The Fetal Spinal Cord Does Not Regenerate after In Utero Transection in a Large Mammalian Model

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OBJECTIVE: Regeneration and functional recovery after spinal cord transection do not occur in mammalian animals and humans postnatally. The goal of this study was to test whether in utero transection of the fetal spinal cord is succeeded by anatomic healing and functional recovery.

METHODS: In five sheep fetuses, at 60 days of gestation and 75 days of gestation (term = 150 d), the spinal cord was completely transected at T10. The animals were delivered near term by cesarean section for clinical evaluation, measurement of cortical somatosensory evoked potentials, and morphological assessment.

RESULTS: The newborn lambs demonstrated sensory-motor paraplegia, were incontinent of urine and stool, and exhibited a spinally generated, ambulatory pattern of the hindlimbs. No cortical somatosensory evoked potentials could be recorded in response to posterior tibial nerve stimulation, although potentials from the ulnar nerve, which enters the cord rostral to the lesion, were normal in all animals. Histologically, no neuronal connections across the transection site were identified. The cord proximal to the lesion was grossly normal, whereas distal to the transection, it appeared slightly smaller but with the cytoarchitecture preserved.

CONCLUSIONS: Unlike in lower vertebrate and avian species, the fetal ovine spinal cord has no detectable spontaneous regenerative capabilities when transected during midgestation. Gap formation after transection, secondary posttraumatic cell death, and missing guiding channels for sprouting axons may be factors involved in the absence of any regenerative response. (Neurosurgery 39:555–561, 1996)

Key words: Axonal sprouting, Fetus, Neural regeneration, Spinal cord

Permanent paralysis, resulting from spinal cord trauma or disease, leads to lifelong and devastating disabilities, including loss of motor and sensory function, neurogenic bladder and rectum, sexual dysfunction, and trophic disorders of the skeleton and soft tissues. All of these sequelae of spinal cord injury substantially contribute to the enormous medical, psychosocial, and economic impact of this disastrous condition. After severe spinal cord damage, spontaneous anatomic regeneration with recovery of function does not usually occur, and there is no current therapy that can significantly improve the established neurological deficit (8, 17, 29).

In contrast to the prevalent opinion that the mature mammalian and primate central nervous system (CNS) does not have regenerative potential, there is growing evidence that at least some regenerative response can be obtained, e.g., by eliminating factors that are inhibitory to regeneration (16, 22, 23), by adding neurotrophic factors known to promote regeneration (25, 30), or by transplanting fetal neural tissue into the site of the wounded adult CNS (14, 19, 24). It is not known why fetal CNS tissue enhances sprouting and elongation of axotomized adult neurons, and there is little knowledge regarding the healing properties and regenerative capacity of the fetal CNS. Based on the apparent promotor effect of fetal CNS transplants in mammalian species and that the embryonic/fetal spinal cord of larval lower vertebrates (3, 18), chick embryos (12, 27), and, questionably, rodent fetuses (4) dem-

onstrates an astonishing regenerative capacity and recovery of function after complete transection, we hypothesized that similar regenerative forces might be present in the fetal CNS of large mammals. The goal of this study was to determine whether anatomic repair and functional recovery occur after complete spinal cord transection in the sheep fetus.

MATERIALS AND METHODS

Fetal spinal cord transection

In sheep fetuses, at 60 days of gestation (n = 5) and 75 days of gestation (n = 5) (term = 150 d), the spinal cord at T10 was completely transected. This level was chosen, because it induces complete sensorimotor paraplegia (the spinal levels for hindlimb innervation range from L3 to S2). Transection was performed as early as possible in this animal model (60 d), to allow the longest possible time for regeneration. The second time point (75 d) was chosen to determine eventual differences in outcome and morphology.

