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ORGANIZATION AND DEVELOPMENTAL PLASTICITY OF SPINOCEBELLAR AXONS. STUDIES USING THE NORTH AMERICAN OPOSSUM, *DIDELPHIS VIRGINIANA*

DISSERETATION

Presented in Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy in the Graduate School of

The Ohio State University

by

Jonathan Richard Terman

*****

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1997

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ABSTRACT

Trauma to the spinal cord of adult mammals, including that of man, results in paralysis and loss of sensation below the affected area which is often due to the inability of injured axons to regenerate. It is reasonable to predict, however, that regeneration might occur during development when axons are programmed for growth and the environment is conducive for it. Indeed, growth of descending axons around or through a lesion of their spinal pathway has been documented during early development. It is interesting, however, that comparable plasticity has not been shown for axons which form ascending pathways. Experiments designed to study developmental plasticity of ascending axons in mammals have only been carried out in neonatal rats, however, and it may be that damaged axons were relatively mature at that age and had lost their potential for plasticity. To determine if ascending axons grow beyond a lesion of their spinal pathway at earlier stages of development would necessitate intrauterine surgery. An alternative strategy would be to employ a species that is born in a more immature state. The North American opossum, *Didelphis virginiana*, is born 12-13 days after conception when it is in a fetal-like state of development. It is possible, therefore, to lesion its spinal cord very early in development without intrauterine surgery. In the studies described
herein, we used the opossum to ask if ascending axons which originate in the spinal cord and project to the cerebellum (spinocerebellar axons) grow around or through a lesion of their spinal pathway, and, if so, to document the critical period(s) for such growth. Initial studies were carried out in adult opossums, to determine the origin, course, and laterality of spinocerebellar axons. Such information was not available for any marsupial and it was necessary for correct interpretation of the developmental plasticity studies. To conduct the plasticity studies, pouch-young opossums of varying ages were anesthetized and subjected to a hemisection or transection of the spinal cord at mid-thoracic levels. Initially, we focused on axons which originated in Clarke’s nucleus (i.e., axons of the dorsal spinocerebellar tract) and it was established that they grow through a lesion of their spinal pathway if it is made at postnatal day (PD) 5-8 but not at PD12 or at later ages. Spinocerebellar axons differ in origin, developmental history, laterality, funicular position, peduncular course, and terminal distribution, however, so it was possible that differences also exist in their potential for plasticity. For that reason, we also asked whether spinocerebellar axons which originate from spinal border cells (i.e., axons of the ventral spinocerebellar tract), neurons within Stilling’s nucleus, and neurons within the ventrolateral nucleus of the sacral and coccygeal cord grow through a comparable lesion. The results indicate that they are able to do so and that the critical period for plasticity is comparable to that for axons which originate within Clarke’s nucleus. Interestingly, the critical period for developmental plasticity of spinocerebellar axons (and other ascending axons) ends earlier than that for most descending spinal axons.
DEDICATION

To my Dad and Mom for their love and support and for giving me "the blessing"
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I want to first say thank you to the Neuroscience Graduate Studies Program at The Ohio State University and the people who are a part of it for giving me the opportunity to pursue these studies. In addition, they have provided a great deal of help and guidance during my graduate training and preparation of my dissertation. I also would like to thank the Chairman of the Neuroscience Program, Dr. James King, for providing support to attend meetings and for always having an open door for discussions about the program.

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Photomicrographs which show neuronal labeling in selected nuclei at lumbar and sacral levels from the case described in Figs. 2 and 3. In each section, the side of the lesion is on the reader's right. A section from the 2nd lumbar segment of the spinal cord is shown in A. Labeled neurons are documented in the dorsal part of lamina VII (closed arrowhead), in the central/ventral part of lamina VII (open arrowhead), and in the dorsolateral (DL) and ventrolateral (VL) nuclei of lamina IX (closed arrows) on the side of the lesion. A section from the 3rd lumbar segment of the spinal cord is shown in B. Labeled cells are documented in the medial part of laminae VI and VII on the side of the lesion (closed arrow) and in the ventromedial part of lamina VII (open arrow) contralateral to it. Photomicrographs of sections through the 2nd segment of the sacral cord are shown in C and D. Labeled neurons can be seen in Stilling's nucleus (SN) (closed arrow, C) and in the ventrolateral nucleus of lamina IX (VLN) ipsilateral to the lesion (closed arrow, D). The scale bar in A can be used for B-D.

Camera lucida drawings of labeled spinocerebellar neurons to show the trajectory of their axons within the grey matter and part of the white matter. Only neurons in major spinocerebellar nuclei were selected for documentation. A drawing of a section through the second cervical segment (C2) of the spinal cord is shown at the upper left and a labeled neuron in the nucleus centrobasalis is illustrated (closed arrow). Its axon can be followed into the dorsolateral funiculus. A labeled neuron in the central cervical nucleus is also illustrated (open arrow) and its axon can be followed across the midline into the ventral funiculus. The drawing of a section
from the sixth cervical segment (C6) illustrates a labeled neuron in the central/lateral portion of lamina VII (closed arrow) and its axon can be followed into the ipsilateral ventrolateral funiculus. A labeled neuron in medial lamina VII is shown in the same section (open arrow) and its axon can be followed across the midline into the contralateral ventral funiculus. The drawing of a section from the 13th segment of the thoracic spinal cord (T13) contains a camera lucida drawing of a labeled neuron in Clarke’s nucleus (closed arrow) the axon of which can be followed into the dorsolateral funiculus. A labeled neuron is also shown in lamina VIII (T13, open arrow) and its axon can be followed into the ventral funiculus. The drawing of a section from the 3rd segment of the lumbar cord (L3) contains a camera lucida drawing of a labeled neuron in the dorsomedial part of lamina VII (closed arrow) and its axon can be followed ventrally, across the midline, and into the contralateral ventral funiculus. A drawing of a labeled neuron in the lateral part of lamina VII is also shown (open arrow) and its axon can be followed medially across the midline and into the ventral funiculus. The drawing of a section from the 2nd sacral segment of the spinal cord (S2) shows a labeled neuron in Stilling’s nucleus (closed arrow) and its axon can be followed across the midline and into the contralateral ventral funiculus. A camera lucida drawing of a labeled neuron in the ventrolateral nucleus is also shown (open arrow) and its axon can be followed across the midline and into the ventral funiculus on the contralateral side. Note its circuitous course.

11 Photomicrographs of two of the neurons drawn in Fig. 10. A photomicrograph of one of the neurons (large arrow) drawn in L3 in Fig. 10 (open arrow) is shown in A. Its axon (small arrows) can be followed medially toward the midline. A photomicrograph of one of the neurons (large arrow) drawn in S2 of Fig. 10 (open arrow) is shown in B. Note the circuitous course its axon (small arrows) takes to reach the contralateral ventral funiculus (Fig. 10, open arrow). The scale bar in A can be employed for B.

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14 Fluorescence photomicrographs from a case subjected to injections of Fast Blue (FB) into the anterior lobe of the cerebellum and injections of Rhodamine B Dextran (RBD) into the posterior lobe are shown. High power photomicrographs through CN at the T13 level are shown in A and B. FB labeled neurons are illustrated in A and RBD labeled neurons are shown in B. Double labeled neurons are indicated by arrows. Labeled neurons in the dorsolateral and ventrolateral nuclei of lamina IX and adjacent lamina VII at L2 are documented in C and D. FB labeled neurons are shown in C and RBD labeled neurons are shown in D. Double labeled cells are indicated by arrows. The scale bar in A can be used for B and that in C can be employed for D. ......................................................... 65

15 A photograph of a PD5 opossum is shown in A and the spinal cord and brain of a pup subjected to a lesion of the thoracic cord at the same age and sacrifice immediately after are shown in B. The arrow in B indicates the lesion. A darkfield photomicrograph of a section rostral to the lesion is provided in C and a section through the lesion site is illustrated in D. The ependymal zone (EZ), the intermediate zone (IZ) and the marginal zone (MZ) are indicated. The scale bar in C can be used for D. ................................................................. 90
Three dimensional reconstruction of Fluoro-Gold spread (Inj) within the cerebellum of the case illustrated in Fig. 17. A. Dorsal view. B. Lateral view. The inferior colliculus (IC), and the primary fissure (arrows) are indicated for reference.

The spinal cord and brain of an animal subjected to hemisection of the thoracic cord on the right side on PD5 and bilateral injections of Fluoro-Gold into the anterior lobe of the cerebellum (double arrows) at maturity are shown in A. The middle arrow (C) points to the level of the lesion (les) and the lines rostral and caudal to it indicate the levels of the sections shown in B and D. In the section rostral to the lesion (B), cut at T4, the open arrows indicate labeled neurons in CN and the solid arrows point to labeled axons in the DSCT. The asterisk in this section, and in the sections shown in C and D, indicate the score used to identify the side opposite the lesion. A section through the lesion (T8) is shown in C. The arrow on the right (lesioned) side indicates labeled axons in the dorsolateral funiculus in what appears to be reconstructed spinal cord, whereas that on the left indicates labeled axons in the same tract on the unlesioned side. A section of spinal cord caudal to the lesion (T13) is shown in D. The open arrows indicate labeled neurons in CN. The scale bar in B can be used for C and D.

Fluorescence photomicrographs of labeled neurons in CN caudal to the lesion (T13) are provided in A and B. Those labeled contralateral to the lesion are shown in A, whereas those labeled on the ipsilateral side are illustrated in B. The dorsal funiculus (DF) is indicated. Fluorescence photomicrographs of dorsal spinocerebellar axons in a section through the lesion site (T8) are shown in C and D. Those illustrated in C were located contralateral to the lesion, whereas those in D were on the ipsilateral side. The scale bar in A can be used for B and that in C can be employed for D.

The mean numbers of labeled neurons in CN on the experimental (Exp.) and Control sides (± standard error of the mean) at T4, rostral to the lesion, and at T13, caudal to it, are provided in A and B. Histograms of the size (µm²) distributions of labeled neurons in CN rostral (T4) and caudal (T13) to the lesion are shown in C and D.
Counts and sizes were taken from one out of every sixth section and only those labeled neurons exhibiting a visible nucleus were measured.

20 The gross brain and spinal cord of an animal subjected to hemisection of the T9 level of the spinal cord on PD13 and bilateral injections of Fast-Blue into the cerebellum (double arrows) 99 days later are shown in A. The middle arrow points to the level of the lesion (les) shown in C and the lines rostral and caudal to it indicate the levels shown in B and D. In the section rostral to the lesion (B), the open arrows indicate labeled neurons in CN and the solid arrows indicate the location of the DSCT. The asterisk indicates the score used to identify the side contralateral to the lesion. A darkfield photomicrograph of a section through the lesion is shown in C and the arrow indicates the ventral median fissure. The ventral (VF) and lateral funiculi (LF) are also indicated and the asterisk marks the side opposite the lesion. A section caudal to the lesion (T13) is shown in D. The open arrows in D point to CN and the asterisk indicates the score marking the side opposite the lesion. The scale bar in B can be used for C and D.

21 A portion of the spinal cord containing the lesion site (arrow) in an opossum subjected to a lesion of the thoracic cord (T9) on PD26 and bilateral injections of Fast-Blue into the cerebellum 89 days later is shown in A. A myelin stained section slightly rostral to the full extent of the lesion is shown in B. The asterisk indicates the side opposite the lesion which is similarly indicated in C and D. Myelin stained sections through the full extent of the lesion and slightly caudal to it are provided in C and D. The ventral median fissure is indicated by the arrows in B and D and the substantia gelatinosa (SG) is labeled in B and D. The scale bar in B can be used for C and D.

22 A photomicrograph of a Nissl stained section of spinal cord (T13) from an animal subjected to lesioning at T8 on PD13 and injections of Fast Blue into the anterior cerebellum 95 days later is shown in A. Labeled neurons were not found caudal and ipsilateral to the lesion prior to removing the coverslip and staining, although they were found on the contralateral (control) side. After staining, however,
small cells could be found within the area judged to be CN on the side of the lesion (open arrows). CN is outlined on the side opposite the lesion. The photomicrograph in B shows another section from the same case which contains a few relatively large neurons in CN caudal and ipsilateral to the lesion (arrows). As in A, CN is outlined on the contralateral side. The dorsal funiculus (DF) and central canal (CC) are indicated. The scale bar in A can be used for B.

23 A photograph of a portion of spinal cord including the lesion site (arrow) from an animal subjected to mid-thoracic hemisection on postnatal (PD) 5 and bilateral injections of Fluoro-Gold into the cerebellum 6 months later is shown in A. A section of thoracic cord (T4) rostral to the lesion from the same case is illustrated in B. Note that labeling of Clarke’s nucleus (CN) (open arrows) was bilaterally symmetrical suggesting that the injections were comparable on the two sides. Labeled axons were found within the lateral and ventral funiculi bilaterally, although they appeared to be fewest in number on the side of the lesion (closed arrows). The asterisk delineates the side opposite the lesion in this section and in those which follow. A section through the lesion site is shown in C and labeled axons were seen on the side of the lesion (arrows, right side) in what appeared to be reconstructed spinal cord. A photomicrograph from the first lumbar section of the cord is shown in D. Notice that spinal border cells (SBC’s) were labeled bilaterally (closed arrows). Labeled neurons were also present within CN on both sides (open arrows). A section from the second sacral segment of the cord is seen in E and it can be seen that labeled cells were present bilaterally in the sacral/coccygeal ventrolateral nucleus (VLN) (arrows). A section of coccygeal cord is seen in F and labeled cells were present bilaterally in Stilling’s nucleus (SN) (arrows). The scale bar in B applies to C-F.

24 A portion of the spinal cord including the lesion site (arrow) from an animal subjected to hemisection of the mid-thoracic cord on PD12 and bilateral injections of Fast Blue into the cerebellum 4 months later is shown in A. A photomicrograph of a section of thoracic cord rostral to the lesion (T4) from the same case is shown in B. Note that labeled cells were present in CN in relatively equal numbers bilaterally (open
arrows) suggesting that our injections were symmetrical. The asterisk delineates the side opposite the lesion in this section and in those which follow. A section through the lesion is shown in C where it can be seen that over half of the cord was involved. The spared lateral funiculus (LF) and ventral funiculus (VF) are indicated. A section through the first segment of the lumbar cord is shown in D. Labeled SBC’s (closed arrows) were present only ipsilateral to the lesion. In addition, labeling in CN (open arrow) was only present contralateral to the lesion. A section from the second segment of the sacral cord is shown in E. Labeled cells were only seen in the VLN (arrows) ipsilateral to the lesion. A section of coccygeal cord is shown in F. Labeled neurons in SN (arrow) were present only on the side of the lesion. The scale bar in Fig. B applies to Fig. C-F.

Fluorescence photomicrographs of labeled axons in a section through the lesion site from the case illustrated in Fig. 23. Labeled axons located contralateral to the lesion are shown in A, while those on the ipsilateral side are shown in B. Notice that labeled axons occupy the lateral and ventral funiculi on both sides, but that they appear less numerous on the lesioned side (B). The scale bar in A can be used for B.

The lesion site in a PD5 pup subjected to transection of the mid-thoracic cord and immediate sacrifice is shown grossly in A (arrow) and in a Nissl-stained, sagittally cut section in B (arrow). The spinal cord (SC), dorsal (D), caudal (C), and the vertebral body (V) are indicated. The animal was sacrificed shortly after lesioning so that the completeness of the lesion could be documented. The lesion site (arrow) from an animal sacrificed six months after a transection of the thoracic cord at PD5 is shown grossly in C and photomicroscopically in a transversely cut, Nissl-stained section in D. Although the site of the lesion was not identifiable grossly, its abnormalities were evident histologically. Observed abnormalities included the paucity of cells in the grey matter and the dorsal positioning of the central canal (arrow). The lateral (LF) and ventral (VF) funiculi are indicated on one side.
Fluorescence photomicrographs from an animal subjected to a transection of the mid-thoracic cord on PD5 and injections of FB into the T13 level of the spinal cord 6 months later. A section through the injection site is documented photomicrographically in A. The injections filled the spinal cord at this level and spread to adjacent segments but it did not reach the lesion site. A photomicrograph documenting labeled spinocerebellar terminals (mosdy fiber rosettes; e.g., open arrows) within the anterior lobe of the cerebellum is provided in B. Note that labeled terminals are restricted to a limited zone of the granule cell (Gc) layer which is demarcated by the closed arrows. The molecular (Mk) and Purkinje cell (Pk) layers are indicated for reference. A high power photomicrograph from an adjacent section illustrating a labeled spinocerebellar axon (small arrows) and its terminal ending (large arrow) is shown in C.

A plot documenting the locations of labeled spinocerebellar terminals within the anterior lobe of the cerebellum from the case described in Fig. 27. Each labeled terminal (mosdy fiber rosette) is indicated with a dot. Labeling was present within 5 zones (arrows) which appeared to be similar in location to those seen in unlesioned controls. Lobules I and VI are indicated.

Photomicrographs of selected sections from a case subjected to a transection of the mid-thoracic cord at PD5 and injections of FB into the lumbar cord 30 days later are shown in B-F. The brain and spinal cord from the same case are shown grossly in A. The site of the injection (INJ) and lesion (LES) are indicated. A fluorescence photomicrograph through the injection site is shown in B. A photomicrograph of a Nissl-stained section through the site of transection is shown in C and it can be compared with a section of normal cord from an adjacent segment in D. Note the poor differentiation of the grey matter and the abnormally positioned central canal in C (open arrow). The ventral median fissure is indicated with a closed arrow in both figures. Labeled axons terminating within the granule cell (Gc) layer of the anterior lobe of the cerebellum are illustrated in fluorescence photomicrographs in E and F. Note that they are present within distinct zones.
(arrows). The scale bar in C can be used for D and that in E can be employed for F.

30 Plots of labeled axons in the cerebellum from the case illustrated in Fig. 29 are shown in A (the anterior lobe) and B (the posterior lobe). The dots indicate labeled spinocerebellar axons in the granule cell layer, not mossy fiber rosettes, because the latter have not formed yet. Labeled terminals were located in 5 zones (arrows; A) within the anterior lobe and three zones (arrows; B) within lobule VIII of the posterior lobe. The zones of labeling were similar in location to those seen in the age-matched controls (Fig. 31), although they were narrower in width and less densely packed with labeled axons. The cerebellum (Cb) and inferior colliculus (IC) are indicated. The scale bar in Fig. 30 can be used for Fig. 31.

31 Plots of labeled terminals in the cerebellum of an unlesioned control subjected to injections of FB into the lumbar spinal cord at an age comparable to that of the case shown in Figs. 29 and 30. Orthogradely labeled axons in the granule cell layer are indicated as dots in the anterior (A) and posterior (B) lobes. As in the experimental cases, labeled axons occupied 5 zones (arrows; A) within the anterior cerebellum and three zones (arrows; B) in the posterior lobe. Note, however, that the labeled zones were wider and more densely packed with labeled axons than in the experimental case (compare with Fig. 30). The cerebellum (Cb) and inferior colliculus (IC) are indicated. The scale bar in Fig. 30 can be employed for Fig. 31.
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1. Summary of animals used in Chapter 2

2. The number (taken from every 6th section) and size (μm²; taken from every 6th section) of labeled neurons in Clarke's nucleus, rostral (T4) and caudal (T13) to the lesion.
CHAPTER 1

THE ORIGIN, COURSE, AND LATERALITY OF SPINOCEREBELLAR AXONS IN THE NORTH AMERICAN OPOSSUM, *DIDELPHIS VIRGINIANA*

Abstract

Spinocerebellar axons have been studied extensively in placental mammals, but there have been no full reports on their origin, laterality, or spinal course in any marsupial. We have employed the North American opossum (*Didelphis virginiana*) to obtain such information and to ask whether spinocerebellar neurons innervate both the anterior and posterior lobes of the cerebellum via axonal collaterals. The results are of comparative interest and they provided information that was needed for correct interpretation of the results obtained from the experiments described in Chapters 2 and 3. Our results indicate that spinocerebellar axons are generally comparable in origin, course, and laterality between the opossum and the placental mammals studied to date and suggest that if differences exist they are relatively minor.

