Adult Opossums (*Didelphis virginiana*) Demonstrate Near Normal Locomotion after Spinal Cord Transection as Neonates

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When the thoracic spinal cord of the North American opossum (*Didelphis virginiana*) is transected on postnatal day (PD) 5, the site of injury becomes bridged by histologically recognizable spinal cord and axons which form major long tracts grow through the lesion. In the present study we asked whether opossums lesioned on PD5 have normal use of the hindlimbs as adults and, if so, whether that use is dependent upon axons which grow through the lesion site. The thoracic spinal cord was transected on PD5 and 6 months later, hindlimb function was evaluated using the Basso, Beattie, and Bresnahan (BBB) locomotor scale. All animals supported their weight with the hindlimbs and used their hindlimbs normally during overground locomotion. In some cases, the spinal cord was retranssected at the original lesion site or just caudal to it 6 months after the original transection and paralysis of the hindlimbs ensued. Surprisingly, however, these animals gradually recovered some ability to support their weight and to step with the hindlimbs. Similar recovery was not seen in animals transected only as adults. In order to verify that descending axons which grew through the lesion during development were still present in the adult animal, opossums subjected to transection of the thoracic cord on PD5 were reoperated and Fast blue was injected several segments caudal to the lesion. In all cases, neurons were labeled rostral to the lesion in each of the spinal and supraspinal nuclei labeled by comparable injections in unlesioned, age-matched controls (79). Evidence for regeneration of the developing spinal cord after crush injury and growth of axons through the lesion site has also been documented in vitro using the South American opossum, *Monodelphis domestica*, and fetal rats (50, 51, 57, 74, 83). In the present study, we asked whether North American opossums subjected to transection of the thoracic cord on PD5 have normal use of the hindlimbs during locomotion as adults and, if so, whether it is dependent upon axons which grew through the lesion. Relatively normal locomotion has been reported in the South American opossum referred to above subsequent to crushing of the first segment of the thoracic cord on PD4–8 (58, 59). We have also used retrograde and orthograde tracing techniques to determine whether descending axons which grow through the lesion during development (79) are still present in the adult animal and, if so, whether they innervate areas which are normal for them. The North American opossum is useful for such studies because it is born in a fetal-like state, 12 days after conception (32), and its spinal cord can be transected early in development without intrauterine surgery. The results reported herein have been described previously in abstract form (5, 76).

**INTRODUCTION**

When the spinal cord of an adult mammal is transected, regeneration of recognizable spinal cord does not occur and axons fail to grow across the lesion site. It appears, however, that both occur when the lesion is made early in development. When the midthoracic cord of the North American opossum (*Didelphis virginiana*) is transected in vivo on postnatal day (PD) 5, the gap at the lesion site becomes bridged by histologically recognizable spinal cord and 30–40 days later injections of Fast blue caudal to the lesion label neurons in each of the spinal and supraspinal nuclei labeled by comparable injections in age-matched, unlesioned controls (79). Evidence for regeneration of the developing spinal cord after crush injury and growth of axons through the lesion site has also been documented in vitro using the South American opossum, *Monodelphis domestica*, and fetal rats (50, 51, 57, 74, 83). In the present study, we asked whether North American opossums subjected to transection of the thoracic cord on PD5 have normal use of the hindlimbs during locomotion as adults and, if so, whether it is dependent upon axons which grew through the lesion. Relatively normal locomotion has been reported in the South American opossum referred to above subsequent to crushing of the first segment of the thoracic cord on PD4–8 (58, 59). We have also used retrograde and orthograde tracing techniques to determine whether descending axons which grow through the lesion during development (79) are still present in the adult animal and, if so, whether they innervate areas which are normal for them. The North American opossum is useful for such studies because it is born in a fetal-like state, 12 days after conception (32), and its spinal cord can be transected early in development without intrauterine surgery. The results reported herein have been described previously in abstract form (5, 76).

**MATERIALS AND METHODS**

Pouch-young opossums were obtained from pregnant females either captured in the wild by a licensed collector in Florida or bred at The Ohio State University. The snout-rump length of pups conceived in the wild was used to estimate their age using the growth curve of Cutts et al. (18) and observations from our own...
Spinal Surgery on Pouch-Young Opossums

Sixteen opossums were subjected to transection of the fourth or fifth segment of the thoracic (T) cord on PD5. Their mothers were anesthetized by a 1-ml intramuscular injection of ketamine (100 mg/ml) followed by metofane inhalation. The pouch sphincter of the mother relaxed during anesthesia exposing the pups. The pups, still attached to the nipple, were anesthetized individually by hypothermia so their spinal cord could be exposed surgically and transected using a Beaver microblade angled at 15°. In order to ensure that the cord was completely transected, the blade was moved from side to side and pushed into the underlying vertebra. After the initial lesion was made, an angled needle was passed several times through the gap at the lesion site. Six of the pups were sacrificed by an overdose of anesthetic shortly after lesioning and perfused with 10% buffered formalin. The cartilaginous vertebral column and spinal cord were dissected from the rest of the body and processed histologically to determine if the lesion was complete. In four cases, frozen sections through the lesion site were cut at 40 µm in the sagittal plane, mounted onto glass slides and stained for Nissl substance. In two cases, the lesion site was embedded in paraffin, sectioned serially in the sagittal plane at 10 µm, and processed by the silver method of Sevier and Munger (62) which impregnates axons less than 1 µm in diameter. The remaining pups were returned to the vivarium with their mothers where they were under the care of an attending veterinarian for approximately 6 months. Opossums were considered to be adult at 6 months of age.