For fetal sheep surgery, we used standard techniques previously described in detail (11). Briefly, the ewe underwent general halothane/oxygen anesthesia, the uterus was exteriorized through an infraumbilical midline laparotomy, and the back of the fetus was exposed via a hysterotomy. T10 was easily identified by taking the visible ribs (based on 13 pairs of thoracic ribs) as markers. By using microsurgical instruments and ×3.5 loupe magnification, the posterior elements of the spine were exposed through a midline incision from T8 to T12. The spinal cord was exposed between T9 and T11 with a complete laminectomy. The spinal cord (including the dura) was then lifted up on a nerve hook and transected with microscissors under direct vision. The completeness of transection was confirmed by immediate gap formation between the two stumps. In three of the five fetuses operated on at 75 days of gestation, the dura was coapted orthotopically with one or two 10-0 nonabsorbable Surgilene sutures (Davis + Geck Inc., Manati, PR); in the other animals, the dura could not be repaired, leaving the stumps in an orthotopic position. Primary wound closure was achieved by coapting the soft tissue elements and skin with interrupted sutures. The fetus was returned to the uterus and, after restoration of the amniotic fluid volume with warm sterile saline, the hysterotomy was closed with a TA-90 stapler (US Surgical Corporation, Norwalk, CT). Closure of the maternal laparotomy was performed in layers, and the ewe was returned to her stall. The animals were delivered near term (at 145 d of gestation) by cesarean section and assessed neurologically, electrophysiologically, and morphologically.

Clinical assessment for neurological deficit

Clinical evaluation of the newborn lambs was performed on Days 1 and 3 of life by a veterinarian, according to a standardized protocol (5). Briefly, general health status, mental status, and neurological function, including cranial nerves, spinal reflexes, postural reactions, gait, and posture, were assessed. Pain perception of forelimbs, hindlimbs, face, and rump was determined by pricking with a needle (superficial pain) and by pinching with a hemostat (deep pain). Finally, the animal was observed for muscle atrophy, micturition, and defecation. Ten normal newborn lambs were examined analogously, as control animals. The clinical assessment of study and control lambs was recorded on videotape to facilitate data comparison.

Measurement of somatosensory evoked potentials (SEPs)

The cortical SEP response to peripheral nerve stimulation is primarily mediated by the dorsal column pathway of the spinal cord (2). Because reflex movements in response to pain or other stimuli could be mediated at segmental spinal levels without implying perception, SEP in response to bilateral fore- and hindlimb stimulation were recorded in addition to the clinical assessment, to conclusively demonstrate the presence or absence of sensory transmission to the brain. On postnatal Day 3, lambs were anesthetized with intravenously administered propofol (Stuart Pharmaceuticals, Wilmington, DE) and posterior tibial and ulnar nerves were stimulated with electrical pulses (200-μs duration, 4.1/s) sufficient to produce a clear muscle twitch. SEPs were recorded from needle electrodes in the scalp overlying the contralateral primary somatosensory cortex (15). Three separate averages (n = 500; bandpass, 50–1000 Hz) were obtained for each of the four stimulation sites to ensure reliability of the responses. Grand average waveforms across all animals in each group were computed for display purposes and to aid in identification of the primary negative and positive peaks. The latency and amplitude of the main negative-positive complex were scored for each response and compared with values obtained from a control group of six normal neonatal lambs (C.D. Yingling, manuscript in preparation). The unpaired t test (two-tailed) was used for all statistical comparisons of SEP latencies and amplitudes.

Morphological assessment

After SEP recording, the animals were killed by administration of Beuthanasia-D (Schering, Kenilworth, NJ) and immediately thereafter perfused with phosphate-buffered saline standard solution (1.5 L/animal) for washout; a 10% formalin solution (3 L/animal) was used for fixation. The site of the spinal cord transection (T7–T13) was removed intact and processed histologically. Serial sagittal sections (T9–T11) and cross-sections (T7–T8 and T12–T13) were stained with hematoxylin and eosin, Gomori's trichrome stain, silver stain, and Luxol fast blue stain.

RESULTS

All 10 animals survived until near term and were delivered by cesarean section. Three animals (with dura repair in two of three) were subsequently excluded from the study because indispensable data points were not obtainable (two animals were subvital and died shortly after birth; for one animal, the tissue block containing the transection site was lost during histological processing).

