Spinocerebellar Projections to the Cerebellar Cortex in the Chicken

（ニワトリの小脳皮質への脊髄小脳路投射）

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General introduction

The most striking and unifying characteristic of all birds is their ability to fly. Since birds can fly freely in three dimensional spaces, birds possess a large cerebellum for outstanding motor regulation. The mammalian cerebellum consists of mediolaterally the central vermis and the lateral hemispheres and rostrocaudally the anterior lobe (folia I to V), the posterior lobe (folia VI to IX) and the flocculonodular lobe (folia X). Meanwhile, the avian cerebellum corresponds macroscopically to the vermis of the mammalian cerebellum. The auricle in the avian cerebellum consisting of the lateral parts of folia V through VIII has been suggested to correspond to the cerebellar hemisphere [Whitlock, 1952; Dubbeldam, 1998; Pakan et al., 2007). The avian vermis consists of ten folia as well as the mammalian vermis.

The mammalian direct spinocerebellar (SC) tracts bring impulses from the spinal cord to the cerebellar cortex. These tracts are originated from many SC neuron groups, which are basically divided into the dorsal and ventral SC neuron types. The dorsal SC neurons are located in the Clarke's column (CC) in the thoracic and rostral lumbar cord and give origin to uncrossed fibers of the dorsal SC tract. This ascends the dorsolateral funiculus and enters the cerebellum passing through the inferior cerebellar peduncle. The ventral SC neurons are located in the intermediate substance (laminas V, VI, VII) and the dorsolateral margin of the ventral horn (spinal border cells, SBC) in the lumbar to sacral cord and give origin to crossed fibers of the ventral SC tract. This ascends the ventrolateral funiculus and enters the cerebellum passing through the superior cerebellar peduncle. From a functional point of view both the ventral and dorsal SC tracts are concerned mainly with hindlimbs and trunk. The dorsal tract is believed to carry impulses concerned with fine co-ordination of muscle controlling posture, and with movements of individual muscle. On the other hand the ventral SC tract is concerned with movements of the limb as a whole regulating of the central pattern generator. The dorsal and ventral SC tracts have been known well and correspond with the hindlimb (Singh, 2006). The SC tracts responsible for the forelimb are the cuneocerebellar tract originating in the external cuneate nucleus (Cooke et al., 1971; Massopust et al., 1985) and the rostral SC tract originating in the nucleus centrobasalis in the lower cervical cord (Matsushita and Hosoya,

1979). The cuneocerebellar tract and rostral SC tract have been regarded as the dorsal and ventral SC tract types, respectively. Thus, SC neurons excluding the rostral SC tract neurons responsible for the four limbs are located in the more rostral segments than the CE or LSE.

In birds there are some studies on the distribution of SC neurons in pigeons (Necker, 1989, 1992) and chickens (Yamamoto et al., 2000, 2001). According to Yamamoto et al. [2000, 2001], chicken SC neurons are distributed throughout the entire length of the spinal cord. However, these neurons mainly aggregate in the CC, SBC and as ventral spinal border cells (VHv) located in the ventral margin of the ventral horn in both the cervical and lumbosacral enlargements (CE and LSE, respectively) and the ventral marginal nucleus (VMN) located on the ventral surface of the ventral funiculus only in the LSE. In contrast to mammalian SC neurons, the avian SC neurons for the extremities are located in the CE and LSE. This would be the reason why the CC, SBC and VHv are located in both the CE and LSE.

In mammals, there are many studies on SC projections. SC fibers terminate in bilaterally symmetrical, parasagittal bands of the granular layer in the vermis [Apps and Hawkes, 2009]. However, there are a few studies on the distribution of SC fiber terminals in the cerebellar cortex in birds [Vielvoye, 1970; Okado et al., 1987; Necker, 1992]. SC fibers terminate in parasagittal bands also in birds. However, it is not unclear whether these parasagittal bands change in number or pattern by origin of SC neurons and the SC tracts are divided into the dorsal and ventral SC tracts in chicks. Furthermore, it is also obscure whether SC fiber terminals originating from the LSE distribute in common in each lobule. This study aimed to give an answer to these questions.

Chapter 1

Spinocerebellar Prejections from the Cervical and Lumbosacral Enlargements in the Chicken Spinal Cord

Abstract

In birds, SC projections to the cerebellar cortex have not been understood well. We examined spinocerebellar (SC) fiber terminal fields originating from the cervical and lumbosacral enlargements (CE and LSE, respectively) in the chicken. SC fiber terminals show parasagittal bands in the granular layer. Labeled terminals from the CE were distributed primarily in folia II-V and IX. Parasagittal bands of labeled terminals from the CE were not clearly separated in folia II and III but were clearly separated in folia IV and V. In folium IX, labeled terminals were diffusely distributed in all subfolia with no evidence of banding. The numbers of bands were 5 in folium II, 12 in folium III and 7 in folia IV and V at maximum. Labeled terminals from the LSE were distributed primarily in folia II-VI and IX. Labeled terminals from the LSE were arranged in 4 bands in folium II and in 8 bands in folium III at maximum. Parasagittal bands from the LSE in folia IV and V were not clearly separated. In folium VI, the numbers of parasagittal bands was 6 at maximum. In folium IX, labeled terminals were mainly found in subfolium IXc forming 6-8 parasagittal bands. There were more parasagittal bands of labeled terminals from the CE than from the LSE. The topography of SC fiber terminals from the CE was different from that of SC fiber terminals from the LSE.

Introduction

In mammals, the majority of SC fibers project to the vermis of the cerebellum, which is therefore also termed the spinocerebellum. The avian cerebellum corresponds macroscopically to the vermis of the mammalian cerebellum, even though the lateral small parts of the avian cerebellum have been suggested to be homologous to the mammalian hemispheres [Whitlock, 1952; Pakan et al., 2007]. Since the avian cerebellum is well developed and largely corresponds to the spinocerebellum, the avian SC tracts may be significant for studies on the mammalian vermis.

There have been many studies on SC projections to the cerebellar cortex in mammals [Hazlett et al., 1971; Matsushita and Ikeda, 1980, 1987; Matsushita and Okado, 1981; Matsushita and Hosoya, 1982; Matsushita et al., 1984, 1985; Yaginuma and Matsushita, 1986, 1987, 1989; Matsushita and Tanami, 1987; Heckroth and Eisenman , 1988; Matsushita, 1988; Matsushita and Yaginuma, 1989; Voogd, 2003] and some studies in birds [Whitlock, 1952; Vielvoye and Voogd, 1977; Okado et al., 1987; Necker, 1989, 1992]. The mammalian cerebellar cortex is divided into the vermis, intermediate zone and hemispheres in a mediolateral direction. Furthermore, these longitudinal zones can be subdivided into multiple parasagittal bands based on climbing fiber projections, corticonuclear projections and various cytological markers such as zebrin [Hawkes and Herrup, 1996; Herrup and Kuemerle, 1997; Oberdick et al., 1998; Voogd, 2003]. The zonal organization of the cerebellar cortex is a highly conserved feature throughout vertebrates

[Voogd, 2003]. SC fiber terminal fields also show parasagittal bands in the granular layer, of which the boundary is ill-defined in comparison to climbing fiber terminal fields or antigenic parasagital bands such as zebrin II [Tolbert et al., 1993; Wu et al., 1999; Voogd et al., 2003; Pakan et al., 2010]. It is pivotal for elucidation of cerebellar function to determine whether parasagittal bands of SC fiber terminals have a close correlation to the Purkinje cell compartment. An investigation of spatial relationships of mossy fiber terminal fields to zebrin expression of Purkinje cells or climbing fiber terminal fields has progressed gradually [Matsushita et al., 1991; Ji and Hawkes, 1994, 1996; Gravel and Hawkes, 1990; Voogd et al., 2003; Pijpers et al., 2006; Pakan et al., 2010].

The topography of SC fiber terminals differs markedly different by spinal level of origin. Although there have been many studies on mammalian SC projections, these studies do not cover SC projections originating from all spinal segments (SS). Understandably, the parasagittal organization of SC projections to the avian cerebellum has been less well understood. Furthermore, Purkinje cell compartmentation as revealed by zebrin II has been reported recently in birds [Pakan et al, 2007; Iwaniuk et al., 2009; Marzban et al., 2010]. Thus, further data on SC projections in birds are wanting. In this study, we attempted to analyze the parasagittal organization of SC projections to the cerebellum originating from the CE and LSE in the chicken.

Materials and Methods

Six White Leghorn chickens at ages of 1.5-3 months were used for this study. The original research reported herein was performed under guidelines established by the Animal Care and Use Committee of Tottori University.