Spinal Surgery on Adult Opossums

Three of the opossums lesioned on PD5, plus three unlesioned controls, had their thoracic cord transected at about 6 months. In the animals lesioned on PD5, a transection was made at the site of the original lesion or just caudal to it. In the control cases, it was made at approximately the same level. All animals were anesthetized by intraperitoneal injections of sodium pentobarbital (40 mg/kg) and their spinal cord was exposed surgically using sterile techniques. The spinal cord was then transected and care was taken to ensure that the transection was complete. The surgical exposure was extended far enough laterally so that the end of a watch-makers forceps could be inserted between the spinal cord and bone. The spinal cord was then pulled to the side so that a No. 11 surgical blade (Paragon) could be inserted between the bone and cord until its tip touched the bottom of the spinal canal. The blade was then pulled completely through the spinal cord in one motion. This procedure was repeated several times until the meninges were cut and the ends of the spinal cord retracted 5–10 mm from the lesion site. Once the floor and walls of the spinal canal were exposed, the surgical blade was scraped across them until the bone was scored. This was done to ensure that all axons of the ventral and lateral funiculi were cut. Completeness of the lesion was verified histologically (see below). After lesioning, the gap at the site of transection was packed with gelfoam, the surgical exposure was closed by suturing the deep back muscles and skin, and the animals were treated for postoperative pain by subcutaneous injections of Buprenorphine (0.028 mg/kg). Body temperature was maintained with a heating pad during and immediately after surgery and by a heat lamp for variable time periods thereafter. Body temperature was monitored by a rectal thermometer. All animals received Ringer’s solution shortly after surgery and on subsequent postoperative days as needed. The animals were checked several times a day for urine retention and urine was examined for blood and signs of infection. One milliliter of sterile penicillin G procaine (300,000 units/ml) was administered daily beginning 1 day before surgery and continuing for 10 days thereafter. The animals were weighed weekly and examined daily for signs of autophagia, decubitus ulcers, and distress.

Behavioral Studies

The animals used in these experiments were divided into four groups. One group (n = 7) was subjected to transection of the midthoracic cord on PD5 only (the PD5TX group). The second group (n = 3), also lesioned on PD5, had a second transection of the midthoracic cord at 6 months (the ReTX group). The third group (n = 3) was subjected to transection of the midthoracic cord at 6 months only (the AdultTX group) and the fourth group (n = 3) consisted of nonlesioned controls (the Control group). Locomotor performance was assessed and videotaped at approximately 6 months of age in all groups. The Control and PD5TX groups were tested once at 6 months of age, whereas the ReTX and Adult TX groups were tested just prior to lesioning, the day after the lesion was made, and twice a week for 30–45 days. All animals were shaved over the lower back and hindlimbs so that movement could be observed more clearly.

In order to evaluate use of the hindlimbs in overground locomotion, a behavior presumably mediated by supraspinal axons (31), we employed the 21-point Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (2) (Table 1). The BBB scale has been shown to be a sensitive and reliable tool for assessment of locomotor deficits in rats after spinal contusion (2, 3, 4, 69), hemisection (10, 14), or transection (3). In a previous experiment, we found that components of the BBB scale
scale revealed deficits in hindlimb function ipsilateral to the lesion after spinal cord hemisection in opossums (unpublished observations).

In the present study, experimental and control animals were encouraged to locomote in a large flexible-sided child’s swimming pool (diameter, 205 cm; wall height, 38 cm) for 4 min. Two examiners observed each animal without knowing the group to which it belonged and a score was derived for each hindlimb. The hindlimb scores were averaged to yield a single locomotor score for each animal.

The BBB scale produces semiquantitative, ranked scores that traditionally require nonparametric statistical analysis. However, Abelson and Tukey (1) suggested that scaling procedures like those used in the BBB scale yield data that are more than simple rank order. Under these conditions, nonparametric tests are unnecessarily restrictive, sacrifice power, and fail to take into account the full breadth of information contained in the data (1). Therefore, we used both parametric and nonparametric analyses to compare locomotor scores at 6 months of age for the PDSTX, the AdultTX, and the Control groups (Parametric, one-way ANOVA with Scheffe post hoc tests; nonparametric, Kruskal-Wallis test with Dunn’s multiple comparison post hoc text). For the AdultTX group, the BBB scores on the final day of testing at 4 weeks posttransection were used in the ANOVA. One opossum in the AdultTX group was sacrificed 2 weeks after transection due to poor wound healing. In that case, we used the highest BBB score obtained for statistical analysis at 4 weeks. To determine whether retransection of the cord significa-

