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Fetal spina bifida: Loss of neural function *in utero*

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Abstract

The devastating neurological deficit associated with myelomeningocele (MMC) has previously been assumed to be a direct and inevitable consequence of the primary malformation, failure of neural tube closure. An alternative view, however, is that secondary damage to the pathologically exposed spinal cord tissue *in utero* is responsible for the neurological deficiency. If the latter mechanism was shown to be correct, it would provide an objective rationale for the performance of *in utero* surgery for MMC, since coverage of the exposed spinal cord could be expected to alleviate or perhaps prevent neurodegeneration. To examine this question, we studied the development of neuronal connections and neurological function during fetal and neonatal stages in a mouse genetic model of open lumbosacral spina bifida (SB). During early gestation (embryonic days 13.5 to 16.5), the persistently open spinal cord of fetuses carrying both *curly tail* (*ct*) and *loop-tail* (*Lp*) mutations exhibited essentially normal anatomical and functional hallmarks of development, including sensory and motor projections to and from the cord. A significant proportion of early fetuses with SB exhibited sensorimotor function identical to that seen in unaffected age matched controls. With ongoing gestation, however, we detected increasing neurodegeneration within the SB lesion, and this was paralleled by a progressive loss of neurological function. These findings provide support for the hypothesis that neurological deficit in human MMC arises following secondary neural tissue destruction and loss of function during pregnancy.

Keywords

Myelomeningocele; Spina bifida; Neural tube defect; Neurological function; Fetal surgery

INTRODUCTION

Myelomeningocele (MMC) is the most severe form of spina bifida (SB) which, in most cases, leads to lifelong and devastating physical and developmental disability. This includes, but is 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 MMC with, in the majority of cases, a disorder of primary neurulation leading to failure of neural tube closure [4,7].

A widely supported hypothesis is that the neurological deficiency in survivors with MMC is related to neurodegeneration *in utero* of the exposed spinal cord. Studies of therapeutically aborted fetuses with MMC have demonstrated damage to the open spinal cord that appears

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to be acquired *in utero* [16,21,23]. These findings have been supplemented by surgical production of MMC-like lesions *in utero* in the monkey [26], sheep [24,25], rabbit [14,30,31] and rat [5,12], all of which demonstrate progressive loss of spinal cord tissue with increasing gestation. Moreover, 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 *et al* [12] to propose a "two-hit" hypothesis in which failure of neural tube closure (the first "hit") is followed by *in utero* spinal cord degeneration, resulting in the typical neurological deficit of MMC.

A requirement of the "two-hit" hypothesis is that the early dysraphic spinal cord initially develops normal function. Indeed, the rationale for prenatal surgical repair in early MMC is that prevention of neurodegeneration can preserve the functional competence present in the early open spinal cord. In seeking evidence for functional development of the dysraphic spinal cord, we cannot rely on data from animal models in which an MMC is created artificially, but must instead look to naturally occurring MMC lesions. Early gestation human embryos and fetuses have been found to exhibit normally differentiated spinal cord tissue at the site of the MMC lesion [23]. Moroever, ultrasound examination has detected leg movements in 16-17 week fetuses with MMC [20]. Mouse genetic models provide the main source of naturally occurring SB among experimental animals [4]. Here, several studies have shown that the dysraphic mouse neural plate is initially composed of healthy neuroepithelial cells that appear to undergo neuronal differentiation according to the normal developmental timetable [33].

What is lacking, to date, is any evidence on whether mouse fetuses with SB develop neurological function at the site of the lesion, and then lose this function as neurodegeneration occurs *in utero*. Alternatively, it is possible that function is absent throughout gestation at the level of mouse SB. In the present study, we studied a mouse genetic model with naturally occurring open SB [32,34] in order to distinguish between these two possibilities.