The animals were anesthetized with xylazine (8 mg/kg) followed by midazolam (2 mg/kg) and ketamine (25 mg/kg) and were placed in a stereotaxic apparatus. The CE or LSE (SS 14 or SS 26-28, respectively) was exposed by laminectomy. Pressure injections of 5% wheat germ agglutinin-horseradish peroxidase (WGA-HRP; Toyobo, Osaka, Japan) dissolved in physiological saline were made through a glass micropipette connected to a 1 μl Hamilton syringe. Injections of a total of 0.6 μl were made bilaterally into CE or LSE. In all cases, the gray matter of both sides was almost entirely labeled. After a survival period of 2-3 days, the animals were deeply anesthetized with ketamine (35 mg/kg) and pentobarbital sodium (20 mg/kg). After administration of heparin, perfusion was started sequentially with 500 ml of physiological saline, 500 ml of a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) and finally with 800 ml of 20% sucrose in the same buffer through the left ventricle. The cerebellum and the injected SS were removed, stored in the sucrose solution, embedded in 15% gelatin in sucrose solution and then hardened in 20% formalin in the sucrose solution for 12 h at 4°C. Each cerebellar folium was serially cut transversely at 60 μm and the injected SS was cut transversely at 90 μm with a freezing microtome (Komatsu Electron,

Japan). Every second section from the cerebellum and intermittent sections from the injection site were processed for visualizing HRP with tetramethyl benzidine according to Mesulam [1978]. Labeled terminals were plotted on a sheet with the aid of a drawing tube (BH2-DA, Olympus, Japan) and the number of labeled terminals was counted. A two-dimensional distribution of labeled terminals in each folium was reconstructed using Adobe Photoshop 5.0 Limited Edition (Adobe Systems, Tokyo, Japan).

Results

WGA-HRP was injected into a large portion of the gray matter in the CE and LSE (Fig. 1-1a). In the granular layer of the cerebellum, labeling of SC terminals was easily distinguished from labeled fiber fragments because of the coarse expansions of SC terminals (Fig. 1-1b). Figures 1-1c and d are a section of folia II and IX, respectively, which show SC terminal fields originating from the LSE. In folium IX, SC terminals were located in the deep portion of the granular layer. SC tracts are located in the lateral and ventral funiculi in the CE and in the ventral funiculus in the LSE. Uptake of tracer by SC fibers of passage would have been minor in the CE and negligible in the LSE because injections were made through the dorsal funiculi.

SC Projections originating from the CE

Few or no labeled terminals were seen in folia I, VI-VIII and X. Although there were more labeled terminals in folia III and V than in the other folia.

All transverse sections were observed in 3 cases. Typical results of these cases are shown in figures 1-2 and 1-3.

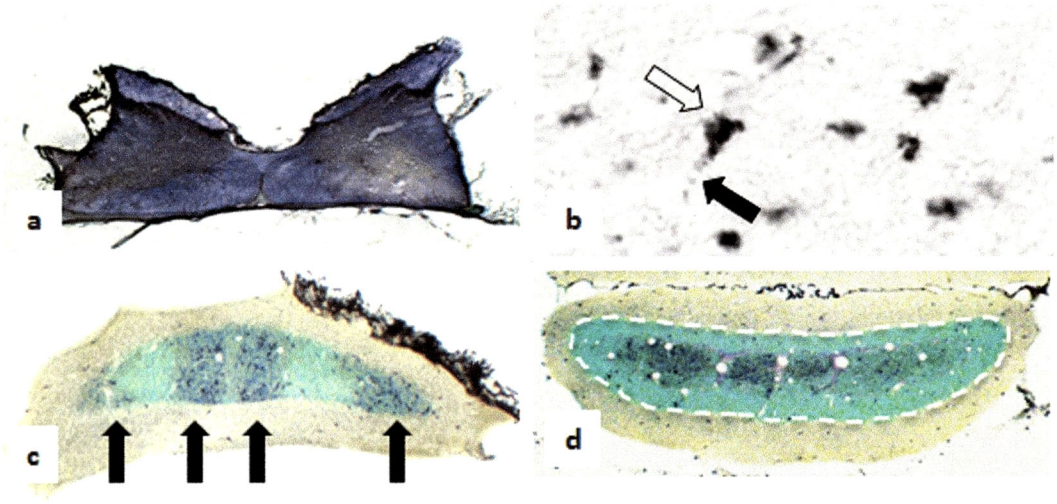

*Figure 1-1. The LSE is almost entirely labeled and a reaction product blurs a distinction between the gray and white matters (**a**). Labeled terminals (white arrow) showing coarse expansion (**b**). Labeled SC terminals are easily distinguished from labeled fiber fragments because of coarse expansions of SC terminals. Black arrow = labeled SC fiber. (**c**) A section of folium II showing 4 parasagittal bands (arrows) of SC terminals originating from the LSE. (**d**) A section of subfolium IXc. SC terminals are located in the deep portion of the granular layer. Dotted line = borderline between the granular and molecular layers.*

In folium II (Fig. 1-2a), labeled terminals were symmetrically arranged in the mediolateral extent as parasagittal bands, one on the midline and the second more laterally in the apical portion. In the intermediate portion of folium II, a third band appeared laterally at either side of the second band.

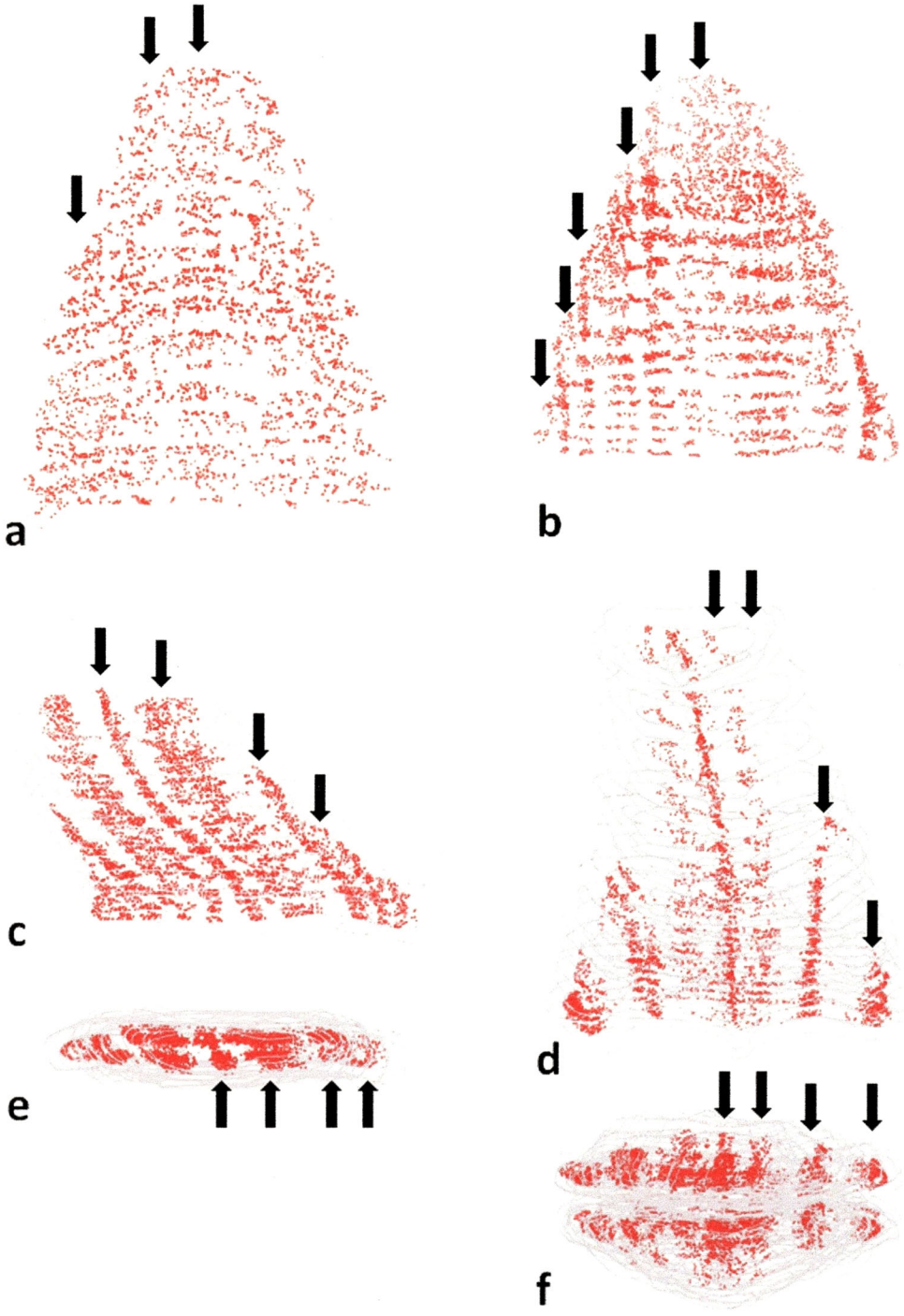

Figure 1-2. Reconstructed images of labeled terminals originating from the bilateral CE showing parasagittal bands in the lateral half (arrows). ***(a)*** *Lobule II.* ***(b)*** *Lobule III.* ***(c)*** *Lobule IV.* ***(d)*** *Lobule Va.* ***(e)*** *Lobule IV. Dorsal view* ***(f)*** *Lobule Va (bottom) and Vb (top). Dorsal view.*

Parasagittal bands were recognized, though not clearly separated, especially in the basal portion.

In folium III (Fig. 1-2b), labeled terminals formed several bands in a symmetric manner and the median band lay astride the midline. Parasagittal bands were more clearly separated than those in folium II. There were 3 or 4 bands in the apical portion of the folium, 6 or 7 bands in the intermediate portion, and 10 or 12 bands in the basal portion. In the basal portion, the most lateral bands consisted of a small number of terminals.

