of a spina bifida fetus immediately after insertion of DiI crystals *(arrowheads)*. *Box outline* in panel A indicates the lumbosacral area, including the spina bifida lesion and adjacent closed spinal cord, enlarged in panel B. *Arrowhead* on the left side (panel B) indicates DiI crystal within the myelomeningocele. Two other crystals have been inserted into the closed spinal cord rostral to the spina bifida lesion. Note the 20-gauge needle tip for a size comparison (B). C–H: Specimens obtained several weeks after insertion of DiI crystals. *Arrowheads* indicate a peripheral nerve exiting the spinal cord and running into the hindlimb in both control (C) and spina bifida (D) fetuses, with a similar appearance in fetuses at embryonic Day 15.5 (E and F). At embryonic Day 17.5 (G and H), a nerve exiting the spinal cord and projecting into the hindlimb *(arrowhead)* is visible in the normal fetus (G), whereas neural projections into the hindlimb from the spina bifida lesion are no longer visible (H), although nerves can be seen exiting the spinal cord at levels rostral to the spina bifida lesion (*arrowheads* in H). Bar = 1 mm (A), 0.7 mm (B), 0.35 mm (C and D), 0.7 mm (E), 0.55 mm (F), and 0.65 mm (G and H).

TABLE 1

*Summary of the results of in vivo neurological testing of spina bifida fetuses at various stages of gestation**

Pain Stimulus	Gestational Age	No. of Spina Bifida Fetuses w/ Normal Pain Reaction (%)
forepaw pinching	E16.5	20 (100)
	E17.5	20 (100)
	E18.5	20 (100)
	P1	20 (100)
hindpaw pinching	E16.5	11 (55)
	E17.5	10 (50)
	E18.5	9 (45)
	P1	17 (85)
tail pinching	E16.5	4 (20)
	E17.5	1 (5)
	E18.5	0 (0)
	P1	0 (0)

* E = embryonic Day; P = postnatal Day.

logical function. This biphasic natural history of spina bifida has been termed the two-hit hypothesis by Heffez and colleagues.[11,12] According to this hypothesis, the failure of neural tube closure comprises the first hit, whereas secondary degeneration of the exposed spinal cord is the second hit that eventually results in the neurological disability (myelomeningocele). Our primary aim was to explicitly address the question of whether mouse fetuses at early gestational stages develop normal neurological function, despite the presence of spina bifida. Such findings would be informative in relation to the further development of human in utero surgery to repair myelomeningocele prior to the onset of the neurodegenerative phase.

In this study of mice with both loop-tail and curly tail gene mutations, all fetuses with spina bifida exhibited failure of neural tube closure in the lumbosacral region. The wing-shaped, persistently open spinal cord was not covered by meninges or other tissue and was directly exposed to the amniotic cavity. At early stages of gestation, there was no sign of significant traumatic or degenerative changes within the exposed spinal cord that appeared to have developed in close parallel with the normal spinal cord of controls. In particular, gray and white matter was clearly discernible, whereas dorsal and ventral nerve roots and dorsal root ganglia appeared normal. Tracer studies using DiI demonstrated functionally intact neuronal connections between the spinal cord and peripheral targets, particularly the hindlimbs. Considering all findings together, we found that the only abnormal feature of the early gestational spina bifida lesion was failed neural tube closure.

Other investigators have reported similar morphological findings in splotch-delayed and curly tail mouse fetuses at early gestation, and have shown convincingly that the spinal cord tissue within the early spina bifida lesion is intact and undergoes neuronal differentiation.[18,33] Moreover, we and others have shown in previous studies of early gestational human fetuses with myelomeningocele that apart from failure of neural tube closure, all anatomical hallmarks of a well-developed spinal cord are present and intact.[9,16,23,28,29]

Our results in mouse fetuses with spina bifida at a later gestational stage provide evidence for secondary degenerative changes within the pathologically exposed spinal cord tissue acquired in utero. These changes progress with ongoing gestation and culminate in partial or complete loss of all exposed neural structures by birth. These findings are consistent with traumatic and/or toxic destructive processes that occur within the amniotic cavity during late gestation. Apart from toxicity of the amniotic fluid,[8] these processes may also include mechanical shearing and abrasive stresses on the surface of the delicate neural tissue, particularly late in gestation when amniotic fluid volume is low and fetal movements are enhanced. The implication of these findings is that all functional capacity, potentially present earlier in gestation, would be lost around birth at the latest.

Keller-Peck and Mullen[18] also described neurodegeneration in the spina bifida lesion of splotch-delayed and curly tail mouse fetuses, beginning at 17 days of gestation, in close agreement with our findings. On the other hand, McLone and colleagues[22] did not observe late gestational neurodegeneration with splotch-delayed mice. The negative findings of this latter study might be due to the relatively mild phenotype of splotch-delayed mice on some genetic backgrounds,[2] which might have minimized in utero degenerative changes.