Clinical evaluation

Of the survivors, four were operated on at 60 days of gestation (with dura repair in none of four) and three were operated on at 75 days of gestation (with dura repair in one of three). Clinically, all animals were paraplegic but otherwise normal. Briefly summarized, voluntary or coordinated movements of the hindlimbs were never observed, the animals were not able to stand or walk, sensory function of the hindlimbs was absent, and there was urine and stool incontinence. All animals had similar, spinally mediated, reflex patterns and showed "automatic" cyclic ambulatory movements (the neural circuitry of the lumbar spinal cord can generate alternating flexion and extension movements of the hindlimbs without supraspinal control) (7). When the animal was lifted off the ground or when an external stimulus (pinching or gentle shaking) was applied to the recumbent animal, the animal started to rhythmically move its hindlimbs as if it were walking. No hindlimb-forelimb coordination was noted during these phases nor was weight-bearing present. There was no change of the neurological deficit between the first and second examinations. The clinical diagnosis in all animals was complete sensory-motor paraplegia below level T10.

Electrophysiological evaluation

Repeatable SEPs in response to ulnar stimulation were obtained from all experimental animals. Latencies of the primary negative and positive waves in response to left ulnar stimulation were as follows: negative, 16.1 milliseconds; positive, 23.4 milliseconds (controls: negative, 16.6 ms; positive, 24.3 ms; not significant). On the right, the corresponding latencies were as follows: negative, 17.7 milliseconds; positive, 23.5 milliseconds (controls: negative, 18.1 ms; positive, 23.5 ms; not significant). The peak-to-peak SEP amplitudes were 1.16 μV on the left (controls: 1.26 μV, not significant) and 0.53 μV on the right (controls: 1.29 μV, not significant). The right SEP amplitude was somewhat lower than that of the control group, but this difference was not statistically significant because of the large interindividual variation in SEP amplitudes.

No consistent responses could be obtained from any of the T10-transected animals to stimulation of the posterior tibial nerve (*Fig. 1*). Clear posterior tibial nerve responses were obtained from all animals in the control group, with (as expected) longer latencies (mean: negative, 29.9 ms; positive, 38.7 ms) and lower amplitudes (mean peak-to-peak amplitude, 0.49 μV) than those from ulnar stimulation.

Histological evaluation

In all seven study animals, both ends of the transected spinal cord were sealed off by glial tissue and no continuity of neural tissue between the rostral and caudal stumps was present. In three animals, the subarachnoid space was continuous across the level of transection but the glial tissue completely enveloped both ends of the cord and so prevented direct contact of the neural tissue (*Figs. 2* and *3*). In four animals, the pia was fused to the dura between the two stumps and there was no continuity of the subarachnoid space across the transection site (*Fig. 4*). This fusion of dura and pia produced a conical configuration of the cord ends, and these two cones were bound to each other by connective tissue strands. Six animals had a syringomyelic cavity at the rostral end and four at the caudal end of the cord. In two instances, the central canal of the spinal cord was in continuity with the cavity (*Fig. 5*), and in three others, the close approach of the central canal to the cavity suggested that a similar arrangement was present. Discrete proliferations of glial tissue (glial scar) were present in the area of the conical cord stumps or around the syringomyelic cavities (*Fig. 3*). Axonal growth or sprouting was not recognizable in these areas. Examination of the serial histological sections and the silver stains demonstrated that there was neither continuity of

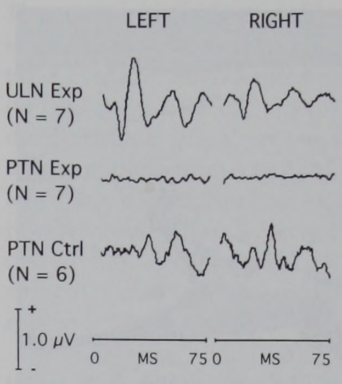

FIGURE 1. Cortical SEP from bilateral ulnar nerve (*ULN*) and posterior tibial nerve (*PTN*) stimulation. *Top*, grand average SEP from the experimental group (*Exp*) in response to ulnar stimulation, showing responses equivalent to those obtained from the control group of normal neonatal lambs (not shown). *Center*, grand average SEP from the experimental group in response to posterior tibial nerve stimulation, showing the absence of any cortical response. *Bottom*, grand average SEP from the control group (*Ctrl*) in response to posterior tibial nerve stimulation, showing bilateral responses at longer latency than those to ulnar stimulation. The first 5 milliseconds of each trace have been deleted to eliminate the electrical stimulus artifact.