MATERIALS AND METHODS

Mating and collection of specimens

All animal experiments were performed in accordance with the regulations of the Animals (Scientific Procedures) Act 1986 of the UK Government. Mutant *curly tail* (*ct*) and *loop-tail* (*Lp*) mice were maintained on a 12 hour light-dark cycle (light from 07.00 to 19.00). Overnight matings were performed between doubly heterozygous males (*Lp*/+; *ct*/+) and homozygous curly tail females (+/+; *ct/ct*). The finding of a vaginal plug the following morning was taken as evidence of mating, with midday being designated embryonic day (E) 0.5 (term = E19.5). Such matings generated litters containing fetuses with three different phenotypes: on average, 34% of fetuses had open lumbosacral SB together with a curly tail, 25.5% had curled or looped tails in the absence of SB, and 40.5% were entirely normal, with straight tails. The latter served as within-litter controls. To collect experimental fetuses, pregnant females were killed by cervical dislocation on E12.5 through E18.5, whereas neonates (P1) were collected immediately after vaginal delivery.

Fetuses were dissected from the uterus in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, UK) containing 10% fetal calf serum (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 SB and one control fetus was selected for neurological assessment (described below). After the neurological assessment, umbilical cord and placenta were removed and fetuses were killed on a frozen metal plate. Neonates were killed by injecting an overdose of Midazolam/Fentanyl (Hypnorm®/Hypnovel®). Fetuses were rinsed in

phosphate-buffered saline (PBS), then processed either for histology, immunohistochemistry, or immunofluorescence studies.

Histology

A total of 85 fetuses/neonates were processed for histological analysis as follows: E13.5: 2 SB, 2 control; E14.5: 4 SB, 3 control; E15.5: 6 SB, 5 control; E16.5: 8 SB, 7 control; E17.5: 7 SB, 8 control; E18.5: 6 SB, 7 control; P1: 10 SB, 10 control. Specimens were fixed in Bouin's solution (Sigma) for 2-7 days depending on age, serially dehydrated, and embedded in paraffin wax. Sections were cut with a rotary microtome (Microm HM330), either in a transverse or a sagittal plane to the body axis, at 10-12 μm thickness. Slides were dewaxed with Histo-Clear (National Diagnostics), serially rehydrated to distilled water, stained with Ehrlich's hematoxylin and eosin for 10 and 5 minutes respectively, dehydrated, mounted in DPX and coverslipped.

Immunohistochemistry (neurofilament staining)

Specimens were collected from E12.5 to E18.5, and on P1 (n=56; 3-4 fetuses of each stage and phenotype) and fixed in 4% paraformaldehyde/PBS for 2-7 days depending on age, then dehydrated through an ethanol series and embedded in paraffin wax. Transverse sections (6 or 8 μm thickness) were dewaxed, rehydrated and unmasked with Declere (Cell Marque) in a microwave oven for 20 minutes. Endogenous peroxidase was quenched by treatment with 0.6% H_2O_2/Tris buffered saline (TBS) for 7.5 minutes and non-specific epitopes were blocked using 10% FCS/TBS. Primary monoclonal anti-neurofilament 68 antibody (Sigma, mouse IgG1) was diluted 1:400 in TBS containing 1% FCS and 0.5% Triton and applied to sections overnight at 4°C. Then, sections were washed in 1% FCS/0.5% Triton/TBS and exposed for 1 hour to secondary biotinylated rabbit anti-mouse antibody (DAKO; diluted 1:500 in 1% FCS/0.5% Triton/TBS). 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 addition of 3,3′-diaminobenzidine (DAB, Sigma). Negative control sections had primary antibody substituted by 1% FCS/TBS, with negligable background staining.

Immunofluorescent neuronal tracing studies

Specimens were collected from E12.5 to E18.5, and on P1 (n=97; 5-6 fetuses of each stage and phenotype) and fixed in 4% paraformaldehyde/PBS at 4°C for 2-5 days, depending on age. Under a light microscope, the fetal/neonatal spine was exposed and gently perforated with the tip of a forcep to create small holes at different thoracic and lumbar levels. Single crystals of 1,1′-dioctadecyl-3,3,3′3′-tetramethyl indocarbocyanine perchlorate (DI "large crystals"; Molecular Probes) were inserted into the holes. In negative control preparations, the mid-thigh and/or the spine rostrally and caudally to the insertion site was transsected following crystal insertion, in order to demonstrate the prevention of dye diffusion after axonal interruption. Fetuses/neonates were then placed in 2% formalin/PBS and incubated at room temperature (younger specimens) or 37°C (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 (1-2 of each stage and phenotype) were embedded in 10% agarose and sectioned transversely at 100-150 μm thickness on a vibratome (Series 1000, Agar Scientific). Sections were mounted on glass slides, covered with glycerol/PBS (1:1), and examined under fluorescence illumination (Axiophot, Zeiss). Thereafter, specimens were stored at 4°C in the dark.