### Table 1
Basso, Beattie, and Bresnahan Locomotor Rating Scale

<table>
<thead>
<tr>
<th>Point</th>
<th>Description</th>
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<tbody>
<tr>
<td>0</td>
<td>No observable hindlimb (HL) movement</td>
</tr>
<tr>
<td>1</td>
<td>Slight movement of one or two joints, usually the hip and/or knee</td>
</tr>
<tr>
<td>2</td>
<td>Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint</td>
</tr>
<tr>
<td>3</td>
<td>Extensive movement of two joints</td>
</tr>
<tr>
<td>4</td>
<td>Slight movement of all three joints of the HL</td>
</tr>
<tr>
<td>5</td>
<td>Extensive movement of two joints and slight movement of the third</td>
</tr>
<tr>
<td>6</td>
<td>Extensive movement of all three joints of the HL</td>
</tr>
<tr>
<td>7</td>
<td>Sweeping with no weight support; or plantar placement of the paw with no weight support</td>
</tr>
<tr>
<td>8</td>
<td>Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional weight supported dorsal stepping and no plantar stepping</td>
</tr>
<tr>
<td>9</td>
<td>Occasional weight supported plantar steps, no FL-HL coordination</td>
</tr>
<tr>
<td>10</td>
<td>Frequent to consistent weight supported plantar steps, no FL-HL coordination</td>
</tr>
<tr>
<td>11</td>
<td>Frequent to consistent weight supported plantar steps and occasional FL-HL coordination</td>
</tr>
<tr>
<td>12</td>
<td>Frequent to consistent weight supported plantar steps and frequent FL-HL coordination</td>
</tr>
<tr>
<td>13</td>
<td>Consistent weight-supported plantar steps, consistent FL-HL coordination, predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping</td>
</tr>
<tr>
<td>14</td>
<td>Consistent plantar stepping, consistent FL-HL coordination during gait, toe clearance occurs frequently during forward limb advancement Predominant paw position is parallel at initial contact and rotated at lift off</td>
</tr>
<tr>
<td>15</td>
<td>Consistent plantar stepping, consistent FL-HL coordination during gait, toe clearance occurs consistently during forward limb advancement Predominant paw position is parallel at initial contact and rotated at lift off</td>
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<tr>
<td>16</td>
<td>Consistent plantar stepping, consistent FL-HL coordination during gait, toe clearance occurs consistently during forward limb advancement Predominant paw position is parallel at initial contact and rotated at lift off</td>
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<tr>
<td>17</td>
<td>Consistent plantar stepping, consistent FL-HL coordination during gait, toe clearance occurs consistently during forward limb advancement Predominant paw position is parallel at initial contact and rotated at lift off</td>
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<tr>
<td>18</td>
<td>Consistent plantar stepping, consistent FL-HL coordination during gait, toe clearance occurs consistently during forward limb advancement Predominant paw position is parallel at initial contact and rotated at lift off</td>
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<tr>
<td>19</td>
<td>Consistent plantar stepping, consistent FL-HL coordination during gait, toe clearance occurs consistently during forward limb advancement Predominant paw position is parallel at initial contact and rotated at lift off</td>
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<tr>
<td>20</td>
<td>Consistent plantar stepping, consistent FL-HL coordination during gait, toe clearance occurs consistently during forward limb advancement Predominant paw position is parallel at initial contact and rotated at lift off</td>
</tr>
<tr>
<td>21</td>
<td>Consistent plantar stepping, consistent FL-HL coordination during gait, toe clearance occurs consistently during forward limb advancement Predominant paw position is parallel throughout stance, trunk stability; tail consistently up</td>
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Note. Definitions: Slight, partial joint movement through less than 1/2 the range of joint motion. Extensive, movement through more than half of the range of joint motion. Sweeping, rhythmic movement of HL in which all three joints are extended and then fully flex and extend again; animal is usually sidelying and plantar surface of paw may or may not contact the ground; weight support across the HL is evident. No weight support, no contraction of the extensor muscles of the HL during plantar placement of the paw; or no elevation of the hindquarter. Weight support, contraction of the extensor muscles of the HL during plantar placement of the paw; or elevation of the hindquarter. Plantar stepping, the paw is in plantar contact with weight support then the HL is advanced forward and plantar contact with weight support is reestablished. Dorsal stepping, weight is supported through the dorsal surface of the paw at some point in the step cycle. FL-HL coordination, For every FL step a HL step is taken and the HLs alternate. Occasional, less than or equal to half; 51–94%. Frequent, more than half but not always; 95–100%. Trunk instability, lateral weight shifts which cause waddling from side to side or a partial collapse of the trunk.
cantly disrupted locomotion, we used a one-way ANOVA for repeated measures and Tukey's HSD post hoc tests.

Confirmation of the Lesion in the ReTX and Adult TX Groups

Once behavioral analysis was complete, the animals in the ReTX and AdultTX groups were anesthetized deeply, as described previously, and sacrificed by perfusion with a 10% buffered formalin solution. Their spinal cord was then exposed and the lesion site was photographed before and after removal. In order to help verify that the lesion was complete, blocks of spinal cord which included the lesion site were embedded in paraffin and sectioned serially at 20 μm in the horizontal plane. In all but one case, every section was impregnated for axons by a modified Bodian technique (40). In one case, most sections were impregnated for axons but selected sections were stained for Nissl substance.

Studies Using Retrograde and Orthograde Tracing Techniques

After behavioral analysis, four of the animals in the PDSTX group were given bilateral injections of 2% Fast Blue (FB) (38) into the spinal cord at T13. Up to 3 μl of FB was injected on each side and it was distributed evenly in three injections per side differing only in depth. The intent of the injections was to label neurons rostral to the lesion that supported axons caudal to it. Comparable injections were made at the same level in the three unlesioned, age-matched controls. All animals were anesthetized as described above so that the spinal cord could be exposed surgically. The vertebral column was stabilized in a stereotaxic frame and the injections were made using a glass micropipette attached to a 1-μl Hamilton syringe which was positioned with a microdrive system. Surgical closure and treatment for postoperative pain were as described above. The operated animals were maintained for 5 to 7 days before being reanesthetized with sodium pentobarbital and sacrificed by transcardiac perfusion with 0.9% saline followed by a citrate-buffered solution containing 10% formaldehyde. The spinal cord and brain were removed, photographed, and immersed in 30% sucrose citrate buffer for approximately 24 h at 4°C. All brains and cords were scored by a shallow cut on one side so that laterality of the tissue sections could be determined after mounting. Frozen sections of the spinal cord and brain were cut at 40 μm in the coronal plane, mounted onto glass slides using a nonfluorescent mounting medium, and coverslipped with Entellan (Merck). Sections were viewed using a Leitz (Orthoplan) fluorescence photomicroscope equipped with the A cube of the Ploem illumination system (excitation wavelength, 340-389 nm). Darkfield illumination was employed for nuclear identification and to make drawings of the sections.