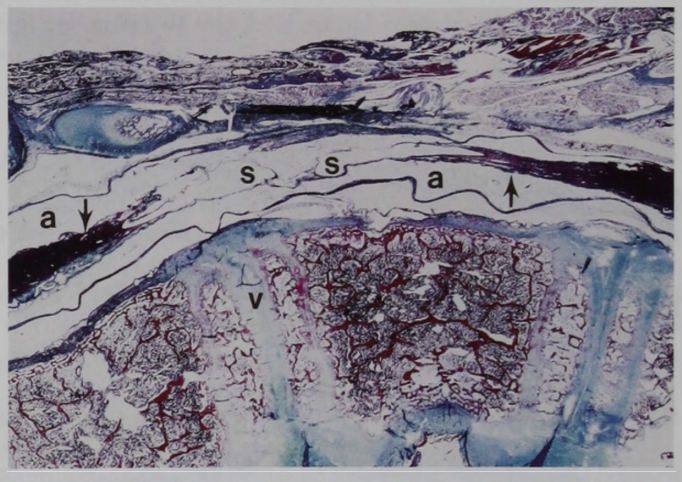

FIGURE 2. Sagittal section through the area of spinal cord transection. The rostral end is on the right, and there is kyphosis of the vertebral column (*v*), presumably secondary to the laminectomy (*above*). There is continuity of the subarachnoid space (*a*) across the transection site. At both stumps (*arrows*) there is a syringomyelic cavity (*s*) covered by pial tissue and lined internally by a thin layer of glial tissue (Gomori trichrome; original magnification, ×10).

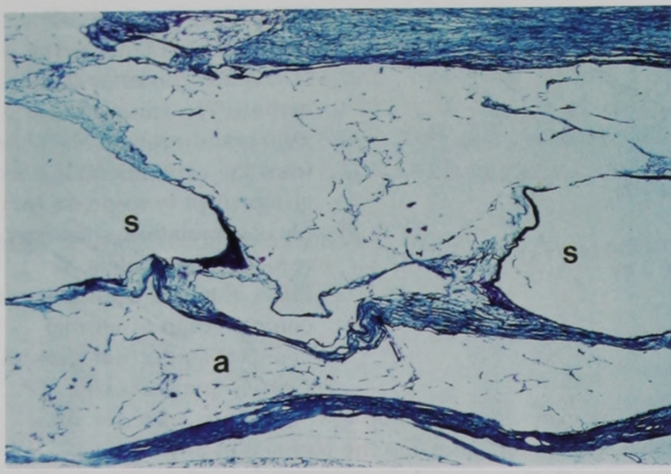

FIGURE 3. Higher magnification of the same section shown in *Figure 2*. The close approach of the syringomyelic cavities (*s*) is shown in the same orientation. The rostral-caudal continuity of the subarachnoid space (*a*) is clearly seen below (Gomori trichrome; original magnification, ×100).