Neurological assessment in vivo

Specimens were collected from E16.5 to E18.5, and on P1 (n=160; 20 animals of each phenotype and stage). Fetuses of E15.5 and younger were not investigated because a pilot series revealed that no reaction was elicited in unaffected controls (data not shown), probably owing to 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 culling the mother, while the fetus was maintained on placental support *ex utero*, in order to ensure the vital reactions of the examined fetuses. Thus, only one animal with SB and one phenotypically normal control fetus could be examined from each litter. In order to elicit a classical pain reaction, the following tests were performed in a standardized way in all animals: pinching of both forelimbs, both hindlimbs, and tail using microforceps. All tests were repeated three times. Results were only rated conclusive and termed “normal pain reaction” if an identical response was obtained in all three consecutive test series.

RESULTS

A combination of histology, immunohistochemistry and DiI tracing was used to determine the time course of spinal cord development in the region of the SB lesion in affected fetuses, by comparison with the normal control spinal cord.

Early spinal cord development exhibits normal morphology in the SB lesion

The normal sequence of spinal cord development between E13.5 and P1 was clearly revealed by H&E staining (Figure 1A,C,E,G) and immunohistochemistry for neurofilaments (Figure 2A,C,E,G). Of particular note, in transverse sections through the lower trunk of normal control fetuses, was the marked progression of neuronal differentiation within the spinal cord, the development of sensory and motor roots, and the establishment of dorsal root ganglia.

SB fetuses at early gestation (E13.5) demonstrated a spinal cord lesion that was directly exposed to the amniotic cavity as a result of the failure of neural tube closure. The neuroepithelium was not covered by meninges or other tissues. At this early time point, the cytoarchitecture of the spinal cord appeared to have developed normally, and there were no signs of traumatic or degenerative alterations (Figure 1B). Immunohistochemistry for neurofilaments showed that sensory and motor roots, and dorsal root ganglia, had all developed with apparently normal morphology (Figure 2B). The only obvious deviation from normal was a marked kinking of the sensory roots (as opposed to a straighter course in controls). This seemed to be the result of the extremely lateral position of the dorsal horns in the open SB lesion.

Progressive degeneration of spinal cord morphology during later SB development

With ongoing gestation, H&E sections through the SB lesion (Figure 1D,F,H) revealed signs of progressive neural tissue damage, ranging from superficial abrasion, erosion, and hemorrhage (E16.5) to tissue avulsion and degeneration (E18.5), and finally to complete loss of all formerly exposed spinal cord tissue at birth (P1). Neurofilament staining revealed good preservation of the sensory and motor roots, and of the dorsal root ganglia, at E15.5 and E17.5, despite progressive alteration of the spinal cord (Figure 2D,F). At P1, however, only tiny remnants of sensory and motor roots and dorsal root ganglia were identifiable in some sections (Figure 2H), whereas in other sections no neural tissue remained at the SB site.

Development and subsequent loss of neuronal connections between spinal cord and periphery in SB fetuses

DiI tracer studies (Figure 3A,B) were performed to demonstrate the presence or absence of functionally intact neuronal connections between spinal cord and peripheral tissues. Nerves exiting the spinal cord and running to the hindlimb could be identified in all control fetuses between E12.5 and E17.5 (Figure 3C,E,G). At later stages, nerves could not be demonstrated by this technique, owing to the thickness of overlying soft tissues.