In order to identify spinal neurons that project to the cerebellum, we employed the retrograde transport of Fluoro-Gold (FG) from the anterior lobe, the main target of spinocerebellar axons. In some cases, cerebellar injections of FG were combined with
hemisections at rostral cervical or mid-thoracic levels so that laterality of
spinocerebellar connections could be established. In all cases, neurons were labeled
throughout the length of the spinal cord but they differed in laminar distribution and
laterality of their projections. Neurons which projected contralaterally to the
cerebellum were present within the central cervical nucleus (CCN) from cervical (C)1-
C4, lamina VIII of Rexed (and adjacent parts of laminae VII and IX) from C1-C8 and
lumbar (L)1-L3, the dorsomedial and ventral parts of lamina VII from thoracic (T)2-
T8, the spinal border cell (SBC) region from T10-L3 (lamina VII as well as the
dorsolateral [DL] and ventrolateral [VL] nuclei of lamina IX), the medial part of
laminae VI-VII from L3-L5, the medial/central part of lamina VII from L4-L6, the
lateral part of lamina IV-VII from T12-coccygeal (Cx)5, as well as within Stilling’s
nucleus (SN) and the ventrolateral nucleus (VLN) from L6 - Cx5. Neurons which
projected ipsilaterally to the cerebellum were present within the nucleus centrobasalis
(NCB) from C1-C8, the dorsal horn dorsal spinocerebellar tract (dhDSCT) area from
C1-L5, the medial part of lamina VII and central/lateral part of lamina VII from C5-
T1, Clarke’s nucleus (CN) from T1-L2 (including neurons in the dorsal funiculus and
neurons which projected into the dorsal columns), lamina VIII (and adjacent areas)
from T6-L2, and the medial part of lamina VII from T12-L3. Neurons scattered within
lamina I and lamina X projected bilaterally to the cerebellum.

Cerebellar injections of FG also retrogradely labeled spinocerebellar axons
allowing us to document their locations in the grey and white matter. Spinocerebellar
axons were present within the periphery of the lateral and ventral funiculi at all levels.
The contribution of axons to the dorsal spinocerebellar tract (DSCT) and ventral spinocerebellar tract (VSCT) was similar to that described in other mammals and a segmentotopic organization appeared to exist for both tracts. Spinocerebellar axons which were not part of the classically defined DSCT and VSCT were also present in the ventral and lateral funiculi. In addition, spinocerebellar axons were documented in the dorsal funiculus and they appeared to originate from neurons in the dorsal part of CN from T9-L1.

Although most spinocerebellar axons innervate the anterior lobe, some project to the posterior lobe. In order to determine whether single neurons project to both the anterior and posterior lobes, injections of Fast Blue into the anterior lobe were combined with injections of DiamidinoYellow or Rhodamine B dextran into the posterior lobe, or vice versa. Double labeled neurons were found within the CCN, the NCB, the central/lateral part of lamina VII from C5-T1, CN, the region containing dhDSCT neurons, the area containing SBC's (neurons of lamina VII in the thoracic and lumbar cord, and the DL and VL nuclei of lamina IX), the medial part of laminae VI-VII from L3-L5, the lateral part of laminae IV-VI at sacral and coccygeal levels, SN, and the VLN.
Introduction

The origin, course, laterality, and distribution of spinocerebellar axons have been studied extensively in placental mammals. Such studies have employed the mouse (e.g., Caddy et al, 1977; Heckroth and Eisenman, 1988), the rat (e.g., Snyder et al, 1978; Matsushita and Hosoya, 1979; Arsenio-Nunes and Sotelo, 1985; Shirao, 1987; Gravel and Hawkes, 1990; Yamada et al, 1991; Berretta et al, 1991; Tolbert et al, 1993), the guinea pig (e.g., Tracey et al, 1988), the rabbit (e.g., Grottel, 1975), the cat (e.g., Snyder et al, 1978; Matsushita et al, 1979; Grant et al, 1982; Xu and Grant, 1988; Yaginuma and Matsushita, 1989; Xu and Grant, 1994), the dog (e.g., Horax, 1915; Petras and Cummings, 1977; Cummings and Petras, 1977), and certain primates (e.g., Yoss, 1952; Yoss, 1953; Voogd, 1969; Petras, 1977; Snyder et al, 1978; Culberson et al, 1985a). In contrast, little is known about these connections in marsupials. The distribution of spinocerebellar axons has been described in the North American opossum, *Didelphis virginiana*, by Hazlett et al, (1971) and in the brush tailed opossum, *Trichosurus vulpecula* by Watson et al, (1976) but except for two abstracts (Culberson et al, 1985ab), there have been no reports on their origin, course, or laterality in any marsupial. Since the distribution of spinocerebellar axons is different in the marsupials and placental mammals studied to date (compare Hazlett et al, 1971 and Watson et al, 1976 with Voogd, 1969), it might be predicted that differences also exist in other aspects of their organization. In the studies reported herein, we used the retrograde transport of Fluoro-Gold (FG) from the cerebellum to
determine the origin of spinocerebellar axons in the North American opossum (Fig. 1) and in some cases cerebellar injections of FG were combined with spinal lesions to establish their laterality. An unexpected advantage of FG was that it retrogradely labeled spinocerebellar axons as well as their neuronal somata so it was also possible to study the course of spinocerebellar axons. In the study of Hazlett et al (1971), it was shown that spinocerebellar axons innervate both the anterior and posterior lobes. In the present study we used Fast Blue (FB) in combination with Diamidino yellow (DY) or Rhodamine B dextran (RBD) to ask whether spinal neurons which project to the anterior lobe also innervate the posterior lobe. To our knowledge, such studies have not been done in any marsupial and those performed in placental mammals gave different results (Xu and Grant, 1988; Berretta et al, 1991). The results of our study are of comparative interest, but they also provided data necessary for interpreting the results of studies designed to determine if spinocerebellar axons grow around or through a lesion of their spinal pathway during development (Chapters 2 and 3: Terman et al, 1994, 1995, 1996abc, 1997abc).
Materials and Methods

I. Studies Designed to Determine the Origin of Spinocerebellar Axons

Five adult opossums were anesthetized with sodium pentobarbital (40 mg/kg) and placed in a stereotaxic frame to immobilize their head. The cerebellum was exposed surgically using sterile techniques and multiple injections of Fluoro-Gold (FG; Fluorochrome, Inc.) were made unilaterally (N=3) or bilaterally (N=2) into the anterior lobe, the primary target of spinocerebellar axons (Hazlett et al. 1971). The injections were made by pressure using a glass micropipette which was attached to a 1μl Hamilton syringe. A microdrive system was employed to position the pipette and the injections were placed in two locations on each side of the midline and at three depths for each position. A total of 6μl of a 5% aqueous solution of FG was injected on each side per animal in an attempt to label as many spinocerebellar neurons as possible. In all cases, care was taken to keep FG from spreading into the subarachnoid space. After the injections were complete, the meninges were replaced and the craniotomy was covered by suturing the temporalis muscle, the overlying fascia, and the skin. Following treatment for post-operative pain with Buprenorphine (0.028 mg/kg), the animals were returned to the vivarium under care of a veterinarian. The animals were allowed to survive 7-12 days before being reanesthetized, sacrificed by an overdose of sodium pentobarbital, and perfused with 4% paraformaldehyde. The spinal cord and brain were then removed, immersed in 30% sucrose for 48 hours at
4°C, sectioned in the transverse plane at 40μm using a freezing microtome, mounted onto gelatin coated slides, and coverslipped using Entellan (Merck). All brains and spinal cords were scored on the left side for orientation after mounting. Sections through the cerebellum were examined to determine the locations and spread of the injections and every section of the spinal cord was examined for labeled neurons.

Selected sections through the brainstem and cerebellum and all segments of the spinal cord were drawn using an image analysis software program (Neurolucida, MicroBrightField, Inc.) connected by a video camera (Optronics) to a Leitz fluorescence photomicroscope (Orthoplan 2) equipped with the A cube (excitation wavelength 340-380 nm) of the Ploem illumination system. The locations of labeled neurons in the spinal sections were plotted using the same system. Labeled neurons were also documented photographically at all spinal levels. Segmentation of the spinal cord was based on Voris (1928).

In some animals, all neurons had a light, yellow fluorescence that was apparently due to the presence of lipopigment granules (Dowson et al, 1982; Xu and Grant, 1988). FG could easily be distinguished from lipopigment granules, however, but to be conservative we have only included neurons in our plot that were heavily and clearly labeled by FG. It is possible, therefore, that some of our plots underestimated the number of neurons labeled by FG. It should be noted, however, that injections of FB, RBD, or DY into the cerebellum (see section IV) produced the same pattern of spinal labeling as FG.
After labeled cells had been plotted from selected sections, the coverslips were removed and the sections stained for Nissl substance so that Rexed's laminae (Rexed, 1952) could be identified using the criteria of Boyles (1979). When it was difficult to ascribe a labeled neuron to a specific lamina, the neuron in question was captured in a video image so that its location could subsequently be studied in the same section after Nissl staining.

II. Studies Designed to Determine the Laterality of Spinocerebellar Axons in the Spinal Cord.

To determine the laterality of spinocerebellar axons, six adult opossums were subjected to a right-sided hemisection of the spinal cord at upper cervical (C) levels (C2 [N=2]; C4 [N=2]) or mid-thoracic (T) levels (T7 [N=2]) and subsequent injections of FG into the anterior lobe of the cerebellum on the contralateral side. In these experiments, the animals were placed in a stereotaxic frame to immobilize their vertebral column at the appropriate level. The spinal cord was then exposed surgically using sterile techniques and hemisected on the right side using a #11 surgical blade (Paragon). Immediately following closure of the incision by suturing the overlying muscle, fascia, and skin, the animals were placed in a stereotaxic frame to immobilize their head for injections of FG into the anterior lobe of the cerebellum on the left as described in section I. Following closure of the craniotomy and treatment for post-operative pain (see section I), the animals were returned to the vivarium under the care
of a veterinarian. Seven to twelve days later, they were sacrificed and perfused as already described and the spinal cord and brain were removed and placed in 30% sucrose for 48 hours at 4°C. All brains and spinal cords were scored by a shallow cut on the side opposite the lesion (the left side) to determine laterality of the tissue sections after mounting. Sections through the cerebellum and brainstem, the lesion site, and all segments of the spinal cord caudal to the lesion were cut in the transverse plane as described in section I. Sections through the cerebellum were examined for the location and spread of the injections and plotted using the system already described. Sections through the lesion site were studied to verify completeness of the hemisection. Labeled neurons were documented photographically and their positions plotted from every 12th section in each segment caudal to the lesion as described in section I.

III. The Locations of Spinocerebellar Axons in the Spinal Cord

Since injections of FG into the cerebellum labeled spinocerebellar axons as well as the neuronal somata that supported them, sections of spinal cord were examined for labeled axons in all of the cases referred to above. Labeled axons in the grey matter could often be followed from their cells of origin for long distances within a single section and sometimes through several adjacent sections. The results were
documented photographically and plotted with the image analysis system described in section I. This image analysis package allowed us to follow axons through multiple sections and reconstruct their trajectory.

The locations of spinocerebellar axons in the white matter were also studied. In those cases in which a hemisection was made, only axons on the unlesioned side of the cord were plotted. Our plots document the positions of axons which project to the anterior lobe, but it is possible that axons which innervate the posterior lobe are also included. The positions and densities of spinocerebellar axons were plotted using a technique developed with the image analysis system described above. FG labeled axons, in contrast to labeled soma, quickly lose their fluorescence so it is difficult to plot their positions and density. To help minimize fading while documenting their position, high magnification video images were made of the labeled axons in one plane of section. Labeled axons could then be plotted from the video images and simultaneously checked for accuracy with the microscope. In addition, selected segments of the spinal cord were cut at 40μm in the horizontal or sagittal plane. The positions of labeled axons in these sections were examined and documented photographically.
IV. Studies Designed to Determine the Origin of Spinal Axons Which Innervate Both the Anterior and Posterior Lobes of the Cerebellum

In the opossum (Hazlett et al. 1971), as in other species (see review, Bloedel and Courville, 1981; for additional refs., see Tolbert et al, 1993), spinocerebellar axons terminate in the posterior lobe of the cerebellum as well as the anterior lobe. To investigate whether individual spinal neurons innervate both lobes, three adult opossums were anesthetized as described above, placed in a stereotaxic head frame, and subjected to unilateral injections of 3μl of 2% Fast Blue (FB; Sigma) into the anterior lobe and 3μl of either 3% Diamidino Yellow (DY; Sigma) or 10% Rhodamine B dextran (RBD; Molecular Probes [D-1841]) into the pyramis and paramedian lobules, the primary targets of spinocerebellar axons in the posterior lobe (Hazlett et al, 1971). In two additional adults, DY or RBD was injected into the anterior lobe and FB was injected into the posterior lobe. The injected markers could be distinguished from one another with relative ease. FB is blue in color and fills the cytoplasm, whereas DY, which is viewed at the same wavelength, is yellowish - green in color and found within the nucleus. RBD is red and visualized using a different wavelength. FB was employed rather than FG, since it spreads less and appears to have the same sensitivity (unpublished observations). In addition, FB is less toxic to the animal than FG. RBD and DY were chosen since they are easily distinguished from FB, they are sensitive markers, and they spread very little following injection. Experiments were also carried out using Rhodamine isothiocyanate (RITC; Sigma) and Rhodamine latex.
beads (Rbeads), but RITC spread too much and Rbeads spread too little. In addition, Rbeads did not give the quality of spinal labeling produced by RBD or DY. All injections were made with the aid of a Hamilton syringe as described above. Following closure of the craniotomy by suturing the temporalis muscle, fascia, and skin, the animals were treated for post-operative pain as described in section I and returned to the vivarium under the care of a veterinarian.

Ten to fourteen days after surgery, the animals were sacrificed and perfused with 4% paraformaldehyde and their brain and spinal cord were removed and immersed in 30% sucrose for 48 hours at 4°C. The cerebellum and underlying brainstem were cut in the sagittal plane at 40μm so that separation of the injections could be documented. Sections through all levels of the spinal cord were cut transversely at 40μm and examined for neurons labeled by one or both tracers. A Leitz fluorescence photomicroscope (Orthoplan 2) equipped with the A cube (excitation wavelength 340-380 nm) for visualizing FB and DY and the N2.1 cube (excitation wavelength 515-560) for visualizing RBD was used to examine all sections. Labeled neurons were documented photographically and their positions plotted as described above. 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

I and II. The Origin and Laterality of Spinocerebellar Axons.

Labeled neurons were located in the same areas of the spinal cord in all cases subjected to injections of FG into the anterior lobe of the cerebellum. Therefore, we have limited our description to one of the cases with a C2 hemisection because it supplied definitive information regarding laterality of spinocerebellar projections as well as the location of spinocerebellar neurons. In the case chosen for presentation, the hemisection interrupted all axons on one side of the cord (Fig. 2A). Involvement of the dorsal funiculus is not obvious in Fig. 2A, but it was clear in adjacent sections. The injections filled most of the anterior lobe on the side contralateral to the lesion and they spread across the midline and into the posterior lobe (Fig. 2B; Fig. 3). Care was taken to minimize leakage into the subarachnoid space, but there was some spread into the posterior aspect of the inferior colliculus due to its proximity to the anterior lobe. There was no spread into the pons or medulla. FG filled the cytoplasm of labeled neurons and extended into their processes making them easy to distinguish from unlabeled cells.

The plots in Figs. 4-7 illustrate the locations of labeled neurons in the C2 hemisection case referred to above. Such neurons were plotted from every 12th section in each of the segments indicated and all of the plotted neurons were included on a
drawing of a single section. Figs. 8 and 9 provide photographic documentation of selected labeling in the same case. In all figures the side of the lesion is on the reader’s right.

Dorsal root ganglia at several levels were examined for labeled neurons but none were seen. Dorsal root ganglion neurons have been reported to project to the cerebellum in amphibians (Joseph and Whitlock, 1968; Székely et al. 1980) and reptiles (Corvaja et al., 1979; Künzle, 1982), but their presence has not been documented in mammals (see however, Holbrook and Wilcox, 1964) or birds (Necker, 1992).

The distribution and laterality of labeled neurons at cervical levels (Fig. 4).

Labeled neurons were found within the medial part of lamina VII from C1-T1. Those in C1-C4 were particularly abundant, and below the lesion (at C3 and C4) they were found only ipsilateral to it in what appeared to be the central cervical nucleus (CCN) (Fig. 4; Fig. 8A, closed arrow) described for placental mammals (Wiksten, 1975; Matsushita and Ikeda, 1975; Cummings and Petras, 1977; Snyder, 1978). From C5-T1, however, labeled neurons in the medial part of lamina VII were found contralateral to the lesion (Fig. 4, closed arrows; Fig. 8B, closed arrow).

From C1-T1, labeled neurons were also numerous in the dorsal part of lamina VII and within adjacent parts of laminae V and VI, contralateral to the lesion. Based on position they appeared to comprise Stilling’s cervical nucleus or the nucleus
centrobasalis (NCB) (Fig. 4; Fig. 8A. open arrow) described in other species (Stilling, 1859; Petras and Cummings, 1977; Matsushita et al, 1979). At lower cervical levels this nucleus and the labeled neurons within it were positioned more laterally than at rostral cervical levels (Fig. 4).

Another prominent group of labeled neurons was found in the central and lateral part of lamina VII on the side opposite the lesion from C5 through T1 (Fig. 4. open arrows; Fig. 8B, open arrows). These cells will be referred to as the central/lateral lamina VII group although some of them appeared to be within lamina IX (e.g., C6, C8 in Fig. 4).

A few scattered neurons were labeled in the ventromedial part of the ventral horn ipsilateral to the lesion from C1-C8. For the most part, these neurons were found within the medial part of lamina VII or within lamina VIII (e.g., C4-C6 in Fig. 4), but some of them appeared to be located within the ventromedial part of lamina IX (e.g., C7 and C8 in Fig. 4).

At all cervical levels, labeled neurons were scattered in the lateral part of laminae IV-VI and within the adjacent white matter, contralateral to the lesion (Fig. 4). A few labeled neurons were also found within lamina I contralateral to the lesion at all cervical levels (Fig. 4), and within lamina X mainly contralateral to the lesion from C3-C5 (Fig. 4).
The distribution and laterality of labeled neurons at thoracic (Fig. 5) and lumbar 1-5 levels (Fig. 6).