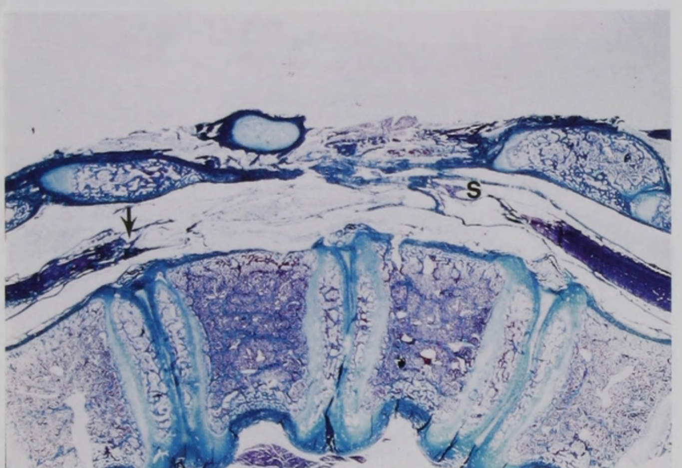

FIGURE 4. Sagittal section through the transection site of another animal (oriented as in *Fig. 2*). The rostral-caudal continuity of the subarachnoid space has been lost by the fusion of the pia to the dura. A syringomyelic cavity (*s*) is present only at the rostral stump. The caudal stump (*arrow*) shows only a conical proliferation of glial tissue surrounded by pial tissue (Gomori trichrome; original magnification, ×200).

neural tissue across the point of transection nor axons within the tissues connecting the ends of the transected cord. The spinal cord proximal to the transection-related changes appeared grossly normal, whereas the caudal cord appeared slightly smaller in some cases. The cytoarchitecture in both areas was intact.

DISCUSSION

This study demonstrates that early second-trimester and midgestational spinal cord transection in sheep fetuses does not result in structural restoration of the neural tissue or functional recovery, as evidenced by permanent sensorimotor

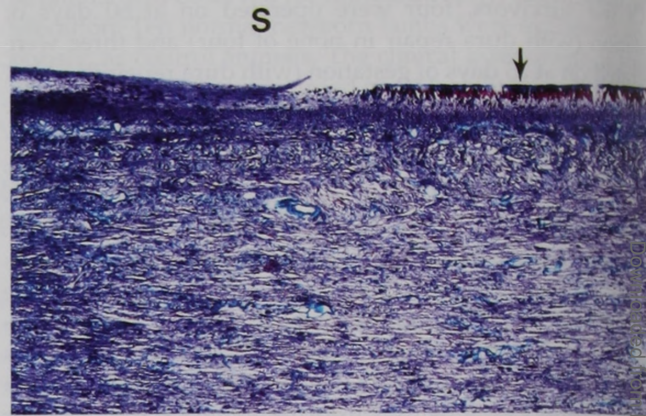

FIGURE 5. Ventral wall of a rostral syringomyelic cavity (*s*) from a third animal, near the point of transection. Ependyma (*arrow*) lines the rostral end of the cavity (*right*), demonstrating continuity with the central canal. The syringomyelic cavity extended to the level of the transection site. The axons of the cord appear normal (Gomori trichrome; original magnification, ×200).

paraplegia, absent hindlimb SEP, and absent neuronal connections across the transection site. Also, dura repair (performed in one of the evaluated animals) did not seem to have an impact on regeneration. Moreover, light microscopy did not reveal any signs of axonal sprouting at the transection site. These findings are not consistent with our hypothesis and raise questions about the causes of regeneration failure.

In theory, there are four possible explanations for nonregeneration. First, the axotomized neurons and the neurons with a direct lesion of the cell body may have died from the inflicted mechanical injury and/or neurotrophic factor deprivation (13). Although we did not apply sophisticated methods to prove or exclude massive neuronal death, there was little if any gross evidence for significant neuronal loss at spinal levels distant from the transection site. Longitudinal sections (*Figs.* 2 and 4) and cross-sections rostral and caudal to the transection site demonstrated an amazingly well-preserved spinal cord, which did not differ significantly from sections through a normal spinal cord in terms of size and cellular architecture. On the other hand, localized neural loss limited to the areas adjacent to the transection site presumably occurred shortly after severing of the cord; the gaps found at birth (10–20 mm) were considerably longer than just after transection (1–2 mm). Possibly, retraction of the spinal cord stumps and positive allometric growth of the vertebral column in relation to the spinal cord might also have contributed to the formation of these long gaps (10). However, recent reports provide strong evidence supporting the local neural necrosis paradigm. Traumatic cord lesions often become greatly enlarged as a consequence of massive secondary cell necrosis, which includes neurons as well as glial cells (6). Although the underlying mechanisms are not understood in detail, posttraumatic loss of vascular autoregulation with decreased microcirculation, leading to local ischemia and infarc-

tion of the injured tissue and adjacent areas, may be an important factor (28).