In SB fetuses, nerves connecting the open spinal cord to the hindlimbs were identifiable up to E15.5 (Figure 3D,F) indicating the development of intact neuronal continuity between spinal cord and periphery despite failure of neural tube closure. In contrast, at E17.5, neural projections from the SB lesion to the hindlimbs were barely visible, indicating a largely disrupted neuronal continuity between spinal cord and periphery at this stage in SB fetuses (Figure 3H).

Development and subsequent loss of sensorimotor function in the lower spinal cord of SB fetuses

A standard set of pinching tests was performed to check for sensorimotor function in fetuses maintained on placental support *ex utero* (Table 1). In control fetuses, pinching of the fore or hind paw resulted in an immediate (i.e. within 2 seconds) withdrawal movement of the respective limb, squirming of the ipsilateral body side, and silent mouth opening in fetuses, or audible vocalisation in neonates. Similarly, pinching of the tail resulted in an immediate motor response of all 4 extremities, together with trunk squirming, and mouth opening in fetuses or audible vocalisation 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 (E16.5 to P1). Therefore, these reaction patterns were interpreted as normal physiological fetal or neonatal pain reactions.

Among SB fetuses, fore paw pinching elicited a normal pain reaction (identical to that observed in age-matched controls) in all fetuses at all gestational stages (E16.5 to P1). A normal pain reaction following hind paw pinching was recorded in 55% of SB fetuses at E16.5, in 50% at E17.5, in 45% at E18.5 and in 85% of neonates (Table 1). Tail pinching produced a normal pain reaction in 20% of SB fetuses at E16.5 and in 5% at E17.5. All six of these ‘responsive’ SB fetuses also had a positive hind paw response. In contrast, none of the E18.5 SB fetuses and none of the SB neonates responded to tail pinching. These results demonstrate that an apparently normal neurological function is present in a considerable proportion of affected E16.5 fetuses despite SB, as evidenced by normal pain reactions after both hind paw and tail pinching.

DISCUSSION

In the present study, we used a mouse genetic model in which SB develops spontaneously, and with many similarities to human MMC [32,34], to address two questions. First, does spinal cord development, with the establishment of functional nerve connections, proceed relatively normally at early gestational stages of SB? Second, does neurodegeneration during later fetal stages ultimately lead to loss of spinal cord tissue, neuronal connections and neurological function? This bi-phasic natural history of SB has been termed the “two hit” hypothesis by Heffez and colleagues [11,12]. According to this idea, failure of neural tube closure comprises the first “hit”, while secondary degeneration of the exposed spinal cord is the second “hit” that ultimately results in the neurological disability of MMC. Our main aim was to explicitly address the question of whether early gestation mouse fetuses develop

normal neurological function, despite the presence of SB. Such findings would be informative in relation to the further development of *in utero* surgery to repair MMC prior to the onset of the neurodegenerative phase.

In a study of mice doubly mutant for the *curly tail* and *loop-tail* genes, all fetuses with SB exhibited failure of neural tube closure in the lumbo-sacral 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 which appeared to have developed in close parallel with the normal spinal cord of controls. In particular, grey and white matter were clearly discernable, while dorsal and ventral nerve roots, and dorsal root ganglia, appeared normal. DiI tracer studies demonstrated functionally intact neuronal connections between the spinal cord and peripheral targets, especially the hindlimbs. Taken together, we found that the only abnormal feature of the early gestation SB lesion was failed neural tube closure.

Other investigators have reported similar morphological findings in early gestation *splotch-delayed* and *curly tail* mouse fetuses and have shown convincingly that the spinal cord tissue within the early SB lesion is intact and undergoes neuronal differentiation [18,33]. Moreover, we and others have shown in previous studies of early gestational human fetuses with MMC 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 with later gestational stage mouse SB fetuses provide clear-cut evidence for secondary, *in utero* acquired, degenerative changes within the pathologically exposed spinal cord tissue. These changes progress with ongoing gestation and culminate in sub-total or complete loss of all exposed neural structures by birth. These findings are consistent with traumatic and/or toxic destructive processes occurring within the amniotic environment during late gestation. The implication 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 SB lesion of *splotch-delayed* and *curly tail* fetuses, beginning at 17 days of gestation, in close agreement with our findings. On the other hand, McLone et al [22] working with the *splotch-delayed* mouse strain did not observe late gestational neurodegeneration. Perhaps the negative findings of this latter study relate to the relatively mild phenotype of *splotch-delayed* mice on some genetic backgrounds [2], which might have minimised *in utero* degenerative changes.