In folium IV (Figs. 1-2c, e), labeled terminals were distributed in parasagittaly-oriented bands, which were similar to those in folium III. These bands were clearly separated. The central band was located on the midline as a thin band. There were 3 bands in the apical portion, 5 bands in the intermediate portion and 7 bands in the basal portion.

Labeled terminals in subfolium Va (Figs. 1-2d, f) were similar to those in subfolium Vb in distribution. In subfolium Va, each band was clearly separated. The central band was located in the midline. There were 3 or 5 bands in the apical half and 5 or 7 bands in the basal portion.

In folium IX, labeled terminals were found in all subfolia, namely subfolia IXa, IXb and IXc, located in the deep portion of the granular layer. Labeled terminals were scattered through the subfolia with no evidence of banding (Fig. 1-3).

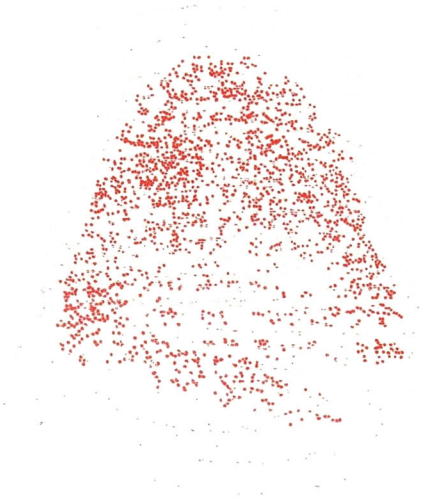

Figure 1-3. Reconstructed image of labeled terminals in lobule IXc originating from the bilateral CE.

SC Projections originating from the LSE

Typical results of these cases are shown in figures 1-4 and 1-5. Few or no labeled terminals were seen in folia I, VII, VIII and X. A large number of labeled terminals were found in folium V following folia III and IV. In contrast to the distribution of labeled terminals from the CE, labeled terminals from the LSE were concentrated in folia III, IV and V.

In folium II, labeled terminals were arranged in 2 bands in the lateral half. The innermost bands astride the midline separated into 2 bands in the apical portion of this folium and united in the middle to deep portions. Lateral bands were clearly detached (Figs. 1-4a, c).

In folium III (Figs. 1-4b, d), 2 innermost bands were clearly separated on either side of the midline. There were 4 bands in the apical portion, and 6 or 8 bands in the intermediate to basal portions. The most lateral bands in the

bottom of the granular layer consisted in a small number of terminals. Parasagittal bands were clearly separated.

In folium IV (Figs. 1-4e, g), labeled terminals tended to be arranged into 5 parasagittal bands, though these bands were not clearly defined.

In subfolia Va (Figs. 1-4f, h) and Vb (Fig. 1-4h), numerous labeled terminals were found, but parasagittal bands were not clearly defined, especially in the three-dimensional reconstructed image. Parasagittal bands for labeled terminals, however, tended to be clear in the case of relatively few labeled terminals. Subfolium Va was similar to subfolium Vb in number and distribution of labeled terminals.

Subfolium VIa (Figs. 1-5a, c) contained more labeled terminals than those in subfolium VIb, but the two subfolia were similar in distribution pattern of labeled terminals. The innermost bands frequently were split 2 bands on either side of the midline. There were 4 bands of labeled terminals in the apical and intermediate portions and 6 bands in the basal portion. Most of the lateral bands in the basal portion consisted of only a small number of terminals.

Folium IX was composed of subfolia IXa, IXb and IXc. Labeled terminals were rare in subfolia IXa and IXb but numerous in subfolium IXc (Figs. 1-5b, d). In subfolium IXc, labeled terminals were distributed in the deep part of the granular layer forming 6-8 parasagittal bands.

Apicobasal distribution of labeled terminals in each folium

In the apicobasal direction of the anterior folia, the projection area from

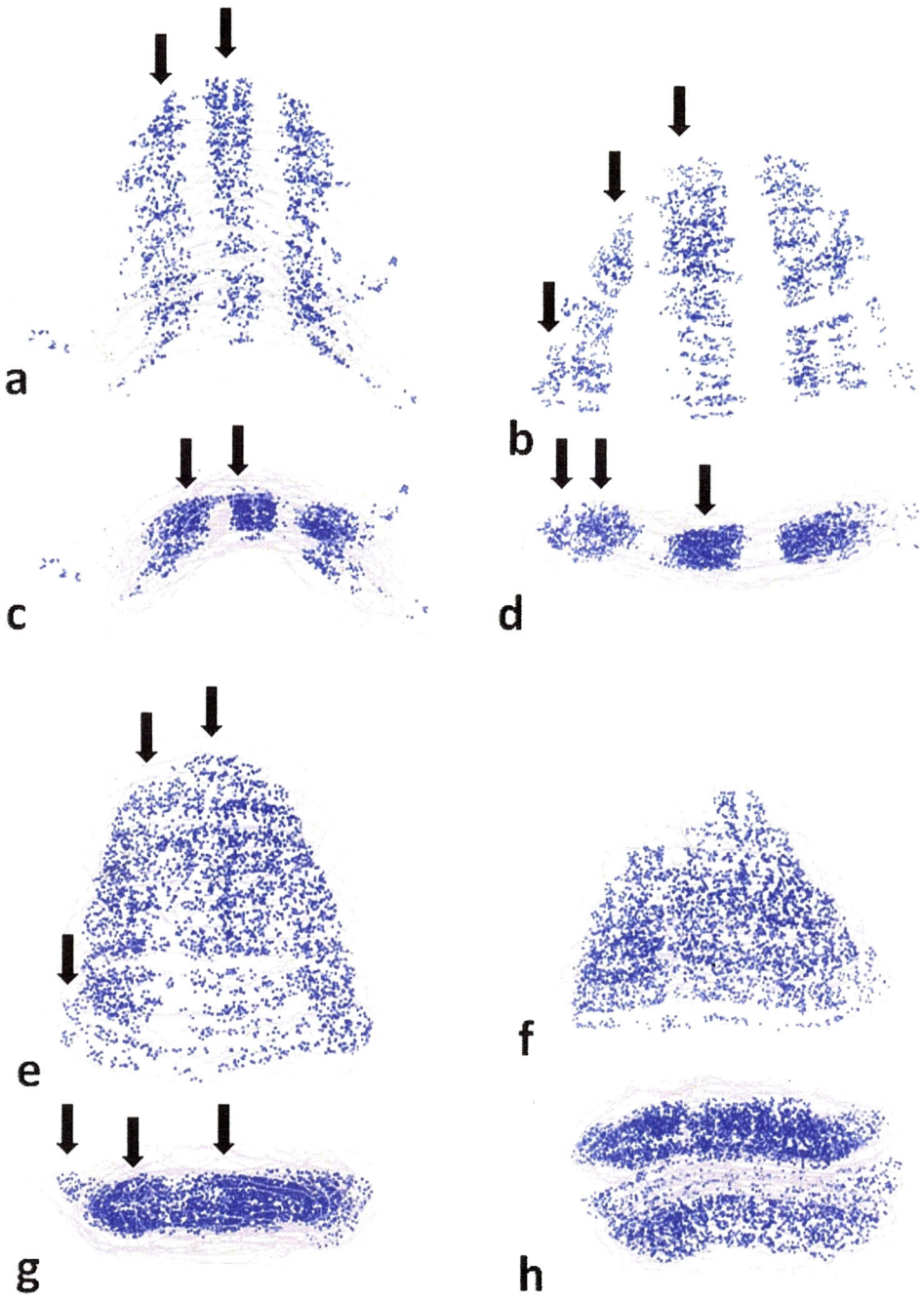

Figure 1-4. Reconstructed images of labeled terminals originating from the bilateral LSE showing parasagittal bands on 1 side (arrows). ***(a)*** *Lobule II.* ***(b)*** *Lobule III.* ***(c)*** *Lobule II. Dorsal view.* ***(d)*** *Lobule III. Dorsal view.* ***(e)*** *Lobule IV.* ***(f)*** *Lobule Va.* ***(g)*** *Lobule IV. Dorsal view.* ***(h)*** *Lobule Va (top) and Vb (bottom). Dorsal view.*