At all thoracic levels (and the caudal portion of C8) and extending into L2, a column of labeled cells was found in lamina VII, contralateral to the lesion, which extended into lamina V at most levels and, when present, lamina VI (Figs. 5 and 6; Figs. 8C and D, closed arrows). Based on position and laterality we assume that the neurons in question belong to the thoracic nucleus (Clarke's nucleus [CN]) described in other mammals (see review by Mann, 1973). CN was largest in diameter and number of cells at T12 and T13 (Figs. 5 and 6; see also Boyles, 1979; Terman et al. 1996a). From T9-L1, a group of labeled neurons was aggregated in the dorsal part of CN (Fig. 8D, closed arrowhead). These cells could be distinguished from those within CN proper by their large size and the fact that they appeared to project into the dorsal columns (Fig. 8D, small arrows; also see the section on course of spinocerebellar axons in the cord and Figs. 13B, C, and D) rather than the lateral funiculus, the normal location of axons from CN. Occasionally, labeled neurons also were present within the dorsal funiculus, contralateral to the lesion (e.g., Fig. 5, T13) from T9-L1. These cells appeared to correspond to the dorsal funiculus (DF) neurons described in other species (see Sherrington, 1893; Beal et al, 1990). In the rat, such neurons are derived from the same precursor pool as CN neurons and they project into the lateral funiculus (Beal et al, 1990).
At thoracic and lumbar levels, labeled neurons were also numerous contralateral to the lesion in the lateral part of laminae IV-VII and in the adjacent white matter (Figs. 5, 6 and 8D, open arrowhead). This cell group appeared to be a continuation of the one present in the same area at cervical levels. Neurons within this area have been referred to as dorsal horn dorsal spinocerebellar tract (dhDSCT) neurons in the cat (Tapper et al. 1975; Randic et al. 1976; Edgley and Gallimore, 1988). In the opossum, they decreased in number by L5.

Labeled neurons were found throughout lamina VII from T1 - L6, ipsilateral to the lesion. From T1-L3, they were present within the dorsal part of lamina VII (e.g. Fig. 5, T2-T8, closed arrowheads; Fig. 9A, closed arrowhead) and from T1-L6, within the ventral/central part of the same lamina (Fig. 5, e.g., T2-T8, L4-L6, open arrowheads; Fig. 9A, open arrowhead). From T10 - L3, cells were also labeled along the lateral parts of lamina VII, along the border between the grey and white matter (Figs. 5, 6, open arrows; Fig. 8D, open arrow), within the white matter (Fig. 5: T12, T13; Fig. 6: L1, L2), and, at L2 and L3, within the ventrolateral (VL) and dorsolateral (DL) cell groups of lamina IX (Fig. 6: Fig. 9A, closed arrows). The labeling in these areas was most extensive at L2. The labeled cells within laminae VII and IX from T10 - L3 appeared comparable to the spinal border cells (SBC's) described in other species. SBC's were first described by Cooper and Sherrington (1940) as large cells in the ventrolateral grey matter which project to the cerebellum through the ventral spinocerebellar tract (VSCT). Ha and Liu (1968) found that cells which give rise to the VSCT (the "border cells" of Cooper and Sherrington) were widely distributed in
the ventral grey (medially and laterally) and suggested that the term “border cells” was misleading. Nonetheless, SBC’s has remained the term to describe neurons in lamina VII and IX (DL, VL) in the lower thoracic and lumbar cord which project contralaterally to the cerebellum via the VSCT. Labeled neurons were also found within lamina VIII (and adjacent areas of laminae VII and IX) from L1-L3, ipsilateral to the lesion. It is likely that such neurons were a continuation of neurons labeled in comparable areas from C1-C8 (see Matsushita et al., 1979).

Labeled neurons also were found in lamina VII contralateral to the lesion, although they were limited in distribution. Most of them were clustered dorsomedially within lamina VII from T12 through L3 (Figs. 5, 6; Fig. 9B, open arrow), but labeled neurons also were present in the ventromedial part of the same lamina from T6 through L2 (Figs. 5, 6; Fig. 8C, open arrow). Most of the neurons labeled in the ventromedial part of the ventral horn were actually within lamina VIII (Fig. 8C, open arrow), but some appeared to be located within laminae VII and IX.

From L3-L5, labeled neurons were present ipsilateral to the lesion in the medial part of laminae VI and VII (Fig. 6, closed arrow; Fig. 9B, closed arrow) in a position similar to that occupied by CN more rostrally. They could be distinguished from neurons within CN, however, based on size and laterality of labeling.

Labeled neurons were found from T12 to the caudal end of the cord in an area similar to that occupied by dhDSCT neurons (lateral part of lamina IV-VII), except they were located ipsilateral to the lesion (Figs. 5, 6, and 7). Labeled neurons in this area were most numerous at the caudal levels (see Fig. 7).
A few cells were labeled in the dorsal/central part of lamina VII at L5 contralateral to the lesion (Fig. 6) and a few labeled neurons were also found within laminae I and X at all levels. Labeled neurons within lamina I were usually found contralateral to the lesion, while those in lamina X were present primarily ipsilateral to it (Figs. 5 and 6).

The distribution and laterality of labeled neurons from lumbar 6 to coccygeal levels (Fig. 7).

Beginning in the lower portions of L5 and continuing through the most caudal segments, labeled neurons were grouped in three main areas ipsilateral to the lesion. One group, located in the lateral part of laminae IV-VII (Fig. 7), appeared to be a continuation of that present in the same location at lower thoracic and lumbar levels (Figs. 5 and 6). A second group was present in the medial part of lamina VII beginning at L6. These neurons appeared to belong to Stilling's sacral nucleus (SN) (Fig. 7; Fig. 9C, arrow) as described in placental mammals (e.g. Stilling, 1859; Chang, 1951; Petras, 1977; Matsushita et al, 1979).

Labeled neurons were also found in the ventrolateral part of lamina IX and adjacent parts of lamina VII ipsilateral to the lesion at sacral and coccygeal levels (Fig. 7; Fig. 9D, arrows). We will refer to these neurons as within the ventrolateral nucleus of the sacral and coccygeal cord (VLN). Similar neurons have been identified in placental mammals (Snyder et al, 1978; Matsushita and Hosoya, 1979; Matsushita et
al., 1979; Grant et al., 1982). A few cells also were labeled in what appeared to be the ventromedial nucleus of lamina IX (e.g., Fig. 7, closed arrow in L6 and SI). In addition, neurons were labeled within laminae I and X (Fig. 7). Except for a few cells in laminae I and V, labeled neurons at these levels were located ipsilateral to the lesion.

III. The Course of Spinocerebellar Axons in the Grey and White Matter of the Spinal Cord

Spinocerebellar axons were labeled in our preparations, making it possible to follow individual axons from their origin in the grey matter toward their initial position in the white matter. The case used to document the position and laterality of spinocerebellar neurons (see above, Figs. 2-9) was also employed to study labeled axons in the grey and white matter.

The location of selected spinocerebellar axons in the grey matter.

The axons illustrated in Fig. 10 were representative of all others observed to originate in the same areas. Axons of labeled neurons in the NCB (Fig. 10, C2, closed arrow) were followed directly into the ipsilateral dorsolateral funiculus, whereas those from labeled neurons in the CCN (Fig. 10, C2, open arrow) extended across the midline and into the contralateral ventral funiculus. Labeled axons originating in the
central/lateral part of lamina VII of the cervical enlargement could be followed into the ventrolateral funiculus on the same side (Fig. 10, C6, closed arrow), whereas those associated with labeled neurons in the ventromedial part of lamina VII or lamina VIII (Fig. 10, C6, open arrow) projected dorsally across the midline and into the contralateral ventral funiculus. Labeled neurons within CN projected into the dorsal part of the ipsilateral lateral funiculus (Fig. 10, T13, closed arrow), and those within lamina VIII (Fig. 10, T13, open arrow) projected into the ventral funiculus on the same side. Labeled axons which originated in the dorsomedial part of lamina VII could be traced ventrally across the midline and into the contralateral ventral funiculus (Fig. 10, L3, closed arrow) and, at the same level, labeled axons originating in the lateral part of lamina VII (from a SBC) could be followed medially across the midline and into the contralateral ventral funiculus (Fig. 10, L3, open arrow). The latter mentioned axon and the neuron giving rise to it are illustrated photomicrographically in Fig. 11A.

Labeled axons originating in SN (Fig. 10, S2, closed arrow) and the VLN (Fig. 10, S2, open arrow) extended through the ventral commissure and into the contralateral ventral funiculus. A portion of the axon originating in the VLN in Fig. 10 (S2; open arrow) is documented photographically in Fig. 11B. It should be noted that the axon illustrated for the VLN cell has a particularly circuitous course; others projected more directly across the midline.
The location of labeled axons in the white matter.

Labeled spinocerebellar axons were found in the same areas of the white matter in all cases. Those on the unlesioned side in the hemisection case used for the preceding descriptions are shown in Fig. 12 where it can be seen that they were present in different positions and densities at all levels. At cervical and thoracic levels, they were densest in the dorsal part of the lateral funiculus, but at all levels they were found within the periphery of the lateral and ventral funiculi.

At sacral levels (Fig. 12, S2), labeled axons were relatively sparse but they were present throughout the periphery of the lateral and ventral funiculi. It is likely that most of the axons within the ventral funiculus originated from neurons in SN and the VLN (e.g., S2 in Fig. 10) because they could be followed through the ventral grey/white commissure and into that region from the contralateral side. Labeled axons in the lateral funiculus most likely originated from more caudal levels since none were observed to enter it at this level. Labeled axons were more numerous at L1 than at S2. At L1, they were relatively abundant in the ventral funiculus and could be followed into that area from neurons within lamina VII and IX (SBC's) (open arrow, Fig. 10, L3) and other areas on the contralateral side (e.g., closed arrow, Fig. 10, L3). Some of the labeled axons in the ventral funiculus originated from the same side of the cord, however (open arrow, Fig. 10, T13). The labeled axons in the dorsal part of the lateral funiculus most likely originated from neurons at more caudal levels and from the caudal part of CN. Labeled axons from caudal levels apparently move dorsally as they
ascend (see below). By T12 (Fig. 12, T12), labeled axons were abundant in the dorsal part of the lateral funiculus and most of them likely originated from CN at T12 and T13 (closed arrow, Fig. 10, T13). Labeled axons were still found crossing in the ventral commissure and those in the ventral funiculus were relatively abundant. By T6 (Fig. 12, T6), relatively few axons were labeled in the ventral funiculus. They were readily apparent in the ventrolateral funiculus, however, and some of them appeared to have reached that position from the ventral funiculus more caudally. Labeled axons in the dorsolateral funiculus were particularly numerous and they extended dorsally to a position immediately adjacent to the dorsal horn. It should be noted that the axons labeled in the dorsolateral funiculus were situated along the edge of the spinal cord at this level and throughout the cervical cord. This was not the case at lumbosacral levels. By C6 (Fig. 12, C6), labeled axons in the dorsolateral funiculus were more tightly packed than at caudal levels, but they were no longer directly adjacent to the dorsal horn. Labeled axons were still relatively numerous in part of the ventrolateral funiculus and some of them could be seen to originate from the central/lateral part of lamina VII on the ipsilateral side (closed arrow, Fig. 10, C6). A few axons were labeled in the ventral funiculus and some of them could be followed into that area from neurons in lamina VIII on the contralateral side (open arrow, Fig. 10, C6). By C2 (Fig. 12, C2; Fig. 13A), most labeled axons were densely packed at the periphery of the lateral funiculus. Those axons that were not tightly packed likely originated from nuclei at those levels (e.g., NCB, closed arrow, Fig. 10, C2). As was the case at C6, labeled axons in the dorsolateral funiculus were not as close to the dorsal horn as they
were at thoracic levels. Labeled axons were more numerous in the ventral funiculus than at lower thoracic levels and it appeared that they originated primarily from neurons in the CCN (open arrow, Fig. 10, C2).

A few labeled axons were found in the dorsal funiculus and most of them appeared to originate from neurons in the dorsal portion of CN between T9 and L1 (Fig. 8D, closed arrowhead; Fig. 13B, closed arrow and Fig. 13C, small arrows). When sections were cut in the horizontal plane, the presence of labeled axons in the dorsal funiculus could be verified (Fig 13D) and some of them could be traced to labeled neurons in the vicinity of CN. It is also possible that some of the labeled axons in the dorsal columns originated from the NCB since a few labeled axons could be followed from that region into the dorsal funiculus in horizontally cut sections (data not shown).

IV. The Distribution of Spinal Neurons Which Project to Both the Anterior and Posterior Lobes of the Cerebellum

Comparable results were obtained in all of the double labeling studies so the results will only be described and documented for one case. In this case, FB was injected in the anterior lobe and RBD was injected into those areas of the posterior lobe which are innervated by spinal axons (Hazlett et al, 1971). Although the injections were intentionally large, they did not overlap. By using two filter sets, FB and RBD labeled neurons could be distinguished from one another. FB labeled
neurons displayed a blue fluorescence in their cytoplasm and proximal dendrites using the A filter set and RBD labeled neurons showed a bright red fluorescence in the same areas when the N2.1 filter set was employed. Neurons labeled by FB were found in all areas of the spinal cord labeled by comparable injections of FG (see Figs. 4-9). Neurons labeled with RBD were found in the same areas, but they were fewer in number. Only major spinocerebellar cell groups were examined for double labeling because some groups were only sparsely labeled by RBD and it was difficult to identify all of them with certainty because lesions were not made to determine laterality. Double labeled neurons were found in each area examined, however, including the CCN, the NCB, the central/lateral part of lamina VII from C5-T1, CN, the lateral part of laminae IV-VII from C1-L5 (dhDSCT neurons), lamina VII including the DL and VL nuclei of lamina IX from T10-L3 (SBC's), the medial part of laminae VI-VII from L3-L5, the lateral part of laminae IV-VII from T12-Cx5. SN, and the VLN. Double labeled neurons are illustrated in CN in Figs. 14A and B and within the VL nucleus in Figs. 14C and D. It was noted that double labeled neurons were less numerous than single labeled neurons.
Discussion

To the best of our knowledge this is the first full report on the origin, course, and laterality of spinocerebellar axons in any marsupial. In addition, we have documented the presence of spinal neurons which project to both the anterior and posterior lobes of the cerebellum. Some aspects of spinocerebellar organization have been studied in the North American opossum (Voris and Hoerr, 1932; Larsell, 1936; Hazlett et al, 1971), the Australian brush-tailed possum (Clezy et al., 1961; Magni and Oscarrsson, 1962ab; Watson et al., 1976), and the Quokka (Blumer, 1963), but information on their origin, course, and laterality is limited. Voris (1929) described the spinal cord of the North American opossum but found no evidence for CN. CN was identified in the opossum (Hazlett et al., 1971), however, and it was subsequently shown to innervate the cerebellum by Martin et al. (1983). Culberson and colleagues (1985ab) have published the most complete description of spinocerebellar neurons in the North American opossum in two abstracts. After large injections of wheat germ agglutinin - horseradish peroxidase (WGA-HRP) into the anterior lobe of the cerebellum, labeled neurons were present at cervical levels in the CCN, in lamina VI-VII, at the lateral edge of lamina IV and V, and scattered throughout the ventral horn. At thoracic and upper lumbar levels they were found within CN, in the lateral portion of lamina V and VII (which they called thoracic border cells), and in the ventral horn. In the lumbar enlargement they labeled spinal border cells, neurons in the ventrolateral funiculus, and neurons in the intermediate grey of the lumbar enlargement. We have
confirmed their findings and added to them by documenting the existence of additional spinocerebellar neurons and by establishing the laterality of spinocerebellar projections. We have assumed that neurons labeled ipsilateral to the hemisection in our experiments projected contralaterally, whereas those labeled on the contralateral side projected ipsilaterally. Based on that assumption, we conclude that neurons which project contralaterally to the cerebellum are present within the CCN from C1-C4, lamina VIII (and adjacent laminae) from C1-C8 and L1-L3, the dorsomedial and ventral portion of lamina VII from T2-T8, the SBC region from T10-L3 (including lamina VII and the DL and VL nuclei of lamina IX), the medial part of laminae VI-VII from L3-L5, the medial/central part of lamina VII from L4-L6, the lateral part of lamina IV-VII from T12-Cx5, and within SN and the VLN from L6 - Cx5. Neurons which projected ipsilaterally were documented within the NCB from C1-C8, the dhDSCT area from C1-L5, the medial part of lamina VII and the lateral part of lamina VII from C5-T1, CN from T1-L2 (including neurons in the DF and neurons which projected into the DF), lamina VIII (and adjacent areas) from T6-L2, and the medial part of lamina VII from T12-L3. Neurons within laminae I and X were labeled bilaterally in our experiments, but spread to the inferior colliculus may have accounted for the lamina I labeling (see Grant et al., 1982). It is unclear why Culberson and colleagues did not describe some of the cell groups labeled in our study, but it is possible that the greater sensitivity of FG, the marker used in our experiments, over WGA-HRP, the marker used by Culberson et al., may have been a factor. In addition,
laterality studies were not performed by Culberson and colleagues, so it is likely that labeled cells in some areas were grouped together whereas they were separated in our study based on the laterality of their projections.

From a comparative point of view, it is of interest to discuss our results in light of those described for placental mammals. Differences have been reported between eutherian and metatherian mammals in the distribution of spinal axons in the cerebellum (the North American opossum, Hazlett et al., 1971; the brush-tailed possum, Watson et al., 1976; the cat, the ferret, the hedgehog, the tree shrew, the loris, and the macaque monkey, Voogd, 1969) and their electrophysiological properties (the rabbit, the cat, and the brush-tailed possum, Magni and Oscarsson, 1962ab; Mann, 1973), so it is possible that other differences exist. It should be emphasized, however, that differences in techniques and the methods used to document results may account for what appear to be species differences. Variations in the way laminar boundaries were drawn in different studies may also be a factor. In the cat, for example, lamina VIII stretches across the ventral third of the ventral horn at all spinal levels except the cervical and lumbar enlargements where it is restricted to the medial half of the ventral horn (Rexed, 1952). In the opossum, however, lamina VIII is limited to a thin sliver along the medial part of the ventral horn at all levels (Boyles, 1979). It is possible, therefore, that a labeled neuron within medial lamina VII of the opossum would be described as within lamina VIII in the cat. Furthermore, in our study, it was often difficult to determine whether labeled neurons in the medial part of the ventral horn were within lamina VII, lamina VIII, or medial lamina IX even after the sections which
contained them were stained for Nissl substance. Likewise, neurons plotted within the lateral nuclei of lamina IX at cervical levels may actually be within an adjacent lamina. Spinocerebellar neurons have not been previously described within the ventromedial nucleus of lamina IX at cervical, thoracic, or lumbar levels, or within the lateral nuclei of lamina IX at cervical levels (although, see Wiksten et al. 1979).