Once it was established that FB had not spread to the lesion site, selected sections through the injection and lesion sites (experimental cases), one of three sections from each segment of the spinal cord rostral to the lesion, and every section from the brain stem were drawn using an X-Y plotter attached to the microscope stage by position transducers. The positions of labeled neurons were recorded on the drawings using the same plotting system and photomicroscopic documentation of labeling was taken from the plotted sections. When plotting and photography were complete, the coverslips were removed and the sections were stained for Nissl substance to help clarify laminar and nuclear boundaries.

Profiles of labeled neurons were counted bilaterally from the plots of one of three sections in the medial part of the pontine reticular nuclei, the dorsal part of the lateral vestibular nuclei, and the red nuclei in both experimental and control cases. These nuclei were chosen for quantitative analysis because axons which originate within them reach the spinal cord at different ages (45), they occupy different funiculi (42), and they grow around or through a lesion of their pathway at different stages of development (43, 46, 77, 79, 84, 85). Each labeled profile was counted because it was difficult to examine all of them for a nucleus without losing fluorescence. Since the diameter of labeled profiles never exceeded the thickness of three sections and they were counted from one of three sections, it is not likely that they were counted more than once. All counts were expressed as the mean ± SEM. Analysis of the data was carried out by using the two-way repeated measures analyses of variance to determine whether statistically significant (P < 0.05) differences existed between the two groups for each nucleus studied. In all cases, counts from the right and left sides were not statistically different. Statistical analysis was performed using SYSTAT for Windows (Version 5.04).

Three of the animals in the PDSTX group were used for orthograde transport experiments after behavioral analysis was complete. Each animal was anesthetized as described above and a craniotomy was performed over the desired area of the brain using sterile techniques. Stereotaxic injections (6 to 8 μl of a 10% solution delivered in three closely placed injections) of Fluoro-Ruby (60) were made into either the red nucleus (n = 2) or the ventral gigantocellular reticular nucleus (n = 1) using a glass micropipette attached to a 1-μl Hamilton syringe. Coordinates for the injections were modified from Oswald-Cruz and Roja-Miranda (52). After the injections were complete, the meninges were reflected back over the brain and the surgical exposure was closed by suturing the temporalis fascia and overlying skin. The animals were treated for postoperative
pain as described above and approximately 30 days later, they were anesthetized again and sacrificed by transcardiac perfusion with a 10% citrate-buffered 10% formalin solution. Frozen sections of the removed brain and spinal cord were cut coronally, mounted onto glass slides using a nonfluorescent mounting medium, covered-slipped with Entellan (Merck), and viewed using a Leitz Orthoplan II fluorescence photomicroscope equipped with filter cube N2.1 of the Ploem illumination system (excitation wavelength, 515–560 nm). The injection sites were checked for accuracy and sections of the spinal cord rostral to the lesion, at the lesion site, and caudal to it, were examined for labeled axons. The locations of labeled axons were charted on drawings of selected sections and recorded photographically. Comparable injections were made in agematched, nonlesioned controls (the red nucleus, n = 3; the ventral gigantocellular reticular nucleus, n = 3) using the methods described for the experimental cases. Rexed’s laminae (54) were demarcated according to Boyles (9) and brain stem nuclei were identified and designated according to Oswaldo-Cruz and Rocha-Miranda (52). All experiments were performed in compliance with the requirements of The Laboratory Animal Care and Use Committee of The Ohio State University and the U.S. Public Health Service.

RESULTS

The histological appearance of the normal thoracic cord at PD5 is documented in Fig. 1A. When the thoracic cord was transected at PD5 and the pups were sacrificed shortly thereafter, the site of transection could be identified grossly (Fig. 1B), and its completeness verified histologically (Figs. 1C–1F). Every section through the lesion site was examined in all cases and it was clear that the lesioning instrument had passed completely through the spinal cord and into (Fig. 1C) or through (Figs. 1D and 1F) the developing vertebral body. When the silver-impregnated sections were examined at high power (Fig. 1E and inset of Fig. 1F), impregnated axons could be discerned clearly and none of them traversed the lesion site. Pups subjected to comparable lesions were maintained until they were approximately 6 months old when they and age-matched, unlesioned controls were used for the behavioral and anatomical studies described below.

Behavioral Studies

The BBB locomotor scores of all groups are shown in Fig. 2. Opossums subjected to transection of the thoracic cord at PD5 only (the PD5TX cases) had remarkable use of the hindlimbs in locomotion at 6 months. All animals supported their weight, stepped consistently, and demonstrated normal coordination of the forelimbs and hindlimbs (Figs. 3A–3C). Most animals (80%) maintained normal position of the paw throughout the stance phase of gait (Figs. 3A and 3C) and did not display the external or internal rotation of the paw commonly observed in rats after partial lesions of the spinal cord (2, 3, 10). The swing phase of gait also appeared normal in most animals although two of them dragged their hindpaws during forward movement of the limb. Although the PD5TX cases locomoted well as adults and their BBB scores were high (19.15 ± 1.92 SD), residual deficits were apparent when compared to normal control cases (control). Most of the animals in the PD5TX group demonstrated trunk instability or walked with their tail down part of the time (Fig. 3A), deficits that were not observed in normal animals. Transection of the spinal cord at 6 months only (AdultTX) resulted in loss of locomotion and marked paralysis of the hindlimbs which persisted for the entire testing period (mean BBB, 1.33 ± 1.04 SD). Thus the BBB scores were significantly different across groups using either parametric (F = 151.3; P < 0.001) or nonparametric (P = 0.006) tests. As expected, parametric and nonparametric, post hoc comparisons showed that the locomotor scores of the AdultTX group were significantly lower than those in the other groups. Interestingly, the locomotor scores of the PD5TX group were not significantly different from those of normal controls (control) despite the residual deficits referred to above (parametric and nonparametric post hoc tests, P > 0.05).