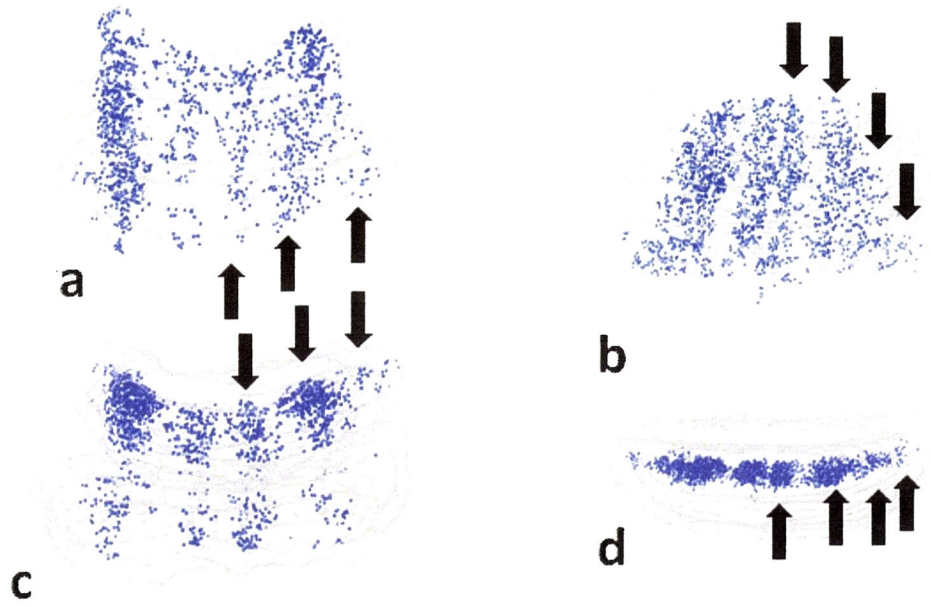

Figure 1-5. Reconstructed images of labeled terminals originating from the bilateral LSE showing parasagittal bands on one side (arrows). ***(a)*** *Lobule VIa.* ***(b)*** *Lobule IXc.* ***(c)*** *Lobule VIa (top) and VIb (bottom). Dorsal view.* ***(d)*** *Lobule IXc. Dorsal view.*

the CE mainly occupied the middle to basal parts, whereas that of the LE occupied the apical part. SC terminals in folium IX from the CE and LSE were most abundant in the middle part of the apicobasal extent.

Discussion

Comparison of SC projections from the CE and LSE

In the present study, we examined the anterograde projection pattern of SC fibers following WGA-HRP injections in the CE or LSE. Main SC neurons

are located in the CC, SBC and VHv in the CE and LSE and in VMN only in the LSE (Yamamoto et al., 2000). Thus, the CE is similar in origins of SC neurons to the LSE.

Labeled terminals were mainly found from folia II to V originating from both enlargements. Meanwhile, threre were plenty SC terminals in folium VI originating from the LSE but few from the CE. In addition, SC fibers projected to subfolia IXa, b and c from the CE but only to subfolium IXc from the LSE. Similar topographic organization has been found in the pigeon by electrophysiological recordings. In folia V, VI and IX of the pigeon cerebellum, SC responses are found in folia V and IXa, b from the wing and in folia VI and IXc, d from the leg [Schulte and Necker, 1998].

Labeled terminals formed parasagittal bands in the granular layer of folia II to VI. These bands were bilaterally symmetric and increased in number from the apical to basal portions in each folium. There were more parasagittal bands from the CE than from the LSE. Furthermore, parasagittal bands originating from the LSE tend to be more clearly defined than those from the CE. Also in the rat and cat, the extent of parasagittal bands of SC terminals varies with spinal level, and the propensity of the SC terminals to form sharp parasagittall bands reduces from the caudal to rostral spinal levels (Matsushita et al., 1985; Yaginuma and Matsushita, 1987, 1989; Ji and Hawkes, 1994).

Spinocerebellar projections in the bird

An anterograde labeling study of WGA-HRP originating from the cervical,

thoracic and lumbar SS (SS 8-10, 18-20 and 26, respectively) was carried out using young chickens [Okado et al., 1987]. According to Okado et al. [1987], labeled terminals were found densely in folia I-V and sparsely in folia VI and IX from the LSE, densely in folia I-VI and sparsely in folia VII-IX from the thoracic segments, and densely in folia II-V and lightly in folia VI-IX from the cervical segments. Labeled terminals are mediolaterally arranged in 3 parasagittal bands on each side of folia II-IV originating from the LSE and thoracic segments, in 2 parasagittal bands on each side of folia II and III, and in 4 parasagittal bands on each side of folia IV and V from the cervical segments. Folium I was projected heavily from the LSE and lightly from the thoracic and cervical segments. The distribution of SC fibers has been observed in chicks and pigeons e.g. by a silver impregnation degeneration technique. Degenerated SC fibers in folium I were found in 2 studies [Vielvoye, 1970; Vielvoye and Voogd, 1977] but not in another study in birds [Whitlock, 1952]. In mammals, SC fibers terminate principally in lobules II-V of the anterior lobe and lobule VIII of the posterior lobe [Grant, 1962; Matsushita and Ikeda, 1987; Gravel and Hawkes, 1990; Ji and Hawkes, 1994]. It is true that some SC fibers terminate in lobule I, the number in lobule I is small [Matsushita and Ikeda, 1985; Yaginuma and Matsushita, 1987, 1989]. In this study, labeled terminals were not found in lobule I from either the CE or LSE. It may be exceptional in birds as well as mammals that SC fibers terminate heavily in lobule I.

In this study, parasagittal bands from the CE consisted of 1 at the midline in folia II-V and a totally of 5 (folium II), 10 or 11 (folium III) and 7 (folia IV and

V) on either side. The numbers of parasagittal bands from the LSE were 3 or 4 in folium II, 6-8 in folia III and IXc, and 6 in folium VI at maximum on either side. SC fiber projections from the LSE in this study are similar to those reported by Okado et al. [1987]. While they did not observe SC fiber projections from the CE, they found more parasagittal bands from the cervical cord (segments 8-10) than from the LSE in the anterior lobe. This tendency is similar to this study.

There is controversy as to whether parasagittal bands are formed in folium IX of the avian cerebellum. Negative results were obtained in chickens [Whitlock, 1952; Okado et al, 1987] and in pigeons [Necker, 2001] showing that labeled terminals are distributed diffusely in the middle part of the granular layer. Positive results were obtained in pigeons [Vielvoye, 1970] showing 2 symmetrical bands. In this study, parasagittal bands of SC fiber terminals in folium IX of LSE origin, but not of CE origin,were found.

Chapter 2

Trajectories in the Spinal Cord and the Mediolateral Spread in the Cerebellar Cortex of Spinocerebellar fiber terminals from the Unilateral Lumbosacral Enlargement in the Chicken

Abstract

SC neurons in the LSE give rise mainly to crossed fibers and generally terminate in parasagittal bands in the granular layer of the chicken cerebellar cortex. However, parasagittal bands for mossy fiber terminals have not always been clear in some cerebellar folia. The present study aimed at (1) observing the course in the spinal cord of the SC tracts, (2) confirming whether SC fibers originating from the unilateral LSE terminate in parasagittal bands, and (3) elucidating the relationship between the ventral and lateral funicular parts of the SC tracts in the CE using anterograde and retrograde labeling methods. The SC tracts were located in the medial part of the ventral funiculi in the SS 27, the full width of the ventral funiculi in the SS 22, the lateral and ventral funiculi in the SS 14 and in the lateral funiculi from the SS 10 rostralward. Projection areas in the cerebellar cortex of SC fibers were studied following unilateral injections of WGA-HRP into the LSE. As a result, SC fibers from the LSE terminated bilaterally in parasagittal bands of folia II - VI and IXc. Labeled terminals in the injected side were similar in number to those in the other side in folia II - IV and IXc and more than those in the other side in folia V and VI. Following ablation of the left (contralateral) lateral funiculus of the CE, the

same tracer was injected into the right (ipsilateral) LSE or into the anterior or posterior cerebellar lobe. As a result, anterogradely labeled SC fibers passing through the ventral funiculus in the CE mainly terminated in the contralateral cerebellar cortex in folia II, III and IV and in the ipsilateral cerebellar cortex in folia V, VI and IX. Following ablation of the unilateral lateral funiculus, retrogradely labeled neurons in the contralateral LSE were found in all SC neuron groups showing marked reduction in number. Thus, the ventral and lateral funicular parts of the SCTs in the CE were not pathways for specific SC neuron groups but different in projection areas.

Introduction

In mammals, the direct SC tracts for the hindlimb consist of two main groups, the dorsal and ventral SC tracts. The dorsal SC tract originates from the CC in the base of the dorsal horn and ascends on the same side as its origin. The ventral SC tract originating from SBC located mainly laterally in lamina VII gives rise largely to crossed axons in the lateral funiculus of the opposite side. The dorsal and ventral SC tracts are different in location, trajectory and function. The dorsal SC tract mediates mainly receptor-specific information from muscles, tendons, joints and skin in the trunk and the lower extremities. The ventral SC tract conveys information about the activity of spinal interneurons [Brodal, 1998]. Although there are many other SC neuron groups, all SC neurons could be divided into the type of dorsal or ventral SC neuron.

According to Yamamoto et al. [2000, 2001], chicken SC neurons are distributed throughout the entire length of the spinal cord. However, these neurons mainly aggregate in the CC, SBC and VHv in both the CE and LSE and the VMN in the superficial layer of the ventral funiculus only in the LSE.

The axons of the cervical CC and SBC are uncrossed while the axons of the lumbar CC, SBC and VMN are crossed. The axons of the cervical and lumbar VHv are a mixture of crossed and uncrossed fibers.

In this study we found that the SC tracts in the cervical enlargement consisted of two parts, the ventral and lateral funicular parts. With these results in mind, we wanted to determine whether there are anatomically great differences between the ventral and lateral parts of SC tracts in the CE. For example, we wanted to determine whether the lateral and ventral parts of SC tracts correspond to uncrossed and crossed SC fibers or to the dorsal and ventral SC tracts in mammals, respectively.