Evidence from rat, rabbit and sheep models with surgically induced MMC similarly demonstrate 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 MMC have generated compelling evidence for neural tissue damage consequent to intrauterine exposure and progressive with ongoing pregnancy [16,23,28]. Taken together, the findings of the present and previous studies demonstrate beyond doubt the occurrence of secondary acquired damage within the unprotected spinal cord of the MMC lesion *in utero*.

By maintaining mouse fetuses for short periods on placental support *ex utero*, we found that a significant proportion of early gestation mice with SB exhibit identical spinal cord function to their normal litter mates. At the earliest possible testing timepoint (E16.5), we observed a normal pain reaction in 55% of SB fetuses after hind paw pinching and in 20% of the same fetuses after tail pinching. lt is possible that the SB lesions in our mice predominantly affected sacral levels, with hindlimb innervation largely 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 certainly possible.

Regardless of the explanation for the axial level-specific difference in pain response, our finding that 20% of early SB fetuses respond to tail pinching, in a manner that is indistinguishable from control litter mates, when tested using a standardised protocol, argues strongly that sensorimotor spinal cord function can develop at lumbosacral levels despite the presence of SB. Moreover, our findings demonstrate a graudal loss of neurological function with advancing gestation in close parallel to the increasing tissue damage of the exposed spinal cord.

Prenatal surgical coverage or repair of human MMC is currently practised in three centers worldwide [1,3,10,36,39]. After around ten years of experience of this surgical strategy, more than 250 of these patients have been born. The published results indicate a significant reduction in the incidence of hydrocephalus requiring shunting, with a frequency of 50-60% prenatally operated newborns needing shunts compared with more than 90% of patients following standard postnatal care [3,10,36,40]. Moreover, herniation of the hindbrain through the foramen magnum (i.e. Chiari type II malformation), which is thought to be a key factor leading to obstructive hydrocephalus, is often reversible after early prenatal MMC repair [3,36,39]. Data on sensorimotor function of lower extremities and function of the bladder and bowel are less clear, with some patients exhibiting better function than expected from the level of the MMC lesion [17], but others having 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 National Institutes of Health (USA) is now conducting a multi-center prospective randomized controlled clinical trial (Management of Myelomeningocele Study, MOMS: www.bsc.gwu.edu) [27,35] to determine the efficacy of *in utero* repair of MMC at 19-25 weeks of gestation compared with standard postnatal repair.

In conclusion, our study is the first, to our knowledge, to demonstrate acquisition of neurological function at the level of the spinal cord lesion in a genetic mouse model of naturally occurring SB. Our findings illustrate the two-stage natural history of MMC *in utero*, and provide support for the concept of fetal surgery to attenuate the irreversible neurological consequences of *in utero* acquired spinal cord damage in MMC.

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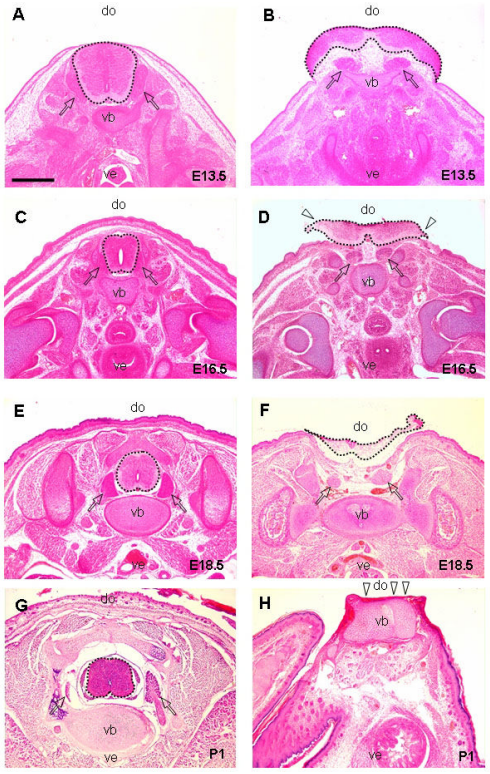

Figure 1.