In order to determine whether axons which grew across the lesion site played a role in the hindlimb function described above, we retransected the spinal cord in three of the animals initially lesioned on PD5 (the ReTX group). The hindlimbs of opossums in the ReTX group were paralyzed after surgery and for 10–14 days only isolated movements of one or two joints were seen (Figs. 4A–4D). It appeared that active movement was limited to the hip joint with only passive flexion of the knee, but active flexion at the knee could not be ruled out. By 14 days, however, the retransected animals began to support weight with the hindlimbs. Improvement continued for 3–4 weeks when occasional stepping was observed (Figs. 4E and 4F). One animal progressed particularly well and 5 weeks after retranssection developed the ability to stand consistently on all four limbs and to step frequently with the hindlimbs throughout the 4-min test period. Use of the hindlimbs was never coordinated with that of the forelimbs, however. The locomotor scores for the ReTX group (Fig. 2) were significantly different across days (P < 0.001). Post hoc comparisons showed that retranssection resulted in significantly lower locomotor scores from 1 day postoperatively (P < 0.01) to 45 days postoperatively (P < 0.01) when compared to preoperative 6-month scores. The apparent recovery of some stepping after retranssection resulted in significantly higher locomotor scores at day 28 and thereafter than immediately following retranssection on day 1 (P < 0.05).
FIG. 1. A Nissl-stained section of the normal midthoracic spinal cord from a PD5 opossum is shown in A. The ependymal zone (EZ), the intermediate zone (IZ), and the marginal zone (MZ) are indicated. The lesion site (arrow) in an opossum subjected to transection of the midthoracic spinal cord (SC) on PD5 and sacrificed shortly thereafter is shown in B. A Nissl-stained section through the lesion site (arrow) in the same case is shown in C. Note that the lesioning instrument passed through the spinal cord and into the developing vertebral body. A low power photomicrograph of a silver-impregnated section through the lesion site (arrow) in another case is illustrated in D. Note that the lesioning instrument passed through the spinal cord and through the developing vertebral column. The photomicrograph in E shows a higher power view of the area contained within the rectangle in D and the arrow indicates impregnated axons. The low power photomicrograph in F shows a section lateral to that in D and the lesion site is indicated by the arrow. The insert is a higher power photomicrograph of the area outlined by the rectangle and the arrow points to impregnated axons.
Documentation of the Lesion in the ReTX and AdultTX Groups

Subsequent to transection, the spinal cord retracted from the lesion site in both the ReTX and the AdultTX cases and a 5- to 10-mm gap could be seen grossly when the animals were sacrificed. The gap is documented in Figs. 5A and 5B for the ReTX case that showed the greatest recovery and in Figs. 5B and 5C for one of the AdultTX cases. In the latter case, the gap is filled with gelfoam.

Retrograde Tracing Studies

After behavioral analysis, three of the animals in the PD5TX group received bilateral injections of FB at T13 and the results from a representative case are illustrated in Figs. 6–11. The site of transection could be identified at the time of sacrifice by its pale appearance (Fig. 6A), and histologically it could be distinguished by the relative paucity and poor differentiation of the gray matter (Fig. 6B). Distinct dorsal horns were not present and, in some cases, the central canal was abnormally positioned (arrow, Fig. 6B). In some sections, the gray matter was difficult to distinguish as such and only a few neurons could be identified. The injections were large, bilaterally symmetrical (Fig. 6C), and limited primarily to the segment injected. Examination of sections rostral to the lesion site revealed that labeled neurons were present in each of the supraspinal and

![Graph showing BBB scores for different groups](image)

**FIG. 2.** BBB scores for the normal (control), the postnatal day 5 transected (PD5TX), the adult transected (AdultTX), and the retranssected (ReTX) groups (***P < 0.001 vs control, PD5TX). Scores for the ReTX animals are shown for day 1 (ReTX1d) and 6 weeks (ReTX6w) after the second lesion and are compared to the scores obtained prior to retranssection (**P < 0.01).

![Images of opossums showing weight support and stepping](image)

**FIG. 3.** Video images of 6-month-old opossums previously subjected to transection of the midthoracic spinal cord on PD5. Opossums in this group demonstrated consistent weight support (A–C) and stepping (A and C) with the hindlimbs. The position of the paw at the beginning (right hindlimb) and end (left hindlimb) of stance was normal and parallel to the body (C). The animals were shaved over the lower trunk and proximal hindlimb so that movement could be observed more clearly.
spinal nuclei labeled in the age-matched, unlesioned controls. A plot of the labeling present in representative sections of the hypothalamus and brain stem is illustrated for one case in Fig. 7 and photomicroscopic documentation of selected labeling is provided for the same case in Fig. 8. Labeled neurons were found bilaterally within the paraventricular nucleus of the hypothalamus; the dorsal hypothalamic area; the lateral hypothalamic (Fig. 7A); the interstitial nucleus of the medial longitudinal fasciculus; the nucleus of Edinger-Westphal; the interstitial and deep tegmental nuclei of the midbrain; the red nucleus (Figs. 7B and 8A); the locus coeruleus (Fig. 7D and 8B); the locus coeruleus pars alpha; the pontine reticular nucleus (Figs. 7D and 8C); the lateral, medial, and inferior vestibular nuclei (Figs. 7E and 7F and Fig. 7D); the gigantocellular and ventral gigantocellular reticular nuclei (Figs. 7G and 7H and Figs. 8E and 8F); the raphe magnus, obscurus, and pallidus (Figs. 8E and 8F); the ventral and dorsal reticular nuclei of the medulla (Fig. 7I); the solitary complex; and the hilum of the nucleus cuneatus (Fig. 7I). As expected, labeling was not present in the cerebral cortex. In the opossum, cortical axons do not extend beyond rostral levels of the thoracic cord (42).

