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A Study on the Spinoreticulocerebellar Tract in Chickens

Il-kwon Park, Moo-kang Kim1, Imagawa Tomoro and Uehara Masato*

Faculty of Agriculture, University of Tottori, Tottori 680-8550, Japan
1College of Veterinary Medicine, Chungnam National University, Daejeon 305-764, Korea

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Abstract

The spicoreticulocerebellar (SRC) tract is an indirect spinocerebellar tract formed by the reticular formation (RF), which is connected to the cerebellum and spinal cord. The RF receives ascending fibers to both the spinal enlargement and sends descending fibers to the cerebellum. This study demonstrated that the connectivity of the neurons in the RF is concerned to the cerebellum and spinal cord using the anterograde projection with biotinylated dextran amine (BDA) and retrograde labeling with wheat germ agglutinin-horseradish peroxidase (WGA-HRP). Until now, a preliminary study in mammals has dealt with the afferent and efferent pathways in separating groups of neurons in the RF. There are only few reports on chickens. This study examined the SRC tract in chickens. Following bilateral injections we injected BDA into chicken spinal cord (lumbosacral enlargement) and WGA-HRP into the cerebellum. Both of single- and double-labeled cells were found within the RF. The spinoreticular axons were mainly distributed from the potomedullary junction to the rostral medulla in the rostro-caudally RF levels, for example, nucleus of reticularis (n. r.) pontis oralis, locus coeruleus, n. r. pontis caudalis, n. r. pars gigantocellularis, n. r. gigantocellularis and n. r. parvocellualris. Reticulocerebellar labeling by the WGA-HRP was found in the same place as well as that of the BDA-projection. We observed that the proportion and location of double labeling cells in the chicken were almost similar in each level, comparing to the rodents. These results suggest that the reticular formation is strongly related to the spicoreticulocerebellar tract in chickens.

Key words: chicken, spinoreticulocerebellar tract, WGA-HRP, BDA, double labeling

*Corresponding Author: Uehara Masato
Faculty of Agriculture, University of Tottori, Tottori 680-8550, Japan
Tel: +81-857-31-5419; Fax: +81-857-31-5419
E-mail: muehara@muses.tottori-u.ac.jp

Introduction

Neurons of the crossed spinoreticulocerebellar (SRC) pathway originate from the cervical cord and pass through the reticular formation (RF) in the ventrocaudal medulla oblongata. This is an important precerebellar relay center that provides a significant number of mossy fibers in the granule cell layer that inputs into the cerebellum [6]. The initiation of locomotion in mammals involves the mesencephalic and reticulospinal neurons, and a primary pathway in the spinal cord [13]. Until now, the SRC tract has been investigated separately with several other methods in a few animals including cats [3, 4, 9, 10, 11, 21, 23, 33, 34], rats [1, 6, 12, 13, 15, 17, 20, 30], rabbits [31], pigeons [5, 16] and opossums. However, the SRC tract in chickens [7, 8, 36, 37] has only recently been known by labeling methods. The RF of the brain stem is considered to comprise the medulla, pons, and mesencephalon, which are characterized structurally by a diffuse aggregation of cells with different types and sizes, and separated by nerve fibers transversing the region in all directions [10]. The RF is involved in behavioral arousal, regulating muscle reflexes, coordinating the autonomic functions, and modulating pain sensation [8]. Subdivisions of the RF form premotor networks that organize several complex behaviors [24]. There are many prenuclei involving the RF and these pre-cerebellar nuclei mainly consist of the n. r. lateralis (RL), the n. r. tegmenti pontis, and the n. r. paramedianus (RpaM). The RL is subdivided into three subdivisions including the parvicellualr division, the magnocellualr division, and the lateral group. The inferior olive and the RL are two brain stem nuclei whose projections to the cerebellar cortex have been shown to terminate in this way [2]. The same neurons also received a bilateral somatosensory input from the periphery.