The origin of spinocerebellar axons has been studied extensively in placental mammals including the mouse (Caddy et al., 1977, Heckroth and Eisenman. 1988), the rat (Snyder et al., 1978; Matsushita and Hosoya, 1979; Kitamura and Yamada, 1989; Skinner et al., 1989; Verburgh et al., 1989; Rivero-Melián and Grant, 1990), the guinea pig (Tracey et al., 1988), the cat (Snyder et al., 1978; Matsushita et al., 1979; Grant et al., 1982; Wiksten, 1985; Grant and Xu, 1988; Xu and Grant, 1988; Verburgh et al., 1989), the dog (Cummings and Petras, 1977; Petras and Cummings, 1977), the bushbaby (Culberson et al., 1985a), the squirrel monkey (Snyder et al., 1978), and the rhesus monkey (Petras, 1977). However, most studies did not document their origin and laterality at all spinal levels, making it difficult to compare their results with ours. Even when hemisections were employed to study laterality (the rat: Snyder et al., 1978. Matsushita and Hosoya, 1979; the guinea pig: Tracey et al., 1988; the cat: Snyder et al., 1978, Matsushita et al., 1979, Grant et al., 1982; and the squirrel monkey: Snyder et al. 1978), it was sometimes difficult to know whether the lesions were complete. Our ability to make species comparisons is also compromised by the fact that differences in origin and laterality of spinocerebellar axons have been reported in the same species. That being said, it should be noted that our results compare favorably with those
reported for the cat. Matsushita et al (1979) documented 11 major spinocerebellar cell
groups in the cat and all but one appear to be present in the opossum. In addition,
most of the minor cell groups reported in their studies and more recently by others
(e.g., Grant et al, 1982; Wiksten, 1985; Grant and Xu, 1988) appear to be present in
the opossum. Differences appear to exist in the spinal segments at which these cell
groups are found, however. Cerebellar projecting neurons reported for the cat that
have not been found in the opossum include: 1) ipsilaterally projecting neurons in the
central portion of lamina VII from L4-L6 (Aoyoma et al, 1973; Matsushita et al, 1979),
2) contralaterally projecting neurons in the medial part of lamina VII from C5-T1, and
3) ipsilaterally projecting neurons in the ventromedial part of lamina VII at L5-L6.
Cerebellar projecting neurons in the opossum (present study) that have not been
reported in the cat or any other species included 1) ipsilaterally projecting neurons
within the medial part of lamina VII from C5-T1, 2) ipsilaterally projecting neurons
within lamina VIII and adjacent areas (medial lamina IX and ventromedial lamina VII)
from T6-L2, 3) ipsilaterally projecting neurons within the medial part of lamina VII
from T12-L3, 4) neurons within that part of CN which projects into the dorsal
funiculus (see below), and 5) contralaterally projecting neurons within the
central/ventral part of lamina VII from L4-L6. It should be noted, however, that in the
opossum, some of the cells within CN at L2, or within adjacent areas in the lateral
portion of lamina IV-VII (some of the cells we have called dhDSCT neurons), may
correspond to those reported in the central portion of lamina VII from L4-L6 in the cat
(although, see Edgley and Gallimore, 1988). Likewise, cells in the medial part of
lamina VII from T12-L3 of the opossum may be comparable to those reported in the ventromedial part of lamina VII at L5-L6 in the cat based on location and laterality of their projections.

Spinocerebellar neurons which innervate both the anterior and posterior lobes have been reported in the mouse (Heckroth and Eisenman, 1988), the rat (Berretta et al., 1991), and the cat (Xu and Grant, 1988) and our results show that they also exist in the opossum. In the mouse, such neurons were reported to be present throughout the spinal cord but their position was only described for the largest population (lamina VII neurons of the lumbar enlargement) which probably include SBC's (Heckroth and Eisenman, 1988). In the rat, neurons which project to both the anterior and posterior lobes were found in the CCN, CN, and dorsolateral part of lamina VII at lumbar levels (SBC's) (Berretta et al., 1991). We found them in the same areas in the opossum, but they were also present within the NCB, the central/lateral part of lamina VII from C5-T1, CN, the lateral part of laminae IV-VII from C1-L5 (dhDSCT neurons), the DL and VL nuclei of lamina IX at L2-L3, the medial part of laminae VI-VII from L3-L5, the lateral part of laminae IV-VII from T12-Cx5, SN, and the VLN. In the cat (Xu and Grant, 1988), the spinal cord was only examined from L2 to Cx1. At those levels, however, their results were comparable to ours, with the exception that we found double labeled neurons in the ventrolateral nucleus of lamina IX at lumbar levels and in the lateral part of lamina VII in the lower thoracic and lumbar cord. Neurons
corresponding to those reported in the medial lamina VI group from L4-L6 in the cat
(Aoyoma et al, 1973; Matsushita et al, 1979) were not labeled by either marker in our
experiments (also, see above).

Our results on the locations of spinocerebellar axons in the spinal cord of the
opossum appear comparable to those reported in the rat (Shirao, 1987: Yamada et al.
1991), the cat (Grant, 1962; Matsushita et al, 1979; Xu and Grant, 1994), the dog
(Sherrington and Laslett, 1903; Horax, 1915), the macaque monkey (Yoss, 1952:
1953), and man (Smith, 1957). We are convinced that the axons labeled in our studies
were spinocerebellar axons. Although axons which project to the spinal cord originate
within the cerebellum in the opossum (Martin et al, 1974), and in other mammals (the
tree shrew: Ware and Mufson, 1979; the rat: Bentivoglio and Kuypers, 1982; the cat:
Matsushita and Hosoya, 1978; the monkey: Asanuma et al, 1983), they are few in
number and limited to the cervical cord. It is unlikely, therefore, that their presence
would have confounded our results even if they were labeled. It is theoretically
possible that collaterals of neurons in the brainstem that project to both the cerebellum
and spinal cord were labeled orthogradely in our material. Double-labeling studies
suggest that such neurons are few in number (Martin and Waltzer, 1984), however.
Our ability to follow labeled axons from their cells of origin in the grey matter of the
spinal cord and into the white matter also helped assure that the axonal labeling in the
spinal cord resulted from retrograde transport within spinocerebellar axons.
Classically, spinocerebellar axons have been described as organized into 3 pathways: the dorsal spinocerebellar tract (DSCT), the ventral spinocerebellar tract (VSCT), and the rostral spinocerebellar tract (RSCT) (for reviews, see Bloedel, 1973; Bloedel and Courville, 1981). The dorsal spinocerebellar tract (DSCT) has long been known to ascend within the dorsolateral funiculus (DLF) and to originate from CN (Mann, 1973), and probably dhDSCT neurons (Tapper et al., 1975; Randic et al., 1976; Edgley and Gallimore, 1988). At cervical levels, a segmentotopic arrangement exists in the DSCT such that axons originating from the lumbar cord are located in the dorsal part of the DLF and axons from rostral levels occupy more ventral positions (Sherrington and Laslett, 1903; Yoss, 1952; Grant, 1962; Xu and Grant, 1994). The VSCT has been defined as contralaterally projecting axons which originate from SBC’s within the caudal thoracic and lumbar cord and ascend in the periphery of the ventrolateral funiculus (VLF) (Cooper and Sherrington, 1940; Ha and Liu, 1968; Burke et al., 1971; Brodal, 1981). The VSCT has been reported to move laterally as it ascends so that it is positioned in the VLF and the ventral portion of the dorsolateral funiculus (DLF) at cervical levels (Xu and Grant, 1994). A segmentotopic arrangement has also been reported for the VSCT (Yoss, 1953; Xu and Grant, 1994). The RSCT has been defined as originating at cervical levels from neurons in the central/lateral part of lamina VII and the NCB (Oscarsson, 1965; Hirai, 1976; Matsushita et al., 1979; Wiksten, 1985). Axons originating in lamina VII project ipsilaterally into the VLF and axons of the NCB project ipsilaterally into the DLF (Xu and Grant, 1994). Contemporary tract tracing and electrophysiological methods have shown that
additional spinocerebellar axons are present in the DLF and the VLF which are not considered part of the three pathways described above (e.g., Shirao, 1987; Yamada et al., 1988; Grottel et al., 1991; Xu and Grant, 1994). For example, axons from contralaterally projecting neurons of the sacral and coccygeal cord (e.g., SN and the VLN) are present in the DLF and axons of the CCN are present in the VLF. Our results in the opossum are in agreement with those described above.

A few differences appear to exist between the opossum and the placental mammals studied to date, however, in the location of spinocerebellar axons. In the opossum, spinocerebellar axons in the DLF are found along the ventrolateral border of the dorsal horn only at mid-thoracic levels, whereas in the cat, they abut the dorsal horn at all thoracic and cervical levels (Xu and Grant, 1994). In the rat, they do not approximate the dorsal horn at all (Shirao, 1987, Yamada et al., 1991). In the opossum (present study) and rat (Shirao, 1987, Yamada et al., 1991), spinocerebellar axons are situated in the DLF at sacral levels, but they are not found in that area at the same levels in the cat (Xu and Grant, 1994). Finally, in the opossum, a dorsal column spinocerebellar tract appears to exist. To the best of our knowledge, spinocerebellar axons in the dorsal funiculus (DF) have not been described previously in adult mammals, although they may exist during development (Grishkat and Eisenman, 1995). Spinocerebellar axons have been reported in the DF in amphibians (Joseph and Whitlock, 1968, Székely et al., 1980) and reptiles (Corvaja et al., 1979).
Ebbesson and Goodman, 1981, Kunzle, 1982), however. In the opossum, axons in the DF appear to originate from clusters of neurons in the dorsal portion of CN and possibly from the NCB.

Concluding Remarks

Differences in central nervous system organization exist between marsupials and placental mammals. For example, neocortical axons interconnect the two hemispheres via the anterior commissure in marsupials rather than by a dorsally placed corpus callosum, as in placental mammals (for review, see Johnson, 1977; Johnson et al, 1982ab), somatosensory and somatomotor representations in the neocortex appear to overlap more extensively in marsupials than in placental mammals (for review, see Johnson, 1977; Johnson et al, 1994), and corticospinal axons are limited to cervical or cervical and thoracic levels in marsupials, whereas they extend the length of the cord in many placental mammals (for review, see Johnson, 1977). Spinocerebellar axons also differ at least in their terminal distribution. In the opossum (Hazlett et al, 1971) and brush-tailed possum (Watson et al, 1976), spinal axons innervate 5 longitudinal zones within the anterior lobe of the cerebellum, whereas they project to a variable number of zones in placental mammals (see reviews by, Voogd, 1969; Bloedel and Courville, 1981). It appears, however, that spinocerebellar axons of the opossum and the placental mammals studied to date are generally comparable in origin, course, and laterality. If differences exist they are relatively minor.
FIGURE 1

Photograph of an adult North American opossum (*Didelphis virginiana*) with young.
A darkfield photomicrograph of the lesion site from a case subjected to a C2 hemisection prior to injections of Fluoro-Gold (FG) into the contralateral anterior cerebellum is shown in A. When all sections through the lesion site are examined, it is clear that the lesion interrupted all axons on the right side. The ventral (VF) and lateral funiculi (LF) on the spared side are indicated for reference and the arrow points to the ventral median fissure. A section through the injection site from the case described above is indicated in B. This section corresponds to that seen in Fig. 3C; the large open arrow in B indicates the border of the area filled with black in Fig. 3C (large open arrow) and the small closed arrow indicates the border of the cerebellum similarly demarcated in Fig. 3C. The IV ventricle (IV) and the genu of the facial nerve (g) are indicated. In the cerebellum, lobules I, V, and VI are indicated.
FIGURE 3

Plots of sections through the cerebellum illustrating the extent of the FG injection in the case described in the text. The sections are arranged from rostral (A) to caudal (E). The black area indicates the region containing the highest concentrations of the injected marker when viewed under a 2.5x objective, whereas the hatched lines indicate additional areas which can be seen to contain FG when viewed using a 4x objective. The section drawn in C corresponds to that shown photographically in Fig. 2B and the arrows in C indicate the borders similarly demarcated in Fig. 2B. The inferior colliculus (IC) is indicated in A. In the cerebellum, lobules I, II, III, V, IX, and X and the paramedian lobule (PML) are indicated.
FIGURE 3
FIGURE 4

Plots of the neuronal labeling in the cervical spinal cord from the case referred to in Figs. 2 and 3. All labeled neurons in every 12th section of the segments indicated have been drawn on a single section. Each dot represents one labeled cell. The side of the lesion is on the reader’s right (LESION SIDE). The scale bar in Fig. 4 can be used for Figs. 5 and 6.
FIGURE 5

Plots of the neuronal labeling at thoracic levels of the spinal cord in the case described in Figs. 2 and 3. All the labeled neurons in every 12th section of the segments indicated have been drawn on a single section. Each dot represents one labeled cell. The side of the lesion is on the reader’s right (LESION SIDE). The scale bar in Fig. 4 can be used for Figs. 5 and 6.
FIGURE 5
FIGURE 6

Plots of the neuronal labeling at lumbar levels of the spinal cord in the case described in Figs. 2 and 3. All the labeled neurons in every 12th section of the segments indicated have been drawn on a single section. Each dot represents one labeled cell. The side of the lesion is on the reader's right (LESION SIDE). The scale bar in Fig. 4 can be used for Figs. 5 and 6.
FIGURE 6
FIGURE 7

Plots of the neuronal labeling at sacral/coccygeal levels of the spinal cord in the case described in Figs. 2 and 3. All the labeled neurons in every 12th section of the segments indicated have been drawn on a single section. Each dot represents one labeled cell. The side of the lesion is on the reader's right (LESION SIDE).
FIGURE 8

Photomicrographs showing selected spinal labeling at cervical and thoracic levels from the case described in Figs. 2 and 3. In each section, the side of the lesion is on the reader’s right. A photomicrograph of a section from the 3rd cervical segment of the spinal cord is shown in A. Labeled neurons are present in the central cervical nucleus (CCN), ipsilateral to the lesion (closed arrow), and the nucleus centrobasalis (NCB) (open arrow), contralateral to it. A photomicrograph of a section from the 6th cervical segment of the spinal cord is shown in B. Labeled cells are documented in the lateral part of lamina VII (open arrows) and in the medial part of lamina VII (closed arrow), contralateral to the lesion. A photomicrograph of a section through the 12th segment of the thoracic cord is shown in C. Labeled neurons are documented in Clarke’s nucleus (CN) (closed arrow) and in lamina VIII (open arrow) contralateral to the lesion. A section through the 12th segment of the thoracic cord is shown in D. Labelling is documented in CN (closed arrow), in dorsal horn dorsal spinocerebellar neurons (dhDSCT) (open arrowhead), and in the large cells in the dorsal part of CN (closed arrowhead) on the side opposite the lesion. The latter neurons appear to project into the dorsal funiculus (small arrows). A labeled cell can also be seen in the grey/white border region of lamina VII on the side of the lesion (open arrow). The scale bar in A can be used for B-D.
FIGURE 8
FIGURE 9

Photomicrographs which show neuronal labeling in selected nuclei at lumbar and sacral levels from the case described in Figs. 2 and 3. In each section, the side of the lesion is on the reader’s right. A section from the 2nd lumbar segment of the spinal cord is shown in A. Labeled neurons are documented in the dorsal part of lamina VII (closed arrowhead), in the central/ventral part of lamina VII (open arrowhead), and in the dorsolateral (DL) and ventrolateral (VL) nuclei of lamina IX (closed arrows) on the side of the lesion. A section from the 3rd lumbar segment of the spinal cord is shown in B. Labeled cells are documented in the medial part of laminae VI and VII on the side of the lesion (closed arrow) and in the ventromedial part of lamina VII (open arrow) contralateral to it. Photomicrographs of sections through the 2nd segment of the sacral cord are shown in C and D. Labeled neurons can be seen in Stilling's nucleus (SN) (closed arrow, C) and in the ventrolateral nucleus of lamina IX (VLN) ipsilateral to the lesion (closed arrow, D). The scale bar in A can be used for B-D.
FIGURE 9
Camera lucida drawings of labeled spinocerebellar neurons to show the trajectory of their axons within the grey matter and part of the white matter. Only neurons in major spinocerebellar nuclei were selected for documentation. A drawing of a section through the second cervical segment (C2) of the spinal cord is shown at the upper left and a labeled neuron in the nucleus centrobasalis is illustrated (closed arrow). Its axon can be followed into the dorsolateral funiculus. A labeled neuron in the central cervical nucleus is also illustrated (open arrow) and its axon can be followed across the midline into the ventral funiculus. The drawing of a section from the sixth cervical segment (C6) illustrates a labeled neuron in the central/lateral portion of lamina VII (closed arrow) and its axon can be followed into the ipsilateral ventrolateral funiculus. A labeled neuron in medial lamina VII is shown in the same section (open arrow) and its axon can be followed across the midline into the contralateral ventral funiculus. The drawing of a section from the 13th segment of the thoracic spinal cord (T13) contains a camera lucida drawing of a labeled neuron in Clarke's nucleus (closed arrow) the axon of which can be followed into the dorsolateral funiculus. A labeled neuron is also shown in lamina VIII (T13, open arrow) and its axon can be followed into the ventral funiculus. The drawing of a section from the 3rd segment of the lumbar cord (L3) contains a camera lucida drawing of a labeled neuron in the dorsomedial part of lamina VII (closed arrow) and its axon can be followed ventrally, across the midline, and into the contralateral ventral funiculus. A drawing of a labeled neuron in the lateral part of lamina VII is also shown (open arrow) and its axon can be followed medially across the midline and into the ventral funiculus. The drawing of a section from the 2nd sacral segment of the spinal cord (S2) shows a labeled neuron in Stilling's nucleus (closed arrow) and its axon can be followed across the midline and into the contralateral ventral funiculus. A camera lucida drawing of a labeled neuron in the ventrolateral nucleus is also shown (open arrow) and its axon can be followed across the midline and into the ventral funiculus on the contralateral side. Note its circuitous course.
Photomicrographs of two of the neurons drawn in Fig. 10. A photomicrograph of one of the neurons (large arrow) drawn in L3 in Fig. 10 (open arrow) is shown in A. Its axon (small arrows) can be followed medially toward the midline. A photomicrograph of one of the neurons (large arrow) drawn in S2 of Fig. 10 (open arrow) is shown in B. Note the circuitous course its axon (small arrows) takes to reach the contralateral ventral funiculus (Fig. 10, open arrow). The scale bar in A can be employed for B.
FIGURE 12

Drawings of selected spinal cord sections showing the distribution of labeled axons in the case described in Figs. 2 and 3. Each drawing illustrates the labeling present in one section and each dot represents a labeled axon.
FIGURE 13

A photomicrograph of the C2 section plotted in Fig. 12 is shown in A. The lateral funiculus (LF) is indicated for reference. Labeled neurons in the dorsal portion of CN (large arrow) which project into the dorsal funiculus are shown in B (small arrows). The dorsal funiculus (DF) is labeled for reference. The scale bar in B can be used for D. A labeled neuron within the same nuclear group is shown at a different level in C (closed arrow). The small arrows indicate its axon as it approaches the dorsal funiculus (DF). Clarke’s nucleus is indicated (CN). A horizontal section of the spinal cord cut through the dorsal funiculus from a case subjected to Fluoro-Gold injections into the anterior lobe of the cerebellum is shown in D. Labeled spinocerebellar axons (arrows) are indicated.
FIGURE 13
Fig. 14  Fluorescence photomicrographs from a case subjected to injections of Fast Blue (FB) into the anterior lobe of the cerebellum and injections of Rhodamine B Dextran (RBD) into the posterior lobe are shown. High power photomicrographs through CN at the T13 level are shown in A and B. FB labeled neurons are illustrated in A and RBD labeled neurons are shown in B. Double labeled neurons are indicated by arrows. Labeled neurons in the dorsolateral and ventrolateral nuclei of lamina IX and adjacent lamina VII at L2 are documented in C and D. FB labeled neurons are shown in C and RBD labeled neurons are shown in D. Double labeled cells are indicated by arrows. The scale bar in A can be used for B and that in C can be employed for D.
FIGURE 14
CHAPTER 2

GROWTH OF DORSAL SPINOCEREBELLAR AXONS THROUGH A LESION OF THEIR SPINAL PATHWAY DURING EARLY DEVELOPMENT IN THE NORTH AMERICAN OPOSSUM, Didelphis virginiana

Abstract

Supraspinal axons grow around or through lesions of their spinal pathway during specific critical periods of mammalian development, but comparable plasticity has not been documented for axons which form ascending tracts. In the present study, we asked whether axons of the dorsal spinocerebellar tract (DSCT) are capable of such growth. The spinal cord of the North American opossum, Didelphis virginiana, was hemisectioned at mid-thoracic levels between postnatal day (PD) 5 and 68 and after varying survival times, bilateral injections of Fluoro-Gold or Fast Blue were made into the anterior lobe of the cerebellum, the major target of DSCT axons. Seven days later, the pups were sacrificed and their spinal cord processed for fluorescence microscopy. In animals lesioned between PD5 and 9, and allowed to survive for 37-269 days, neurons were labeled bilaterally in Clarke's nucleus (CN) caudal to the lesion, but they were fewest in number and smallest in size on the lesioned side. Since the DSCT originates almost entirely within CN on the ipsilateral side, we conclude that the
neurons labeled ipsilateral and caudal to the lesion supported axons which grew around or through it. Histological examination revealed that recognizable spinal cord was present at the lesion site and that labeled spinocerebellar axons were located in their normal position ipsilateral to the lesion. It appears, therefore, that growth occurred through the lesion. In animals lesioned between PD13 and 68, labeled neurons were not found in CN caudal and ipsilateral to the lesion although they were present on the contralateral (control) side. We conclude that DSCT axons, like axons which form descending tracts, grow through a lesion of their spinal pathway if it is made early in development. Interestingly, the critical period for such growth was shorter than that for most descending spinal axons.
Introduction

When the spinal cord is injured in adult mammals, including man, axons fail to grow around or through the lesion (Puchala and Windle, 1977; Kiemon, 1979; Freed et al, 1985; Björklund, 1994) resulting in paralysis and loss of sensation caudal to the damaged area. When the same injury occurs during development, however, supraspinal axons extend caudal to the lesion. Cortical axons grow around and possibly through lesions of their spinal pathway in neonatal rats (Bernstein and Stelzner, 1983; Schreyer and Jones, 1983; Bregman et al, 1989; Firkins et al, 1993) and cats (Bregman and Goldberger, 1982, 1983ab) and in kittens, anatomical plasticity has been associated with sparing of function (Bregman and Goldberger, 1982, 1983ab). Anatomical plasticity has also been reported for brainstem-spinal axons. In opossums, rubral, reticular and vestibular axons extend around or through a lesion of their spinal pathway if it is made early enough in development (Martin and Xu, 1988: Xu and Martin, 1989, 1991; Xu and Martin, 1991; Treherne, et al, 1992; Martin et al, 1994; Wang et al, 1994).