SC fiber terminals are largely distributed in parasagittal bands of the granular layer. However, in previous studies on SC tracts, duplicate distributions of terminals originating from the right and left halves of the spinal cord were observed because of the bilateral injection in the spinal cord (Okado et al., 1987). The purpose of this study is (1) to observe the location of the SC tracts in the spinal cord, (2) to confirm whether SC fibers originating from the unilateral LSE terminate in parasagittal bands, and (3) to elucidate the relationship between the ventral and lateral funicular parts of the SC tracts

in the CE using anterograde and retrograde labeling methods.

Materials and Methods

Animals and experimental design

White Leghorn chickens aged 1.5-3 months were used for this experiment. The animal study was approved by the Animal Care and Use Committee of Tottori University. All animals received an injection of 5 % WGA-HRP in physiological saline as tracer.

Experiment 1: in order to observe the locations of the SC TRACTs through the entire length of the spinal cord, 10 animals received a tracer injection into the cerebellum. *Experiment 2:* in order to observe SC projections originating from the unilateral LSE, 8 animals received an injection into the right half of the SS 26-27 normally in contact with the glycogen body. *Experiment 3:* three of eight animals of experiment 2 were cut the left lateral funiculus of SS 14 (CE) with a scalpel blade prior to the spinal injection for observation of SC projections passing through the ventral funiculus of the CE. *Experiment 4:* in order to observe the distribution of SC TRACT neurons in the LSE with axons passing through the ventral funiculus of the CE, 6 animals

received the cerebellar injection following ablation of the left lateral funiculus of the CE.

Injection of WGA-HRP

The injection of WGA-HRP was performed under deep anesthesia with xylazine (8 mg/kg) followed by midazolam (2 mg/kg) and ketamine (25 mg/kg) administered intramuscularly. After placing the animals in a stereotaxic apparatus, the cerebellum and spinal cord were exposed by craniotomy or laminectomy, respectively. In the spinal cord, the glycogen body was removed by suction prior to a spinal injection. In experiment 1, several bilateral pressure injections of total 6 μl of WGA-HRP were delivered into the cerebellum with a glass micropipette fitted to a 10-μl Hamilton syringe under visual control so as to label the cerebellum extensively. In experiments 2 and 3, single spinal injection of 0.5 μl of WGA-HRP was delivered with a glass micropipette fitted to a 1 μl Hamilton syringe under visual control. In experiment 4, pressure injections of total 1 μl of WGA-HRP were delivered twice into the anterior or posterior lobe of the cerebellum with a glass micropipette fitted to a 1 μl Hamilton syringe under visual control. Following all

injections the pipettes were left in place for a period of 10 min before withdrawal to reduce reflux up the track.

Tissue preparation for light microscope observation

After a survival period of 2-3 days, the animals were deeply anesthetized with ketamine (35 mg/kg) and pentobarbital sodium (20 mg/kg). Perfusion was started with 500 ml of physiological saline, followed by 500 ml of a fixative consisting of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) and finally with 20% phosphate-buffered sucrose. In order to distinguish the right side from the left side of the cerebellum and spinal cord, these were unilaterally applied India ink after these were removed and cryoprotected by immersion in 20% phosphate buffered sucrose for 5 h at 4°C. The cerebellum and spinal cord were embedded in 15% gelatin in 20% sucrose, hardened in 20% formalin in 20% sucrose and kept in the freezer. Frozen sections were cut sagittally at 100 μm in the cerebellum and transversely at 100 μm in the SS 2, 4, 6, 8, 10 and 12-30 in experiment 1, transversely at 60 μm in the cerebellum and transversely at 90 μm in the WGA-HRP injection site of ss in experiments 2

and 3, and transversely at 50 μm in SS 21-30 and sagittally at 100 μm in the cerebellum in experiment 4 using a freezing microtome. Sections were processed for visualizing HRP with TMB according to Mesulam [1978]. Sections were mounted onto gelatin-coated slides and coverslipped following stabilization with methyl salicylate.

The labeled terminals were drawn using a drawing device. Illustrated labeled terminals in experiments 2 and 3 were reconstructed by Adobe Photoshop 5.0 Limited Edition.

Results

Experiment 1 - Location of the SC tracts within the spinal cord (Fig. 2-1)

There were few SC fibers in SS 30 and a small number of SC fibers in the medial part of the ventral funiculi of SS 28. As the SC tracts ascended, these extended laterally in the ventral funiculi to SS 22, and then dorsally in the lateral funiculi in addition to the ventral funiculi to SS14. In the CE, the SC tracts consisted of the ventral and lateral funicular parts. The SC tracts in the ventral funiculi diminished gradually and disappeared in SS 10 or more rostral

segments. Thus, in SS 10 and more rostral segments, the SC tracts lay exclusively in the lateral funiculi.

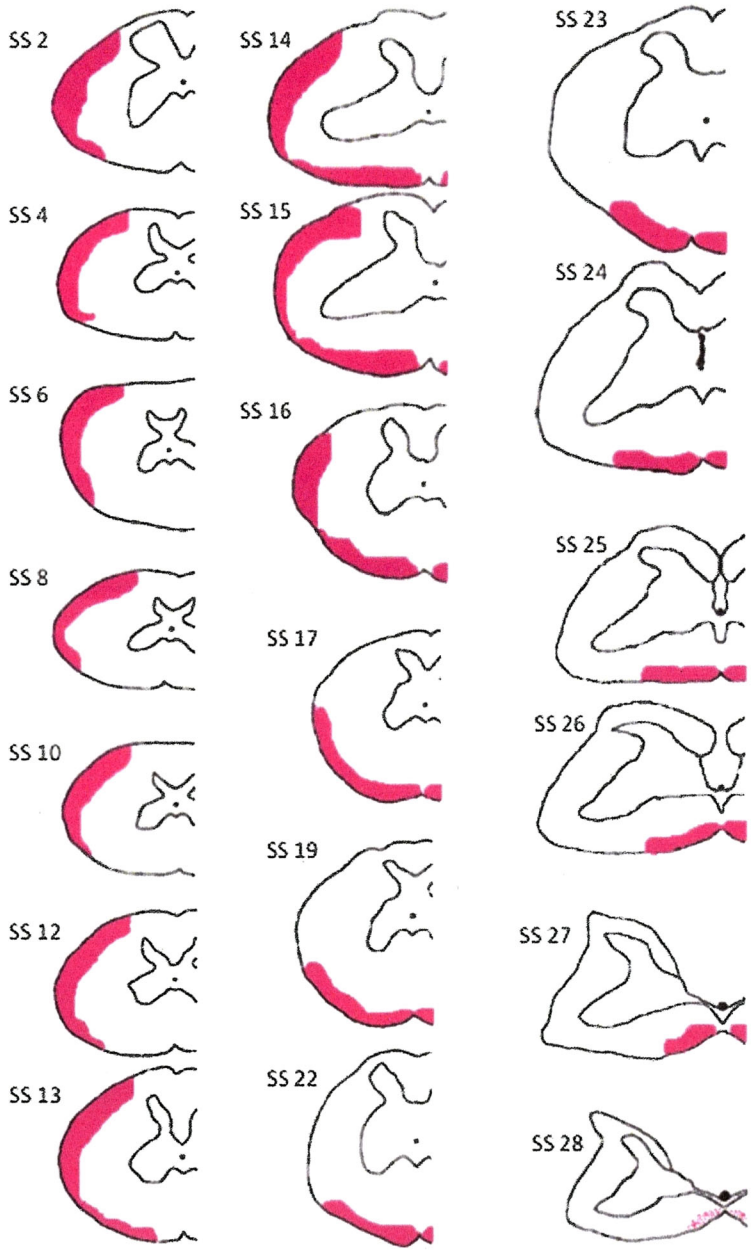

Figure 2-1. Location of retrogradely labeled SC tracts *after injection of WGA-HRP into the whole cerebellum. The SC* tracts *are clearly divided into*

lateral and ventral funicular parts in the CE (SS 13-16). In SS 27, both halves of the spinal cord are only connected by a narrow ventral commissure, because of the well developed-glycogen body.

Experiment 2 – SC projections from the unilateral (right) LSE (SS 26-27)

Labeled terminals were mainly found in folia II-VI and IXc, and they were abundant in folia IV-VI with the largest number in folium V.

Folium I: there were few labeled terminals.

Folia II and III: labeled terminals were distributed primarily in 3 (folium II) or 5 (folium III) parasagittal bands, one at the midline and the others disposed laterally on both sides. The median band tended to be divided into two bands in the apical part of the folium. Parasagittal bands were clearly separated from each other (Fig. 2-2a). Most lateral bands were located only in the deep part of folia consisting of a small number of labeled terminals. Although the labeled terminals in the injected side were fewer than those in the other side, there was not much difference between the numbers of labeled terminals in both sides. The ratio of labeled terminals in the injected side to those in the other

side was 9:10 in folium II and 7:10 in folium III except for labeled terminals in the midline band.