Evidence from rat, rabbit, and sheep models (in which myelomeningocele is surgically induced) shows similarly progressive destruction of the spinal cord that is directly exposed to the intrauterine environment.[5,11,12,14,24–26,30] Moreover, studies of therapeutically aborted human fetuses with myelomeningocele have generated compelling evidence for neural tissue damage consequent to intrauterine exposure and progressive with ongoing gestation.[16,23,28] The combined findings of the present and previous studies provide strong support for the two-hit hypothesis,[11,12] demonstrating the occurrence of secondary acquired damage within the unprotected spinal cord of the myelomeningocele lesion in utero.

By maintaining mouse fetuses for short periods on placental support ex utero, we found that a significant proportion of mice with spina bifida at early gestation exhibit identical spinal cord function compared with their normal littermates. At the earliest possible testing point (embryonic Day 16.5), we observed a normal pain reaction in 55% of spina bifida fetuses after hindpaw pinching and in 20% of the same fetuses after tail pinching. It is possible that the spina bifida lesions in these mice predominantly affect sacral levels, whereas hindlimb innervation is more often intact, thus explaining this axial difference in response. Alternatively, ascending spinal cord damage might affect sacral levels first, with lumbar levels only affected later in gestation, thus causing the neurological deficit to progress in a caudal-to-cranial direction over time. A combination of these two explanations is also certainly possible.

Regardless of the explanation for the axial level–specific difference in pain response, our finding that 20% of spina bifida fetuses in early gestational stages respond to tail pinching in a manner that is indistinguishable from control littermates demonstrates strongly that sensorimotor spinal cord function can develop at lumbosacral levels despite the presence of spina bifida. Moreover, we documented a gradual loss of neurological function with advancing gestation. We currently cannot entirely rule out the possibility that defects such as hydrocephalus, Chiari malformation Type II, or other malformations frequently associated with neural tube defects in humans could have contributed to this

loss of neurological function. Nevertheless, the close parallel between loss of neurological function and the increasing tissue damage of the exposed spinal cord strongly suggests that the two processes are causally linked.

A surprising finding of this study was that a significantly higher proportion (85%, $p = 0.01$) of spina bifida fetuses at postnatal Day 1 were responsive to hindpaw stimulation, whereas only 45% of fetuses at embryonic Day 18.5 responded. Although only the most severely affected spina bifida fetuses were always used for testing from in utero litters, it was not always possible to ensure that the most severely affected spina bifida neonates were similarly tested. Our experience has been that neonates with the most extensive spina bifida lesions die earliest because of larger hemorrhage and fluid loss through the lesion. These neonates would often have been cannibalized by their mother after nocturnal delivery, before testing was possible. It therefore appears likely that the apparently higher response rate to hindpaw stimulation at postnatal Day 1 was due to the testing of a slightly less severely affected group of mice than at fetal time points.

Prenatal surgical coverage or repair of human myelomeningocele is currently practiced in three centers worldwide.[1,3,10,36,39] This surgical strategy has been used for approximately 10 years, and more than 250 of these patients have been born. The published results indicate a significant reduction in the incidence of hydrocephalus requiring placement of a cerebrospinal shunt: 50 to 60% of newborns undergoing prenatal operations needed shunts, compared with more than 90% of patients treated with standard postnatal care.[3,10,36,40] Furthermore, herniation of the hindbrain through the foramen magnum (Chiari malformation Type II), which is thought to be a key factor leading to obstructive hydrocephalus, is often reversible after early prenatal myelomeningocele repair.[3,36,39] Data on sensorimotor function of lower extremities and function of the bladder and bowel are less clear, as some patients exhibit better function than expected from the level of the myelomeningocele lesion,[17] whereas others have no apparent improvement in function.[13,38]

To date, comparisons between the results of fetal surgery and standard postnatal care have been based entirely on historical comparisons. To provide more reliable data, the US National Institutes of Health is now conducting a multicenter, prospective, randomized controlled clinical trial (Management of Myelomeningocele Study)[27,35] to determine the efficacy of in utero repair of myelomeningocele at 19 to 25 weeks of gestation compared with standard postnatal repair.

Conclusions

To our knowledge, this study is the first to demonstrate acquisition of neurological function at the level of the spinal cord lesion in a genetic mouse model of naturally occurring spina bifida. Our findings illustrate the two-stage natural history of myelomeningocele in utero, and provide support for the practice of fetal surgery to attenuate the irreversible neurological consequences of spinal cord damage in myelomeningocele acquired in utero.

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Manuscript submitted April 17, 2006.
Accepted November 17, 2006.
This research was supported by grants from the Wellcome Trust, United Kingdom (A.J.C.), and from the University of Zurich, Switzerland (D.S.).

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