Orthograde Tracing Studies

Labeled axons could be traced caudal to the lesion site in the PD5TX cases after injections of Fluororuby...
into the red nucleus as adults. The injection sites in one animal are shown in Fig. 12A and spinal labeling in the same case is illustrated in Figs. 12B–12D. At cervical and rostral thoracic levels of the cord, labeled axons were present in the dorsal part of the lateral funiculus contralateral to the injections (Fig. 12B) and within laminae V–VII of the gray matter. In sections through the lesion site (Fig. 12C) and caudal to it (Fig. 12D), they were present in the same areas. When injections of Fluororuby were made into the red nucleus of age-matched, unlesioned controls, labeled axons were found in comparable areas, but caudal to the lesion they

**FIG. 5.** A and B illustrate the lesion site (arrows) grossly and microscopically in the ReTX animal that showed the greatest recovery. The lesion site is similarly shown for one of the AdultTX in C and D. C and D, gelfoam is still present between the cut ends of the cord.
appeared to be more numerous and more closely aggregated than in the experimental cases (data not shown). In both experimental and control cases, axonal labeling could be traced to T13.

Labeled axons were also present caudal to the site of transection in the one PD5TX case subjected to injections of Fluororuby into the ventral gigantocellular reticular nucleus as an adult. As can be seen from Fig. 13A, the injection spread dorsally to include the gigantocellular reticular nucleus. Rostral to the lesion site, labeled axons were found bilaterally in the ventral and lateral funiculi (Fig. 13B) and within laminae I–X of the gray matter. Labeling was found in the same areas at the lesion site (Fig. 13C) and caudal to it (Fig. 13D). The pattern of spinal labeling in the control cases was similar to that in the experimental animal, but labeled axons appeared to be more numerous caudal to the lesion (data not shown). In both the experimental and the control cases, labeled axons could be followed to T13.

DISCUSSION

Our results show that nearly normal overground locomotion is present in adult opossums which had their midthoracic cord transected on PD5. Use of the hindlimbs in locomotion is at least partially dependent upon intact brain stem axons which innervate the lumbar cord (3, 7, 11, 12, 13, 56), so it is likely that descending axons which grew through the lesion during development (79) helped support use of the hindlimbs at maturity. It should be noted, however, that ascending axons also grow through lesions made at PD5 (70, 71, 72, 73, 78) and they are still present in the
adult animal (70, 71, 72). It is possible, therefore, that they also played a role. The presence of paralysis after retranssection supports our view that axons which grew through the lesion were significant in sparing of function. Relatively normal use of the hindlimbs at maturity has also been reported after crushing the first thoracic segment of the cord between PD4 and 8 in the South American opossum, *M. domestica* (58, 59). Taken together, these results indicate that the spinal cord of developing opossums is capable of functional repair as reported for the chick (65, 67) and other developing, nonmammalian vertebrates (6, 19–23, 25, 26, 29, 48, 63, 64, 66, 87).

Interpretation of our results is dependent upon the assumption that the spinal cord was transected completely on PD5. It is well known that incomplete

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**FIG. 7.** Plot of supraspinal neurons labeled by injections of FB eight segments caudal to the lesion site in the case illustrated in Fig. 6. Labeled neurons have been plotted as dots on selected sections through the hypothalamus (A) and brain stem (B–I from rostral to caudal). Each dot represents one labeled profile in the section depicted. Indicated are the cerebral aqueduct (aq), the dorsal cochlear nucleus (CcD), the superior central nucleus (CeS), the inferior colliculus (IC), the locus coeruleus (Coe), the superior colliculus (CS), the cuneate nucleus (Cu), the lateral cuneate nucleus (CuL), the dorsal nucleus of the lateral lemniscus (DLLd), the facial nucleus (Fac), the fastigial nucleus (Fast), the dorsal lateral geniculate nucleus (GLD), the medial geniculate nucleus (GM), the hypoglossal nucleus (Hg), the medial habenular nucleus (HM), the lateral hypothalamus (HyL), the lingula (Lg), the fourth ventricle (IV), the inferior olive (OI), the superior olive (OS), the cerebral peduncle (ped), the retroambiguus nucleus (RA), the gigantocellular reticular nucleus (RGc), the ventral gigantocellular reticular nucleus (RGcv), the red nucleus (RN), the pontine reticular nucleus (RP), the ventral reticular nucleus (RV), the motor root of the trigeminal nerve (RVM), the caudal spinal trigeminal nucleus (TrSc), the oral spinal trigeminal nucleus (TrSO), the facial nerve (VII), the ventral nucleus of the lateral lemniscus (VLLv), and the lateral vestibular nucleus (VstL).
FIG. 8. Fluorescence photomicrographs of labeled neurons in the red nucleus (RN in A), the locus coeruleus and adjacent areas (Coe in B), the medial part of the pontine reticular nucleus (RP in C), the dorsal part of the lateral vestibular nucleus (VstL in D), the ventral gigantocellular reticular nucleus and raphe magnus (RGcv and RaM in E), and the gigantocellular reticular nucleus and raphe obscurus (RGc and RaO in F) are illustrated from the case plotted in Fig. 7. The fourth ventricle (IV), the mesencephalic tract of the trigeminal nerve (rV), the restiform body (cr), the pyramidal tract (pyr), and the inferior olive (Ol) are indicated for reference. The arrows at the bottom in E and F point to the midline.
Our finding that animals with spinal cord lesions early in development have greater sparing or recovery of function than animals with comparable lesions at adulthood replicates earlier work in the rat (41, 49, 68) and cat (11, 12, 35, 56). At least two explanations have been offered for this phenomenon: (i) immaturity of descending systems at the time of lesioning (41, 68) and (ii) lack of inhibition within segmental systems in the spinal cord (56). The maturation hypothesis of Stelzner and colleagues holds that there is a gradual shift during development from segmental control of spinal motor systems to control by supraspinal systems, a process termed spinal encephalization (41, 68). Behavioral evidence for greater function after spinal cord transection in neonatal rats than in neonatal cats supports the role of maturation since the rat CNS is less mature than that of the cat at birth as evidenced by differences in maturation of the corticospinal tract (36, 39) and the degree of myelination (53, 61). In addition, when encephalization is delayed by blocking NMDA receptor activation of supraspinal systems, the critical period for sparing of function after lesioning is extended (41). A role for disinhibition in sparing of function after transection of the developing spinal cord has been suggested by Robinson and Goldberger (56). These investigators showed that blocking inhibition with bicuculline, a GABA receptor antagonist, in adult cats whose spinal cord had been transected at birth or 2 weeks later resulted in no improvement in segmentally driven locomotion (bipedal locomotion). However, cats with spinal cord transection in adulthood demonstrated significant improvement in bipedal locomotion after similar treatment. These findings suggest that inhibitory circuits within the spinal cord develop with age and that they produce at least some locomotor deficits after transection.