Second, although the axotomized cells would intrinsically have regenerative capabilities, sprouting and elongation of axons could have been blocked by an environment inhospitable to neurite outgrowth. For example, severe glial scarring (17, 21), oligodendrocytes and CNS-myelin-associated molecules (1, 26), and lack of guiding channels (9) are factors reported to inhibit regeneration in adults. However, none of these factors are likely to have played a prominent role in our fetal experiments. Severe gliosis (or scar) was not found at the transection site. In particular, the glial tissue that sealed off the cut ends of the cord was very thin and virtually identical to the pia that normally envelops the spinal cord. This finding, together with the complete absence of axonal sprouting in this area, conveys the impression that the glial seal occurred because of the inert cut end. Because myelination of the CNS is a phenomenon that starts late in gestation and is completed only postnatally, it is not likely that myelin-associated proteins inhibited regeneration at midgestation (26). This view is further supported by the virtual absence of myelin in the fetal sheep spinal cord around midgestation (data not shown). Finally, although only minimal gap formation occurred immediately after transection, indicating the absence of relevant tension, these gaps could have been further enlarged by subsequent fiber retraction. This might have had a negative impact, because it is known that regenerating axons do not likely bridge long gaps in the absence of appropriate guiding channels (24).

Third, the affected neurons intrinsically may not have a regenerative capability. Although we were not able to identify signs of regeneration, it would be inappropriate to simply conclude that regeneration was inherently impossible, because many recent studies demonstrated regeneration of CNS neurons in various experimental settings (8, 12, 14, 16, 19, 22–25, 27, 30). In particular, one recent study in rats raised interest (and controversy) by reporting restoration of spinal cord function after excision of neonatal spinal cord segments, which were replaced by orthotopically transplanted fetal spinal cord segments (14).

Fourth, transection may have been performed "too late" in gestation, i.e., at a time point at which the hypothetical ability to regenerate was already lost. In our model, this aspect cannot be clarified because we have already chosen the earliest time point (60 d of gestation) at which this type of experiment is feasible. However, the time point of 60 days of gestation in sheep (term = 150 d) compares favorably with the embryonic Day 14 time point in rats (term = 21 d), which is generally considered the turning point after which fetal spinal cord transplants are less efficient (19).

Spinal cord transections during development have been performed in a variety of species. In lower vertebrates (e.g., sea lamprey), the transected larval spinal cord regenerates and normal neurological function is restored (3, 18). Studies in chick embryos are the only experiments in higher vertebrates to conclusively demonstrate significant axonal regeneration and restoration of normal motor function after complete thoracic transection on embryonic Day 10 (term = 21 d) (12, 27). The mechanisms driving this powerful regeneration machinery are unknown. In the past 2 decades, spinal cord transections in mammalian fetuses have been performed in fetal mice (9) and fetal rats (4); however, no study has conclusively demonstrated neural regeneration and restoration of function after complete transection. Notably, the reported recovery of hindlimb motor function (4) does not critically depend on regeneration, because the well-known ability of the disconnected caudal spinal cord to generate cyclic output that results in a walking pattern has also been documented in fetal rodents (20).

In conclusion, our findings suggest that fetal spinal cord transection relatively early in gestation does not elicit any detectable regenerative response in sheep, and we assume that this holds true for mammals in general. Factors such as massive secondary cell death at the site of transection and the absence of guiding channels because of gap formation may be involved, although further investigations are needed to clarify the underlying mechanisms leading to the complete absence of regeneration. Our model can be used to test the effect of growth factors known to promote neural regeneration or to test the effect of feto-fetal spinal cord transplants to bridge spinal cord gaps.