In light of the evidence available for developmental plasticity of descending spinal axons, it is surprising that comparable plasticity has not been documented for axons which form ascending tracts. It appears, in fact, that ascending spinal axons do not grow rostral to a lesion of their spinal pathway in neonatal rats (Cummings et al, 1981; Stelzner and Cullen, 1988; Leclerc et al, 1988; Lahr and Stelzner, 1990; Buyan Dent and Stelzner, 1996). Ascending spinal axons are relatively mature at birth in rats.
(Lahr and Stelzner, 1990; Ashwell and Zhang, 1992), however, and they may have lost their potential for plasticity. In order to determine whether ascending axons grow around or through a lesion of their spinal pathway at earlier stages of development in rats, it would be necessary to perform intrauterine surgery. An alternative strategy would be to employ a species which is born at a more immature state of development. The North American opossum, *Didelphis virginiana*, is born 12-13 days after conception and the entire development of its ascending spinal tracts occurs postnatally (Martin et al, 1983). Immediately after birth, opossums climb into an external pouch where most of their central nervous system development takes place (McCrady, 1938; Ulinski, 1971). In the present study we used the opossum to determine whether one group of ascending spinal axons, those which form the dorsal spinocerebellar tract (DSCT), grow rostral to a lesion of their spinal pathway and, if so, to establish the critical period for that plasticity.

Axons of the DSCT were chosen for study because they originate within a well defined nucleus (Clarke's nucleus) in the thoracic and rostral lumbar cord, they course almost exclusively ipsilaterally (see Chapter 1), and they are located in the dorsolateral funiculus (Hazlett et al, 1971; see Chapter 1) where they can be cut with minimal difficulty. Lesions which include the DSCT axotomize neurons within Clarke's nucleus (CN) almost exclusively ipsilateral and caudal to the lesion (see Chapter 1), allowing CN on the contralateral side to serve as an internal control. CN rostral to the lesion serves as an additional control. Our results suggest that DSCT axons grow through a lesion of their spinal pathway during early development and that the critical
period for such growth ends earlier than that for comparable plasticity of most descending spinal axons. Interestingly, there was no evidence for growth of DSCT axons around a lesion at any age.
Materials and Methods

Opossum pups were obtained from females either captured by licensed collectors in Florida or bred at The Ohio State University. Animals conceived in the wild were aged from the growth curve of Cutts et al (1978) and data from our own collection. The North American opossum is born 12 or 13 days after conception (McCready, 1938; Cutts et al. 1978) and we considered the first day in the pouch to be postnatal day (PD) 1.

In order to transect the DSCT, we attempted to hemisect the spinal cord on the right side between the 7th and 9th thoracic (T) segment in 41 pouch-young opossums ranging in age from PD5 to PD68. Prior to lesioning, the mothers were anesthetized with an intramuscular injection of ketamine (100mg/ml) followed by Metafane inhalation. During anesthesia, the pouch sphincter relaxed and the mothers were placed in a supine position to expose the pups. The pouch-young, still attached to the nipple, were anesthetized individually by hypothermia so that their spinal cord could be exposed surgically and lesioned with a microblade (Beaver). Multiple incisions were made through the same area of the cord by inserting the microblade in a position slightly to the left of the midline and pulling it laterally across the midline. To insure that the lesioning was complete, a microdissection needle with a 90° angle was drawn through the lesion cavity. The incisions were then closed by suturing and the pups were returned to the vivarium with their mother. In order to verify the effectiveness of our lesioning method, two of the PD5 pups were reanesthetized shortly after lesioning.
and sacrificed by perfusion with a 0.2M citrate buffer-10% formaldehyde solution. In each case, the spinal cord was removed and photographed and the lesion site was embedded in paraffin, sectioned at 20μm in the frontal plane, and stained for Nissl substance.

Eleven of the pups lesioned between PD5 and 9 were maintained until they were considered to be adults (2.5-3.0kg; PD173-271; Table 1). At an appropriate time, they were anesthetized by an intraperitoneal injection of sodium pentobarbital (40mg/kg) so that their cerebellum could be exposed using sterile techniques for bilateral injections of Fluoro-Gold (FG) (Fluorochrome Inc., Englewood, CO) into the anterior lobe, the major target of DSCT axons (Hazlett et al, 1971). FG was used because it holds up well under ultraviolet light allowing us to count and measure labeled neurons before losing fluorescence. The animals were placed in a stereotaxic head frame and the injections were made in two positions on each side of the midline and at three depths for each position using a micropipette attached to a 1μl Hamilton syringe. The distances of the injections from the midline and their depths depended on the size of the animal. A total of 7.5μl (3.75μl each side) of a 5% aqueous solution was injected/animal. After the injections were complete, the meninges were replaced and the craniotomy was covered by suturing the muscle, overlying fascia and skin. Prior to recovery from the anesthetic, a 0.028mg/kg subcutaneous injection of Buprenorphine was given subcutaneously to alleviate postoperative pain. The animals were then returned to the vivarium for 7 days to allow for retrograde transport of FG to neurons in CN, the origin of DSCT axons. After the appropriate survival, they were
reanesthetized with sodium pentobarbital and killed by transcardial perfusion with a 0.9% saline solution followed by a phosphate buffered solution containing 4% paraformaldehyde.

Thirty one pups subjected to lesions between PD6 and PD68 were maintained 30 days or until their cerebellum was large enough for accurate placement of injections into the anterior lobe (Table 1). Several pups were injected on PD36 but it was found that more accurate injections and better survivals were obtained in older animals (Table 1). If the pups were still attached to a nipple at the time of injection, they and the mother were anesthetized as described for the spinal cord lesions. If they were weaned, Metafane inhalation was used as the anesthetic. After an appropriate level of anesthesia was established, the cerebellum was exposed surgically so that 10.0µl (5.0µl each side) of a 2.5% aqueous solution of Fast-Blue (FB; Sigma) or FG could be injected bilaterally into the anterior lobe as described previously (Table 1). Older animals were placed in a stereotaxic head frame as described previously. FB was used in all but one animal because it did less damage at the injection site. FB does not hold up as well as FG to ultraviolet light, but it was not our intent to count or measure labeled neurons in this series of pups. After surgery, Buprenorphine was administered to alleviate postoperative pain as described above. The pups were maintained 7 days before being anesthetized with sodium pentobarbital and perfused transcardially with hypertonic saline and a 0.2M citrate buffered - 10% formaldehyde solution.
In both sets of experiments the brain and spinal cord were removed, photographed in some cases, and immersed in a 30% sucrose citrate buffer for approximately 48 hours at 4°C. All brains and spinal cords were scored by a shallow cut on the side opposite the lesion to determine laterality of the tissue sections after mounting. Serial sections of the cerebellum, the brainstem, and selected levels of the spinal cord (the lesion segment, T4, and all segments caudal to the lesion which contained CN) were cut at 40μm in the coronal plane using a freezing microtome, mounted onto gelatin coated slides, and coverslipped using Entellan (Merck). Sample sections were also cut at levels other than those mentioned. All sections were examined and appropriate ones were photographed using a Leitz (Orthoplan or Orthoplan 2) fluorescence photomicroscope equipped with the A cube (excitation wavelength 340-380 nm) of the Ploem illumination system. In some cases, the coverslips were removed and the sections were stained for Nissl substance after photography and plotting (see below) were complete. Some of the lesion sites were embedded in paraffin, sectioned at 20μm, and stained for myelin and Nissl substance. The sections were then examined and photographed, in some cases, using brightfield or darkfield microscopy.

The spinal cord sections were studied to verify the extent of the lesion and the presence or absence of labeling in CN, while sections through the cerebellum and brainstem were examined to determine the locations and spread of the injections. Cases with a documented lesion and injections which produced labeling in CN caudal and contralateral to the lesion (the control side) as well as on both sides rostral to it
were placed into one of ten groups: those lesioned on PD5-9 (N=16), PD12-14 (N=7), PD19-22 (N=4), PD26 (N=3), PD33 (N=2), PD40 (N=2), PD47 (N=2), PD54 (N=1), PD61 (N=1), and PD68 (N=4) (Table 1). Photomicroscopic documentation of labeling, or the lack of it, was carried out at different levels of the cord. Sections through the injection sites, the lesion site, and the spinal cord rostral and caudal to the lesion were drawn using a computer-based image analysis software program (Neurolucida, MicroBrightField) connected by a position transducer (Boeckeler) to the stage of a Leitz photomicroscope (Orthoplan 2). The locations of labeled neurons in CN were plotted, using the same system, on the drawings of spinal cord sections. In one case, every 6th section through the brainstem and cerebellum was drawn using the system described above. Plots of the areas with the highest concentration of injectate were made on the same sections. The drawings were then aligned, lofted into a three dimensional object and rendered using a software package developed at The Ohio State University. The cerebellar injections could then be visualized in three dimensions.

Quantitative analyses were carried out on six of the cases lesioned between PD5 and 9 and allowed to survive until they were adults (180-269 days; 2.5-3.0 kg). Each labeled neuronal profile in CN on both sides of the cord was counted from plots of every 6th section in segments rostral (T4) and caudal (T13) to the lesion. There was a mean of 178 sections through the T4 level of the cord and a mean of 240 sections through the T13 level. All labeled profiles were counted, including those not sectioned through the nucleus, because a single neuron never extended more than 3 sections.
addition, the circumference of labeled neuronal profiles was measured in the same sections using the image analysis program described previously and soma areas were derived from them using the same system. Only labeled profiles in which a nucleus could be identified were measured. The counts and sizes of labeled neurons in CN rostral to the lesion (T4) helped to evaluate the effectiveness of the injections. It was assumed that such neurons would be labeled in roughly equal numbers if the injections were bilaterally symmetrical and that they would be roughly equal in size on the two sides. A bilaterally injected, unlesioned animal also served as a control. Data for each variable (cell number and size) for all 6 cases were grouped together on the lesioned and control side, rostral (T4) and caudal (T13) to the lesion, and were expressed as the mean ± the standard error of the mean (SEM). The paired t-test was used to analyze the numerical data for side to side differences at T4 and T13, for mean cell size of labeled neurons, and for the mean number of labeled neurons. Statistical analyses were performed using the mainframe version of the Statistical Package for the Social Sciences (SPSS) computer program at The Ohio State University. Stereological techniques and correction factors were not employed because we did not seek to estimate the total number of labeled neurons in CN. Based on the magnitude of the differences observed, however, it is not likely that the results of such studies would have altered our conclusions. All of the experiments described above 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

Figure 15A shows a PD5 opossum and Figure 15B illustrates the spinal cord and brain from a pup subjected to an attempted hemisection of the thoracic cord at the same age and sacrificed shortly thereafter. Figures 15C and D are photomicrographs of sections rostral to the lesion and through its deepest part, respectively. It can be seen that over half the cord was missing at the lesion site. When the DSCT was cut using the same method in PD5 through 9 pups and injections of FG or FB were made into the anterior lobe of the cerebellum 30 to 262 days later, labeled neurons were present bilaterally in CN caudal to the lesion. The results from one of the PD5 cases are documented in Figs. 16-18. In this case, the lesion was made at T8 and bilateral injections of FG were made into the anterior lobe of the cerebellum 171 days later. Figure 16 shows a three dimensional reconstruction of the injected cerebellum and it can be seen that FG filled the anterior lobe and spread well into the posterior lobe. The apparent symmetry of the injections was substantiated by the presence of bilaterally comparable labeling in CN at T4, rostral to the lesion (Table 2; Figs. 17B and 19A). At sacrifice, the site of the hemisection was difficult to find grossly (Fig. 17A), but it was obvious histologically (Fig. 17C). A section through the lesion is illustrated in Fig. 17C where the lesioned side is on the reader’s right. Note that recognizable spinal cord was present on the lesioned side although it was abnormal in appearance (e.g., the dorsal horns were fused, the grey matter had an uncharacteristic shape, the dorsal columns were smaller than usual and the white matter was thinner). Labeled neurons
were present in CN caudal and ipsilateral to the lesion (Fig. 17D, right side; Fig. 18B), but they appeared to be fewer in number and smaller in size than those on the contralateral side (Figs 17D, left side; Fig. 18A).

In order to verify our qualitative impression, labeled neurons in CN were counted and measured in six of the animals lesioned between PD5 and 9 and maintained until PD180 to 269 when they were considered to be adults (Table 2 and Fig. 19). Neurons were studied on both sides at T13, caudal to the lesion, and at T4, rostral to it. Detailed analyses caudal to the lesion were performed at T13 because labeled neurons in CN were most numerous at that level. The mean of the total number of labeled neurons at T13 ipsilateral to the lesion was 43% less than that on the contralateral (control) side (statistically significant difference: p<.01; Table 2; Fig. 19B) and labeled neurons on the lesioned side had mean soma areas which were 15% smaller than those on the unlesioned side (statistically significant differences: p<.01; Table 2). When the same analyses were done on labeled neurons in CN rostral to the lesion (T4), they were found to be comparable in number and size bilaterally (Table 2; Fig. 19A). Figures 19C and D show the size distributions of labeled cells at T4 and T13. The distributions were similar on the two sides at T4; but at T13 a greater percentage of small cells and a lower percentage of large cells were present on the lesioned side. In the non-lesioned control subjected to bilateral injections in the anterior cerebellum, labeled neurons were present in similar numbers and sizes at both T13 and T4 (data similar to control side of lesioned animal and not shown).
Labeled axons were present bilaterally in the DSCT rostral to the lesion (Fig. 17B, arrows), at the lesion site (Fig. 17C, arrows; Figs. 18C and D) and caudal to it (present in Fig. 17D but not seen at this magnification), but at all levels they appeared to be fewer in number on the lesioned side. It should be noted, however, that the labeled axons present on the lesioned side occupied their normal position, even in sections through the lesion (Fig. 17C, right side; Fig. 18D).

When a hemisection of the thoracic cord was attempted between PD12 and 68 and FB or FG was injected bilaterally into the anterior lobe of the cerebellum 30 to 131 days later, labeled neurons were not present in CN caudal and ipsilateral to the lesion although they were present in the same area contralaterally (control side). Figure 20A shows the spinal cord and brain of an animal lesioned on PD13 and injected with FB 99 days later. As in the PD5 - 9 cases, the lesion was not obvious grossly (Fig. 20A). After sectioning, however, it was clear that over half the cord was involved in the lesion (Fig. 20C). The normal size of the spinal cord was apparently due to abnormal thickness of the white matter contralateral to the lesion (Fig. 20C). Examination of sections rostral to the lesion indicated that labeling in CN was essentially equal bilaterally (Fig. 20B). In contrast, labeling was not present in CN caudal and ipsilateral to the lesion, although it was normal on the contralateral (control) side (Fig. 20D). The labeled neurons on the side of the lesion in Fig. 20D are spinal border cells which project contralaterally (see Chapter 1). As might be expected, few axons were labeled in the DSCT rostral and ipsilateral to the lesion although they were abundant on the contralateral (control) side (Fig. 20B). The labeled axons that were present
rostral and ipsilateral to the lesion most likely originated from CN rostral to it, since labeled axons were not found caudal and ipsilateral to the lesion (data not shown). Similar results were seen in the animals lesioned at older ages, although the lesion site was more obvious grossly. Figure 21 shows the lesion site in a case subjected to a lesion at T9 on PD26 and cerebellar injections of FB 89 days later. Figure 21C shows a section through the fullest extent of the lesion and Figures 21B and D illustrate sections 300µm rostral and 580µm caudal to it. Labeled neurons were not present in CN caudal and ipsilateral to the lesion although they were present on the contralateral (control) side and on both sides rostral to the lesion (the labeling appeared comparable to that in Figs. 20B and D and was therefore not shown). The coverslips were removed and sections caudal to the lesion were stained for Nissl substance in many of the cases subjected to hemisection at PD12 and at later stages of development. In all cases, cells could be identified in the area judged to be CN on the lesioned side (Figs. 22A and B. right side) but they were relatively small (compare right and left sides of Fig. 22A). Only an occasional larger neuron was found (Fig. 22B).
Discussion

Our results suggest that axons of the DSCT grow rostral to a lesion of their spinal pathway during early development in the opossum and that they reach the cerebellum. DSCT axons are located on the surface of the dorsolateral funiculus (see Hazlett et al, 1971; Chapter 1), so it is unlikely that our lesions failed to include them. In addition, the effectiveness of our lesioning method was documented in the animals sacrificed shortly after lesioning and in all of the cases lesioned between PD12 and PD68. Although the lesion site was not apparent grossly at the time of sacrifice in animals lesioned prior to PD12, its presence could be verified histologically. Since DSCT axons originate almost entirely within CN on the same side, it is reasonable to conclude that neurons labeled in CN caudal and ipsilateral to the lesion supported axons which grew around or through it. From the appearance of the spinal cord at the lesion site and the location of labeled axons at that level, we conclude that they grew through the lesion in what appears to be regenerated tissue. Repair of the injured spinal cord has been described in non-mammalian species (see review; Martin et al, 1994) and it has recently been documented in neonatal opossums (Treherne et al. 1992; Martin et al, 1994; Nicholls et al, 1994) and fetal rats (Saunders et al, 1992; Nicholls et al, 1994). The mechanisms which underlie such repair are not completely understood.