Folium IV: labeled terminals were distributed diffusely in the apical part of the folium, but in the middle to deep parts, 4-5 parasagittal bands were observed in the lateral half. Most medial bands were found on each side of the midline. The lateral 2 or 3 bands consisted of a small number of labeled terminals (Fig. 2-2b). Labeled terminals tended to be fewer on the injected side than on the other side. The ratio of labeled terminals on the injected side to those in the other side was 7:10.

Folia V and VI: in subfolia Va and Vb, 3 parasagittal bands were roughly recognized on each side of the midline. However, these bands were unclear in the apical to middle parts of the folium because of continuation of these bands (Fig. 2-2c). Furthermore, in the case of a large number of labeled terminals, parasagittal bands were barely recognized. Markedly more labeled terminals were found on the injected side than on the other side. The ratio of labeled terminals in the injected side to those in the other side was 24:10 in folium V. Folium VI was similar to folium V in distribution of labeled terminals. The ratio of labeled terminals in the injected side to the other side was 33:10.

Subfolium IXc: subfolia IXa and b contained few labeled terminals. In subfolium IXc, there were many labeled terminals locating only in the deep part of the granular layer. Three parasagittal bands were recognized in the unilateral half of subfolium IXc (Fig. 2-2d). There was no significant difference between the numbers of labeled terminals in the right and left halves of subfolium IXc. The ratio of labeled terminals in the injected side to those in the other side was 16:10.

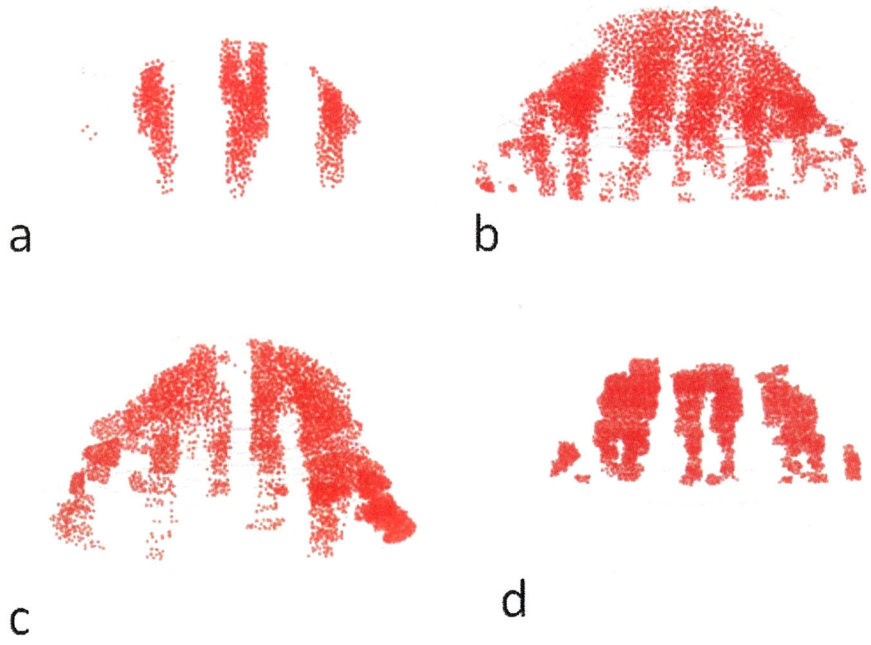

Figure 2-2. Reconstructed images of labeled terminals originating from the unilateral LSE. Ellipses are an earmark for reconstruction and both ends of

ellipses correspond to both mediolateral ends of the cerebellum in section. In spite of the unilateral injection, labeled terminals are distributed bilaterally in a, b and d in similar proportions. More labeled terminals are found in the injected side than in the other side in c. The injected side of the tracer is shown on the right; top to bottom in each image corresponds to apical to basal in each folium. (a) Folium II. (b) Folium III. (c) Subfolium Va. (d) Subfolium IXc.

Experiment 3 - SC projections from the right LSE (SS 26-27) following ablation of the left lateral funiculus of SS 14

In all cases used, the left ventral funicular part of the SC tracts was intact and the left lateral part disappeared in the spinal segments which were more rostral to the surgical segment. There were few labeled fibers in the right half of the spinal cord because the extent of injection area of WGA-HRP was limited to the right half LSE (Figs. 2-3a, b). We describe mainly results different from experiment 2.

Folia II, III and IV: median bands had a considerably smaller number of labeled terminals than the number in the lateral bands and never showed any sign of bifurcation (Figs. 2-4, 2-5a). Labeled terminals dramatically decreased

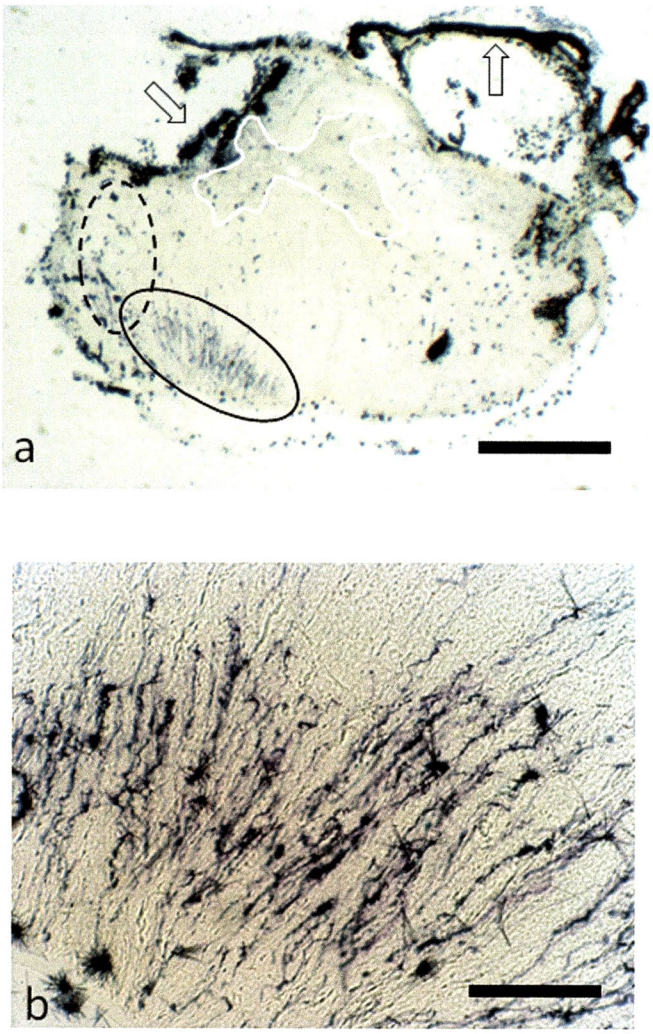

Figure 2-3 Photomicrographs of anterogradely labeled SC fibers in SS 13 originated from the right half of SS 26 following ablation of the left lateral funiculus in SS 14. (a) Labeled fibers are found in the left ventral funiculus (solid circle) but not in the lateral funiculus (dashed circle). The right lateral and ventral funiculi are free of labeled fibers. Arrows show India ink as marker for SS. Scale bar = 1 mm. (b) Magnified microphotograph of the left ventral funiculus in a. Scale bar = 100 μm.

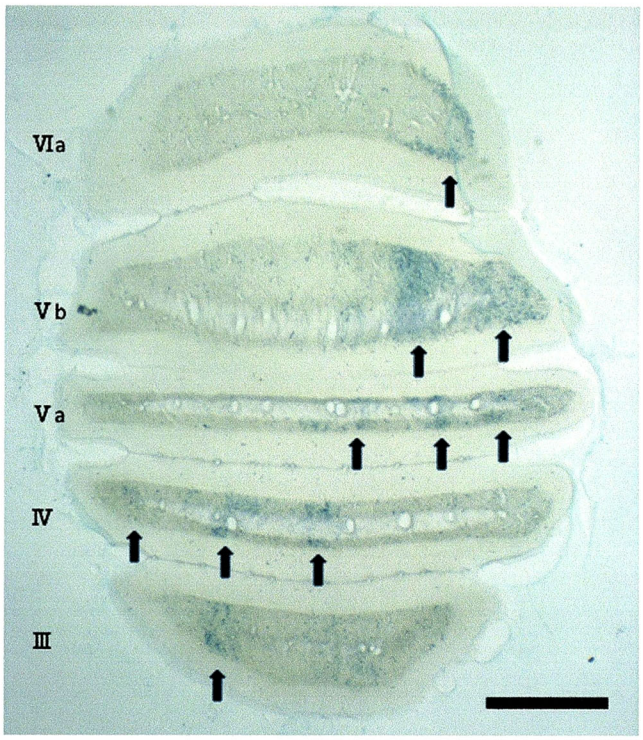

Figure 2-4. Photomicrograph of SC terminal fields originating from the right half of SS 26 following ablation of the left lateral funiculus in SS 13. Parasagittal labeled bands are clear in the left (contralateral) half of folia III and IV, and in the right (ipsilateral) half of folia V and VI. Scale bar = 1 mm.

on the injected side. The ratios of labeled terminals on the injected side to those on the other side, except for the terminals in the midline, were 3:10 in folium II and 1:10 in folium III. In folium IV, labeled terminals in the injected side had largely disappeared (Fig. 2-5b). The ratio of labeled terminals in the injected side to those on the other side was 1:10.