Hematoxylin and eosin staining of sectioned control and SB fetuses at various stages of gestation. (**A,C,E,G**) Cross-sections through the lumbar spinal area from E13.5 to P1 in control animals showing normal development of the spinal cord (dotted line), with laterally located dorsal root ganglia (open arrows), and surrounding skeletal and soft tissues. (**B,D,F,H**) Sections through the central part of age-matched litter mate fetuses/neonates with SB. At E13.5 (B), an intact dorsally-open spinal cord (dotted line) faces the amniotic cavity. The grey matter (purple), located dorsally, can be distinguished from the ventrally located white matter (light red). Dorsal root ganglia are located ventrally (open arrows) as a consequence of the failure of neural tube closure. At E16.5 (D), the dorsally-exposed spinal cord (dotted line) exhibits mild to moderate superficial neural tissue abrasion and hemorrhage, mostly located laterally (open arrowheads), probably resulting from contact with the uterine wall. At E18.5 (F), considerable loss of spinal cord tissue (dotted line) is visible, with only a few remnants of grey matter (purple) remaining. In contrast, more ventrally located white matter (light red) and dorsal root ganglia (open arrows) are mostly present. At birth (H), all exposed neural tissues have been lost and dorsal root ganglia are no longer discernible. The bare dorsal aspect of the vertebral body (open arrowheads) faces the amniotic cavity. Abbreviations: do, dorsal; vb, vertebral body; ve, ventral. Scale bars: 250 μm in A and B; 500 μm in C-H.

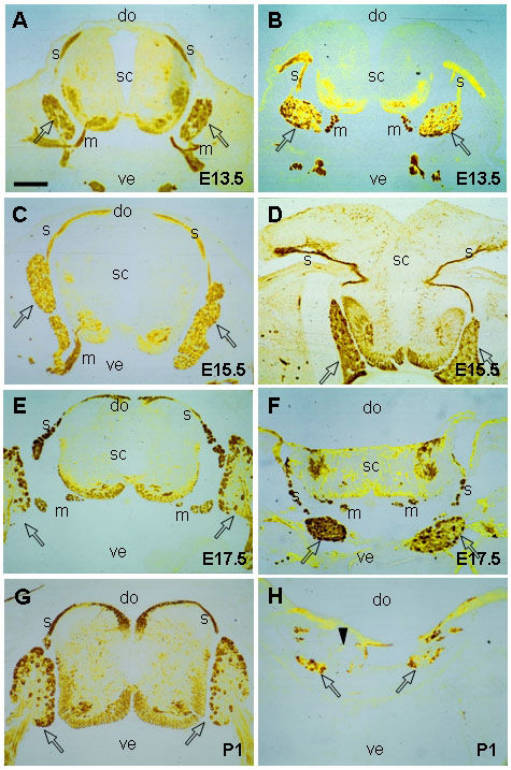

Figure 2.

Neurofilament immunostaining of sectioned control and SB fetuses at various stages of gestation. (**A,C,E,G**) Cross-sections through the lumbar spinal area from E13.5 to P1 in control animals. The neurofilament staining method allows good visualization of sensory roots (s) projecting into the dorsal part of the spinal cord, motor roots (m) exiting the spinal cord ventrally, and dorsal root ganglia (open arrows) located ventro-laterally to the spinal cord. These structures characteristically exhibit a brownish coloration, whereas the spinal cord tissue stains light yellow. (**B,D,F,H**) Sections through the central part of age-matched litter mate fetuses/neonates with SB. At gestational stages E13.5, E15.5 and E17.5 (B,D,F), sensory roots, motor roots, and dorsal root ganglia (open arrows) 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 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 controls. At birth (H), only remnants of neural tissue remain. Note the few neural bundles (probably motor root) that persist (arrowhead). Dorsal root ganglia (open arrows) are much smaller than in controls at this stage (G). Scale bars: 200 μm.