It is not known whether the plasticity observed in our experiments resulted from regeneration of cut axons or late growth. Axons from caudal levels of the cord reach the cerebellum by at least PD7 in *Didelphis* (Martin et al, 1983), so it is likely
that DSCT axons were cut by hemisecting the thoracic cord between PD5 and 9. That conclusion is supported by our finding that neurons in CN caudal and ipsilateral to the lesion were generally smaller than those on the contralateral (control) side since neuronal shrinkage and/or preferential loss of large neurons is seen in CN after DSCT transection (Richardson, 1984; Himes et al, 1994). It is possible, therefore, that regeneration of cut axons contributed to growth of DSCT axons through the lesion. Axons are added to the DSCT over time, however (Martin et al. 1983; Arsenio Nunes and Sotelo, 1985; Ashwell and Zhang, 1992; Qin et al. 1993), so it is likely that late growth also contributed. Both mechanisms are involved in extension of rubrospinal and corticospinal axons around a lesion of their pathway in neonatal opossums and rats (Xu and Martin, 1991; Bates and Stelzner, 1993), in growth of supraspinal and propriospinal axons through the lesion after transection of the spinal cord in the opossum (Wang et al, 1997b) and in growth of supraspinal axons through a lesion of their spinal pathway in the developing chick (Hasan et al, 1993).

Although DSCT axons grew through lesions made between PD5 and 9, labeled neurons in CN caudal and ipsilateral to the lesion were fewer in number than on the contralateral (control) side, suggesting that some neurons failed to survive axotomy. Alternatively, some of the surviving neurons may not have supported axons which reached the cerebellum or transported the marker. We favor the first explanation, since CN neurons have been reported to be particularly sensitive to axotomy during development (Stelzner et al. 1975; Loewy and Schader, 1977; Smith and Castro, 1979; Bryz-Gornia and Stelzner, 1986; Himes et al, 1994). Failure of developing neurons to survive axotomy may be due to decreased availability of target derived trophic factors.
(Snider et al, 1992). That explanation is supported by the observations that placement of embryonic spinal cord or cerebellum into the lesion cavity rescues axotomized neurons in CN (Himes et al, 1994) and that application of neurotrophin-3 to the lesioned area has a protective effect (Diener and Bregman, 1994). It is also possible that axotomy-induced cell death is produced by the cytotoxic effect of excitatory amino acids (McDonald and Johnston, 1990; Choi, 1992; Lowrie and Vrbova, 1992; Mentis et al, 1993; Greensmith et al, 1994). Glutamatergic dorsal root axons provide a major input to CN (Mann, 1973; Maxwell et al, 1990; Walmsley, 1991) and in adult animals, axotomy induced cell death can be prevented by cutting dorsal root axons (the cat: Sanner et al, 1993) or treating the animal with the NMDA receptor blocker, MK-801 (the rat; Sanner et al, 1994). In this regard, it should be noted that dorsal root axons are present within CN very early in development (the rat: Snider et al, 1992; the Brazilian short-tailed opossum; Qin et al, 1993; the North American opossum; Wang et al, 1997a). The neurotrophic and cytotoxic hypotheses are not mutually exclusive, however, since neurotrophins are protective against the effects of excitotoxins (Lindvall et al, 1994).

Differences in the ability of ascending and descending axons to grow through a lesion of their spinal pathway appear to exist. Our results provide evidence for growth of DSCT axons through a lesion in the developing opossum, but the critical period for that plasticity ends earlier than that for comparable plasticity of most descending spinal axons in the same species (Martin et al, 1994). Differences in the ability of ascending and descending axons to grow through a lesion of their spinal pathway have also been reported in other species. In some frogs (Rana catesbeiana and Xenopus laevis).
growth of ascending axons through a transection of the spinal cord does not appear to occur (Forehand and Farel, 1982; Campbell et al. 1984; Beattie et al. 1990), but growth of descending spinal axons through a comparable lesion is well documented (Forehand and Farel, 1982; Lee, 1982; Beattie et al. 1990; Brenner and Stehouwer, 1991; ten Donkelaar, 1994). In adult salamanders, dorsal column axons fail to traverse a spinal cord transection (Stensaas, 1983; Davis et al., 1989), but descending axons do so readily (Davis et al., 1989, 1990). In fish, spinocerebellar and Rohon-Beard axons terminate in the vicinity of the lesion after transection of the spinal cord (Bunt and Fill-Moebs, 1984), but growth of supraspinal axons beyond the lesion is extensive (Coggeshall, et al., 1982, 1983; Bunt and Fill-Moebs, 1984; Sharma et al., 1993; Zottoli et al., 1994). Even in larval lampreys, axons of dorsal cells and giant interneurons only grow for a short distance rostral to a transection of the spinal cord (Yin and Selzer, 1983), but brainstem axons extend for long distances caudal to the same lesion (Yin and Selzer, 1983; Croop et al., 1987; Davis and McClellan, 1994). The reasons for these differences are not known.

Differences also exist in the ability of ascending and descending axons to grow around a lesion. There was no evidence for growth of DSCT axons around the lesion in our experiments, although such growth has been well documented for descending spinal axons (Kalil and Reh, 1979, 1982; Bregman and Goldberger, 1982, 1983ab; Bernstein and Stelzner, 1983; Schreyer and Jones, 1983; Martin and Xu, 1988; Xu and Martin, 1989, 1991; Firkins et al., 1993; Wang et al., 1994). Apparently, DSCT axons
have lost their potential for growth and/or the environment has become less conducive for it (see below) by the time spinal cord repair no longer occurs and growth past the lesion necessitates circumnavigation.

It is appropriate to ask why DSCT axons do not grow through a lesion of their spinal pathway and innervate the cerebellum at PD12 or at later stages of development. Since neurons of CN are particularly susceptible to axotomy (Stelzner et al. 1975; Loewy and Schader, 1977; Smith and Castro, 1979; Bryz-Gornia and Stelzner, 1986; Himes et al, 1994), it is possible that none of them survived to support growth through the lesion. When sections through CN were stained for Nissl substance, however, small neurons were present in the region of CN caudal and ipsilateral to the lesion. Unfortunately, the small size of the immature cerebellum prior to PD12 makes prelabeling experiments unrealistic, so it may be difficult to prove that any of the neurons in question actually supported axons which were cut by our lesions. The degree to which axotomy-induced cell death contributes to the end of the critical period for developmental plasticity deserves further study.

The end of the critical period for extension of DSCT axons through a lesion of their spinal pathway may be due to decreased ability of the neurons which survive axotomy to initiate and/or sustain axonal growth. Axons presumably have more growth potential when they are immature and programmed for growth than at later stages of development or in the adult animal (Black and Lasek, 1979; Pestronk et al. 1980; Fawcett, 1992). It should be noted, however, that axons which originate within CN have some ability to regenerate in adult mammals. In adult rats, axotomized DSCT axons regenerate into peripheral nerve grafts although they do not regenerate
within the spinal cord itself (Richardson et al., 1984). Peripheral nerve grafts are permissive for axonal growth, whereas the adult spinal cord is not. It is likely, therefore, that DSCT axons are capable of growth after the critical period for plasticity defined herein, but that changes in the environment do not allow it.

Environmental factors which inhibit axonal growth include the development of myelin (Caroni and Schwab, 1988; Schwab and Caroni, 1988; McKerracher et al. 1994; Mukhopadhyay et al., 1994), the maturation of glia (Firkins et al. 1993), and the development of a mature glial response to lesioning (Reier and Houle, 1988; Rudge and Silver, 1990). It is interesting, however, that the end of the critical period for growth of DSCT axons through a lesion does not correlate with the appearance of any of these factors. Myelin basic protein-like immunoreactivity (MBP-LI) is not present in the dorsolateral funiculus of the midthoracic cord until PD26-33 (Ghooray and Martin, 1993), 2-3 weeks after the end of the critical period for DSCT plasticity: mature astrocytes are not found in that area until roughly the same age (Ghooray and Martin, 1993); and a mature glial scar after lesioning is not seen until PD26 (Ghooray and Martin, 1993). Since myelination and glial maturation follow rostral to caudal and ventral to dorsal gradients (Ghooray and Martin, 1993ab), it might be suggested that DSCT axons grow for some distance rostral to a lesion, but that they encounter myelinating oligodendrocytes or mature glia in route to the cerebellum. This does not appear to be the case, however, since MBP-LI is not present in the dorsolateral funiculus at cervical levels until PD24-26 (Ghooray and Martin, 1993) and mature appearing glia are not found in that area until PD21 (Ghooray and Martin, 1993). Neither can be detected in the inferior cerebellar peduncle, the location of DSCT axons
in the medulla, until PD30-33 (unpublished results). Interestingly, recent studies have shown that the appearance of myelin in the optic nerve does not correlate with failure of retinal axons to grow through a lesion of the nerve in another opossum, *Monodelphis domestica* (MacLaren, 1996). Although myelin development, glial maturation, and the development of a mature glial response to lesioning do not appear to be operative in loss of DSCT plasticity, loss of trophic factors (e.g., Lindsay, 1988; Houle et al., 1992; Schnell et al., 1994), and/or changes in cell surface and extracellular matrix molecules (e.g., Hantaz-Ambroise et al., 1987; Snow et al., 1990; Cole and McCabe, 1991; McKeon et al., 1991; Fawcett, 1992; Carbonetto and David, 1993; Milev et al., 1994) cannot be ruled out. These issues should be addressed in future studies.
**TABLE 1. Summary of Animals Used**

<table>
<thead>
<tr>
<th>Lesion Age</th>
<th>Range of Injected Ages</th>
<th>Marker Used</th>
<th>Days Survival After Injection</th>
<th>Number Of Good Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD5-9</td>
<td>PD173-271</td>
<td>FG</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>PD6</td>
<td>PD36-105</td>
<td>FB</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>PD12-14</td>
<td>PD76-117</td>
<td>FB</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>PD19-22</td>
<td>PD115-150</td>
<td>FB</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>PD26</td>
<td>PD112-115</td>
<td>FB</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>PD33</td>
<td>PD122-135</td>
<td>FB</td>
<td>7</td>
<td>2</td>
</tr>
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<td>PD40</td>
<td>PD76-100</td>
<td>FB</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>PD47</td>
<td>PD102-125</td>
<td>FB</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>PD54</td>
<td>PD109</td>
<td>FB</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>PD61</td>
<td>PD112</td>
<td>FB</td>
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<td>1</td>
</tr>
<tr>
<td>PD68</td>
<td>PD98-100</td>
<td>FB,FG</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

Abbreviations: postnatal (PD); Fluoro-Gold (FG); Fast Blue (FB)
A photograph of a PD5 opossum is shown in A and the spinal cord and brain of a pup subjected to a lesion of the thoracic cord at the same age and sacrifice immediately after are shown in B. The arrow in B indicates the lesion. A darkfield photomicrograph of a section rostral to the lesion is provided in C and a section through the lesion site is illustrated in D. The ependymal zone (EZ), the intermediate zone (IZ) and the marginal zone (MZ) are indicated. The scale bar in C can be used for D.
FIGURE 16

Three dimensional reconstruction of Fluoro-Gold spread (Inj) within the cerebellum of the case illustrated in Fig. 17. A. Dorsal view B. Lateral view. The inferior colliculus (IC), and the primary fissure (arrows) are indicated for reference.
FIGURE 17

The spinal cord and brain of an animal subjected to hemisection of the thoracic cord on the right side on PD5 and bilateral injections of Fluoro-Gold into the anterior lobe of the cerebellum (double arrows) at maturity are shown in A. The middle arrow (C) points to the level of the lesion (les) and the lines rostral and caudal to it indicate the levels of the sections shown in B and D. In the section rostral to the lesion (B), cut at T4, the open arrows indicate labeled neurons in CN and the solid arrows point to labeled axons in the DSCT. The asterisk in this section, and in the sections shown in C and D, indicate the score used to identify the side opposite the lesion. A section through the lesion (T8) is shown in C. The arrow on the right (lesioned) side indicates labeled axons in the dorsolateral funiculus in what appears to be reconstructed spinal cord, whereas that on the left indicates labeled axons in the same tract on the unlesioned side. A section of spinal cord caudal to the lesion (T13) is shown in D. The open arrows indicate labeled neurons in CN. The scale bar in B can be used for C and D.
FIGURE 18

Fluorescence photomicrographs of labeled neurons in CN caudal to the lesion (T13) are provided in A and B. Those labeled contralateral to the lesion are shown in A, whereas those labeled on the ipsilateral side are illustrated in B. The dorsal funiculus (DF) is indicated. Fluorescence photomicrographs of dorsal spinocerebellar axons in a section through the lesion site (T8) are shown in C and D. Those illustrated in C were located contralateral to the lesion, whereas those in D were on the ipsilateral side. The scale bar in A can be used for B and that in C can be employed for D.
# TABLE 2

The number (taken from every 6\textsuperscript{th} section) and size (\(\mu m^2\); taken from every 6\textsuperscript{th} section) of labeled neurons in Clarke’s nucleus, rostral (T4) and caudal (T13) to the lesion.
Table 2. Number\(^1\) and Size\(^1\) (\(\mu m^2\)) of Labeled Neurons in Clarke's Nucleus, Rostral (T4) and Caudal (T13) to the Lesion

<table>
<thead>
<tr>
<th>Group</th>
<th>CELL NUMBER</th>
<th></th>
<th></th>
<th>CELL AREA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rostral to Lesion (T4)</td>
<td>Caudal to Lesion (T13)</td>
<td>Rostral to Lesion (T4)</td>
<td>Caudal to Lesion (T13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exp. Side</td>
<td>Control Side</td>
<td>Exp. Side</td>
<td>Control Side</td>
<td>Exp. Side</td>
<td>Control Side</td>
</tr>
<tr>
<td>Hemisection at PD5-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±</td>
<td>35.33</td>
<td>36.17</td>
<td>164.17</td>
<td>286.67(^A)</td>
<td>466.08</td>
<td>471.78</td>
</tr>
<tr>
<td>S.E.M. (n=6)</td>
<td>±3.25</td>
<td>±4.07</td>
<td>±31.56</td>
<td>±20.14</td>
<td>±39.06</td>
<td>±46.11</td>
</tr>
</tbody>
</table>

\(^1\) Taken from every 6th section
\(^A\) Control significantly higher than experimental (Exp.), by paired t-test (t=6.85, df=5, p<.01)
\(^B\) Control significantly higher than experimental (Exp.), by paired t-test (t=4.66, df=5, p<.01)
FIGURE 19

The mean numbers of labeled neurons in CN on the experimental (Exp.) and Control sides (+ standard error of the mean) at T4, rostral to the lesion, and at T13, caudal to it, are provided in A and B. Histograms of the size (µm²) distributions of labeled neurons in CN rostral (T4) and caudal (T13) to the lesion are shown in C and D. Counts and sizes were taken from one out of every sixth section and only those labeled neurons exhibiting a visible nucleus were measured.
A. Number of Labeled Neurons in CN at T4

B. Number of Labeled Neurons in CN at T13

C. Size of Labeled Neurons in CN at T4

D. Size of Labeled Neurons in CN at T13
FIGURE 20

The gross brain and spinal cord of an animal subjected to hemisection of the T9 level of the spinal cord on PD13 and bilateral injections of Fast-Blue into the cerebellum (double arrows) 99 days later are shown in A. The middle arrow points to the level of the lesion (les) shown in C and the lines rostral and caudal to it indicate the levels shown in B and D. In the section rostral to the lesion (B), the open arrows indicate labeled neurons in CN and the solid arrows indicate the location of the DSCT. The asterisk indicates the score used to identify the side contralateral to the lesion. A darkfield photomicrograph of a section through the lesion is shown in C and the arrow indicates the ventral median fissure. The ventral (VF) and lateral funiculi (LF) are also indicated and the asterisk marks the side opposite the lesion. A section caudal to the lesion (T13) is shown in D. The open arrows in D point to CN and the asterisk indicates the score marking the side opposite the lesion. The scale bar in B can be used for C and D.
FIGURE 20
FIGURE 21

A portion of the spinal cord containing the lesion site (arrow) in an opossum subjected to a lesion of the thoracic cord (T9) on PD26 and bilateral injections of Fast-Blue into the cerebellum 89 days later is shown in A. A myelin stained section slightly rostral to the full extent of the lesion is shown in B. The asterisk indicates the side opposite the lesion which is similarly indicated in C and D. Myelin stained sections through the full extent of the lesion and slightly caudal to it are provided in C and D. The ventral median fissure is indicated by the arrows in B and D and the substantia gelatinosa (SG) is labeled in B and D. The scale bar in B can be used for C and D.
FIGURE 22

A photomicrograph of a Nissl stained section of spinal cord (T13) from an animal subjected to lesioning at T8 on PD13 and injections of Fast Blue into the anterior cerebellum 95 days later is shown in A. Labeled neurons were not found caudal and ipsilateral to the lesion prior to removing the coverslip and staining, although they were found on the contralateral (control) side. After staining, however, small cells could be found within the area judged to be CN on the side of the lesion (open arrows). CN is outlined on the side opposite the lesion. The photomicrograph in B shows another section from the same case which contains a few relatively large neurons in CN caudal and ipsilateral to the lesion (arrows). As in A, CN is outlined on the contralateral side. The dorsal funiculus (DF) and central canal (CC) are indicated. The scale bar in A can be used for B.
CHAPTER 3

DEVELOPMENTAL PLASTICITY OF SELECTED SPINOCEREBELLAR AXONS IN THE NORTH AMERICAN OPOSSUM: GROWTH THROUGH A LESION OF THEIR SPINAL PATHWAY AND INNERVATION OF APPROPRIATE AREAS OF THE CEREBELLUM

Abstract

In the North American opossum, axons which originate in Clarke's nucleus (CN), i.e., axons of the dorsal spinocerebellar tract (DSCT), grow through a lesion of their spinal pathway at PD5-8, but not at PD12 or at later ages. Spinocerebellar axons originate throughout the spinal cord, however, and differ in their developmental history, laterality, funicular position, course, and terminal distribution. It is possible, therefore, that differences also exist in their ability to grow through a lesion and the critical periods for such growth. In the present study, we asked if spinocerebellar axons of the ventral spinocerebellar tract (i.e., axons originating from spinal border cells [SBC’s]), as well as those originating within the sacral/coccygeal ventrolateral nucleus (VLN), or Stilling's nucleus (SN) grow through a lesion and, if so, whether the critical period(s) for such growth is the same as that for axons of the DSCT. In
addition, we have asked if spinocerebellar axons grow through a lesion after transection of the thoracic cord and, if so, whether they innervate appropriate areas of the cerebellum.

To determine if spinocerebellar axons originating within SBC's, neurons within the VLN, or neurons within SN grow through a lesion of their spinal pathway, pouch-young opossums were anesthetized and subjected to hemisection of the mid-thoracic cord on the right side at PD5. One to nine months later, bilateral injections of Fast Blue (FB) or Fluoro-Gold were made into the anterior lobe of the cerebellum, the major target of spinocerebellar axons. In all cases, neurons were labeled bilaterally within the SBC region, within SN, and within the VLN. Since the cerebellar projection from each nucleus is contralateral, labeled neurons contralateral to the hemisection must have supported axons which grew past the lesion to reach the cerebellum. Examination of sections through the lesion site revealed that reconstitution of recognizable spinal cord had occurred at the lesion site and labeled axons were found within it in their normal position. When similar lesions were made on PD12 or at later ages, and the animals were allowed to survive for 1-4 months prior to injections of FB, labeling was not present in SBC's, SN, or the VLN contralateral to the lesion although it was present ipsilateral to it.