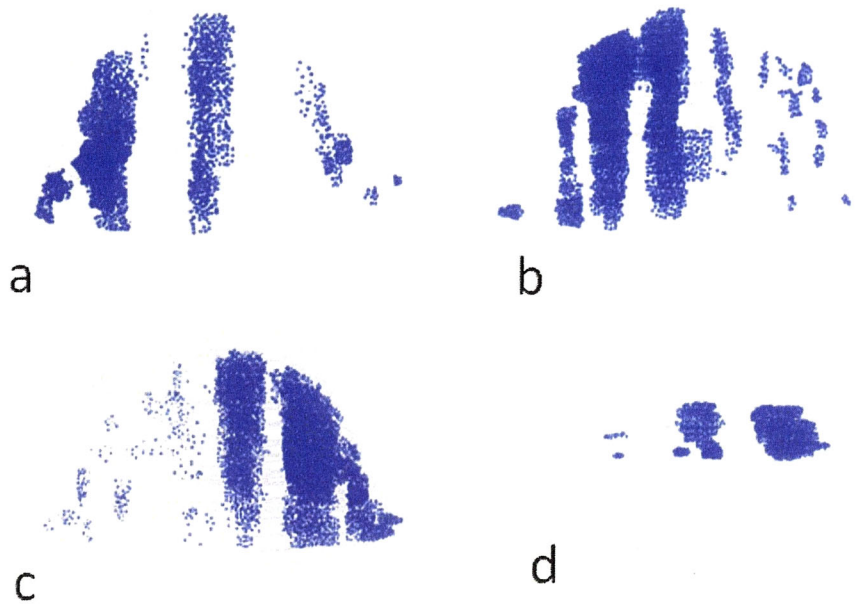

Fig. 2-5. Reconstructed images of labeled terminals originating from the right half of the LSE following ablation of the left lateral funiculus. Following ablation of the left lateral funiculus, (a) and (b) decrease in labeled terminals in the injected side and (c) and (d) decrease or disappear in labeled terminals in the other side. Right, injected side of the tracer is shown on the right; top to bottom in each image corresponds to apical to basal in each folia. (a) Folium III. (b) Folium IV. (c) Subfolium Va. (d) subfolium IXc.

Subfolia Va, Vb and VIa: labeled terminals formed 3 parasagittal bands on both sides but prominently decreased on the uninjected side (Fig. 2-5c). The ratio of labeled terminals on the injected side to those in the other side

was 19:1 in folium V. Labeled terminals in the uninjected side had largely disappeared in folium VI.

Subfolium IXc: labeled terminals prominently decreased on the uninjected side (Fig. 2-5d) and the ratio of labeled terminals on the injected side to those in the other side was 20:1.

Experiment 4.1 - Distribution of SC neurons in the LSE with axons passing through the left ventral funiculi of the CE following tracer injection into folia IV to VI

WGA-HRP was deposited in folia IV-VI except for both lateral ends. In 1 case out of 4 cases, the left half of the cerebellum was more heavily injected than the right half. The left lateral funicular part of the SC tracts was completely cut in the CE (Fig. 2-6). Labeled neurons in the LSE showed a similar distribution, but varied in number through all cases. Two representative cases, in which labeled neurons were numerous, were selected to describe the distribution of labeled neurons.

Retrogradely labeled neurons were found through SS 22-29 we observed. Labeled neurons were largely located in the CC in laminas V and

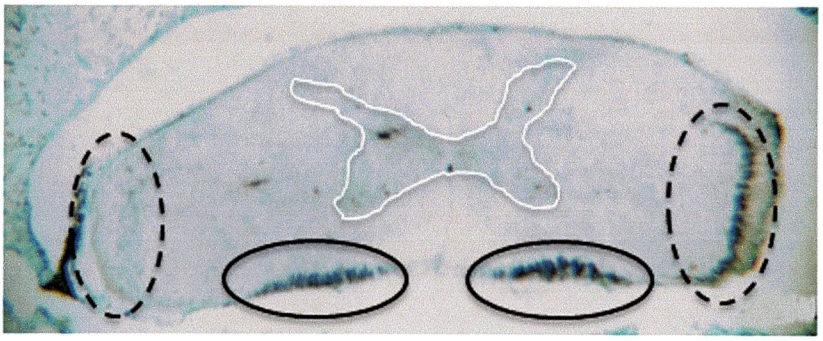

Figure 2-6. Photomicrograph of the retrogradely labeled SC tracts in SS 14 following ablation of the left lateral funiculus of SS 13 and WGA-HRP injections into the cerebellum. Labeled fibers are found in the lateral (dashed circle) and ventral (solid circle) funiculus in the right half but only in the ventral funiculus in the left half. Scale bar = 1 mm.

VI at the base of the dorsal horn, SBC in the lateral border of laminas VI, VII and IX, VHv in the ventral border of laminas VIII and IX, and VMN in the ventral margin of the ventral funiculi.

The CC showed the maximal incidence of labeling (about 63% of the total number of labeled neurons). More labeled neurons were found in the left (operated) half than in the right half of the cord. The ratio of labeled neurons in the operated side to those in the other side was about 3:1. Labeled neurons in the SBC, VHv and VMN accounted for about 14, 19 and 4%, respectively, of

the total number of labeled neurons (Figs. 2-7a, 2-8). However, these proportions were variable in each case. More labeled neurons in these cell groups were found in the operated side than in the other side of the cord. The ratios of labeled neurons in the operated side to those in the other side were of 8:1, 4:1 and 7:1, respectively. These ratios were also variable in each case.

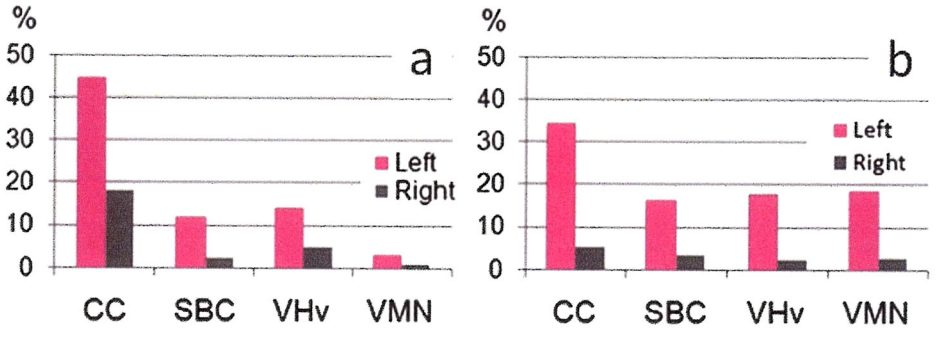

*Figure 2-7. Proportions of retrogradely labeled neurons in SS 22-29 originating from the anterior (**a**) and posterior (**b**) lobes following ablation of the left lateral funiculus in the CE. Left and right halves of SS 22-29 are presented.*

Experiment 4.2 - Distribution of SC neurons in the LSE with axons passing through the left ventral funiculi of the CE following tracer

injection into the posterior lobe

The injection area was mediolaterally symmetric covering folia VIII and

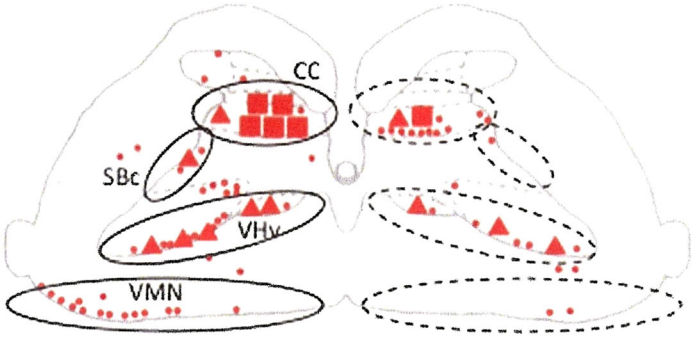

Figure 2-8. Labeled neurons in SS 25 following ablation of the left lateral funiculus in the CE and WGA-HRP injection into folia IV-VI. Labeled cells are mainly located in CC, SBc, VHv and VMN, and there are more labeled cells in the left than in the right half. This line drawing represents the findings in 36 sections. Sections are serially cut at 50 μm and processed for HRP histochemistry every 3 sections. Square represents 50 labeled neurons; triangle represents 10 labeled neurons; small circle represents one labeled neuron.

IX in one case and folium IX in one case except for both lateral ends. The left lateral SC tract was completely cut in the CE. Labeled neurons were mainly

located in the above-mentioned 4 cell groups but were markedly reduced in number compared to those in cases of injection into the anterior lobe. Labeled neurons in the CC, SBC, VHv and VMN accounted for about 40, 19, 20 and 21%, respectively, of the total number of labeled neurons (Fig. 2-7b). In comparison with the data shown in figure 2-8, the percentage of neurons in the CC was lower and the percentage of neurons in the VMN was higher. The percentage of labeled neurons in the operated side was much higher than that in the other side. The ratios of labeled neurons in the operated side to those on the other side in the CC, SBC, VHv and VMN were about 7:1, 8:1, 8:1 and 8:1, respectively. Thus, the majority of SC fibers passed through the lateral funicular part in the CE and this tendency was remarkable in those terminating in the posterior lobe.