In the present study, we report that retransection of the spinal cord in adulthood results in an immediate and prolonged loss of HL locomotion. It is likely, therefore, that axons which grew through the lesion after transection on PD5 were responsible for the near-normal locomotion seen when the animals were tested behaviorally as adults. Loss of HL locomotion after retranssection was not permanent, however, suggesting that segmental systems in the lumbosacral cord underwent substantial reorganization. Evidence for segmental reorganization is twofold: first, the isolated lumbosacral cord produced periods of weight-supported HL stepping in all opossums given two transactions; second, no opossums subjected to a single spinal cord transection in adulthood demonstrated HL weight support or stepping. It is not likely that recovery was due to sparing of white matter by the retranssection because a 5- to 10-mm gap was present between the cut ends of the cord at sacrifice.

The present study does not address whether partial
resolution of HL paralysis and subsequent stepping after retranssection involves maturational and/or inhibitory mechanisms (see above). However, it is plausible that substantial changes occurred after the initial transection in both encephalization of locomotion and the development of segmental inhibition. In any case, the expected behavioral alterations may have been masked or inhibited by axons that grew across the lesion. If so, the modified capabilities of the segmental spinal cord would be apparent after retranssection in adulthood. Further study is needed to determine if spinal reorganization is related to encephalization, inhibition, or other mechanisms.

We have shown previously that descending axons are present in the lumbar cord of the North American opossum 30 days after transection of the midthoracic cord on PD5 (79). The studies reported herein show that axons which grow caudal to the lesion during development are still present in the adult animal and that they innervate appropriate areas. The orthograde tracing studies also suggest that the axons in question grew through reconstituted spinal cord at the lesion site rather than around an incomplete lesion. Growth of brain stem axons around a lesion occurs at later stages of development, however (46, 84). Failure to label rubral and reticular axons caudal to T13 in the experimental cases does not mean that they only reached that level. The caudal extent of labeling was also limited to T13 in control cases so it is probably a function of survival time. We know from previous studies that axons from the red nucleus and ventral gigantocellular reticular nucleus extend the length of the spinal cord (42, 44). In vivo growth of descending axons through the lesion after transection of the spinal cord has also

![ Diagram showing spinal neurons labeled in the case illustrated in Figs. 6–8. The locations of labeled neurons (dots) are indicated on drawings of the second, fourth, sixth, and eighth cervical (C) segments and the second and fourth thoracic (T) segments of the cord. All of the neurons labeled in 1 of 3 sections through the indicated segments are plotted on a drawing of 1 section (49 sections for C2, 40 sections for C4, 17 sections for C6, 31 sections for C8, 38 sections for T2, and 59 sections for T4). Rexed’s laminae (I–X) are demarcated by dotted lines. ](image)

**FIG. 10.** Plot of spinal neurons labeled in the case illustrated in Figs. 6–8. The locations of labeled neurons (dots) are indicated on drawings of the second, fourth, sixth, and eighth cervical (C) segments and the second and fourth thoracic (T) segments of the cord. All of the neurons labeled in 1 of 3 sections through the indicated segments are plotted on a drawing of 1 section (49 sections for C2, 40 sections for C4, 17 sections for C6, 31 sections for C8, 38 sections for T2, and 59 sections for T4). Rexed’s laminae (I–X) are demarcated by dotted lines.
FIG. 11. Fluorescence photomicrographs of labeled neurons in selected segments of the cervical (C2, 5, 6, 7, and 8 in A–E) and thoracic (T1 in F) cord from the case illustrated in Figs. 6–10. Labeled neurons are illustrated in the lateral funiculus (arrow in A), lamina I (arrow in D), lamina V (uppermost arrows in E and F), lamina VII (arrow on the right in B and lower three arrows in E), lamina VIII (arrows on the left in B, C, and F), and lamina X (arrow on the left in B). The dorsal funiculus (DF), the lateral funiculus (LF), the ventral funiculus (VF), the substantia gelatinosa (SG), and the central caudal (CC) are indicated.
been reported in developing birds (33, 65, 67) and amphibians (6, 29) as well as in adult lizards (25, 26, 66), teleost fish (8, 15, 64, 87), and lampreys (17, 21–23, 48, 82). Such growth has also been described in vitro in the developing South American opossum, *M. domestica*, and in fetal rats (50, 51, 57, 74, 83).