To determine if spinocerebellar axons grow through a spinal transection and innervate appropriate areas of the cerebellum, opossum pups were subjected to transection of the thoracic cord at PD5 and injections of FB into the lower thoracic cord one or six months later. In all cases, labeled axons were present rostral to the
lesion and they terminated within 5 bands in the anterior lobe of the cerebellum. In the animals allowed to survive 1 month after transection, the posterior cerebellum was also examined and labeled axons were found within it which terminated within three zones. These results were similar to those obtained after similar injections in age-matched unlesioned controls, although the zones of labeling were smaller in width and less densely packed with labeled axons. In animals lesioned at PD12 and subjected to injections of FB one month later, labeling was not visible in the cerebellum.

Our results suggest that spinocerebellar axons supported by SBC’s, neurons within SN, and neurons within the VLN grow rostral to a lesion of their spinal pathway during early development. The critical period for such growth is comparable to that reported previously for spinocerebellar axons originating within Clarke’s nucleus and for axons of the fasciculus gracilis and the anterolateral system, but shorter than that for most descending spinal axons. It appears, therefore, that differences exist in the ability of ascending and descending axons to traverse a lesion of their spinal pathway during development. Interestingly, the end of the critical period does not correlate with the appearance of myelin. Our results also indicate that spinocerebellar axons which grow through the lesion after transection of the thoracic cord reach those areas of the cerebellum that they normally innervate.
Introduction

Axons fail to grow beyond the lesion after a hemisection or transection of the spinal cord in adult mammals (Larner et al. 1995), but at least some of them do when the lesion is made early in development (see reviews by Martin et al. 1994 and Nicholls and Saunders, 1996). In the North American opossum, Didelphis virginiana, supraspinal axons grow around (Martin and Xu, 1988; Xu and Martin, 1989; Wang et al, 1994) or through (Martin et al, 1994; Wang et al, 1996ab) a lesion of the thoracic cord when it is made prior to postnatal day (PD) 30, but not when it is made at older ages. Axons which grow beyond the lesion find their appropriate targets (Xu and Martin, 1989; Wang et al, 1996b) and when the lesion is made early enough in development they support relatively normal use of the hindlimbs in locomotion (Basso et al, 1996).

Interestingly, the end of the critical period for growth through a lesion is not the same for all axons. For example, the critical period for lateral vestibulospinal and certain pontine reticulospinal axons ends before PD12, yet that for rubrospinal and raphespinal axons continues until PD30 (Xu and Martin, 1989; Wang et al, 1994; Wang et al, 1996ab). Our results indicate that axons which reach the spinal cord first during development lose the ability to grow around or through a lesion before axons which arrive at a later date. It is likely, therefore, that diminished ability to initiate and/or support axonal growth with age is a contributing factor to loss of developmental plasticity. However, there is a temporal correlation between the end of the critical
period for developmental plasticity of descending spinal axons and the development of environmental factors which are known to inhibit axonal growth (Ghooray and Martin, 1993abc). In fact, a cause and effect relationship between loss of developmental plasticity and the appearance of myelin has been established for the chick (Keirstead et al, 1992; Keirstead et al, 1995).

Although growth through or around a lesion of the spinal cord has been documented for axons of most, if not all, descending spinal pathways in developing mammals, it has only been described for a limited number of ascending axons. Using the North American opossum, we have shown that axons which originate in Clarke's nucleus (CN), i.e., axons of the dorsal spinocerebellar tract (DSCT), grow through a lesion of their spinal pathway when it is made between PD5-8, but not when it is made at PD12 or at later ages (see Chapter 2; Terman et al, 1996a). Spinocerebellar axons differ, however, in origin (see Chapter 1; Matsushita and Hosoya, 1979; Matsushita et al, 1979), developmental history (Martin et al, 1983; Arsenio Nunes and Sotelo, 1985; Yaginuma, 1987; Payne and Bower, 1988), laterality (see Chapter 1; Matsushita and Hosoya, 1979; Matsushita et al, 1979), funicular position (see Chapter 1; Shirao, 1987; Yamada et al, 1991; Xu and Grant, 1994), peduncular course (Hazlett et al, 1971; Grant and Xu, 1988; Yamada et al, 1991), and terminal distribution (see Chapter 1; Kitamura, 1988; Xu and Grant, 1988; Berretta et al, 1991). It is possible, therefore, that differences also exist in their ability to grow through or around a lesion of their spinal pathway. In the present study, we’ve asked if spinocerebellar axons originating from spinal border cells (SBC’s; i.e., those in the ventral spinocerebellar
tract [VSCT]) (see Chapter I; Cooper and Sherrington, 1940; Snyder et al. 1978), neurons within the sacral/coccygeal ventrolateral nucleus (VLN) (see Chapter 1: Snyder et al. 1978; Matsushita and Hosoya, 1979), or neurons within Stilling's sacral nucleus (SN) (see Chapter I; Stilling, 1859; Chang, 1951; Petras, 1977) are capable of such growth and, if so, whether the critical period(s) for it is the same as that for axons of the DSCT. We have also asked whether axons which grow through the lesion after transection of the thoracic cord reach areas of the cerebellum they normally innervate. In the adult opossum, spinocerebellar axons terminate within 5 zones in the anterior lobe of the cerebellum and 3 zones of the posterior lobe (Hazlett et al. 1971). Our results indicate that axons originating from SBC's, the VLN, and SN grow through a lesion of their spinal pathway and that the critical period for such plasticity is similar to that documented previously for DSCT axons. In addition, it appears that spinocerebellar axons grow through the lesion after transection of the thoracic cord and that they innervate areas of the cerebellum that are appropriate for them.
Materials and Methods

I. Studies Designed to Determine If Spinocerebellar Axons from SBC's, the VLN, or SN Grow Beyond a Lesion of Their Spinal Pathway

Some of the animals used in this study have been employed previously and a detailed description of the methods can be found elsewhere (Terman et al. 1996a: see Chapter 2). Opossums with appropriately aged young (PD5 (N=7), PD8 (N=4), PD12 (N=8), PD20 (N=2), and PD26 (N=2)) were anesthetized with ketamine (100mg/ml) and Metafane and placed in a supine position to expose their pouch. During anesthesia, the pouch sphincter relaxes making it possible to anesthetize the pups by hypothermia. The spinal cord of the anesthetized pups was exposed surgically and hemisected at mid-thoracic levels using a microblade (Beaver). The lesion was made by inserting the microblade slightly to the left of the midline and pulling it to the right until the right half of the cord was severed. To ensure that the hemisection was complete, a microdissection needle with a 90° angle was drawn through the lesion cavity. In addition, 3 of the PD5 pups were sacrificed shortly after lesioning and examined for the presence of a complete hemisection. In these cases the spinal cord was removed after perfusion and the lesioned segment was embedded in paraffin, sectioned at 20μm in the frontal plane, and stained for Nissl substance. In each of these animals the hemisection was obvious grossly and histologically. The remaining animals were maintained 4-9 months before being anesthetized using sodium
pentobarbital (40mg/kg) for bilateral injections of 5% Fluoro-Gold (FG) or 2% Fast Blue (FB) into the anterior lobe of the cerebellum, the primary target of spinocerebellar axons (Hazlett et al. 1971). No differences have been observed in our laboratory between the ability of FB or FG to retrogradely label neurons (unpublished results: see Chapter 1 and 2). Following the injections, the craniotomy was closed, and the animals were treated for post-operative pain with subcutaneous injections of Buprenorphine (0.028 mg/kg). After a 7-10 day survival, each animal was reanesthetized and sacrificed by perfusion so its brain and spinal cord could be removed and sectioned using a freezing microtome. Every section of the spinal cord caudal to the lesion site was examined for labeling of SBC’s (lower thoracic and upper lumbar levels), neurons in the VLN (sacral and coccygeal levels), and neurons in SN (sacral and coccygeal levels). Since the cerebellar projections of these nuclei are crossed in the opossum (see Chapter 1), as in other species (Snyder et al. 1978; Matsushita et al. 1979; Matsushita and Hosoya, 1979), labeled neurons contralateral to the lesion were considered to have supported axons which grew through or around the lesion to the cerebellum. Labeled neurons ipsilateral to the lesion were used as internal controls. In addition, the cerebellum and the spinal cord rostral to the lesion were sectioned and examined for symmetry of the injections and labeling. In four of the cases lesioned at PD5, profiles of labeled neurons in each of the above mentioned nuclei were counted in every sixth section on the experimental and control sides.
Results were expressed as the mean ± the standard error of the mean and analyzed using the paired t-test for significant differences in the number of labeled neurons on the two sides.

II. Studies Designed to Determine If Spinocerebellar Axons Grow Through the Lesion after Transection of the Thoracic Cord and Reach the Appropriate Areas of the Cerebellum

Opossums with appropriately aged pouch-young (PD5 [N=18]; PD8 [N=8]; PD12 [N=7]; PD20 [N=8]) were anesthetized so that the pups could be prepared for surgery as described in section I. The spinal cord of the pups was transected at mid-thoracic levels with a microblade (Beaver) angled at 15°. To ensure that the cord was transected, the microblade and then a microdissection needle with a 90° angle were passed through the cord several times. In addition, 4 of the PD5 pups were sacrificed shortly after lesioning and examined for the presence of a complete transection. In these cases the spinal cord was removed after perfusion with 10% formaldehyde, sectioned on a freezing microtome at 20µm in the sagittal plane, and stained for Nissl substance. The remaining animals were split into two groups based on their length of survival after the transection.
One group of animals (PD5 [N=4]; PD8 [N=5]) was allowed to survive for six months prior to bilateral injections of FB into the T13 level of the spinal cord. FB is best known as a retrograde tracer, but it is also labels axons which originate at the injection site and probably axons which pass through it (Qin et al. 1993, Wang et al. 1997a). It was our objective to label spinocerebellar axons which originate caudal to the lesion to determine if they grow through it and, if so, whether they innervate appropriate areas of the cerebellum. All animals were anesthetized with sodium pentobarbital as described in section I so that the spinal cord could be exposed surgically. The vertebral column was stabilized in a stereotaxic frame and injections of 3μl of FB were made on each side in an attempt to fill the spinal cord at that level. Injections were made with a glass micropipette attached to a 1μl syringe (Hamilton) as described above. The surgical incision was then closed by suturing the deep back muscles and skin, and the animals were treated for postoperative pain as described above. All animals were sacrificed after 7 days by being reanesthetized with sodium pentobarbital and perfused transcardially with physiological saline and a 0.1 M citrate buffered - 10% formaldehyde solution. Following fixation, the spinal cord and brain were removed, photographed, and immersed in a 30% sucrose-citrate buffer solution for approximately 24 hours at 4°C.

All brains and spinal cords were scored so that the laterality of the tissue sections could be determined after mounting. Frozen sections through the injection and lesion sites were cut at 40μm in the coronal plane and processed as described above. Once it was determined that FB had not spread to the level of the lesion, the
rest of the spinal cord and brain was sectioned. In some cases, sections through the lesion site were stained for Nissl substance to help clarify the cytoarchitecture within it. The cerebellum was examined for labeled axons and they were documented on plots using an image analysis software program (Neurolucida, MicroBrightField) connected by a video camera (Optronics) to a Leitz fluorescence photomicroscope (Orthoplan 2) equipped with the A cube (excitation wavelength 340-380 nm) of the Ploem illumination system. Labeled axons were also documented photographically in selected sections. In addition, 3 unlesioned adult controls were injected with FB into the T13 level of the spinal cord and their brains and spinal cords were processed as described above. Labeling in such cases was compared with that present in the experimental cases.

The second group consisted of animals (PD5 [N=10]; PD8 [N=3]; PD12 [N=7]; PD20 [N=8]) that were allowed to survive for only 30-40 days after transection. After an appropriate survival, the mothers with lesioned pouch young were reanesthetized as described above. The pups were subsequently anesthetized by hypothermia so that their spinal cord could be exposed for bilateral injections of 0.1-0.2μl of 2% FB approximately four segments caudal to the site of transection. The operated animals were returned to the vivarium with their mothers for 3-7 days before being killed by an overdose of anesthetic and perfused transcardially with a 0.1M citrate buffered - 10% formaldehyde solution as described above. The spinal cord and brain were removed, photographed, and processed as described above. In addition.
unlesioned age-matched controls in the PD5 age group (N=6) were subjected to injections of FB into the spinal cord as described for the experimental cases. The brain and spinal cords of these animals were processed in a similar manner to the experimental cases.
Results

I. Studies Designed to Determine If Spinocerebellar Axons from SBC's, the VLN, or SN Grow Beyond a Lesion of Their Spinal Pathway

When the thoracic cord was hemisected on PD5 or PD8 and bilateral injections of FB or FG were made into the cerebellum 4-9 months later, labeling was present bilaterally within SBC’s, within neurons of the VLN, and within neurons of SN. The results from one case are shown in Fig. 23. The site of the lesion was not evident grossly (Fig. 23A, arrow) and after sectioning, recognizable spinal cord was present on the lesioned side (Fig. 23C; right side) although it was abnormal in appearance. Observed abnormalities included fusion of the dorsal horns, decrease in the size of the dorsal columns, an uncharacteristic appearance of the grey matter, and a relative thinness of the white matter on the lesioned side. Examination of the injection sites and the presence of bilaterally symmetrical labeling of CN rostral to the lesion (open arrows, Fig. 23B) suggested that the injections were comparable on the two sides. Caudal to the lesion, bilateral labeling was found in SBC’s (Fig. 23D, closed arrows), neurons of the VLN (Fig. 23E, arrows), and neurons of SN (Fig. 23F, arrows). Counts from every sixth section revealed that labeled profiles were fewest in number contralateral to the lesion and the results were statistically significant (P<0.01; data not shown). It will be recalled that neurons in each of the above nuclei project contralaterally to the cerebellum (Chapter 1). Note that bilateral labeling was also
present in CN caudal to the lesion (Fig. 23D, open arrows) and that labeled neurons were fewest ipsilateral to the lesion (right side) (see Chapter 2: Terman et al. 1996a). It will be recalled that the DSCT projects ipsilaterally to the cerebellum.

Following hemisection of the mid-thoracic spinal cord on PD12 or at later stages of development and bilateral injections of FB or FG into the cerebellum 4-6 months later, labeling was not found in SBC's, within neurons of the VLN, or within neurons of SN contralateral to the lesion, although it was present in neurons within the same areas on the ipsilateral side. The results from a PD12 case are documented in Fig. 24. The lesion site was identifiable grossly (Fig. 24A, arrow) and histologically it was seen to include over half the cord (Fig. 24C). Examination of the injection sites and labeling in CN rostral to the lesion (open arrows, Fig. 24B) suggested that the injections were symmetrical bilaterally. Caudal to the lesion, labeling in SBC’s (Fig. 24D, closed arrows), the VLN (Fig. 24E, arrows), and SN (Fig. 24F, arrow) was only present ipsilateral to the lesion. As expected (see Chapter 2: Terman et al. 1996a), labeling in CN caudal to the lesion (Fig. 24D, open arrow) was limited to the side contralateral to the lesion.

In all of the PD5 and 8 cases, labeled spinocerebellar axons were present in their normal position ipsilateral to the lesion in what appeared to be regenerated spinal cord at the lesion site (arrows, Fig. 23C; Fig. 25B), as well as in normal spinal cord rostral (arrows, Fig. 23B), and caudal (not seen at this magnification) to it. Although they were not counted, labeled axons ipsilateral to the lesion (Fig. 23B and C; Fig. 25B) appeared to be fewer in number than those on the contralateral side (Fig. 23B and
A few labeled axons also were present rostral and ipsilateral to the lesion in the PD12 cases. Such axons likely originated rostral to the lesion, however, since axonal labeling was not found at the lesion site or caudal to it on the ipsilateral side.

II. Studies Designed to Determine If Spinocerebellar Axons Grow Through the Lesion after Transection of the Thoracic Cord and Reach Areas of the Cerebellum That Are Appropriate for Them

A portion of the spinal cord, including the lesion (arrow), from a PD5 pup subjected to transection of the spinal cord at mid-thoracic levels and sacrifice immediately afterwards is shown grossly in Fig. 26A. Histologically, it can be seen that the lesion was complete (Fig. 26B, arrow). When the spinal cord was transected at PD5 using the same method and the animals were allowed to survive 6 months, the lesion was difficult to identify grossly (Fig. 26C, arrow) and histologically it contained recognizable spinal cord (Fig. 26D). It should be noted, however, that spinal cord tissue at the lesion site was abnormal in appearance (Fig. 26D). Observed abnormalities included a thin and poorly differentiated grey matter and a dorsally positioned central canal (Fig. 26D, arrow).

In all cases lesioned on PD5 and subjected to injections of FB into the spinal cord at T13, 6 months later, labeled axons were present within the cerebellum. Figures 27 and 28 illustrate the results from the case whose lesion site is shown in Figs. 26C and 120.
D. Examination of sections through the injection site and sections caudal to the lesion revealed that FB filled the cord at T13 (Fig. 27A) and traces of FB could be found two segments rostrally. It was clear, however, that FB did not reach the lesion site. Labeled axons were present caudal to the lesion and they could be followed through it into the rostral spinal cord, brainstem, and cerebellum. Fig. 27B documents labeled spinocerebellar terminals (mossy fiber rosettes) within the anterior lobe of the cerebellum (e.g., open arrows) and Fig. 27C illustrates a single terminal at higher power within an adjacent section. The labeled mossy fiber endings were restricted to specific zones of the granule cell layer (a zone of labeled terminals is delineated by the arrowheads in Fig. 27B). Five such zones of terminal label were found within the anterior lobe (Fig. 28) and they were similar in location to those present in the unlesioned age-matched controls. In the control cases, however, the zones of label appeared to be wider and more densely filled with labeled terminals. Such differences were more easily documented in the PD5 transected animals allowed to survive for only 30 days (see below). Labeled terminals were also present within the cerebellar nuclei in experimental and control cases (not shown).

Labeled axons were also present in the cerebellum in the PD5 transected cases which received injections caudal to the lesion 30 days later. Figs. 29 and 30 depict the results seen in one case. The brain and spinal cord, including the site of the lesion (LES) and injection (INJ), are shown grossly in Fig. 29A. Our injections were large and bilaterally symmetrical (Fig. 29B), but they did not spread to the lesion site. The lesion was difficult to identify grossly (Fig. 29A, LES) but it was readily apparent.
histologically (Fig. 29C). Although recognizable spinal cord was present in some sections through the lesion (Fig. 29C), it was abnormal in appearance and easy to distinguish from normal appearing spinal cord rostral (Fig. 29D) or caudal it. Note the poor differentiation of the grey matter and the abnormally positioned central canal (Fig. 29C). Labeled axons were present caudal to the lesion site which could be followed through it and into the rostral spinal cord and brainstem. Labeled axons also were present within the cerebellum (Figs. 29E and F) where they terminated within 5 distinct zones of the anterior lobe (Fig. 30A) and three zones of the posterior lobe (Fig. 30B). The labeling pattern was similar to that in the control cases (Figs 31A and B), but the zones of terminal label were not as wide or as densely packed (compare Figs. 30 and 31). As in the cases allowed to survive 6 months following transection, labeled terminals were present within the cerebellar nuclei (not shown).