Discussion

For unilateral injection of the tracer, we chose SS 27, which houses the maximal portion of the glycogen body, a structure that is peculiar to the avian lumbosacral cord. Since at its maximal portion the central canal is taken into the glycogen body, the right and left halves of the spinal cord are connected to

each other only by the ventral commissure. Thus, the injected tracer is confined to the unilateral half of the SS adjacent to the maximal portion of the glycogen body.

The chicken SC tracts from the LSE project mainly to folia II-VI and subfolium IXc. In previous studies, Vielvoye (1970) reports that SC fibers in folium IX terminate in parasagittal bands in pigeons, whereas others do not determine these bands in folium IX in chickens [Whitlock, 1952; Okado et al., 1987] and also in pigeons [Necker, 2001]. We also observed that parasagittal bands of SC fibers from the bilateral LSE often were unclear (Furue et al., in press). However, SC fiber terminals originating from the unilateral LSE were arranged in parasagittal bands also in subfolium IXc. It is likely to cause unclear parasagittal bands that a couple of bands formed by SC fiber terminals originating from the unilateral and contralateral cords could be slightly different in position. Thus, it was confirmed that chicken SC fibers terminate in parasagittal bands through all folia which are main targets of the SC tracts, at least in the SC tracts from the LSE.

In experiment 1, we showed that the SC tracts in the CE divided into lateral and ventral funicular parts. Experiments 2-4 were executed to

determine whether the two parts have a close relation to a specific SC neuron group or a specific projection area.

SC terminals from the unilateral LSE were slightly fewer in the injected side of folia II-IV than those in the other side and were the opposite left-and right-relationship in folia IX. However, SC fibers from the unilateral LSE may project bilaterally in these folia, because there were only a few differences between the injected and other sides. Meanwhile, these were considerably more in the injected side of folia V and VI than those in the other side. Single mossy fibers from the lateral reticular nucleus in rats have a complex branching pattern in the cerebellar cortex. They enter the ipsilateral side of the cerebellum or often enter the contralateral side by crossing the midline. Finally, they branch into a wide mediolateral spread, usually bilaterally, with multiple parasagittal bands in multiple lobules in the rat cerebellum [Wu et al., 1999]. Chicken mossy fibers from SC tracts may also have a branching pattern similar to that from reticulocerebellar fibers. If they do, there should be SC fibers with bilateral (type B of Wu et al., 1999) and unilateral collaterals. Furthermore, the unilateral type should consist of the contralateral type (type U-C) and ipsilateral type (type U-I), which cross once more within the

cerebellum. Although type B fibers may be divided into (1) those with evenly branched collaterals, (2) those with predominant terminals to the ipsilateral side, and (3) those with predominant terminals to the contralateral side, we assumed that type B fibers give rise evenly to branched collaterals in the mediolateral spread. It is likely that SC fibers, that entered the contralateral side of folia II-IV, consist of 3 types and type U-I is similar in number to type U-C. After unilateral ablation of the lateral funiculus, the numbers of labeled terminals in folia II-IV dramatically decreased in the injected side. Thus, it is thought that type U-I and type U-C are the dominant type passing through the contralateral lateral and ventral funiculi in the CE, respectively. Type B may be a few because if there are many type B, labeled terminals passing through, the lateral funiculus of the CE must decrease prominently on both sides or labeled ones passing through the ventral funiculus of the CE do not decrease dramatically on the injected side.

In this study, labeled terminals from the unilateral LSE in folia V and VI were considerably more frequent in the injected side than in the other side. After ablation of the unilateral lateral funiculus, labeled terminals decreased prominently in the other side. Thus, it can be concluded that in folia V and VI,

SC fibers consist of type U-I passing through the contralateral ventral funiculus and type U-C passing through the contralateral lateral funiculus, and that there are few type B, if any. According to results of axonal degeneration experiments on chicken SC tracts, the distribution of degenerated terminals in folium V can vary depending on the level of hemicordotomy [Vielvoye, 1977]. If the origins of SC fibers change, the proportions of the 3 types of SC mossy fibers may also change.

Labeled terminals in subfolium IXc were approximately even in number through both halves after unilateral injection but largely disappeared in the other side after ablation of the contralateral lateral funiculus. Thus, it is thought that in subfolium IXc, type U-I passing through the contralateral ventral funiculus is similar in number to type U-C passing through the contralateral lateral funiculi in the CE, and type B are few.

The pigeon SC tracts from the LSE cross at the segmental level in the cord. Most CC and SBC fibers recross at brainstem levels, so that they terminate ipsilaterally in the cerebellum. VHv fibers show partial recrossing, so that they terminate in both sides of the cerebellum [Necker, 1992]. In this study, however, chicken SC fibers from the LSE were mainly type U

terminating in both sides of the cerebellum. Thus, almost all chicken SC fibers from the LSE cross to the contralateral side in the spinal cord and then terminate in the ipsilateral cerebellum after recrossing and in the contralateral cerebellum without recrossing. It seems reasonable to conclude, therefore, that the lateral and ventral funicular parts of the SC tracts in the chicken CE have different projection areas, and type B is not the major type fiber in the chicken SC tracts.

Retrogradely labeled neurons from the anterior or posterior cerebellar lobe were located in all major SC neuron groups, the CC, SBC, VHv and VMN, and similar in occurrence ratio in these cell groups to the previous studies (Yamamoto et al., 2000, 2001). Since the CC is uncrossed passing through the dorsal SC tract and SBC with crossed axons passed through the ventral SC tract in mammals (Brodal, 1998), each SCT corresponds to a specific SC tract nucleus. In this study, retrogradely labeled neurons with axons passing through only the ventral funiculus were found in all SC neuron groups showing marked reduction in number. In addition, retrogradely labeled neurons from the anterior lobe were similar in distribution to those from the posterior lobe. Thus, the lateral and ventral funicular parts of the SC tracts in the chicken CE

were not corresponding to a specific SC tract neuron group. However, the ventral funicular part of the SC tracts in the chicken CE was clearly different in projection areas from the lateral funicular part. During SC fibers from the LSE move from the contralateral ventral funiculus to the lateral funiculus as they ascend, they have to be rearranged depending on their projection areas.

Summary

1. Distribution of terminals of spinocerebellar fibers arising from the cervical and lumbosacral enlargements was studied by the anterograde transport of wheat germ agglutinin-horseradish peroxidase in the chicken.
2. Labeled terminals originating from the cervical enlargement were distributed primarily in folia II-V and IX. Labeled terminals in folia IV and V accumulated in clearly separated parasagittal bands in the granular layer. Labeled terminals were distributed in unclear parasagittal bands in folia II and III and diffusely in folia IX.
3. Labeled terminals originating from the lumbosacral enlargement were distributed primarily in folia II-V and IXc. Labeled terminals were few in subfolia IXa and IXb. Labeled terminals accumulated in parasagittal bands. However, parasagittal bands in folia V were unclear.
4. There were more parasagittal bands arising from the cervical enlargement than from the lumbosacral enlargement. Furthermore, parasagittal bands originating from the lumbosacral enlargement tend to be more clearly defined than those from the CE.
5. In order to clarify laterality of spinocerebellar fibers and mediolateral spread of spinocerebellar fiber terminals arising from the lumbosacral enlargement, we performed the following experiments.
6. The spinocerebellar tracts were located in the medial part of the ventral funiculi in the SS 27, the full width of the ventral funiculi in the SS 22, the lateral and ventral funiculi in the SS 14 and in the lateral funiculi from the SS 10 rostralward. There were a few in the medial part of the ventral funiculi in the SS 30.
7. Following injections into the unilateral half of the 27th spinal segment in the lumbosacral enlargement, labeled terminals were bilaterally distributed in parasagittal bands of folia II - VI and IXc. Labeled terminals in the injected side were similar in number to those in the other side in folia II - IV

and IXc and more than those in the other side in folia V and VI. Following unilateral injections, parasagittal bands of labeled terminals were more clearly separated than those of bilateral injections. Thus, it is suggested that at least spinocerebellar fiber terminals from the lumbosacral enlargement are parasagittally distributed in all folia.

8. Following injections into the unilateral (right) half of the 27th spinal segment after ablation of the contralateral (left) lateral funiculus of the cervical enlargement, labeled terminals decreased sharply in the ipsilateral (injection side) half of folia II-IV, and the contralateral half of folia V, VI and IXc.
9. It is suggested that SC fibers arising from the LSE are principally crossed, predominantly terminate in the unilateral half of the cerebellar cortex, SC fibers through the lateral funicular part of the CE terminate mainly in the ipsilateral half of folia II to IV and in the contralateral half of folia V, VI and IXc, and SC fibers through the ventral funicular part terminate mainly in the contralateral half of folia II to IV and in the ipsilateral half of folia V, VI and IXc.
10. Following injections into the cerebellum after ablation of the unilateral funiculus of the cervical enlargement, retrogradely labeled neurons in the contralateral half of the lumbosacral enlargement showed marked reduction in number through all SC neuron groups. Thus, the ventral and lateral funicular parts of the SC tracts in the CE were not pathways for specific SC neuron groups but different in projection areas.

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