Therefore, the RL appears to be composed of two comparatively independent parts, which represents the medullary relay for ascending the SRC tract [22]. The RL mainly receives the afferent connections from the bilateral spinal cord and from various supraspinal centers such as the cerebral cortex, the red nucleus, the nuclei medialis and the fastigial nucleus and the cerebral cortex [1, 18, 19, 25, 26, 28]. Thus, the RL has important functions in integrating

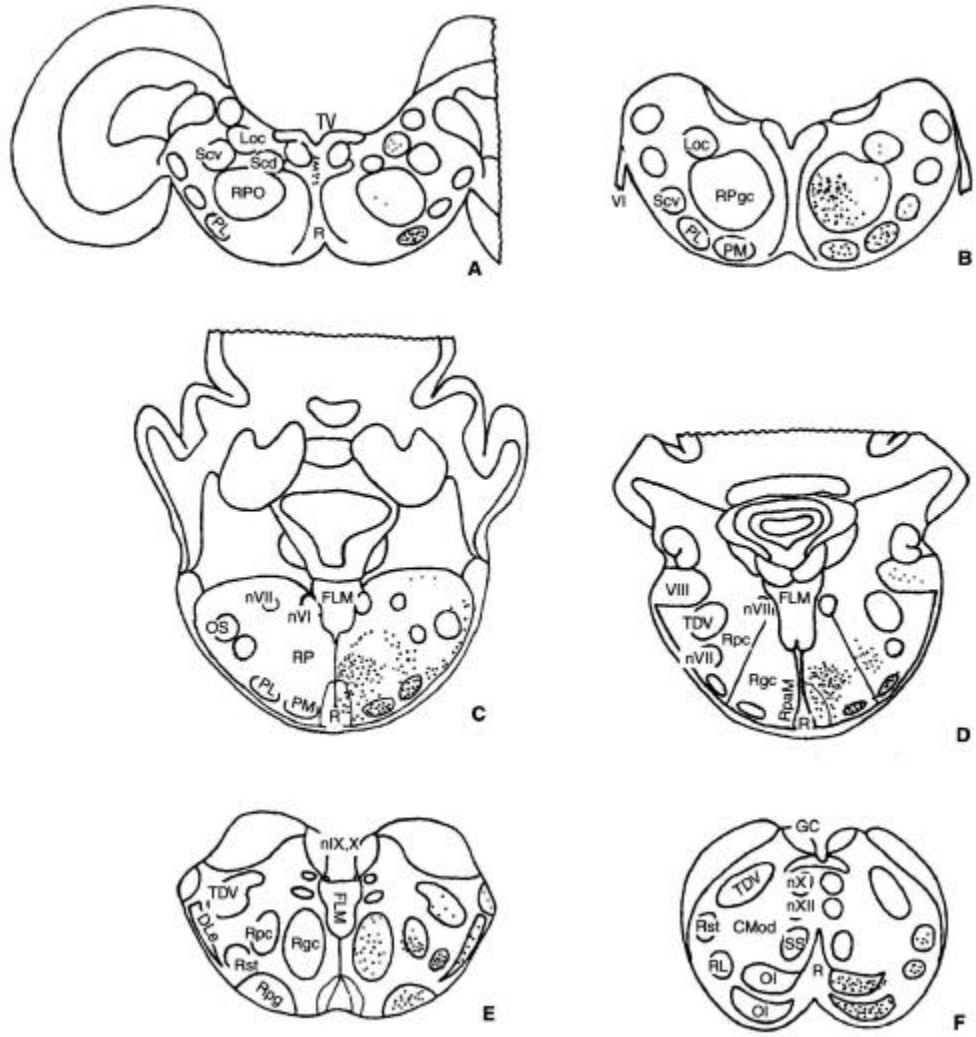

Fig. 1. Diagrams of rostral (A) to caudal (F) transverse sections of the brainstem illustrating the distribution of spinoreticular projections after injection of BDA into lumbosacral enlargement and the reticulocerebellar neurons after injection of WGA-HRP into cerebellum. (A,B) The levels of pons. (C, D) The levels of pontomedullary junction. (E, F) The levels of medulla. ×: BDA-labeled axon terminals. ●: HRP-labeled neurons.

the spinal and supraspinal impulses, and is the main center for modifying the mossy-fiber-mediated spinal input to the cerebellum [25]. There is evidence that the part of the RL that receives the crossed spinoreticular pathway projecting mainly to the corresponding vermal cortex of the cerebellar anterior lobe [22]. The projection of the RL neurons to the cerebellum has been described extensively by studies using various experimental techniques including degeneration [5, 26], fluorescent tracing [3, 6, 16], autoradiographic tracing [11], electrophysiological methods [2, 22], anterograde labeling [12, 20, 24, 25, 29], and retrograde labeling [1, 2, 8, 10, 15, 17, 19, 20, 29, 30, 31, 37]. Despite the strong evidence for the existence of an anatomical link from the RL to the cerebellar cortex in various species, this has not been confirmed in chickens. Transmitters related to such a reticulocerebellar (RC) pathway in chicken remains unclear. The RL and its afferents to the cerebellum are known to contain glutamate-like immunoreactive neurons and axons, respectively [34].