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COMMENTS

Recent studies have shown that myelin possesses potent neurite growth inhibitors and that the blocking of these myelin-associated growth inhibitors promotes spinal cord regeneration in adult rats. From these studies, one would have predicted that fetal and neonatal spinal cords can regenerate. After all, the spinal tracts are already growing (implying the presence of growth factors and the capacity for growth) and the spinal tracts are not myelinated during this period.

This study contradicts the prediction. Meuli-Simmen et al. report that spinal cords transected at 60 to 75 days after gestation do not regenerate. The reason for the lack of regeneration is not clear. The authors considered four explanations for the regenerative failure. First, the injury may have caused massive necrosis at the injury site, including loss of glia and vascular structures, resulting in an inhospitable environment for axonal growth. Second, gliosis or the cavity formation may mechanically block growth. Third, the transection may have caused massive loss of ascending and descending tracts. Fourth, the spinal cord may have lost regenerative capacity by the time of the transection.

The second and third explanations are unlikely. The investigators did not find massive losses of ascending and descending tracts above and below the injury site. Because several investigators have shown that transected axons can grow in the spinal cord when given the appropriate environment, incapacity for growth is unlikely. Furthermore, the spinal cords were growing and developing at the time of transection. The period of 60 to 75 days of gestation is similar to the crucial embryonic Day 14 period in rats, during which axons have been shown to regenerate. One other possibility is that sheep differ from rodents in their spinal cord regenerative capability.

Examination of the histological pictures suggests a reason why growth did not occur. Large gaps were present between the cut ends of the spinal cord. These gaps spanned as much as two vertebral segments. Axons require surfaces to grow on. In the absence of a tissue matrix, growth stops. This view is consistent with the findings of other investigators, who found that bridging the gap with fetal (1, 2) and genetically modified (3) fibroblasts allows substantial axonal ingrowth into the injury site.

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In studies of mammalian CNS regeneration, it is very helpful to have both small- and large-animal models. Most of the advances in our knowledge of mammalian CNS regeneration in the past half-century have come from the use of small-animal models, in particular, rats. The present study performed in sheep showed that there was no regeneration of the spinal cord after transection of the fetal cord at approximately the midterm of gestation. This is a disappointing and somewhat unexpected result, in comparison with the results in other species. The exact cause of the failure to regenerate was not elucidated and may be the result of one or more intrinsic factors, such as myelin-associated inhibitory proteins, or surgically controllable factors, such as retraction and gap formation between the stumps. It is hoped the authors will pursue the issue with additional experiments such as treatment with spinal cord grafts.

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ANNOUNCEMENT

International Society for the History of the Neurosciences

The International Society for the History of the Neurosciences, Inc., was founded by a group of neurohistorians at a meeting in Montreal, Canada, in May 1995. Its mission is to promote the history of the neurosciences by improving communication and education among neurohistorians and to improve the quality and quantity of work in the field. We hope to attract participants from all of the clinical and experimental neurosciences, as well as professional historians and others with related interests. There will be annual meetings (usually in the spring), which will be held in alternate years in North America and on another continent (usually Europe). The Society's official publication is the *Journal of the History of the Neurosciences*, whose founder and continuing editor is F. Clifford Rose, M.D., of the Neurological Centre, London, England.

The founding officers of the Society include President Stanley Finger, Ph.D. (Psychology/Neural Science, Washington University, St Louis, MO); President-Elect, Peter Koehler, M.D. (Neurology, De Wever Hospital, Heerlen, The Netherlands); Duane Haines, Ph.D., Secretary (address below); and Treasurer, Samuel Greenblatt, M.D. (Neurosurgery, Brown University, Pawtucket, RI). Annual dues of $95.00 (U.S. dollars) include a subscription to the *Journal*, published by Swets & Zeitlinger of Lisse, The Netherlands. For information and membership application forms, please contact Duane Haines, Secretary ISHN, Department of Anatomy, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505. Tel: 601/984-1640. Fax: 601/984-1655. e-mail: dehaines@fiona.usmed.edu.