Counts of labeled profiles in the medial part of the pontine reticular nucleus, the dorsal part of the lateral vestibular nucleus, and the red nucleus after FB injections at T13 suggest that labeled neurons were fewer in the animals transected on PD5 than in the age-matched, unlesioned controls. It is possible, therefore, that some neurons failed to survive axotomy or that they survived but failed to support axonal growth through the lesion. Death of developing rubrospinal neurons after axotomy has been reported in the opossum (86) and it may be due to failure to access appropriate trophic factors. Interestingly, BDNF and NT3 rescue axotomized rubrospinal neurons in PD3 rats (24). It is also possible that some of the spinally projecting neurons in the nuclei referred to above were committed to axonal growth at the time of lesioning and that they had not established the critical number of synapses (47) or sustaining collaterals (30) necessary to support them. Of course, the development of synapses and collaterals and incorporation of trophic factors are not mutually exclusive.

It should be emphasized that the counts of labeled...
profiles in our study only provided rough estimates of neuronal numbers (16). We did not use assumption-based or stereological methods because they are difficult to carry out on rapidly fading, FB-labeled neurons. It is unlikely, however, that the results of such studies would have changed our conclusions.

Regeneration of cut axons as well as late arrival of undamaged axons contributed to growth across the lesion in our experiments. Many reticular and vestibular axons reach the lumbar cord by PD3 in the North American opossum (45), so they were obviously cut by transecting the midthoracic cord on PD5. Based on the results of double-labeling studies we have concluded that many reticulospinal and vestibulospinal axons regenerated in our experiments (80). The results of the double-labeling studies also suggested that late growth of undamaged axons occurred. The degree to which the formation of new neuroblasts contributed to late growth is not known. In the unlikely event that our lesions were incomplete (see above), collateral sprouting of spared axons might have taken place. Rubral axons do not reach lumbar levels until PD10–13 (45), so they were not damaged by PD5 lesions. It would appear, therefore, that rubral axons simply grew through the lesion site when they reached the appropriate level. Growth of rubral axons through the lesion after transection of the thoracic cord occurs as late as PD26, however (79), and the results of double-labeling studies indicate that regeneration of cut axons contributes to it when the lesion is made between PD13 and 26 (unpublished results). Regeneration of supraspinal axons has been reported when the spinal cord is transected dur-

FIG. 13. A fluorescence photomicrograph of a Fluororuby injection (INJ) in the ventral gigantocellular reticular nucleus (RGcv) of an adult opossum previously subjected to transection of the midthoracic cord on PD5 is shown in A. Axonal labeling in the spinal cord rostral to the lesion (B), at the lesion site (C), and caudal to it (D) is illustrated for the same case. The facial nucleus (Fac), the gigantocellular reticular nucleus (RGc), the ventral gigantocellular reticular nucleus (RGcv), the pyramidal tract (pyr), and the ventral funiculus (VF), are indicated. The arrows in A–D indicate the midline.
ing the critical period for developmental plasticity in
the chick (33, 65) as well as in adult lampreys (48),
teleost fish (87), certain amphibians (6, 29), and, to a
limited extent, reptiles (66).

We have reported previously that the critical period
for growth of axons from the medial pontine reticular
nucleus and the dorsal part of the lateral vestibular
nucleus through a lesion of their spinal pathway ends
prior to PD12 (79). The critical period for comparable
growth for most supraspinal axons ends later, however,
and for some of them it does not terminate until after
PD26 (79). As discussed previously (79), factors which
dictate the end of the critical period for plasticity
include diminished ability to initiate and/or sustain
axonal growth with age and an increasingly nonpermis-
sive environment for it. Although some spinal axons are
capable of regeneration in adult mammals, a permis-
sive environment must be provided (55) and, even then,
regeneration is not as robust as that seen during
development (28).

One of the major environmental changes during
spinal cord development is the appearance of myelin
and proteins within myelin have been shown to inhibit
axonal growth after transection of the spinal cord in the
developing chick (37) and South American opossum,
M. domestica (75). The presence or absence of extracel-

sharp wall molecules (e.g., laminin, fibronectin, hepa-
рин sulfate proteoglycans) may also influence the end of
the critical period for developmental plasticity.

In summary, our results show that: (i) use of the
hindlimbs in overground locomotion is nearly normal in
adult opossums previously subjected to transection of
the midthoracic cord on PDS, (ii) the sparing of function
observed at maturity in PDS lesioned animals is due in
part to growth of axons through the lesion, and (iii)
supraspinal and descending propriospinal axons that
grow through the lesion after transection of the midtho-
raric cord on PD5 are still present in the adult animal
and they innervate regions which are appropriate for
them. Hopefully, it will eventually be possible to restore
some degree of developmental plasticity to the injured
spinal cord of adult mammals, including man.

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Note added in proof. A behavioral study of adult opossums
(Monodelphis domestica) subjected to transection of the thoracic cord
as neonates has recently been published (Saunders, N. R., J. P.
Development of walking, swimming and neuronal connections after
complete spinal cord transection in the neonatal opossum, Monodel-
was transected on postnatal day 4–6, and the animals were analyzed
behaviorally at 2 or 6 months, overground locomotion was remark-
ably normal. The authors also report that anatomical reconstruction
is less complete after transection than after crush injury.

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