These studies suggest that in chickens, the RF neurons afferent to the cerebellum can be compared from the distribution of the cerebral cortical afferent from the spinal input (lumbosacral enlargement) but did not focus on the afferent direction, for example, ipsilateral or contralateral. The present study, using the BDA anterograde and WGA-HRP retrograde transport as revealed by the DAB and TMB reaction, investigated the location of the spinal neurons at the mediate component of the SRC tract.

Materials and Methods

A total of 11 White Leghorn chickens (*Gallus domesticus*)

weighting about 300 g were used for the present investigation. To determine the distribution in RF, for anterograde transport 5% biotinylated dextran amine (BDA, molecular weight 10,000, Molecular probe), for retrograde transport 5% wheat-germ agglutinin horseradish peroxidase (WGA-HRP; Toyobo, Osaka, Japan) were used. In this experiment, animals were anesthetized with xylazine (5 ㎎/㎏, IM) followed by the midazolam (1 ㎎/㎏, IM) and ketamine (10 ㎎/㎏, IM), and fixed in a stereotaxic apparatus. In animals which were to receive spinal injections, a laminectomy was performed at the appropriate vertebral level (SS 24) bilaterally and 0.3 ㎕ of 5% BDA was injected using a glass micropipette fitted onto a 1 ㎕ Hamilton syringe. Stereotaxic data were at 500 ㎛ lateral to the midline and at a depth of approximately 1,200 ㎛. After a survival time of 2 weeks, the cerebellum was exposed by craniotomy. Injections of 5% WGA-HRP in physiological saline were made into both anterior and posterior cerebellar lobes with a glass micropipette connected to a 10 ㎕ Hamilton syringe. Multiple injections totaling up to 5 ㎕ were made in order to infiltrate all folia in the cerebellum. To avoid undesired residues of the tracer in the cortex, the micropipettes were left in the site of the injection for 10 mins before and after injection. After 3 days, the animals were deeply anesthetized with pentobarbital (20 ㎎/㎏, IV) and then perfused transcardially with saline, followed by a mixture of 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h prior to overnight cryoprotection in 20% sucrose solution (at 4℃). Frozen sections were cut serially at 60 ㎛ by a freezing microtome through the brain stem transversely and the cerebellum sagittally. Every third sections through the RF carried out the only HRP histochemistry with tetramethyl benzidine (TMB) BDA and the third series carried out a double-labeled labeling according to Sakai *et al* [29]. We have in this study used the nomenclature based on Kuenzel and Masson and Nomania Anatomica Avium according to Breazile and Kuenzel. And the laminar organization of the spinal cord was made using the same histological criteria were suggested by Brinkman and Martin [37].

WGA-HRP histochemistry

Sections treated for the WGA-HRP were stained a TMB as the chromogen and ammonium molybdate [18] and stabilized in DAB [27]. Briefly, sections were rinsed in 0.1 M phosphate buffer (pH 6.2) and incubated in a solution of 0.25% ammonium molybdate and 0.005% TMB in 0.1 M phosphate buffer (pH 6.2) and 0.003% hydrogen peroxide. Sections were incubated overnight at 4℃ and then stabilized in a solution of 0.1% DAB, 0.002% cobalt chloride and 0.003% hydrogen peroxide in 0.1 M phosphate buffer, sections processed for WGA-HRP only were mounted onto slides and dried. The sections to be processed for BDA as double labeling were rinsed in 0.1 M phosphate buffer (pH 6.2).

BDA histochemistry

To reveal the BDA, we followed the protocol of Veenam *et al* [33] with minor modification. BDA histochemistry has applied by two protocols, one has only stained for the BDA, and the other has carried for the double staining. The sections were rinsed in 0.1 M phosphate buffer (pH 7.4) and incubated in a 1:500 or 1:50 dilution of avidinbiotinperoxidase complex (Vector ABC Elite kit, PK6001) for 2 h. Sections were rinsed in the same phosphate buffer, transferred to TBS and reacted in 0.1% DAB/0.04% ammonium chloride/0.2% β-D glucose and glucose oxidase for 10 20 min. Both double stained the WGA-HRP and the only BDA- stained sections were then mounted onto slides and dried.