In the cases transected at mid-thoracic levels on PD8, or at later stages of development, and allowed to survive 6 months prior to injections into the T13 level of the spinal cord, labeled axons were not seen within the cerebellum. In all cases the injections filled the T13 level of the cord, however, and labeled axons were observed caudal to the lesion. When the injections were made only 30 days after transection on PD8, a few labeled axons could be traced into the cerebellum. When the lesion was made at PD12, or at later ages, however, and the injections were made 30 days later, cerebellar labeling was not found. In the latter cases, the injections filled the T13 level of the cord and axons were labeled caudal to the lesion.
Discussion

Our results indicate that in the opossum, axons originating from SBC’s, the VLN, and SN, like those which originate in CN, grow through a lesion of their spinal pathway and reach the cerebellum if the lesion is made early enough in development. In addition, we have shown that spinocerebellar axons which grow through the lesion after transection of the thoracic cord terminate within appropriate zones of the cerebellum. It is unlikely that our lesions were incomplete in the PD5-8 cases even though recognizable spinal cord was present at the lesion site. Evidence for a complete hemisection or transection included the abnormal appearance of the spinal cord at the lesion site in the animals that survived for 1 - 9 months and the obvious presence of the appropriate lesion in the PD5 pups sacrificed shortly after surgery. Furthermore, when lesions were made at PD12 or at later ages using the same technique, it was clear that they were complete.

As described previously (Martin et al, 1994; Terman et al, 1994; 1996a; Wang et al, 1996ab), reconstruction of recognizable spinal cord occurs at the lesion site after hemisection or transection of the thoracic cord in the PD5 opossum. Comparable results have been reported after crush lesions of the cervical cord in the South American opossum, Monodelphis domestica (see review, Nicholls and Saunders, 1996). Regeneration of damaged spinal cord is well documented in non-mammalian vertebrates and it is apparently initiated by proliferation of ependymal cells which bridge the gap at the lesion site (see review, Larner et al, 1995). The mechanisms
which underlie spinal cord reorganization in the developing opossum are not known, nor is it known why it no longer occurs at PD12 or at later ages. It is possible, however, that similar mechanisms occur. For example, shortly after injury in early postnatal and adult rats, some of the cells which line the central canal become mitotic (see reviews by Bruni et al. 1985; Del Bigio, 1995). This response is transient, however, and there is little migration and reconstitution of new tissue. These findings suggest that mammalian ependyma contain cells capable of proliferation if the appropriate stimulus is present. Indeed, in vitro studies have suggested that cells in the spinal cord and brain of postnatal and adult mammals are able to enter a germinal state under the right conditions (Reynolds and Weiss, 1992; Weiss et al., 1996; McKay, 1997; Kehl et al., 1997). These conditions may be present during early development in the opossum.

Following hemisection or transection of the thoracic cord on PD5, spinocerebellar axons were labeled in their normal positions at the lesion site. It appears, therefore, that the reconstructed cord provides a favorable substrate for axonal growth and that it contains the cues necessary for growing axons to find their normal position. However, it is not known whether growth through the lesion results from regeneration of cut axons, growth of late arriving axons that were not damaged by the lesion, or both. Both mechanisms contribute to extension of descending axons through the lesion site when the spinal cord is injured during early development in the chick (Hasan et al., 1993) and opossum (Wang et al., 1997b).
Our results indicate that spinocerebellar axons which grow through a lesion innervate the appropriate areas of the cerebellum. In the mammals studied to date, spinocerebellar axons terminate within longitudinal zones of the cerebellum (for review, see Bloedel and Courville, 1981). In the opossum, five such zones are present in the anterior lobe and three are found in the posterior lobe (Hazlett et al, 1971; present study). The results of the present study also suggest that when the spinal cord is transected at PD5 and injections of FB are made caudal to the lesion 1 to 6 months later, spinal axons can be labeled in their appropriate zones in the anterior and posterior lobes. The ability to grow around or through a spinal lesion during early development and to innervate appropriate areas has also been documented for a number of descending spinal axons (Bernstein and Stelzner, 1983; Kalil, 1988; Xu and Martin, 1989; Wang et al, 1996ab) and, more recently, for other ascending axons (fasciculus gracilis, Wang et al. 1997a). The fact that the cerebellar zones innervated by spinocerebellar axons are smaller in width and less densely labeled in experimental animals than in controls suggest that fewer axons grow through the lesion site and into the cerebellum after transection of the cord than are present normally. This suggestion is supported by the observation that fewer spinocerebellar neurons are labeled caudal to the lesion on the experimental sides than on the unlesioned (control) side following a hemisection of the thoracic cord and bilateral injections into the cerebellum (see Chapter 2; Terman et al, 1996a; present study). It remains to be seen if other axons innervate those parts of the zones which are deinnervated by spinal lesions and, if so, whether they compete with spinal axons for postsynaptic targets (e.g., see Pickel et al,
It is possible, of course, that granule cell death occurs after deinnervation and that axons which grow through the lesion find fewer postsynaptic targets. If so, some of them may degenerate. In any case, it should be noted that animals lesioned at PD5 exhibit remarkably normal use of the hindlimbs in locomotion as adults (Basso et al., 1996). In addition, recent studies in the rat have reported that mossy fibers terminate in transversely oriented patches as well as in the longitudinally oriented zones that have been classically described (Tolbert et al. 1993; Alisky and Tolbert, 1997). The existence of these patches has not been verified in the opossum and it remains to be seen if they exist in our experimental animals.

Major differences were not found in the critical periods for developmental plasticity of the spinocerebellar axons studied. In contrast, clear differences exist for descending axons (Xu and Martin, 1989; Wang et al. 1994; Wang et al. 1996ab). For example, lateral vestibulospinal and certain pontine reticulospinal axons do not grow through a lesion when it is made on PD12 or later, but rubrospinal and raphespinal axons do. In fact, some rubral and raphe axons grow through such a lesion when it is made as late as PD30.

The critical period for developmental plasticity of spinocerebellar axons in the opossum is shorter than that for many descending spinal axons. Spinocerebellar axons no longer traverse the lesion site after hemisection or transection of the thoracic cord on PD12 (Terman et al., 1996a; present results); but, as noted above, some supraspinal axons do even when the lesion is made as late as PD30 (Wang et al., 1996ab). The critical period for developmental plasticity of axons within the fasciculus gracilis and
the anterolateral system, other ascending spinal pathways, also ends before PD12 in the opossum (Terman et al., 1996b; 1997a; Wang et al., 1997a). It is possible, therefore, that differences exist in the ability of ascending and descending axons to grow beyond a lesion which transects them. Such differences also have been reported for reptiles (Duffy et al., 1990; Duffy et al., 1993), frogs (Forehand and Farel, 1982; Campbell et al., 1984; Clarke et al., 1986; Beattie et al., 1990), salamanders (Stensaas, 1983; Davis et al., 1990), fish (Bunt and Fill-Moebs, 1984; Becker et al., 1997), and lampreys (Yin and Selzer, 1983; Croop et al., 1987), but the mechanisms which underlie them have not been elucidated.

Loss of developmental plasticity may be due to a diminished ability to initiate and/or sustain axonal growth with age (e.g., Fawcett, 1992; Chen et al., 1995; Li et al., 1995; Rossi et al., 1995; Shewan et al., 1995). That hypothesis is supported in the opossum by the finding that supraspinal axons which reach the spinal cord first lose the ability to grow through or around a lesion before those which arrive at a later date (Xu and Martin, 1989; Wang et al., 1994; Wang et al., 1996ab). Such changes may involve the downregulation of growth promoting molecules and/or receptors for growth promoting ligands as well as, but not limited to, the expression of receptors for inhibitory molecules. It is interesting, however, that the critical periods for developmental plasticity of different spinocerebellar axons appear comparable even though they apparently have different developmental histories (Martin et al., 1983; Arsenio Nunes and Sotelo, 1985; Yaginuma, 1987; Payne and Bower, 1988). Furthermore, DSCT axons retain the ability to regenerate even in adult rats, if a
Peripheral nerve is grafted into the lesion cavity (Richardson et al., 1984) and VSCT axons (i.e., axons of SBC's) grow through a transection of the thoracic cord in the early postnatal rat when embryonic tissue is provided as a substrate (Iwashita et al., 1994). It is likely, therefore, that the development of a non-permissive environment for axonal growth also plays a role in the loss of developmental plasticity of spinocerebellar axons. In the North American opossum, a temporal correlation exists between the appearance of myelin, which is known to contain proteins inhibitory to neurite extension (Caroni and Schwab, 1988; Schwab and Caroni, 1988; McKerracher et al., 1994; Mukhopadhyay et al., 1994), and the end of the critical period for plasticity of descending spinal axons (Xu and Martin, 1989; Ghooray and Martin, 1993; Wang et al., 1994; Wang et al., 1996ab). In fact, evidence for a cause and effect relationship has been provided for the chick (Keirstead et al., 1992; Keirstead et al., 1995). However, the critical period for comparable plasticity of spinocerebellar axons in the opossum (Terman et al., 1996a; present study) ends well before the appearance of myelin (Ghooray and Martin, 1993). If the development of a non-permissive environment is a factor in loss of spinocerebellar plasticity, myelin does not apparently play a role (see also MacLaren, 1996 and Lahr and Stelzner, 1990; Buyan Dent et al., 1996).

It appears, therefore, that the factors which result in loss of developmental plasticity are not the same for all axons. Indeed, it is becoming increasingly clear that differences exist in the ability of axons to grow in the same regions of the brain and spinal cord, and on similar substrates in vitro (e.g., Walter et al., 1987; Letourneau et al., 1992; Snow and Letourneau, 1992; Jhaveri, 1993; Nose et al., 1994; Bagnard et al., 1992).
1995: Colamarino and Tessier-Lavigne, 1995; Guillery et al, 1995; Messersmith et al, 1995; Tuttle et al, 1995). For example, in the brain and spinal cord, the floor plate has been reported to attract some axons to it, while steering others away (Colamarino and Tessier-Lavigne, 1995; Tamada et al. 1995). It is possible that similar mechanisms may also underlie the differences in the ability of ascending and descending spinal axons to grow through a lesion and the critical periods for such plasticity.
FIGURE 23

A photograph of a portion of spinal cord including the lesion site (arrow) from an animal subjected to mid-thoracic hemisection on postnatal (PD) 5 and bilateral injections of Fluoro-Gold into the cerebellum 6 months later is shown in A. A section of thoracic cord (T4) rostral to the lesion from the same case is illustrated in B. Note that labeling of Clarke’s nucleus (CN) (open arrows) was bilaterally symmetrical suggesting that the injections were comparable on the two sides. Labeled axons were found within the lateral and ventral funiculi bilaterally, although they appeared to be fewest in number on the side of the lesion (closed arrows). The asterisk delineates the side opposite the lesion in this section and in those which follow. A section through the lesion site is shown in C and labeled axons were seen on the side of the lesion (arrows, right side) in what appeared to be reconstructed spinal cord. A photomicrograph from the first lumbar section of the cord is shown in D. Notice that spinal border cells (SBC’s) were labeled bilaterally (closed arrows). Labeled neurons were also present within CN on both sides (open arrows). A section from the second sacral segment of the cord is seen in E and it can be seen that labeled cells were present bilaterally in the sacral/coccygeal ventrolateral nucleus (VLN) (arrows). A section of coccygeal cord is seen in F and labeled cells were present bilaterally in Stilling’s nucleus (SN) (arrows). The scale bar in B applies to C-F.
FIGURE 24

A portion of the spinal cord including the lesion site (arrow) from an animal subjected to hemisection of the mid-thoracic cord on PD12 and bilateral injections of Fast Blue into the cerebellum 4 months later is shown in A. A photomicrograph of a section of thoracic cord rostral to the lesion (T4) from the same case is shown in B. Note that labeled cells were present in CN in relatively equal numbers bilaterally (open arrows) suggesting that our injections were symmetrical. The asterisk delineates the side opposite the lesion in this section and in those which follow. A section through the lesion is shown in C where it can be seen that over half of the cord was involved. The spared lateral funiculus (LF) and ventral funiculus (VF) are indicated. A section through the first segment of the lumbar cord is shown in D. Labeled SBC's (closed arrows) were present only ipsilateral to the lesion. In addition, labeling in CN (open arrow) was only present contralateral to the lesion. A section from the second segment of the sacral cord is shown in E. Labeled cells were only seen in the VLN (arrows) ipsilateral to the lesion. A section of coccygeal cord is shown in F. Labeled neurons in SN (arrow) were present only on the side of the lesion. The scale bar in Fig. B applies to Fig. C-F.
FIGURE 24
Fluorescence photomicrographs of labeled axons in a section through the lesion site from the case illustrated in Fig. 23. Labeled axons located contralateral to the lesion are shown in A, while those on the ipsilateral side are shown in B. Notice that labeled axons occupy the lateral and ventral funiculi on both sides, but that they appear less numerous on the lesioned side (B). The scale bar in A can be used for B.
The lesion site in a PD5 pup subjected to transection of the mid-thoracic cord and immediate sacrifice is shown grossly in A (arrow) and in a Nissl-stained, sagittally cut section in B (arrow). The spinal cord (SC), dorsal (D), caudal (C), and the vertebral body (V) are indicated. The animal was sacrificed shortly after lesioning so that the completeness of the lesion could be documented. The lesion site (arrow) from an animal sacrificed six months after a transection of the thoracic cord at PD5 is shown grossly in C and photomicroscopically in a transversally cut, Nissl-stained section in D. Although the site of the lesion was not identifiable grossly, its abnormalities were evident histologically. Observed abnormalities included the paucity of cells in the grey matter and the dorsal positioning of the central canal (arrow). The lateral (LF) and ventral (VF) funiculi are indicated on one side.
FIGURE 27

Fluorescence photomicrographs from an animal subjected to a transection of the mid-thoracic cord on PD5 and injections of FB into the T13 level of the spinal cord 6 months later. A section through the injection site is documented photomicrographically in A. The injections filled the spinal cord at this level and spread to adjacent segments but it did not reach the lesion site. A photomicrograph documenting labeled spinocerebellar terminals (mossy fiber rosettes; e.g., open arrows) within the anterior lobe of the cerebellum is provided in B. Note that labeled terminals are restricted to a limited zone of the granule cell (Gc) layer which is demarcated by the closed arrows. The molecular (Mk) and Purkinje cell (Pk) layers are indicated for reference. A high power photomicrograph from an adjacent section illustrating a labeled spinocerebellar axon (small arrows) and its terminal ending (large arrow) is shown in C.
A plot documenting the locations of labeled spinocerebellar terminals within the anterior lobe of the cerebellum from the case described in Fig. 27. Each labeled terminal (mossy fiber rosette) is indicated with a dot. Labeling was present within 5 zones (arrows) which appeared to be similar in location to those seen in unlesioned controls. Lobules I and VI are indicated.
FIGURE 29

Photomicrographs of selected sections from a case subjected to a transection of the mid-thoracic cord at PD5 and injections of FB into the lumbar cord 30 days later are shown in B-F. The brain and spinal cord from the same case are shown grossly in A. The site of the injection (INJ) and lesion (LES) are indicated. A fluorescence photomicrograph through the injection site is shown in B. A photomicrograph of a Nissl-stained section through the site of transection is shown in C and it can be compared with a section of normal cord from an adjacent segment in D. Note the poor differentiation of the grey matter and the abnormally positioned central canal in C (open arrow). The ventral median fissure is indicated with a closed arrow in both figures. Labeled axons terminating within the granule cell (Gc) layer of the anterior lobe of the cerebellum are illustrated in fluorescence photomicrographs in E and F. Note that they are present within distinct zones (arrows). The scale bar in C can be used for D and that in E can be employed for F.
Plots of labeled axons in the cerebellum from the case illustrated in Fig. 29 are shown in A (the anterior lobe) and B (the posterior lobe). The dots indicate labeled spinocerebellar axons in the granule cell layer, not mossy fibers rosettes, because the latter have not formed yet. Labeled terminals were located in 5 zones (arrows; A) within the anterior lobe and three zones (arrows; B) within lobule VIII of the posterior lobe. The zones of labeling were similar in location to those seen in the age-matched controls (Fig. 31), although they were narrower in width and less densely packed with labeled axons. The cerebellum (Cb) and inferior colliculus (IC) are indicated. The scale bar in Fig. 30 can be used for Fig. 31.
FIGURE 30
FIGURE 31

Plots of labeled terminals in the cerebellum of an unlesioned control subjected to injections of FB into the lumbar spinal cord at an age comparable to that of the case shown in Figs. 29 and 30. Orthogradely labeled axons in the granule cell layer are indicated as dots in the anterior (A) and posterior (B) lobes. As in the experimental cases, labeled axons occupied 5 zones (arrows; A) within the anterior cerebellum and three zones (arrows; B) in the posterior lobe. Note, however, that the labeled zones were wider and more densely packed with labeled axons than in the experimental case (compare with Fig. 30). The cerebellum (Cb) and inferior colliculus (IC) are indicated. The scale bar in Fig. 30 can be employed for Fig. 31.
FIGURE 31
SUMMARY AND CONCLUSIONS

The objective of the present studies was to characterize and better understand the ability of spinal axons to grow beyond a lesion of their spinal pathway. Previous results have shown that descending axons grow around and through a lesion of their spinal pathway but that ability is lost in an age dependent manner. Axons which innervate the spinal cord first, lose their potential for plasticity earlier than axons that grow into it at a later time. In addition, a temporal correlation appears to exist between the appearance of myelin, maturation of glia, and a glial response to lesioning (all of which are known to inhibit axonal growth) and the end of the critical period for plasticity. In the present results, however, and in those reported more recently for other ascending axons (Terman et al, 1996b, 1997a), such factors do not appear to be operative in loss of developmental plasticity of ascending axons. Ascending spinal pathways, like descending ones, develop asynchronously, so it might be predicted that differences would also exist in their critical periods. It appears, however, that the end of the critical period is comparable for all ascending axons.

It is intriguing that the ability of ascending axons to grow through a lesion ends so early in development. The spinal cord is relatively immature during this period and is still characterized by immature glia and growing axons. Ascending axons which do
not grow through a lesion after PD9-11 presumably have available the same extracellular terrain as axons that grow successfully (most descending axons). Most notably, ascending and descending axons which occupy similar areas of the cord and appear to be of comparable stages of maturation show differences in their ability to grow beyond a lesion. Rubrospinal (RS) and dorsal spinocerebellar tract (DSCT) axons serve as the best examples. RS and DSCT axons are very close to one another in the dorsolateral funiculus and are roughly comparable in developmental history (Cabana and Martin, 1984; Martin et al, 1991; Lakke and Marani, 1991; Martin et al. 1983; Arsenio-Nunes and Sotelo, 1985; Ashwell and Zhang, 1992; Qin et al. 1993). However, RS axons grow through a lesion of their pathway as late as PD30, while DSCT axons no longer do so by PD9-11. It seems reasonable to suggest that the factors which prevent DSCT axons from growing through a lesion during early development have little effect on RS axons.

It is important to consider, therefore, that all axons cannot be grouped together as to the cause of their inability to grow through a lesion. Unfortunately, this is rarely done. The next step is to better understand the reasons underlying these differences. The opossum, and in particular RS and DSCT axons, may provide a model in which to
do so. It is our hope that by illuminating these differences it will be possible to understand those factors that are necessary for successful regeneration and to meet the goal of enhancing regeneration and restoration of neurological function after spinal cord injury in man.
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