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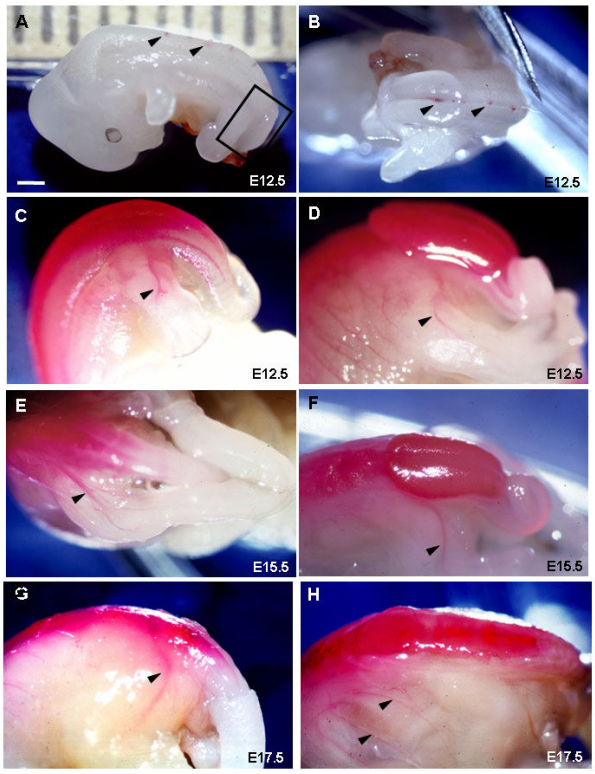

Figure 3.

DiI tracer studies of control and SB fetuses at various stages of gestation. (**A**) Lateral and (**B**) dorsal/caudal views of a SB fetus (E12.5) immediately after insertion of DiI crystals (arrowheads). The lumbo-sacral area including the SB lesion and adjacent closed spinal cord (black box in A) is shown in top view in (B). DiI crystal within the MMC is indicated by left hand arrowhead in B. Two other crystals have been inserted into closed spinal cord rostral to the SB lesion. The dotted lines delineate the hindlimbs. A 20 g needle tip is shown for size comparison (top right in B). (**C-H**) Specimens photographed several weeks following insertion of DiI crystals. E12.5 fetuses (C,D) show a peripheral nerve exiting the spinal cord and running into the hindlimb (arrowheads) in both control (C) and SB (D) fetuses. E15.5 fetuses (E,F) show a very similar appearance as at E12.5, with a nerve entering the hindlimb in control and SB fetuses (arrowheads). At E17.5 (G,H), a nerve exiting the spinal cord and projecting into the hindlimb (arrowhead) is visible in the control fetus (G), whereas neural projections into the hindlimb (dotted line) from the SB lesion are not longer visible (H), although nerves can be seen exiting the spinal cord at levels rostral to the SB lesion (arrowheads in H). Scale bars: 1 mm in A, 0.7 mm in B, 0.35 mm in C and D, 0.7 mm in E, 0.55 mm in F, 0.65 mm in G and H.

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Table 1

Results of *in vivo* neurological testing of normal and SB fetuses at various stages of gestation

Pain stimulus	Gestational Age	Control fetuses: Number (%) with normal pain reaction	SB fetuses: Number (%) with normal pain reaction
A) Forelimb pinching	E16.5	20 (100)	20 (100)
	E17.5	20 (100)	20 (100)
	E18.5	20 (100)	20 (100)
	P1	20 (100)	20 (100)
B) Hindlimb pinching	E16.5	20 (100)	9 (55)
	E17.5	20 (100)	10 (50)
	E18.5	20 (100)	11 (45)
	P1	20 (100)	17 (85)
C) Tail pinching	E16.5	20 (100)	5 (20)
	E17.5	20 (100)	1 (5)
	E18.5	20 (100)	0 (0)
	P1	20 (100)	0 (0)