Results

This study involves the injecting WGA-HRP and BDA into the cerebellum and spinal cord, with the typical injection site being shown in Fig. 2A and 2B. The cell

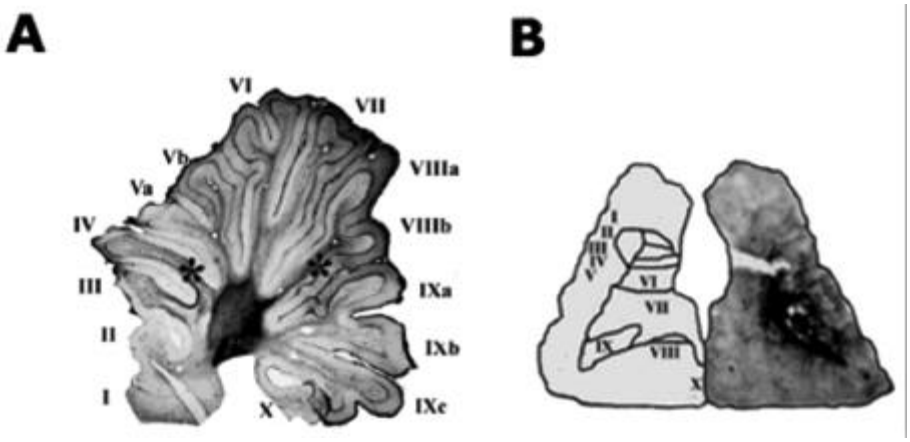

Fig. 2. A photomicrograph of the injection site of wheat germ agglutinin conjugated-horseradish peroxidase (WGAHRP) on a mid-sagittal section through the cerebellum (A) and biotinylated dextran amine (BDA) on a spinal segment 24 of the chicken (B). *: Injection site of BDA and WGAHRP.

properties were identified by their labels. The WGA-HRP-labeled neurons appeared blueblack by TMB as a chromogen, and the BDA-labeled fibers were a brown color by DAB. Dendritic branches of the BDAlabeled neurons often passed into the core of the injection site. These WGA-HRP labeled neurons were often observed in most of the BDA-labeled axons in the RF (Fig. 4).

Retrograde labeling using WGA-HRP

In all cases, the injection area was limited to the cerebellum, which infiltrated every cerebellar lobe including the cerebellar nuclei (Fig. 1A). The WGA-HRP reaction was recognized a blue-black color (Fig. 3B). In the RF, the soma major axis and dendritic arbors of the labeled neurons in the reticularis dorsalis exhibited a pronounced dorsomedial to ventrolateral slant (Fig. 1). Fig. 1 shows the distribution of the RC neurons on the six regions in the RF. The labeled cells appeared bilaterally in the RF and in each half were similar in number. The greater part of the labeled cell numbers (approximately 83%) was located around the levels of the vestibulocochlear nerve (Fig. 1C E). A large number of labeled cells were found in the n. r. pontis caudalis (RP) at the level of the pontomedullary junction and in the Rgc, Rpc, Rpgc, RpaM and subtrigeminalis (Rst) at the level of the medulla oblogata (Figs. 1, 3). At the levels of the vestibulocochlear nerve, there were many large labeled cells arranged in a longitudinal pattern along the lateral edge of the RF (the dorsolateral edge cells, DLe cells) (Fig. 1). A small number of labeled cells were observed in the nucleus centralis, medulla oblongata, pars dorsalis, PM, PL and ventralis (CMod, CMov), and the n. r. lateralis (RL) in the caudal medulla (Figs. 1E, 1F). No labeled cells were detected in the mesencephalic RF. There were a larger number of WGA-HRP-labeled neurons in the pontomedullary junction (Fig. 2D). The results of this study are similar to the results in our laboratory referenced by Hassouna *et al* [8].

Anterograde projection using BDA

Similar results were obtained from 10 chickens that were injected with the BDA into the LSE (Fig. 2B). After injecting the BDA in the LSE, large numbers of anterogradely labeled neurons were found in the RF through the spinoreticular (SR) tract. The transported BDA was visualized with DAB, which produced a homogeneously brown reaction product that filled both axons and varicosities. The fibers of the spinoreticular tract were expressed by an X-mark from the medulla oblongata to the mesencephalon, transverse sections through the chicken brain stem (Figs. 1A-F). Fig. 2 shows the distribution of SR

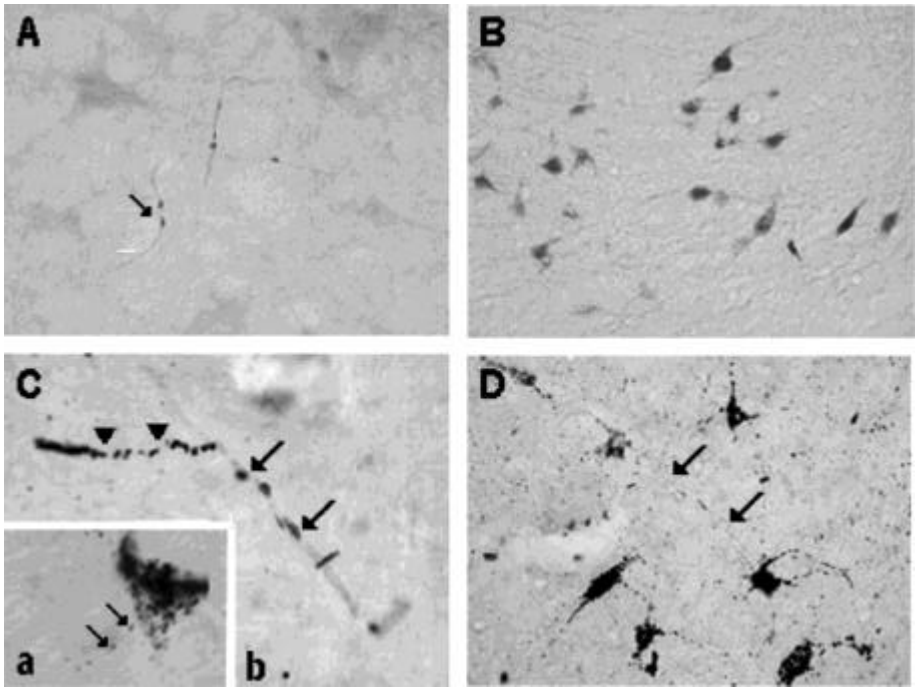

Fig. 3. A: A photomicrograph of a section at the pons in RPgc showing the BDA-labeled axons. ×200. B: A photomicrograph of a section at the pontomedullary junction in RP showing the WGA-HRP-labeled cells. HRP-histochemistry. ×100. C: A photomicrograph of a section at the pontomedullary junction in Rgc showing the double-labeled axon and soma. Blank is that BDA-labeled axon met soma of WGA-HRP labeled. Note that the labeled fibers and varicosities are distributed mainly in the Rgc. Double histochemistry. ×200, ×100. D: A photomicrograph of a section at the pontomedullary junction in PM, Rgc. Axons connceted between WGA-HRP-labeled cells and the other ones. Double histochemistry. ×100.

neurons on the six levels of the RF. As shown in Fig. 2, the labeling was subsequently observed in the brainstem, but little was found in the level of medulla (Fig. 1F). In the mesencephalon, a few reactions appeared in the locus coeruleus (Loc), but no reactions were observed in the RPO. There were few axons, (3 5 fibers were seen) in the mesencephalon. In the pons, labeled profiles of the RPgc were located everywhere and were arranged in several lengths (Fig. 3A). The termination of the labeled axons had a branch-like appearance with a bud and portions of the buds were observed in the dark brown areas (Figs. 3A, 3C, 3D). Labeled varicosities were observed in close proximity to the retrogradely labeled neurons, indicating a reciprocal circuitry.

The pontomedullary junctions contained the most densely labeled cells through the RF. These were also observed dominantly in the Rgc in addition to the Rpam and PM (Fig. 3). In the Rgc, the labeled fibers were arranged rectangular to the surface as a radiation. In the medulla, complex processes were observed in the Rgc, Rpg, Rst and VeP. Occasionally, long and curve-like fibers were observed in the RPO. In both the Rgc and Rpc, the axons were arranged like a string parallel to the surface (Fig. 2D). The BDA-labeled fibers were also numerous in the Rgc and accounted for 28.9% of the total number of labeled axons, followed by the RP (17.8%), Rpc (13.3%), Rpg (8.9%), RpaM (4.2) and Rst (4.5%). No labeled axons were found in the caudal medulla.

Localization of double labeling with BDA and WGA-HRP reaction

In the pontomedullary junction, the axons with a BDA histochemistry near the neurons with a WGA-HRP histochemistry appeared as two types. In one type, the axons were parallel to the neurons (Fig. 3A). Type, the axons contacted the surface of the neurons (WGA-HRP, Fig. 3B). The structure of the connection was divided in two ways, an axosomatic synapse or an axonaxonic synapse (Fig. 3C-a). The BDA projected like a stick with axonal terminals within the Rgc in the pontomedullary junction, which were distinguished from the fibers by their location, morphology, and intensity of staining (Figs. 3, 4).

The distribution of the BDA-labeled axons was almost similar to the pattern of the WGA-HRP-neurons. The labeled cells also appeared bilaterally in the RF, and each half contained a similar number as found in the WGA-HRP retrograde labeling. As seen in Fig. 4, the co-localization of WGA-HRP and BDA demonstrated a relationship of the RF location between the cerebellar cortex and spinal cord.

Discussion

The spinoreticulocerebellar (SRC) tract is an indirect spinocerebellar (SC) tract through the RF. The SC projection arising from all chicken spinal cords were reported by Pompeiano [21]. However, the structure of the cerebeullum in chickens is different from that of mammals. In addition, the SC tract has different pathways. The spinoreticular (SR) tract in mammals has been identified as a bilateral ventral flexor reflex tract. They cross the midline of the spinal cord close to their segmental origin, ascend in the ventral part of the lateral funiculus, and terminate at the level of the RF [1, 16]. Despite the obviousness of this pathway extensive and homologous nature in all major experimental animals, very few attempts have been made to identify this pathway in chickens

The WGA-HRP is extensively incorporated via the endocytosis into the axonal growth cones or the presynaptic terminals in the proximity of the injection site. The BDA is an axonal tracer that is internalized and transported via axons both retrogradely and anterogradely following a pressure injection into the central nervous system [24]. Double-labeling studies have typically utilized the chromogens yielding a brown reaction product as a result of either metal intensified DAB or TMB processing. This study demonstrated the SRC tract in chickens using dual retrograde WGA-HRP

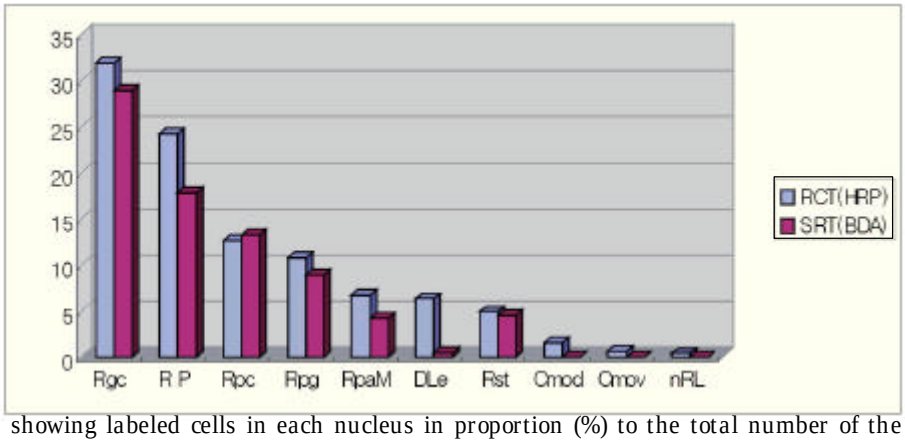

Fig. 4. A histogram showing labeled cells in each nucleus in proportion (%) to the total number of the WGA-HRP labeling cells and BDA projection fibers in the RF. The number of labeled cells was referenced to E. Hassouna *et al.* [8].

labeling and BDA anterograde projections. The neurons activated from the spinal cord occur in all parts of the magnocellular and parvocellular regions of the RL, which suggest that information from the cord reaches a wide variety of areas in the cerebellar cortex. The RL is one of several important precerebellar relay nuclei that provides a mossy fiber projection into the cerebellum and relays the cortical, brainstem and spinal cord [11]. The RL that is related to the cerebellum, plays an important role in the motor activity, primarily dealing with posture, and in the placing reaction. The RL is the most important origin of RC neurons and serves as a relay nucleus for the indirect SRC pathways receiving the main afferent inputs from the entire length of the spinal cord [1, 2, 6-8, 19, 30]. The nRL corresponds to the caudal extension of the RL and is not divided into subnuclei [8]. An anatomical technique based on the anterograde transport of BDA was used to investigate the projections of the spinal cord neurons to the RF in chickens. In chickens, the RL consists of Rgc, Rpc and Rst, and is most important in RC projections. The reason is that it contained approximately half of the total number of RC neurons. The Rgc contains the highest number of RC neurons through the entire RF. Therefore, labeled fibers were found at all RF levels and in particular, large numbers were found in the RPO, Rpgc, Rgc, Rpc, and Rpg. The spinal projection to the RL is essentially contralateral, except for a small ipsilateral projection arising from the cervical cord. The organization of the spinal projection to the RF in chickens was investigated by means of the anterograde pathway tracing method where BDA served as an enzyme marker. These results concur with a previous study [8]. This dorsolateral portion of the magnocellular division of the RL belongs to the termination site of the spinal lumbar projection, and the vermal lobules V-VII receive the afferents from all levels of the RL [1]. There are some differences in the results according to the species of laboratory animal used. These results show that the SR neurons in chickens were distributed in the mesencephalon, pons, pontomedullary region, and the medulla. The majority of fibers for the WGA-HRP projections were the maximum areas of termination, in which the Rpgc, Rpc, RP and Rgc accompany the BDA-labeling fibers. Hassouna [8] suggested that DLe cells were one of the RC nuclei of the RF [8]. However, these results are different despite using the same methods. Hassouna contradicted the nomenclature and made a mistake in naming Rst and Rpg, which needs to be corrected as PL and PM.

In mammals, all cerebellar cortical areas and nuclei receive afferents from one or more of the RF nuclei [10]. However, there are few studies on the distribution of RC neurons in birds. The RC neurons in the chicken mesencephalon contained no labeled cells using the WGA-HRP retrograde labeling in this study. The reaction was almost similar to the results reported by Hassouna et al [8]. No difference was found at the RPgc in the pons by WGA-HRP labeling. The reaction was found in the granular layer in the cerebellum. The WGA-HRP injections were carried out in both anterior and posterior of the cerebellum. The labeled cells were of all sizes, large, medium-sized: and small. The main portion of the RL projected to lobuli I-V, to the rostral part of lobulus VI, the most caudal part of lobulus VII, to lobulus VIII, as well as to the lobulus simplex, the medial parts of the lobulus ansiformis and to the lobulus paramedianus [31, 37]. The distribution of fibers from the RL to the cerebellar cortex was known by their pattern of collateralization in mammals. Although this pattern did not correspond to the avian RF, the location of the reactions was similar to that of the reactivity in mammals. Double retrograde labeling studies would help in determining more clearly whether the RF neurons have axon collaterals between the cerebellar cortex and the lumbosacral region. Approximately 40% of the RL neurons that projected to the cerebellum were non-immunoreactive to glutamine, suggesting that neurochemicals other than glutamine may also participate in RL synaptic control of the vermal neurons [34]. The observed pattern in the RC projection was found to agree partly with that observed in cats and rats. Hassonouna et al. reported that all the RF nuclei (with the exception of the RF of the mesencephalon) send fibers to the cerebellum in chickens as in mammals [7]. The highest number of labeled neurons after the cerebellar injections was found in the caudal RF, particularly within the nucleus reticularis ventralis, the RL and the Rgc. Another region that accumulates labeled cells is the rostral part of the nucleus Rpc. Previous retrograde studies have shown that the vermal lobules V-VII receive afferents from all levels of the RL in cats. WGA-HRP identified an ipsilateral predominance in the RC projections of the Rpam. The Rpam has been shown to be involved in mediating the postural and cardiovascular mechanisms in conjunction with the cerebellum [3]. In this study, a double labeling reaction was observed in the Rpam. Therefore, there is some similarity with mammal studies.

In conclusion, this study showed that the topographic organization revealed the distribution of the spinal afferent fibers to the RL and cerebellar projection to the RL. These results suggest an interaction among RL, cerebellar nucleus and their respective targets, which imply a new role for the RL in controlling cerebellar activities. Overall, this study provides an anatomical foundation on the SRCT regarding the central nervous system of chickens.

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